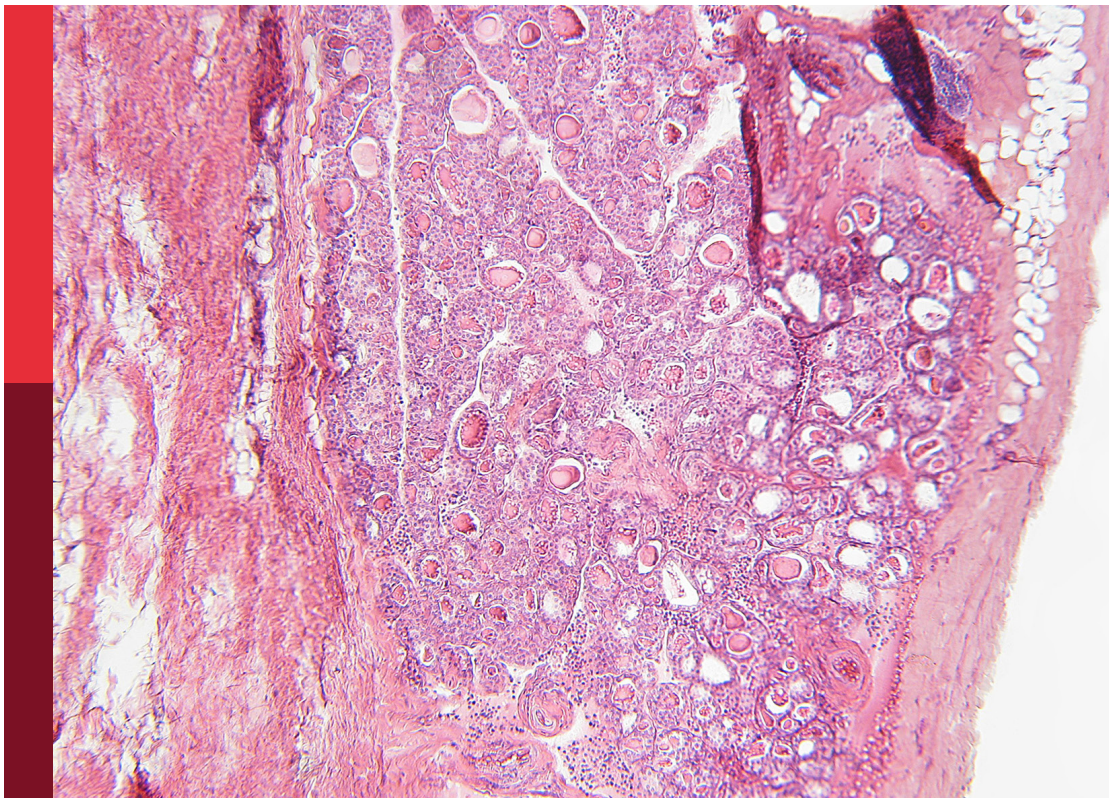
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Advanced approaches in the diagnosis and treatment of diabetes mellitus and secondary complications

Edited by
Pranav Kumar Prabhakar, Ram Prasad and Ponnurengam Malliappan Sivakumar

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Advanced approaches in the diagnosis and treatment of diabetes mellitus and secondary complications

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Editorial: Advanced approaches in the diagnosis and treatment of diabetes mellitus and secondary complications

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Editorial on the Research Topic
Advanced approaches in the diagnosis and treatment of diabetes mellitus and secondary complications

Introduction

Diabetes mellitus, a chronic metabolic disorder, has reached epidemic proportions globally, posing significant challenges to healthcare systems worldwide. With the number of individuals affected by diabetes rising steadily, there is an urgent need for innovative approaches to improve the diagnosis and treatment of this complex condition. Furthermore, the prevention and management of secondary complications associated with diabetes are of paramount importance to enhance patients' quality of life. In this editorial, we delve into the realm of advanced approaches that hold promise in tackling diabetes mellitus and its debilitating secondary complications. Accurate and timely diagnosis is crucial for effective management of diabetes mellitus. Emerging technologies, such as continuous glucose monitoring systems and point-of-care testing devices, have revolutionized diabetes diagnostics. These innovative tools provide real-time information on glucose levels, enabling individuals with diabetes to monitor their blood sugar levels more effectively. Additionally, advancements in genetic testing and biomarker analysis have the potential to enhance risk prediction and personalized treatment strategies for diabetes.

Individualized treatment strategies

Diabetes management is no longer a one-size-fits-all approach. Tailored treatment plans based on individual characteristics, including genetics, lifestyle factors, and

comorbidities, are essential for optimizing patient outcomes. Recent developments in the field of pharmacogenomics have shed light on genetic variations that influence an individual's response to specific antidiabetic medications. This knowledge allows for personalized medication selection, minimizing adverse effects and improving therapeutic efficacy. Moreover, the integration of digital health technologies, such as mobile applications and wearable devices, empowers patients to actively participate in their treatment, promoting self-management and adherence to treatment regimens. Addressing the myriad of secondary complications associated with diabetes requires a comprehensive and multidisciplinary approach. Collaborative efforts among healthcare professionals, including endocrinologists, cardiologists, ophthalmologists, nephrologists, and dietitians, are crucial in preventing and managing complications such as cardiovascular diseases, retinopathy, nephropathy, and neuropathy. The advent of telemedicine and telehealth services has further facilitated interdisciplinary collaboration, enabling remote patient monitoring, consultations, and timely interventions.

Regenerative medicine and novel therapies

Regenerative medicine, with its potential to restore or replace damaged pancreatic beta cells, holds immense promise for individuals with diabetes. Stem cell therapy, tissue engineering, and gene therapy are among the innovative approaches being explored to regenerate insulin-producing cells and restore normal glucose metabolism. Furthermore, advancements in targeted drug delivery systems and nanomedicine offer exciting possibilities for more efficient and precise drug delivery, reducing systemic side effects and enhancing treatment efficacy. Preventing the onset of diabetes and its complications is an essential aspect of comprehensive diabetes care. Public health initiatives focusing on promoting healthy lifestyles, raising awareness, and implementing early screening programs are vital in curbing the diabetes epidemic. Innovative interventions such as digital health interventions, community-based programs, and educational campaigns can empower individuals to make informed decisions regarding diet, physical activity, and overall diabetes management.

The follow-up volume, which began publication in August 2022, focused primarily on areas of the secondary complications of diabetes and their management. It should address the treatment of diabetes, abnormalities of secondary complications, and other diseases associated with diabetes. The original volume's focus was broad.

Submissions were solicited for the article types of Original Research (29), Review (5), Systematic Reviews (7), Case study (2), and Clinical trial (2), and special emphasis/invitation was promoted to the following topics: (1) Diabetic complications and its management; (2) Antibiotics and its involvement in diabetic complications; (3) Circadian rhythm and diabetic complications; (4) Performance of artificial intelligence in diabetic complications; (5) Post-operative complications in diabetic; (6) Hypertension (HTN) and diabetes mellitus type 2; (7) Association of epilepsy, anti-epileptic drugs (AEDs), and type 2 diabetes mellitus (T2DM); (8) Predictors of diabetes and prediabetes; (9) Nanomaterials for diabetic wound healing; (10) impact of oxidative stress-induced mitochondrial dysfunction on diabetic microvascular complications; (11) Case report: Diabetic muscle infarction with diabetic ketoacidosis.

Among the articles, the main focus was on the diagnosis and management of diabetes mellitus and secondary complications. As we know the main issue in the case of diabetes mellitus is secondary complications and it is the main reason for mortality and morbidity. Diabetic Kidney Disease is a prevalent and significant secondary complication often found in individuals with diabetes associated with retinopathies, nephropathies (Xie et al.), and renal failure, cardiovascular issues (Li et al.), and premature mortality. Over time, there has been a growing occurrence of normoalbuminuric diabetic kidney disease in both diabetic and diabetic kidney disease patients. This increase may be attributed to better diabetes management and potential changes in the kidney's pathology in normoalbuminuric diabetic kidney disease (An et al.).

Examining the significance of circadian rhythms in the development of Type 2 Diabetes Mellitus (T2DM) involves assessing the interconnected metabolic processes, their association with circadian rhythms through both lifestyle and molecular angles, and their influence on the pathophysiology of T2DM. These effects have been substantiated through numerous research studies and have given rise to strategies such as time-restricted eating, chronotherapy (treatment timed according to circadian rhythms), and substances that stabilize circadian molecules (Hariri et al.).

The co-occurrence of HTN and T2DM was prevalent among the study participants of Haramaya University which forced us to put rigorous efforts into developing strategies for screening employees to tackle the alarming increase in HTN and T2DM in university employees (Motuma et al.). Chronic type 2 diabetes is linked to the disruption of collagen fiber organization, alterations in the extracellular matrix, and a decline in the biomechanical integrity of the rotator cuff tendon (Xu et al.). Elevated arterial rigidity is a prevalent occurrence among diabetes patients, and inflammation stands out as a primary factor contributing to this heightened arterial stiffness. Neutrophil-to-lymphocyte ratio outperformed platelet-to-lymphocyte ratio, and the combination of neutrophil-to-lymphocyte ratio and monocyte-to-lymphocyte ratio exhibited specific predictive capabilities signifying the rise in arterial stiffness among diabetes patients. These predictive values can facilitate the early detection of heightened arterial stiffness in individuals with diabetes (Ning et al.). Ultra-widefield color fundus photography along with high-speed ultra-widefield swept-source optical coherence tomography angiography showed strong concordance in identifying lesions associated with diabetic retinopathy and assessing its severity when compared to the combination of ultra-widefield color fundus photography and fluorescein angiography (Li et al.).

Diabetic kidney disease (DKD) is a prevalent chronic complication among individuals with diabetes, and its pathological diagnosis has significant shortcomings. Serum insulin-like growth factor-1 and interleukin-6 serve as biochemical markers for diagnosing and assessing the

advancement of DKD and a combination of these two markers can enhance the sensitivity and specificity of the test (Liu et al.). The effect of a single urine C peptide/creatinine ratio can also be used in the estimation and assessment of islet β Cell function of T2DM patients with different renal functions (Zhou et al.). Antibiotic-infused bone cement demonstrates its effectiveness in the treatment of diabetic foot infection wounds, offering substantial savings in terms of medical resources and costs. Promoting its utilization in clinical practice is highly advisable. Moreover, to delve deeper into the topic of film formation induced by trauma bone cement in various stages of diabetic foot management, it is essential to conduct more multicenter trials with larger sample sizes in the future (Dong et al.).

Case studies

One of the major consequences of diabetes that has a high death and disability rate is diabetic foot ulcer. Low-intensity ultrasound and blood microbubbles can improve the local soft tissue's blood perfusion effect, which may help a diabetic ulcer heal more quickly (Zhang et al.). Diabetic muscle infarction, also known as diabetic myonecrosis, is a rare and long-term complication of poorly managed diabetes mellitus. It was discovered that acute diabetes decompensation, such as diabetic ketoacidosis, could also stimulate the occurrence and development of diabetic myonecrosis (Tang et al.).

Clinical trial

According to recent research, the amniotic fluid includes significant amounts of multipotent mesenchymal, hematopoietic, neuronal, epithelial, and endothelial stem cells in addition to the elements necessary for wound repair. After 8-week studies on 92 type 2 diabetic patients between 2019-2022, it was established that the amniotic fluid represents a useful and safe option for treating chronic diabetic foot ulcers (Niami et al). The most prevalent chronic consequence of T2DM, distal symmetric polyneuropathy (DSPN), causes additional major problems such as diabetic foot ulcers, amputations, and decreased life expectancy. While interventional studies show that low-dose vitamin D treatment does not significantly ameliorate neuropathy in DSPN, observational studies suggest that vitamin D insufficiency may be linked to the development of DSPN in T2DM. Additionally, high-dose vitamin D supplementation is necessary for neuropathy healing to maintain optimum vitamin D levels (Chen et al.).

Peripheral nerve damage is a significant issue that disrupts neuronal communication, leading to a diminished quality of life and, in severe cases, permanent disability. Investigating tailored biomaterials, which mimic the extracellular matrix, holds promise for treating peripheral nerve injuries. These specialized biomaterials, owing to their positive biological effects, can accelerate tissue regeneration and act as vehicles for delivering cellular and pharmaceutical therapies. This opens up new avenues for using biomaterials to treat nerve damage (Raghav et al.).

Conclusion

Outstanding improvements are being made in the detection, management, and prevention of diabetes mellitus and its consequences as a result of cutting-edge research and innovative technology. In order to improve patient outcomes and lessen the burden of this global health issue, it is critical that healthcare professionals, researchers, policymakers, and people with diabetes work together and take advantage of these breakthroughs. We can pave the way to a future in which diabetes mellitus and its secondary complications are successfully managed and ultimately prevented by adopting precision diagnosis, individual treatment strategies, multidisciplinary care, regenerative medicine, and a focus on prevention and early intervention.

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Evaluating the role of type 2 diabetes mellitus in rotator cuff tendinopathy: Development and analysis of a novel rat model

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Objective: To establish and validate an intact rotator cuff rat model for exploring the pathophysiological effects of type 2 diabetes on the rotator cuff tendon *in vivo*.

Methods: A total of 45 adult male rats were randomly divided into a control group (n = 9) and type 2 diabetes group (n=36). The rats were sacrificed at 2 weeks (T2DM-2w group, n=9), 4 weeks (T2DM-4w group, n=9), 8 weeks (T2DM-8w group, n=9), and 12 weeks (T2DM-12w group, n=9) after successful modeling of type 2 diabetes. Bilateral shoulder samples were collected for gross observation and measurement, protein expression(enzyme-linked immunosorbent assay, ELISA), histological evaluation, biomechanical testing, and gene expression (real-time quantitative polymerase chain reaction, qRT-PCR).

Results: Protein expression showed that the expression of IL-6 and Advanced glycation end products (AGEs)in serum increased in type 2 diabetic group compared with the non-diabetic group. Histologically, collagen fibers in rotator cuff tendons of type 2 diabetic rats were disorganized, ruptured, and with scar hyperplasia, neovascularization, and extracellular matrix disturbances, while Bonar score showed significant and continuously aggravated tendinopathy over 12 weeks. The biomechanical evaluation showed that the ultimate load of rotator cuff tendons in type 2 diabetic rats gradually decreased, and the ultimate load was negatively correlated with AGEs content. Gene expression analysis showed increased expression of genes associated with matrix remodeling (COL-1A1), tendon development (TNC), and fatty infiltration (FABP4) in tendon specimens from the type 2 diabetic group.

Conclusion: Persistent type 2 diabetes is associated with the rupture of collagen fiber structure, disturbance in the extracellular matrix, and biomechanical decline of the rotator cuff tendon. The establishment of this

new rat model of rotator cuff tendinopathy provides a valuable research basis for studying the cellular and molecular mechanisms of diabetes-induced rotator cuff tendinopathy.

KEYWORDS

rotator cuff, diabetes, tendinopathy, mechanics, animal model

Introduction

Tendinopathy is a common muscle tissue disease with multifactorial pathogenesis that has not yet been fully elucidated. It usually occurs due to overuse, metabolic disturbances, and impact of other factors related to tendon microinjury, one of which is diabetes as a metabolic disorder. Tendinopathy is a common musculoskeletal complication of diabetes mellitus (DM) (1). The effects of type 2 diabetes on tendon structure and homeostasis are often overlooked before serious complications or acute injuries occur because the effects of type 2 diabetes on tendons tend to persist, and tendon-related diseases are often difficult to detect and treat in advance before acute tears occur. The lack of continuous effects of type 2 diabetes before acute injuries leads to pathological changes in tendon tissue, especially on the rotator cuff tendon.

DM is one of the risk factors for rotator cuff injury, and patients with type 2 DM are at a higher risk of tendon rupture (2, 3). The prognosis and healing rates of the rotator cuff after repair are poor (4). Diabetic rats have increased rotator cuff fatty infiltration, poor biomechanics (5), and accelerated steatosis after rotator cuff injury (4). In addition, the detrimental effects of diabetes persist even after the repair of rotator cuff injury, and persistent hyperglycemia impairs tendon-to-bone healing after rotator cuff repair in a rat model, resulting in worse biomechanics and histology (6), which may be associated with the let-7b-5p/CFTR pathway (7). However, whether this leads to poor biomechanics is still controversial. Another study concluded that hyperglycemia alone does not affect the biomechanical properties of the rotator cuff but induces a chronic inflammatory response (5). An increasing number of clinical studies have shown that the incidence of tendinopathy is significantly increased in patients with type 2 diabetes compared with non-diabetic patients (8, 9), and tendinopathy is associated with the duration of type 2 diabetes (10–12). Unfortunately, previous findings have been dominated by clinical studies, none of which described in detail how type 2 diabetes impacts tendon structure, extracellular matrix homeostasis, and biomechanics at different stages of the course of the disease, particularly the supraspinatus tendon of the rotator cuff. In addition, previous studies lacked comprehensive and systematic evaluation and description of time window tendinopathy manifests after diabetes onset.

To this end, we investigated an animal model with an intact rotator cuff. The aim of the present study was to evaluate the effects of type 2 diabetes on rotator cuff tendon structure and homeostasis over the course of the disease by ELISA, histology, biomechanics, and qTR-PCR in a rat model at various time points after induction of non-diabetic and type 2 diabetes, thus providing a more appropriate animal model and theoretical basis for exploring the pathophysiologica l effects of type 2 diabetes on the rotator cuff tendon.

Methods

Experimental animals

A total of 45 adult male clean Sprague-Dawley rats (6 weeks old, 200-250 g, Beijing Vital River) were used for animal modeling. All the animals were housed in an environment with a temperature of 23 ± 2 °C, relative humidity of 50 ± 5%, and a light/dark cycle of 12/12 hr and had free access to food and water. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Qingdao University institutional animal care and conducted according to the AAALAC and the IACUC guidelines.

Animal grouping

A total of 45 rats were numbered and randomly divided into non-diabetic group (NDM group, n = 9) and type 2 diabetic group (T2DM group, n = 36) by random number table. According to different sampling time, rats in type 2 diabetic group were divided into 2-week diabetic group (T2DM-2w group, n = 9), 4-week diabetic group (T2DM-4w group, n = 9), 8-week diabetic group (T2DM-8w group, n = 9) and 12-week diabetic group (T2DM-12w group, n = 9),NDM group rats sacrifice times were the same as T2DM-12w group rats. After purchase, the rats were fed a normal diet in the animal laboratory of Qingdao University, and the diabetic group was fed a high-fat diet for 1 month. The rats in both groups had free access to water, and the bedding was replaced daily. The experimental animal grouping and study design are shown in Figure 1.

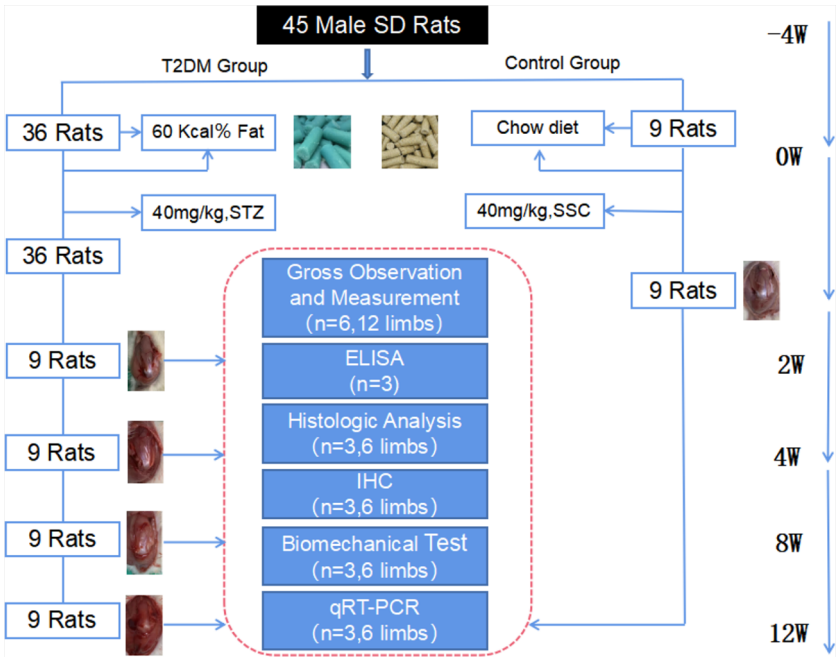


FIGURE 1
Flow chart of the experimental design.

Constructing a rat model of type 2 diabetes

In order to ensure normoglycemia before modeling, blood glucose was measured before the induction of type 2 diabetes in both groups. After 4 weeks of high-fat diet intervention, rats in the type 2 diabetes group were fasted for 12 h, which was followed by intraperitoneal injection of Streptozotocinn(STZ) solution (injection dose of 40 mg/kg) and continuation of high-fat feeding (13). The non-diabetic group was injected with the same amount of citric acid buffer. Three days later, blood samples were collected at fixed times each day, and rats were considered a successful diabetic rat model when fasting blood glucose levels were measured three consecutive times ≥16.7 mmol/L for three consecutive days (14). This model was eliminated and supplemented in a timely manner when random blood glucose was < 16.7 mmol/L.

Obtaining supraspinatus tendon-humerus specimens

At the 2nd, 4th, 8^{th,} and 12th week after modeling, the left forelimb of diabetic rats was divided from the elbow joint and scapula, the excess muscle tissue was removed, the complete supraspinatus tendon-humerus structure was obtained, and the right specimen was removed by following the same method. Tissue samples were stored in a -80° freezer after collection.

Gross tissue assessment and measurement of the supraspinatus tendon

The quality and color of the supraspinatus tendon in the rotator cuff and its adhesion to surrounding tissues were observed. Supraspinatus tendon width, thickness, and weight were measured using a digital micrometer (Syntek, Zhejiang, China).

ELISA

A total of 1.5 mL of venous blood was drawn from the orbital venous plexus of fasting rats in the morning at 2, 4, 8, and 12 weeks after the establishment of the model in the non-diabetic group and type 2 DM. It was rapidly transferred to a biochemical tube and centrifuged at 2500 r/min for 20 min. The supernatant was transferred and stored in a -80°C freezer for examination. Serum levels of IL-6, PGE2, and AGEs were measured by referring to the ELISA kit instructions for rat IL-6 (EK306/3-48, MultiSciences, Zhejiang, China), PGE2 (EK8103/2-48, MultiSciences, Zhejiang, China), and AGEs (CSB-E09413r, Cusabio, Wuhan, China).

Histological evaluation

Bilateral shoulder joints were taken from rats in each group. The muscle parts of the humerus and supraspinatus muscles of the samples were immediately removed, after which the

supraspinatus tendons were fixed in paraformaldehyde for 24 h. The tissues were dehydrated and embedded in paraffin with an automatic tissue processing device. The embedded samples were sectioned at a thickness of 5 mm parallel to the direction of the tendon course. Three stains were performed: hematoxylin and eosin (HampE), picrosirius red (S8060, Solarbio, Beijing, China), and alcian blue staining (DG0041, Leagene, Beijing, China), and the relevant procedures were performed according to previous literature (15).

For immunohistochemical staining, 4 μm sections were dewaxed at 65°C, dewaxed in xylene, treated with graded ethanol, blocked with 3% hydrogen peroxide for 10 min, infiltrated for 15 min, incubated in goat serum, and immediately reacted with primary antibody at 4°C for ≥ 16 h (overnight). Then, samples were washed three times with PBS solution, incubated with the corresponding secondary antibody conjugated to horseradish peroxidase for 30 min at room temperature, and then washed three times with PBS solution. DAB chromogenic solution (volume ratio of concentrated DAB chromogenic solution to buffer 1:500) was reacted for 1 to 5 minutes and washed with pure water 3 to 4 times; Specimens were stained with hematoxylin-eosin and sequentially placed in ethanol at concentrations of 80%, 90%, and 100% for dehydration.Finally, xylene transparent, neutral gum mounting was performedxylene transparency, and neutral gum mounting. Used antibodies included biglycan (Biglycan) antibody (K008381P, Solarbio, Beijing), factor VIII antibody (AB275376, Abcam, UK), MMP-3 (K111452P, Solarbio, Beijing), and IL-6 (KOO9639P, Solarbio, Beijing). HampE, immunohistochemistry, and Alcian blue staining were observed using a Nikon E100 microscope (E100, Nikon, Japan), tissue imaging with Sirius red staining was observed using a polarized light microscope (DM4500, Leica, Germany), and scanning of images was performed using a panoramic scanner (3DHISTECH P250 FLASH, Beijing, China). After staining, five sections were selected from each sample, and three fields of view were selected for densitometric value (OD) analysis at ×400 fields in the tendon region for each section. Microscopically, positive brown positive reaction areas were selected, and positive expression was semiquantitatively analyzed by ImagePro Plus 8.0 software in order to detect cumulative optical density (IOD) and positive area, after which the ratio of the two was taken as the expression level.

Tendinopathy was graded using a modified semi-quantitative Bonar scoring system as previously described (16). A histology scoring system was used for semi-quantitative assessment of the repair interface. The total histological score was calculated by two investigators (KSX and LZ), with higher histological scores indicating more severe tendinopathy.

Biomechanical analysis

Biomechanical tests were performed on the bilateral shoulders of rats in each group (3 rats per group), and the humeral muscles and tissues around the humeral head were carefully removed. Only specimens of the supraspinatus and humeral complex were preserved, and the musculature on the supraspinatus tendon was curetted with a scalpel. The humerus was firmly fixed on the base of the testing machine using polymethyl methacrylate (Thermo Fisher Scientific 's Products, Shanghai, China) (17), and the tendons were flattened, fixed using sandpaper and cyanoacrylate glue (Dongxin' s Products, Shenzhen, China), and then placed in customized serrated grips. After preconditioning with 0.1 N, the traction load was gradually increased until the tendon was completely ruptured at a fixation speed of 10 mm/min (18, 19). Ultimate load and stiffness were recorded. In the load-displacement curve, the slope of its linear segment was used to express its stiffness value.

qRT-PCR analysis

After sampling at predetermined time points, bilateral supraspinatus tendons and muscles were carefully separated, quickly snap frozen in liquid nitrogen, and then stored in a -80° C freezer for testing. Total RNA was extracted by the Trizol method, RNA quality was measured by spectrophotometer, and mRNA expression levels were measured by RT-qPCR. The corresponding cDNA was obtained by reverse transcription of the resulting RNA samples, and the qRT-PCR system was prepared according to the instructions of the SYBR Green kit (Q711-02, Vazyme, Nanjing, China) in this experiment. The cDNA, upstream and downstream primers, SYBR GREEN master mix, and ddH2O were reacted, and quantitative fluorescence analysis was performed using a real-time fluorescence quantifier (Thermo Fisher Scientific, Shanghai, China). Collagen1A1 (COL1A1), Tenascin C (TNC), RUNX2, Scleraxis (SCX), SOX9, and Tenmodulin (TNMD) were tested in supraspinatus tendon samples. Fatty acid binding protein 4 (FABP4) and peroxisome proliferator-activated receptor gamma (PPARγ) were measured in muscle specimens. Three independent replicates were performed for each sample gene, and Gapdh was used as an internal reference value to calculate and count the target gene using the $2^{-\Delta\Delta CT}$ method. The experimental results were averaged. Primer sequences are shown in Table 1.

Statistical analysis

All data analyses were performed using SPSS 21.0 (IBM, Armonk, NY) and GraphPad Prism 8.0 (La Jolla, CA). Student's t-test was used to perform statistical comparisons between biomechanical, ELISA, Banor score, and qRT-PCR data at each time point, and continuous data were presented as mean ± standard deviation. Figures were plotted using Prism 8.0 with mean ± standard error of the mean (SEM). A P value < 0.05 indicated statistical significance.

TABLE 1 Primer information of RT-PCR gene.

Gene	Sequence(5'-3')	Product Length/bp
GAPDH	Gapdh-F: CTGCCTTCTCTTGTGACAAAGTG	148
	Gapdh-R: TTGATGACCAGCTTCCCATTCTC	
COL1a1	Col1a1-F: ATCAAGGTCTACTGCAACATGGA	119
	Col1a1-R: AACCAGACATGCTTCTTCTCCTT	
TNMD	TNMD-F: AACAGTCAGTGATTTGGGTTCCT	104
	TNMD-R: CCAGTACATAGTCACATTGTCGC	
TNC	TNC-F: TATGACAAGGACACAGACTCAGC	112
	TNC-R: TATTGTCCCCATATCTGCCCATC	
SOX9	SOX9-F:GATAAATTCCCAGTGTGCATCCG	120
	SOX9-R: CTTGACGTGTGGCTTGTTCTTG	
RUNX2	RUNX2-F:ATGGCCGGGAATGATGAGAACTA	101
	RUNX2-R:CGGCCTACAAATCTCAGATCGTT	
FABP4	FABP4-F:CCCAACTTGATCATCAGCGTAGA	113
	FABP4-R:GGGGTGATTTCATCGAATTCCAC	
SCX	SCX-F:CGAAAAACCCTGTCGTGTTCATG	179
	SCX-R:CCGTGTTCACGCTGTTGGTG	
PPARγ	PPAR-F:CTCCAGAAGATGACAGACCTCAG	117
	PPARγ-R:CTTGTAGATCTCCTGGAGCAGAG	

COL1A1, collagen1A1; TNMD, tenomodulin; SCX, scleraxis; TNC, Tenascin C; RUNX2, runt-related transcription factor 2; FABP4, fatty acid binding protein 4; PPARγ,peroxisome proliferator-activated receptor gamma.

Results

Evaluation of a rat model of type 2 diabetes

Rats in the T2DM-12w group with random blood glucose > 16.7 mmol/L after modeling showed significant symptoms of polydipsia and polyphagia. They also gained weight slowly compared with the control group. The detailed results are shown in Figures 2A–D.

Gross tissue observation and measurement of supraspinatus tendon in type 2 diabetic rats

None of the rats in any of the groups died. Compared with the control group, the supraspinatus tendon color became darker, and the gloss gradually worsened at 2 weeks, 4 weeks, 8 weeks, and 12 weeks after modeling in diabetic rats. Detailed results are shown in Figure 2E.

Type 2 DM causes persistent histologic tendinopathy of the rotator cuff

Histological findings revealed that sparse arrangement and microtears of collagen fibers were first detected 2 weeks after diabetes induction. As the disease progressed, the number of supraspinatus tendon cells increased, volume of matrix increased, chondroid tissue formation was observed, and tendon collagen fibers showed significant tears and inflammatory cell infiltration by 12 weeks (Figures 3A, C, D). Supraspinatus tendon showed no significant fatty infiltration and calcification. Immunohistochemical results further showed that protein expression involving tissue repair (type I collagen), scar formation (Biglycan), angiogenesis (VIII), and extracellular matrix remodeling marker (MMP-3) was increased in the tendon tissue of rats with type 2 diabetes (Figures 3B, 4A–C), and optical density values (OD) were significantly increased compared with the non-diabetic group (Figures 5A–D). Increased tissue repair, scarring, newly formed vascular invasion, and extracellular matrix remodeling suggested that diabetes induced sustained changes in the rotator cuff tendon. As it was challenging to find manifestations of rotator cuff tendon degeneration in non-diabetic rats, we determined the modified Bonar score as 0 in non-diabetic rats. Type 2 diabetes can lead to persistent tendinopathy for at least 12 weeks after induction. The mean modified Bonar score was significantly higher in the type 2 diabetes group than in the control group (T2DM-2w group: 3.56 ± 0.69; T2DM-4w group: 4.11 ± 0.84; T2DM-8w group: 4.78 ± 0.19. T2DM-12w group: 6.22 ± 0.69). Detailed results are shown in Figure 6.

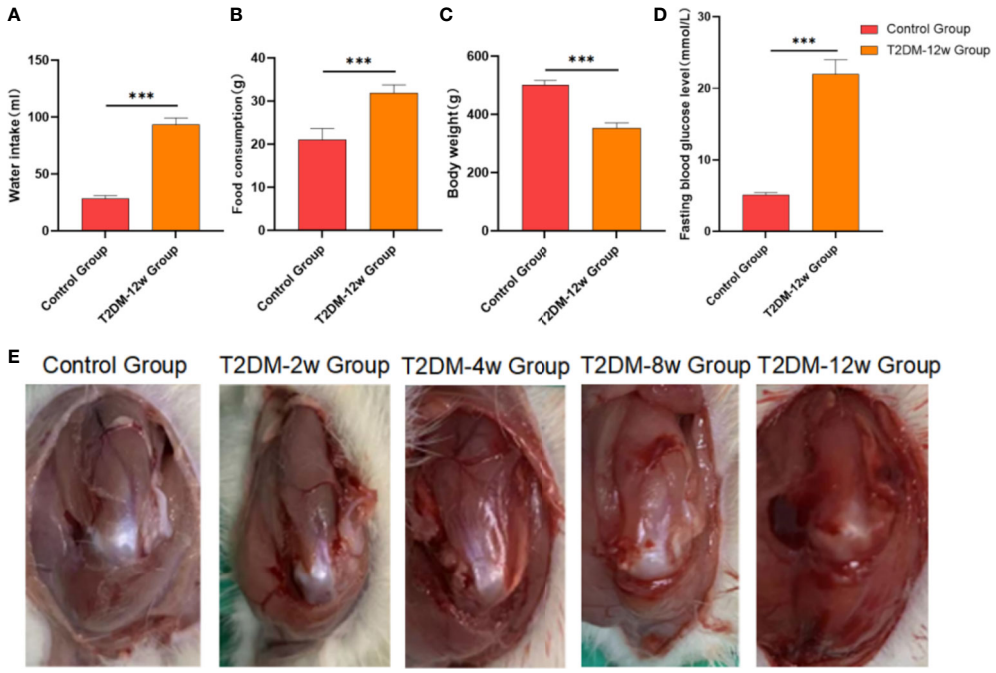


FIGURE 2
Evaluation of a rat model type 2 diabetes. **(A)** Water intake. **(B)** Food consumption. **(C)** Body weight. **(D)** Fasting bloiod glucose level. **(E)** Gross observation of supraspinatus tendon of rotator cuff in each group. ***p < 0.001 versus Control group. The values are presented as means, with the error bars depicting the standard deviation.

Type 2 diabetes leads to an increase in serum inflammatory factors and a decrease in the ultimate load of the supraspinatus tendon of the rotator cuff

ELISA results showed that the expression of serum IL-6 was highest at 2 weeks after induction of type 2 diabetes, followed by a gradual decrease in expression compared with the non-diabetic group (Figure 7A) ($p < 0.05$). There was also a clear upward trend in PGE2 expression, but there was no statistically significant difference (Figure 7B) ($p > 0.05$). The expression of serum AGEs showed a significant increasing trend within 12 weeks after induction of type 2 diabetes; the observed difference was statistically significant (Figure 7C) ($p < 0.05$).

In all specimens, the site of destruction of the supraspinatus tendon was located in the middle of the tendon. The biomechanical evaluation showed that the ultimate load (Figure 7D) and stiffness (Figure 7E) of the supraspinatus tendon had a significant decreasing trend in type 2 diabetic rats compared with the non-diabetic group, and tissue stiffness was restored at 8 weeks. Ultimate load and stiffness were not significantly different between diabetic and non-diabetic groups within 4 weeks after induction of type 2 diabetes ($P < 0.05$). At 8 (14.11 ± 1.43 N) and 12 weeks (10.7 ± 3.47 N) after induction of type 2 diabetes, the mean ultimate load breaking in the non-diabetic group (23.06 ± 4.92 N) was significantly higher than that in the diabetic group ($p < 0.05$). However, there was no significant difference in stiffness of the supraspinatus tendon between the two groups ($p > 0.05$) In addition, there was a significant negative correlation between the ultimate load of the supraspinatus tendon and the expression of serum AGEs ($r = -0.7807$; $p = 0.0006$) (Figure 7H), but no correlation with IL-6 ($r = -0.2844$; $p = 0.3042$) and PGE2 ($r = -0.09291$; $p = 0.7419$) (Figures 7F, G).

Type 2 diabetes causes persistent changes in gene expression profiles of affected tendons

Gene expression trends showed high variability within the diabetic group; however, these qRT-PCR results could still provide preliminary observations on gene expression in type 2 diabetes leading to tendinopathy (Figures 8A–H). Compared with the non-diabetic group, the expression levels of genes involved in tissue repair (COL1A), tenogenesis (TNC, TNMD, SCX), osteogenesis (RUNX2), and chondrogenesis (SOX9) of the supraspinatus tendon of the rotator cuff showed an increasing trend after induction of type 2 diabetes, where the TNC mRNA expression levels of the supraspinatus tendon were significantly increased in the 2-week group after induction of type 2 diabetes

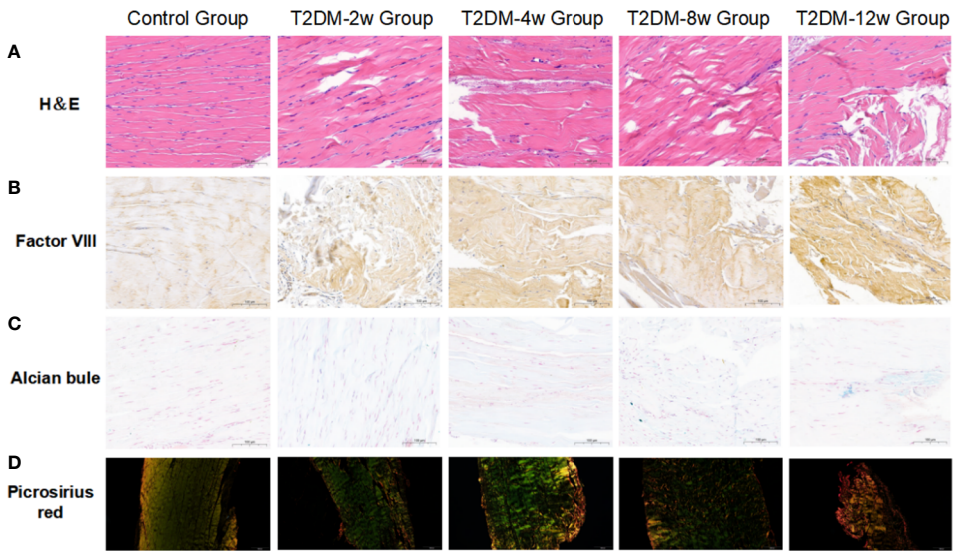


FIGURE 3
Representative images of histology. **(A)** Hematoxylin and eosin(H&E) staining(40X). **(B)** Immunohistochemical staining of factor VIII(40X). **(C)** Alcian-blue staining(40X). **(D)** Picrosirius-red staining(10X). Scale bars depict 100μm.

($p < 0.05$), and the COL1A1 mRNA expression levels of the supraspinatus tendon were significantly increased in the 4-week group after induction of type 2 diabetes ($p < 0.001$). Fat infiltration-related genes (FABP and PPARγ) expression tended to increase in supraspinatus muscle specimens, and fat infiltration developed gradually over time, with FABP4 mRNA expression levels significantly increasing in the 2-week group after type 2 diabetes induction ($p < 0.05$). Although some genes expression (TNMD, SCX, TNMD, SCX, SOX9, PPARγ) in the diabetic group were not statistically significant compared with the non-diabetic group ($p > 0.05$), the gradual increase in tendon gene expression from 2 weeks to 12 weeks after induction of type 2 diabetes showed that the hyperglycemic microenvironment produced long-term chronic changes in the supraspinatus muscle of rats.

Discussion

In the current study, two important findings emerged using protein expression, histological assessment, biomechanical testing, and gene expression. First, we developed a rat model

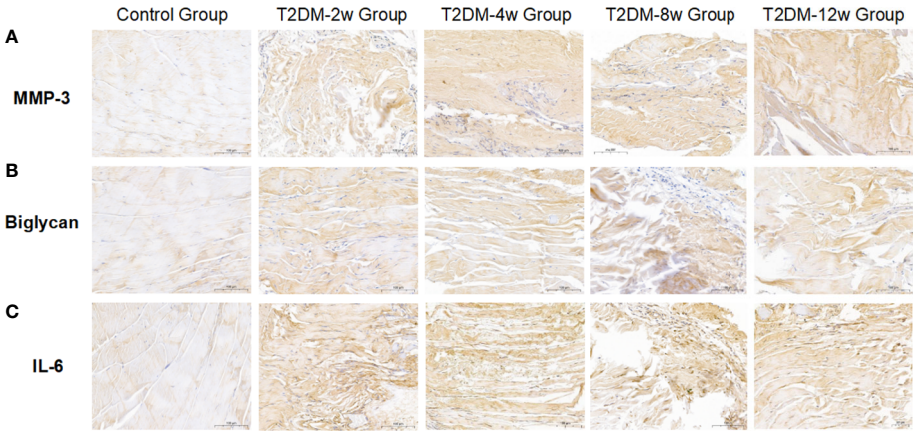


FIGURE 4
Representative images of immunohistochemical staining. **(A)** MMP-3 (40X). **(B)** Biglycan (40X). **(C)** Il-6 (40X). Scale bars depict 100μm.

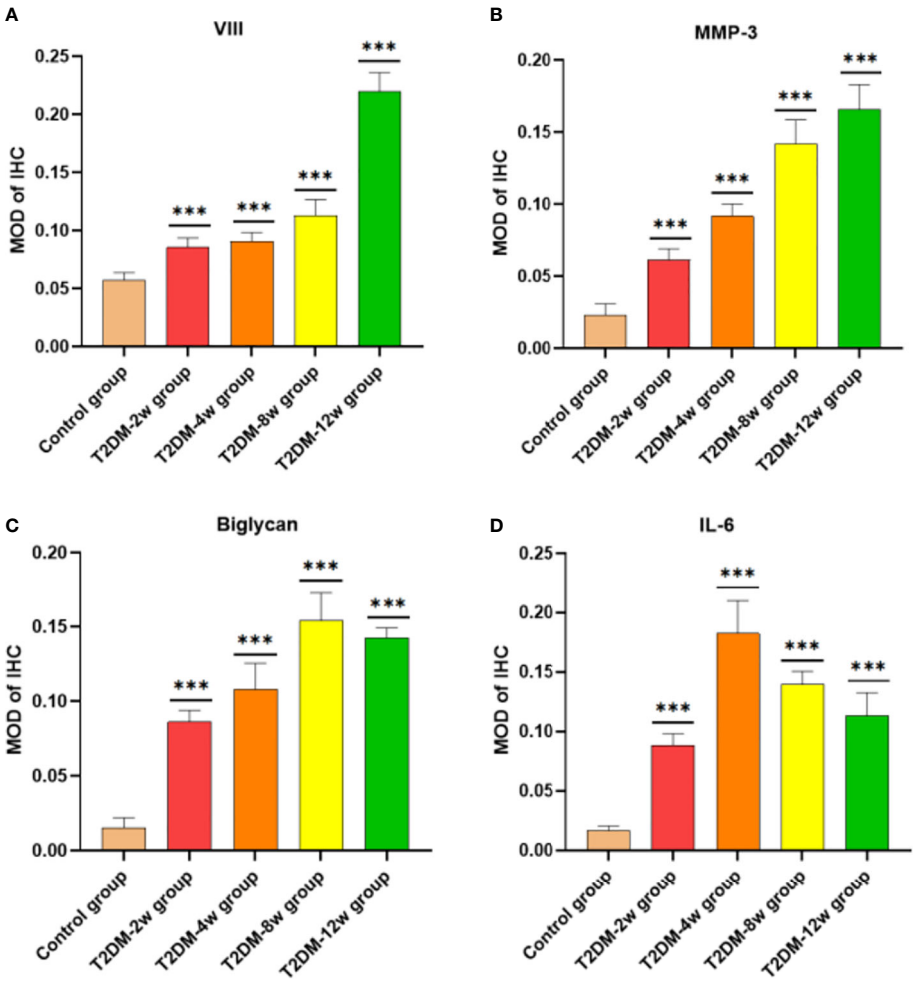


FIGURE 5
Comparison of semi-quantitative MOD after immunohistochemical staining. **(A)** VII. **(B)** MMP-3 (40X). **(C)** Biglycan (40X). **(D)** il-6 (40X). MOD is the ratio of cumulative optical density value (IOD) and positive area (AREA). ***p < 0.001 versus Control group. The values are presented as means, with the error bars depicting the standard deviation.

of rotator cuff tendinopathy induced by type 2 diabetes in rats. To the best of our knowledge, this is the first systematic study that evaluated the role of type 2 diabetes in rotator cuff tendinopathy. In addition, we divided and sampled experimental animals according to the duration of disease after induction of type 2 diabetes and confirmed that type 2 diabetes persistently leads to collagen fiber tears in the rotator cuff tendon, increased histological scores, extracellular matrix disturbances, and biomechanical decline. Moreover, we believe this is the first basic study that assessed the impact of type 2 diabetes on rotator cuff tendinopathy with the progression of the disease.

Two weeks after induction in type 2 diabetic rats, we were the first to detect a sparse arrangement of collagen fibers and a small number of microtears, which are the first features of rotator cuff tendinopathy. It is well-known that tendon structures in diabetic patients show disorganized collagen fibers and microtears (20), tendon thickening (21), and calcification (22) compared with normal tendons. As the disease progresses, collagen fibers become more disorganized and tear further increase. Previous studies reported histopathological changes associated with diabetic tendinopathy. In an earlier study, Kent et al. found that a high glucose environment alters collagen alignment in isolated tendons (23). Ahmed et al. reported similar results, with a marked reduction in collagen organization in injured tendons in diabetic rats (24). Even long-term insulin resistance adversely affects tendons, resulting in decreased collagen fiber density (25, 26), collagen fiber disorganization, and increased vascularity (27). Using immunohistochemical staining, similar studies have confirmed our experimental results that vascular endothelial growth factor expression is increased in tendons of

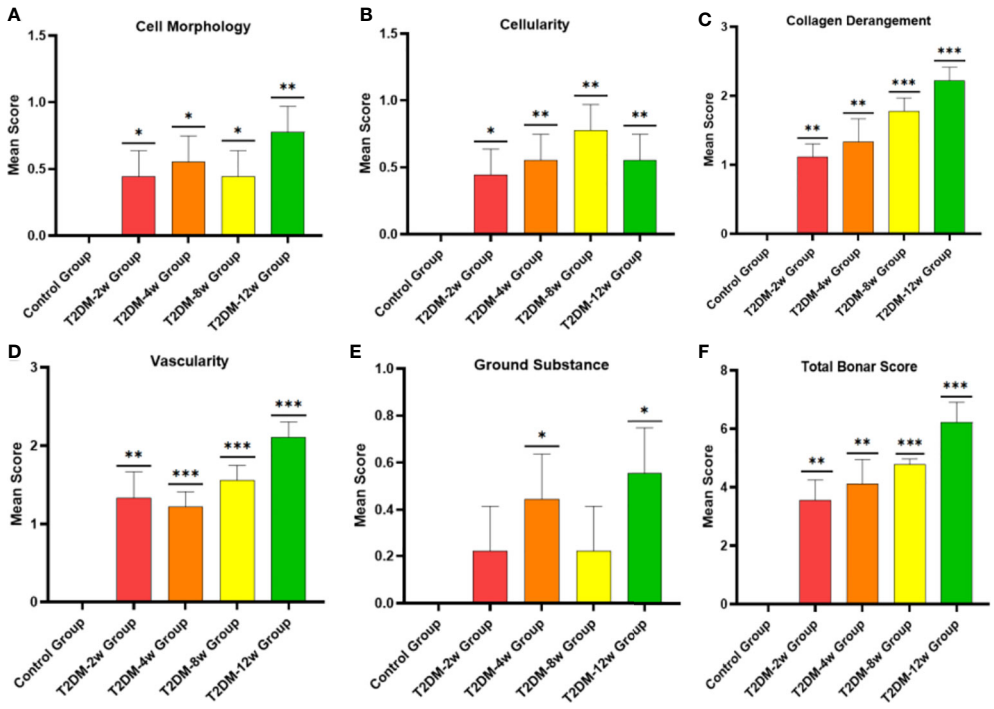


FIGURE 6
Histological specimens were scored using a modified Bonar scoring scheme. **(A-F)**, semiquantitative histological scores showed persistent tendinopathy changes in the rotator cuff tendon due to type 2 diabetes. *p < 0.05 versus Control group. **p < 0.01 versus Control group. ***p 0.001 versus Control group. The values are presented as means, with the error bars depicting the standard deviation.

diabetic rats, which may lead to altered vascularization (20). Our results are similar to previous studies in that we found more neovascularization within collagen fibers in the supraspinatus tendon of the rotator cuff as the course of the disease progressed.

In the current study, gene expression analysis confirmed that COL1A1 and TNC in the supraspinatus tendon of the rotator cuff in type 2 diabetic rats continued to increase in the first four weeks, after which they showed a trend of continuously decreased expression, indicating that there is a continuous remodeling process in the supraspinatus tendon in response to persistent microdamage caused by hyperglycemic conditions. The main component of tendon ECM is type I collagen, which is sensitive to the process of glucose oxidation, while the accumulation of glycogen during glucose oxidation further alters the quality of tendon ECM (28, 29). In addition, severe glycation of type I collagen and other matrix proteins occurs in the tendons of diabetic patients (30), further leading to reduced ECM remodeling (31). In a similar study, Lin et al. (32) found that expression of collagen I (Col1) and tenocyte markers were reduced in cultured tenocytes under high glucose for 48 hours.

Aberrant matrix remodeling is an important part of the pathology leading to the development and progression of tendinopathy. Immunohistochemical results showed that increased MMP-3 expression means dysregulated extracellular matrix turnover and remodeling and increased collagen degradation, both of which are direct evidence of extracellular matrix degradation and disturbance. In their study, Ueda et al. (33) and Tsai et al. (34) found that culturing rat Achilles tendons in high glucose for 72 hours caused increased expression of matrix metalloproteinases and the pro-inflammatory cytokine interleukin-6. In our study, diabetes increased the body's inflammatory response with the progression of the disease. We also detected higher expression of AGEs and IL-6 in the serumof diabetic rats than non-diabetic rats. In addition, immunohistochemical results revealed that the expression of IL-6 levels in the supraspinatus tendon of the rotator cuff was increased in type 2 diabetic rats, and the expression of inflammatory factors, as the first manifestation of tendinopathy, may be related to the disturbance of the extracellular matrix. We also found that AGEs expression in serum was significantly higher in type 2 diabetic rats than in non-diabetic rats; however, we did not investigate AGEs expression in tendons. Previous studies revealed that accumulation of AGEs in collagen could lead to degradation of ECM (35) and matrix metalloproteinases (MMPs) expression levels (11), while expression of MMPs further leads to degradation of type 1 collagen (36), which is consistent with

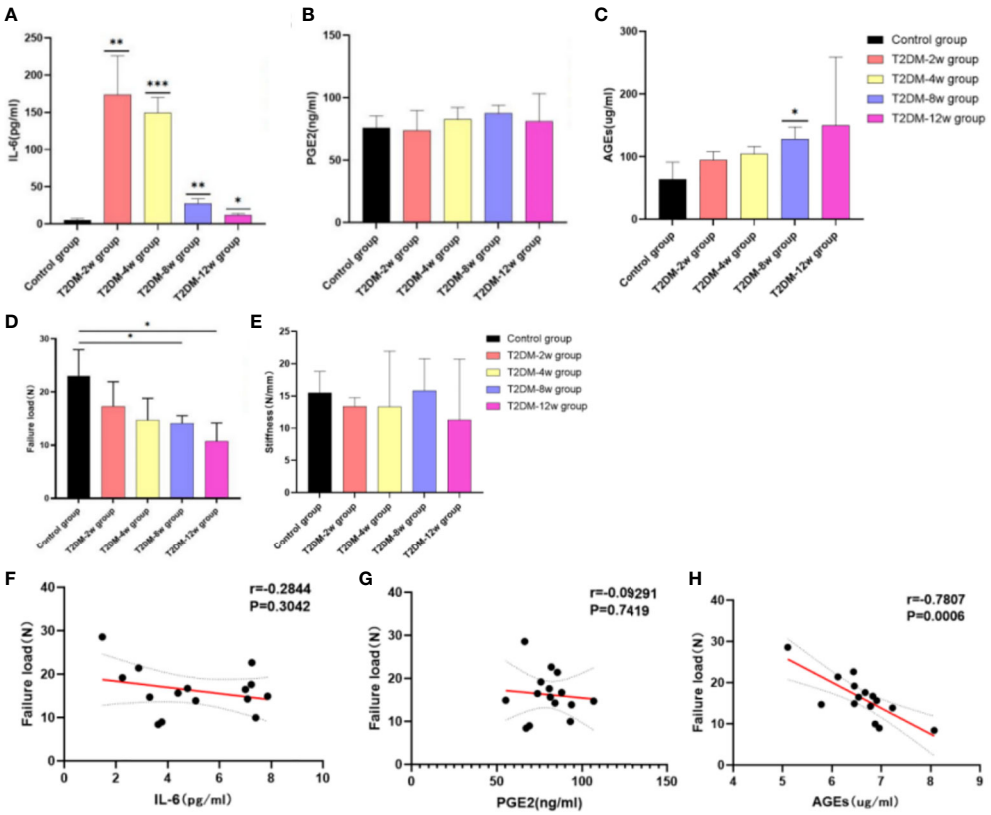


FIGURE 7
Results of ELISA and biomechanical testing. **(A)** Expression of IL-6. **(B)** PGE2 expression. **(C)** Expression of AGEs. **(D)** Failure load. **(E)** Stiffness. **(F)** Correlation analysis between IL-6 and Failure load. **(G)** Correlation analysis between PGE2 and Failure load. **(H)** Correlation analysis between AGEs and Failure load. Pearson correlation analysis was used to analyze the correlation between IL-6, PGE2, AGEs and Failure Load. *p < 0.05 versus Control group, **0.01 versus Control group. ***p < 0.001 versus Control group. The values are presented as means, with the error bars depicting the standard deviation.

our findings. Collectively, these studies suggest that a sustained hyperglycemic state may impair the homeostasis of tenocytes and extracellular matrix, thus further leading to high expression of pro-inflammatory and profibrotic mediators.

Biomechanical tests showed that the rotator cuff tendons of type 2 diabetic rats tended to decrease as the disease progressed, and the difference in ultimate load was statistically significant by week 8, which was the earliest time point at which biomechanical differences were observed. Previous studies have reported conflicting conclusions regarding the effect of diabetes on tendon mechanics. The intact rotator cuff in diabetic rats was biomechanically worse compared with normal rats (6). Another study reported the opposite conclusion, i.e., that hyperglycemia alone does not reduce the mechanical properties of the shoulder (5); nevertheless, our findings were more in line with the former study, and collagen fiber tear and disturbance of the extracellular matrix may be important causes of biomechanical deterioration. Previous studies have investigated and interpreted the decline in biomechanics, with a linear increase in intermolecular distance in glycated tendons (28), reporting that abnormal cross-linking of collagen fibers may occupy space in the ECM, thus affecting biomechanical properties (37). In addition, the high-glucose microenvironment leads to abnormal cross-linking and disarrangement of collagen fibers, resulting in biomechanical decline (38), and these structural abnormalities may be attributed to the deposition of AGEs (39). This conclusion was further confirmed by our correlation analysis between AGEs and ultimate load, where the deposition of AGEs may be one of the factors leading to the biomechanical decline of tendon tissue. In addition, we investigated the repair of scar tissue after supraspinatus tendon injury in type 2 diabetic rats. By immunohistochemical staining, Biglycan protein expression was significantly increased in the rotator cuff tendon of diabetic rats, indicating that the scar tissue reaction was enhanced in the supraspinatus tendon of type 2 diabetic rats. This would affect the reconstruction of tendon mechanical structure and performance recovery and could be related to the increased risk of tendon rupture and decreased biomechanical properties.

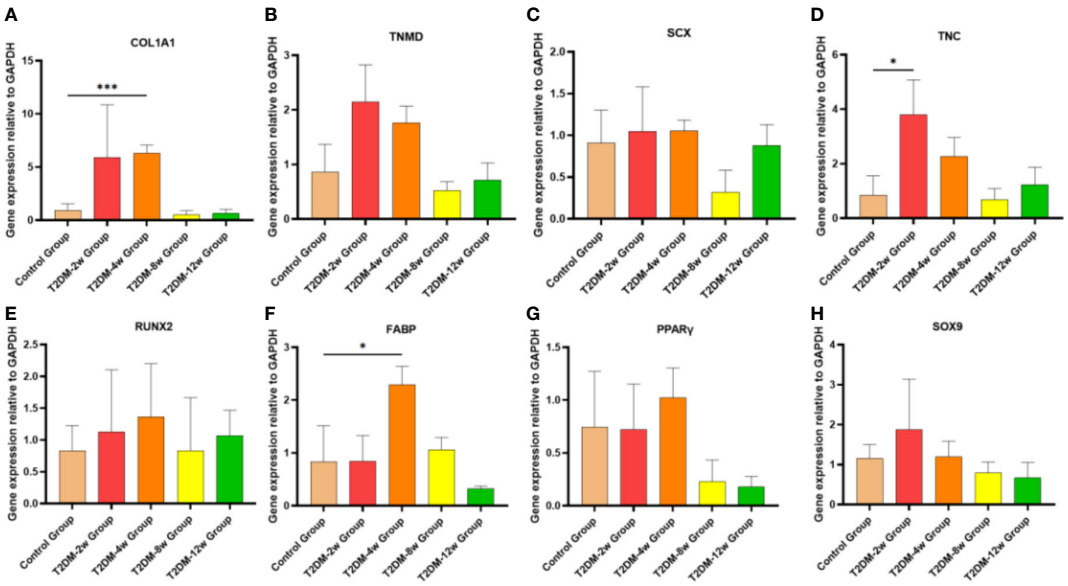


FIGURE 8
Type 2 diabetes leads to persistent alterations in gene expression in tendon tissue. **(A–H)**. qRT-PCR analysis of suspraspinatus tendon associated gene expression (COl1A1, TNMD, SCX, TNC, RUNX2, FABP, PPARγ and SOX9) in diabetic and nondiabetic subjects. COL1A1, collagen1A1;TNMD, tenomodulin; SCX,scleraxis;TNC:Tenascin C; RUNX2, runt-related transcription factor 2; FABP4, fatty acid binding protein 4; PPARγ, peroxisome proliferator-activated receptor gamma. Error bars depict standard error. *p < 0.05 versus Control group. ***p < 0.001 versus Control group. The values are presented as means, with the error bars depicting the standard deviation.

The current study has several limitations. First, the establishment of a rat model of type 2 diabetes cannot completely simulate the pathological state of the human rotator cuff tendon. Nonetheless, this model mimics the natural process of rotator cuff tendinopathy caused by type 2 diabetes to a certain extent, In particular, the tissue microenvironment continues to change at different stages, thus having great value for further study of the causes of rotator cuff tendon injury and the molecular mechanism of healing. Second, we investigated changes in rotator cuff tendon structure and homeostasis throughout the disease, while the molecular mechanisms involved were not explored. Finally, there was variability between samples and variation between individuals, and future studies with bigger sample sizes are needed.

Conclusion

As type 2 diabetes progressed, the development of rotator cuff tendinopathy was demonstrated in a rat model. Moreover, we found persistent histological, biomechanical, and gene expression changes. This model provides a valuable research basis for further evaluation of the underlying cellular and molecular mechanisms of rotator cuff tendinopathy caused by type 2 diabetes.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Ethics Committee for Laboratory Animal Welfare of Qingdao University.

Author contributions

Authors KSX, XZ, and TBY designed the study; LZ and ZKR analyzed the data; KSX and TRW wrote the manuscript; YZZ and TBY supervised the study. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The comparative short-term efficacy and safety of drug-coated balloon vs. drug-eluting stent for treating small-vessel coronary artery lesions in diabetic patients

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Purpose: This meta-analysis aimed to explore the comparative short-term efficacy and safety of drug-coated balloon (DCB) vs. drug-eluting stent (DES) for treating small-vessel coronary artery lesions in diabetic patients.

Methods: We searched PubMed, EMBASE, the Cochrane Library, and China National Knowledgement Infrastructure (CNKI) for retrieving relevant studies regarding the comparison of DCB with DES in treating small-vessel coronary artery lesions in diabetic patients until May 31, 2022. Two independent authors screened study, extracted data, and assessed methodological quality. Then, the meta-analysis was conducted using RevMan software, version 5.4.

Results: We included 6 studies with 847 patients in this meta-analysis. Pooled results showed that DCB was associated with fewer major adverse cardiac events (MACE) [RR, 0.60; 95% confidence interval (CI), 0.39–0.93; $p = 0.02$], myocardial infarction (MI) (RR, 0.42; 95% CI, 0.19–0.94; $p = 0.03$), target lesion revascularization (TLR) (RR, 0.24; 95% CI, 0.08–0.69; $p < 0.001$), target vessel revascularization (TVR) (RR, 0.33; 95% CI, 0.18–0.63; $p < 0.001$), binary restenosis (RR, 0.27; 95% CI, 0.11–0.68; $p = 0.005$), and late lumen loss (LLL) [mean difference (MD), −0.31; 95% CI, −0.36 to −0.27; $p < 0.001$], but was comparable technique success rate, death, minimal lumen diameter (MLD), and net lumen gain (NLG) to DES. There was no difference in long-term outcomes between these two techniques.

Conclusions: This meta-analysis shows that DCB is better than DES in the short-term therapeutic efficacy and safety of small-vessel coronary artery lesions in diabetic patients. However, more studies are required to validate our findings and investigate the long-term effects and safety of DCB.

KEYWORDS

small-vessel coronary artery, diabetes mellitus, drug-coated balloon, drug-eluting stent, meta-analysis

Introduction

Patients who received percutaneous coronary interventions (PCI) usually present small-vessel coronary artery lesions, reporting an incidence of about 40% (1). Although significant advancements in therapeutic techniques, it remains challenging to treat small vessel coronary artery lesions resulting from a higher risk of technical failure, restenosis, and need for repeated revascularization (2, 3). Compared with non-diabetic patients, patients with diabetes mellitus suffered from worse clinical outcomes (e.g., binary restenosis and myocardial infarction) after PCI (4–7) owing to more challenging coronary anatomies (8–10), such as diffuse atherosclerotic plaques and higher frequency of thin-cap fibroatheroma and fibrocalcific atheroma (11).

The drug-eluting stent (DES) remains the cornerstone treatment for small-vessel coronary artery lesions (12) by reducing angiographic and clinical restenosis (13, 14). However, the presence of diabetes mellites significantly increases the risk of adverse outcomes as a significant predictor (15–18) because more stents of longer lengths and smaller diameters were usually required for PCI in diabetic patients (19). Therefore, the need to develop newer devices as alternatives to DES has been emphasized. As a result, drug-coated balloons (DCB) have attracted physicians' attention as a promising therapeutic modality for *de novo* lesions and small-vessel coronary artery lesions because they can deliver the antiproliferative drugs directly into the artery wall without the need for implanting metallic stents in the artery vessels (20).

Currently, several clinical trials and meta-analyses have evaluated the therapeutic role of DCB in treating small-vessel coronary artery lesions, indicating that the therapeutic efficacy and safety of DCB were not inferior to DES (21–25). However, only the meta-analysis by Razzack et al. (23) attempted to evaluate the therapeutic value of DCB in diabetic patients by introducing a subgroup analysis. Notably, this subgroup analysis involved only 3 eligible studies, which provided limited data to investigate the difference between DCB and DES in the treatment of small vessel coronary artery disease in diabetic patients. Meanwhile, most studies were underpowered to evaluate the differences between the DCB and DES in therapeutic efficacy and safety due to limited sample size (26–29). Therefore, we conducted this meta-analysis to investigate the comparative short-term therapeutic efficacy and safety of DCB vs. DES in diabetic patients with small-vessel coronary artery lesions.

Abbreviations: PCI, percutaneous coronary interventions; DCB, drug-coated balloons; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; MACE, major adverse cardiac events; MLD, minimal lumen diameter; LLL, late lumen loss; NLG, net lumen gain; MI, myocardial infarction; TLR, target lesion revascularization; TVR, target vessel revascularization.

Methods

We first designed this meta-analysis's methodological framework, referring to the Cochrane handbook for systematic reviewers (30). Finally, we reported the meta-analysis's results according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist (31). The present study did not require institutional review or patient's informed consent because it was a meta-analysis of published data. However, we must point out that the formal protocol of this meta-analysis was not registered on a public platform.

Search strategy

We systematically searched relevant studies on PubMed, EMBASE, and the Cochrane Library from their establishment date until May 31, 2022. We used the following major terms and their analogs to develop the basic search strategy, including "Coronary," "diabetes," "drug-eluting stent," and "drug-eluting balloon." We modified the basic search strategy to meet the requirements of each database. The detailed search strategy of each target database is summarized in Supplementary Table S1. In addition, we also checked the reference lists of studies included in this meta-analysis to identify those missing from the electronic literature search.

Selection criteria

Two independent authors conducted the study selection by screening the titles, abstracts, and full texts of all retrieved studies according to the selection criteria were as follows: (1) Diabetic patients with small-vessel coronary artery lesions were treated with DCB or DES; (2) Studies reported at least one of the major adverse cardiac events (MACE) outcome, technique success rate, binary restenosis, minimal lumen diameter (MLD), late lumen loss (LLL), and net lumen gain (NLG); and (3) studies were published in English and Chinese, with full texts. We excluded ineligible studies following the exclusion criteria: (1) ineligible study designs, including case reports, experimental studies, reviews, and letters; (2) repeated publications of the same study; (3) essential data were not available after contacting the leading authors.

Definition of outcomes

We defined the MACE as the primary endpoint, which was a composite outcome involving myocardial infarction (MI), target lesion revascularization (TLR), target vessel revascularization (TVR), and death (32). In addition, we defined technique success rate, binary restenosis, MLD, LLL, and NLG as the secondary endpoints. TLR and TVR are treated as two separate outcomes

in this meta-analysis; however, TLR is part of TVR. Specifically, TLR was defined as repeated PCI treatment within the target lesion stent or edge 5 mm, but TVR was defined as PCI in the target lesion coronary vessel outside the stent (33). All outcomes were reported within 12 months after treatment, which were used to reveal short-term therapeutic efficacy and safety.

Data extraction

Two independent authors conducted data extraction using the pre-designed standard information extraction sheet. The following data were extracted from all studies included in this meta-analysis, including the first author's name, publication year, country, study duration, study design, details of comparisons, sample size with the proportion of male patients, patients' mean age, basic reference vessel diameter (RVD), lesion length, diameter stenosis, the number of patients identified with American Heart Association (AHA) type B2/C lesion, and the information on the risk of bias. We calculated the transformed standard deviation (SD) based on the recognized formula (34) when the eligible study reported results as the interquartile range (IQR).

Methodological quality assessment

Two authors assessed the methodological quality of all retrieved studies using the Cochrane risk of bias assessment tool version 2.0 (RoB 2.0) (35). Specifically, the RoB 2.0 quantified the overall methodological quality of a study from five areas, including "randomization process," "deviations from the intended interventions," "missing outcome data," "measurement of the outcome," and "selection of the reported result." Using the RoB 2.0 tool, the overall methodological quality of one study was labeled with "low," "high," or "some concerns." The results of the risk of bias assessment were graphically presented using the "robvis" command (36).

Data analysis

For dichotomous variables, including the MACE outcome including MI, TLR, TVR, and death, technique success rate, and binary restenosis, relative risk (RR) with a 95% confidence interval (CI) was used to express the pooled estimate; however, for continuous variables, including MLD, LLL, and NLG, mean difference (MD) with a 95% CI was used to express the pooled estimate (37). We first tested the level of the statistical heterogeneity across studies using the Cochrane Q test (38) and I^2 statistic (39). Significant heterogeneity was considered if $p < 0.1$ and $I^2 \geq 50\%$ (40) and random-effects model was selected for meta-analysis. On the contrary, the fixed-effects model was selected for meta-analysis when $p > 0.1$ and $I^2 < 50\%$ (40). The publication bias examination was not conducted because only six eligible studies were included in this meta-analysis, which did not meet the criteria for constructing a funnel plot (41). Meta-analysis was conducted using RevMan software, version 5.4 (The Cochrane Collaboration, Copenhagen, Denmark) (42, 43).

Results

Study search

We retrieved 499 studies from the electronic literature search, and one additional study was identified from the reference list. Using the EndNote software, 77 duplicate studies were removed. After screening the titles and abstracts of 423 retaining studies, we excluded 402 ineligible studies. We accessed and screened the full texts of 21 studies, and 15 studies were excluded due to three reasons, including unrelated to the topic ($n = 11$), conference abstract without sufficient data ($n = 3$), and duplicate publication ($n = 1$). Finally, as shown in Figure 1, we included 6 studies (26–29, 44, 45) in this study for meta-analysis.

Characteristics of studies

All studies were randomized controlled trials (RCTs) and were published between 2017 and 2021. Three studies were conducted in China (27–29), two studies in Italy (26, 44), and one study in Germany (45). Three studies (26, 44, 45) were conducted in multiple centers; however, other three studies (27–29) were conducted in a single center. The sample size of individual study ranged from 70 to 252, with an accumulated number of 847. Among the 6 included studies, five studies (26, 27, 29, 44, 45) reported MACE outcome, all studies (26–29, 44, 45) reported technique success, two studies (26, 27) reported binary restenosis, four studies (26–29) reported MLD and LLL, and three studies (26, 27, 29) reported NLG. We can access the remaining basic information of all studies in Table 1.

Risk of bias assessment

One study was high risk in the randomization process, three studies were high risk in the deviations from intended interventions, two studies were high risk in the missing outcome data, and all studies were low or some concerns in the remaining two domains. Finally, the overall methodological quality was rated to be low to moderate. The results of the risk of bias assessment are depicted in Figure 2.

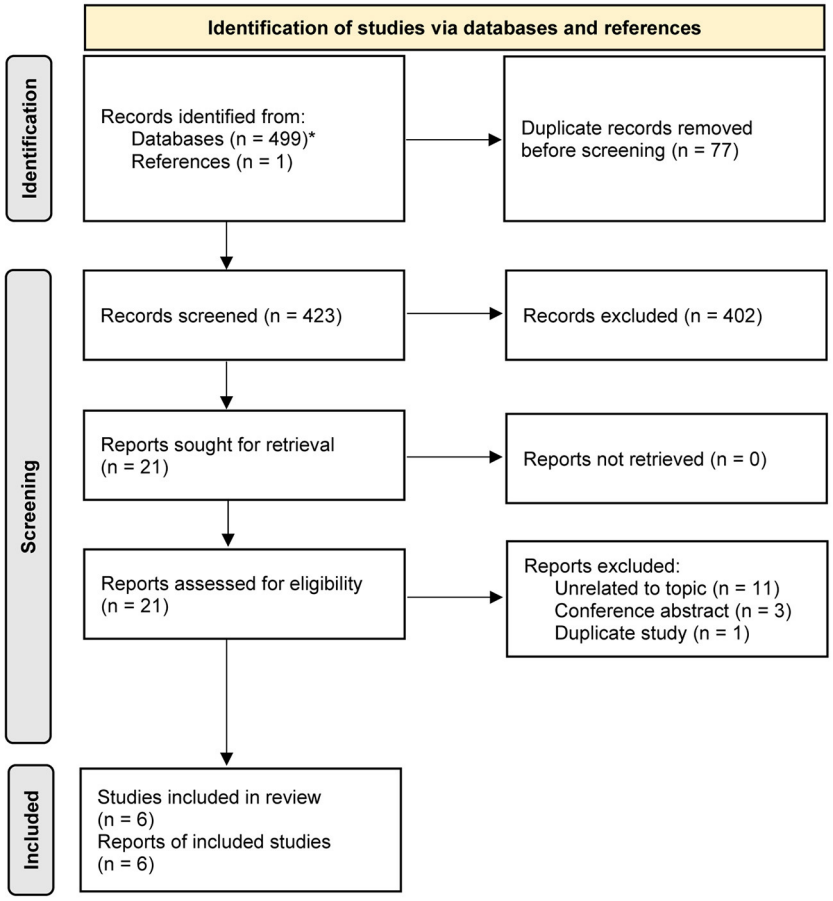


FIGURE 1
PRISMA flow chart of selecting study.

Meta-analysis of MACE outcome

Among the 6 studies included in this meta-analysis, five studies (26, 27, 29, 44, 45) reported the data on the MACE outcome. There was no significant statistical heterogeneity across studies ($p = 0.87$, $I^2 = 0\%$), so we selected a fixed-effects model for meta-analysis. The meta-analysis suggested that DCB was associated with a decreased risk of MACE outcome compared to DES (RR, 0.60; 95% CI, 0.39–0.93; $p = 0.02$; Figure 3).

Furthermore, we conducted a subgroup analysis to investigate the difference between DCB and DES in a single MACE outcome. There was no significant statistical heterogeneity across studies, so we selected a fixed-effects model for meta-analysis. The results of subgroup analysis suggested significant difference between the two techniques in MI (RR, 0.42; 95% CI, 0.19–0.94; $p = 0.03$), TLR (RR, 0.24; 95% CI, 0.08–0.69; $p = 0.008$), and TVR (RR, 0.33; 95% CI, 0.18–0.63; $p = 0.007$), but not in death (RR, 1.60; 95% CI, 0.71–3.59; $p = 0.26$).

In addition, one study (45) also reported the MACE outcome at the 3-years follow-up. However, as shown in Supplementary Figure S1, there was no statistical difference between the techniques regarding the MACE outcome (RR, 0.87; 95% CI, 0.52–1.45; $p = 0.59$) and the single MACE outcome, including MI (RR, 0.65; 95% CI, 0.27–1.60; $p = 0.35$), TVR (RR, 0.56; 95% CI, 0.26–1.19; $p = 0.13$), and death (RR, 1.27; 95% CI, 0.67–2.42; $p = 0.47$).

Meta-analysis of technique success and binary restenosis

All included studies (26–29, 44, 45) reported the data on the technique success rate, and there was no significant statistical heterogeneity across studies ($p = 0.17$, $I^2 = 36\%$). Therefore, we selected a fixed-effects model for meta-analysis, and the pooled result suggested a comparable technique success rate between the two techniques (RR, 1.01; 95% CI, 0.98–1.05; $p = 0.50$; Figure 4). Moreover, two studies (26, 27) reported

TABLE 1 Baseline information of 6 studies included in this meta-analysis.

References	Country	Design	Criteria of small vessel	Group	Sample (male%)	Mean age, years	RVD, mm	Lesion length, mm	Diameter stenosis, %	AHA type B2/C lesion, n	Follow-up duration
Giannini et al. (26)	Italy	Multicenter	RVD < 2.8 mm by visual estimation	PDEB	39 (82.05)	66.0	2.4 ± 0.4	15.3 ± 7.0	83 ± 10	25	12 months
				PES	35 (80.00)	70.0	2.5 ± 0.2	13.9 ± 5.0	84 ± 8	14	
Cortese et al. (44)	Italy	Multicenter	A vessel with a diameter between 2.00 and 2.75 mm with a target lesion ≥ 70%	PDEB	118 (70.34)	64.0	2.2 ± 0.4	13.5 ± 7.3	75 ± 17	n.r.	12 months
				EES	114 (76.32)	66.0	2.2 ± 0.4	14.0 ± 6.9	76 ± 15	n.r.	
Wöhrle et al. (45)	Germany	Multicenter	a small coronary vessel with a diameter between 2 and 3 mm	PDEB	122 (n.r.)	69.9	n.r.	n.r.	n.r.	n.r.	36 months
				EES	130 (n.r.)		n.r.	n.r.	n.r.	n.r.	
Zheng et al. (28)	China	Single-center	A vessel with a diameter between 2.25 and 2.80 mm with a target lesion ≥ 70%	PDEB	58 (75.86)	70.5	n.r.	n.r.	n.r.	n.r.	12 months
				PES	62 (77.42)	71.0	n.r.	n.r.	n.r.	n.r.	
Tang et al. (27)	China	Single-center	A vessel with a diameter < 2.80 mm with a target lesion ≥ 70%	PDEB	36 (69.44)	65.8	n.r.	n.r.	n.r.	n.r.	9 months
				PES	35 (65.71)	67.1	n.r.	n.r.	n.r.	n.r.	
Zhou et al. (29)	China	Single-center	A vessel with a diameter < 2.80 mm with a target lesion ≥ 70%	PDEB	50 (74.00)	61.9	n.r.	16.4 ± 5.5	n.r.	n.r.	12 months
				PES	48 (75.00)	62.4	n.r.	15.4 ± 5.8	n.r.	n.r.	

PDEB, paclitaxel drug-eluting balloon; PES, paclitaxel-eluting stent; EES, everolimus-eluting stent; RVD, reference vessel diameter; AHA, American Heart Association; DS, diameter stenosis; n.r., not reported.

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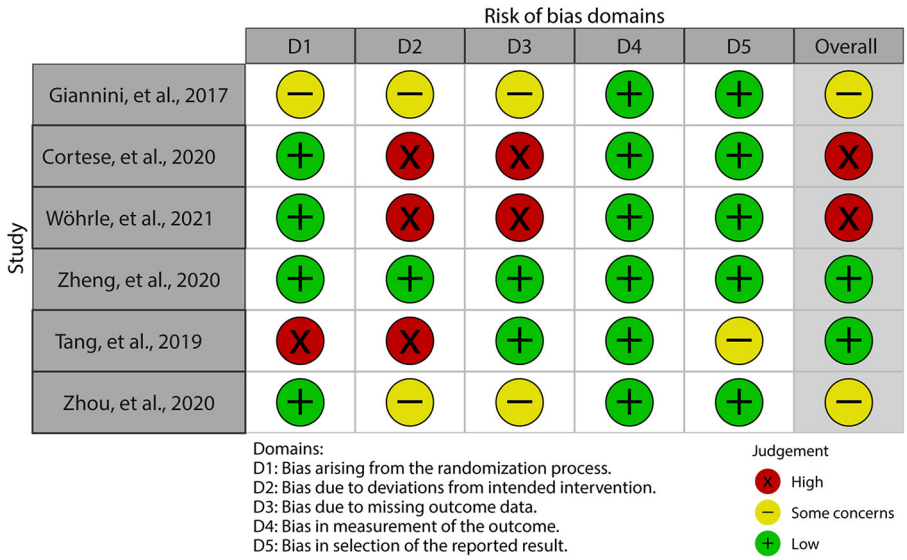


FIGURE 2
Risk of bias summary based on RoB 2.0. RoB, risk of bias.

the data on the binary restenosis. There was no significant statistical heterogeneity across studies ($p = 0.91$, $I^2 = 0\%$), so we selected a fixed-effects model for meta-analysis. The pooled result suggested that DCB was associated with a lower binary restenosis rate than DES (RR, 0.27; 95% CI, 0.11–0.68; $p = 0.005$; Figure 4).

Meta-analysis of MLD, LLL, and NLG

Four studies (26–29) reported the data on the MLD, and there was significant statistical heterogeneity across studies ($p = 0.07$, $I^2 = 57\%$). Therefore, we selected a random-effects model for meta-analysis, and the pooled result suggested no statistical difference between the two techniques in the MLD (MD, 0.05; 95% CI, −0.06 to 0.16; $p = 0.34$, Figure 5). The same four studies (26–29) also reported the data on the LLL, and there was no significant statistical heterogeneity across studies ($p = 0.56$, $I^2 = 0\%$). The result of the meta-analysis based on a fixed-effects model suggested that patients receiving DCB had fewer LLL than patients treated by DES (MD, −0.31; 95% CI, −0.36 to −0.27; $p < 0.001$; Figure 5). In addition, three studies (26, 27, 29) reported the data on the NLG. There was significant statistical heterogeneity across studies ($p < 0.1$, $I^2 = 86\%$), so we selected a random-effects model for meta-analysis. The pooled result suggested no statistical difference between the two techniques regarding the NLG (MD, −0.01; 95% CI, −0.30 to 0.29; $p = 0.95$; Figure 5).

Discussion

In the present meta-analysis, we systematically retrieved all relevant studies comparing DCB with DES in small-vessel coronary artery lesions among patients with diabetes mellitus. In the final data analysis, we included 6 low to moderate quality studies, accumulating a total of 847 patients. The pooled results showed that DCB was comparable to DES regarding technique success rate, MLD, and NLG; however, DCB had a lower risk in MACE outcome and binary restenosis. Subgroup analysis further indicated that DCB had a lower incidence in MI, TLR, and TVR than DES but comparable death to DES. The result from only one study suggested comparable MACE outcomes between the techniques at 3-years follow-up.

Interventional treatment of small-vessel coronary artery lesions is still challenging due to an increased risk of technical failure, restenosis, and the need for repeated revascularization (24), which is especially prominent in diabetic patients (11). DES remains the normative therapeutic strategy for PCI (46); however, implantation of DES will cause arterial wall injury to initiate vascular-proliferative cascade with smooth muscle cell proliferation and migration, resulting in neointimal hyperplasia (47). Compared to DES, DCB can deliver the antiproliferative drug into the vessel wall without the need for the implantation of metal struts, therefore directly inhibiting endothelial proliferation and adverse remodeling (20). From the theoretical perspective, the implantation of DCB will be superior to DES for treating small-vessel coronary artery lesions, which also interprets why the present meta-analysis found that DCB

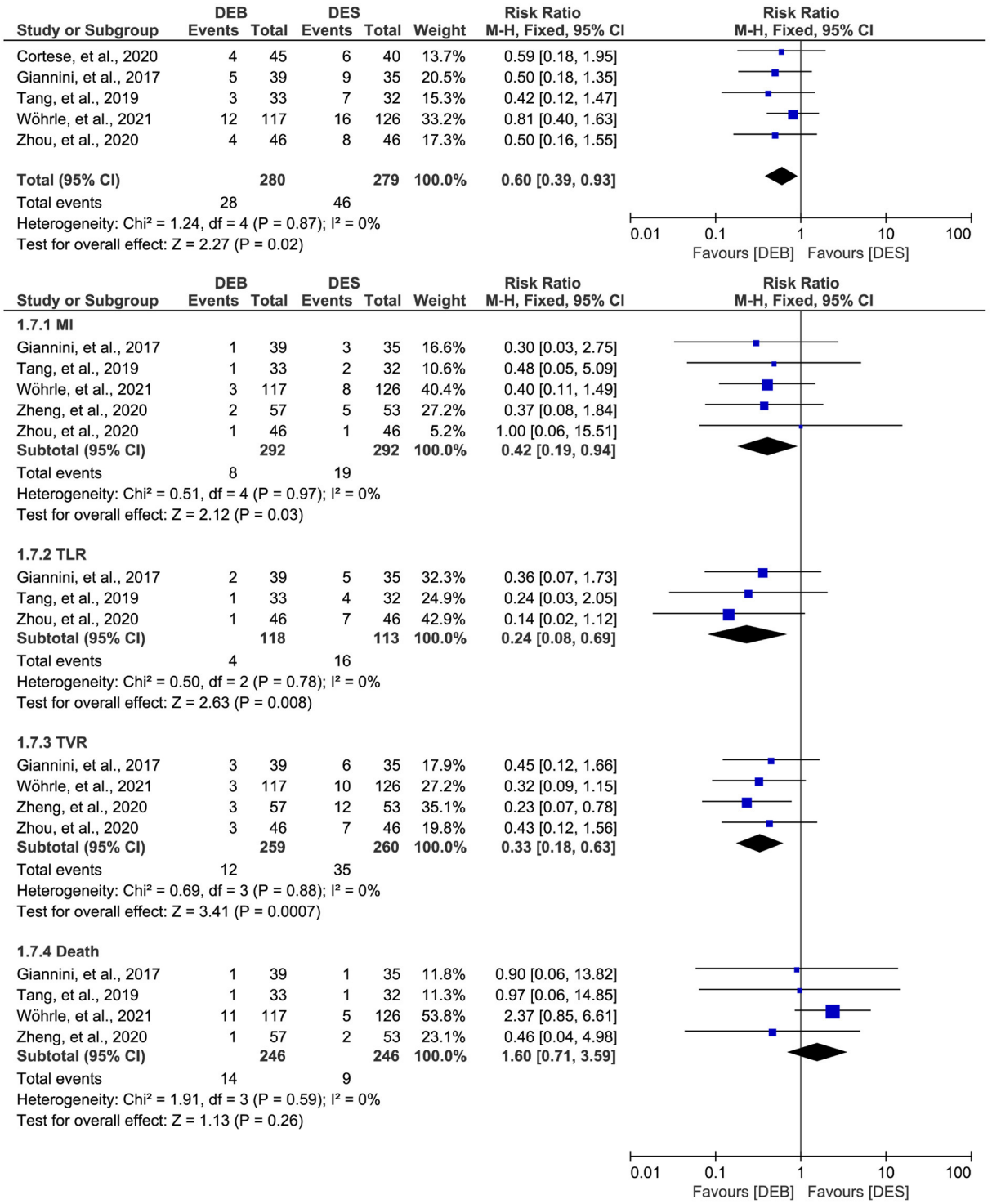


FIGURE 3
Meta-analysis of the MACE outcome. The black diamond represents the pooled result. If the black diamonds are completely to the left of the null line ("1"), it means that DEB is better than DES in terms of MACE results, MI, TLR, TVR, and death; if the black diamonds are completely to the right of the null line ("1"), it means that DEB is inferior to DES in terms of all outcomes; and if the black diamonds crossed through the null line ("1"), it means that DEB is comparable to DES in terms of all outcomes. MACE, major adverse cardiac events; MI, myocardial infarction; TLR, target lesion revascularization; TVR, target vessel revascularization; DCB, drug-eluting balloon; DES, drug-eluting stent; M-H, Mantel-Haenszel.

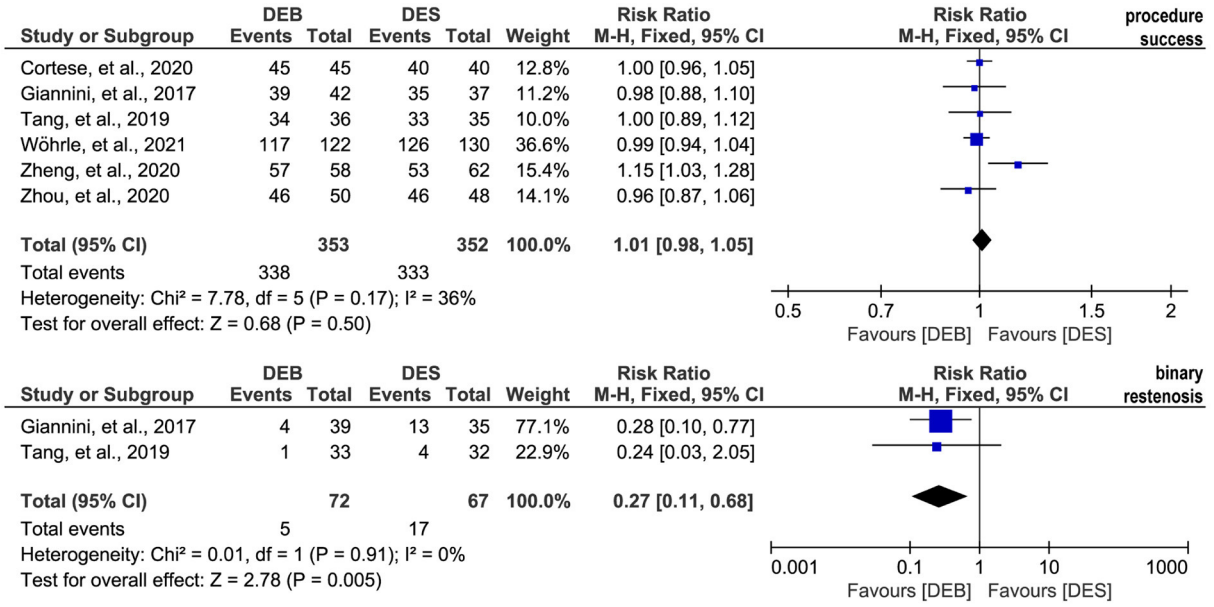


FIGURE 4
Meta-analysis of the procedure success rate and binary restenosis. The black diamond represents the pooled result. If the black diamonds are completely to the left of the null line ("1"), it means that DEB is better than DES in terms of the procedure success and binary restenosis; if the black diamonds are completely to the right of the null line ("1"), it means that DEB is inferior to DES in terms of all outcomes; and if the black diamonds crossed through the null line ("1"), it means that DEB is comparable to DES in terms of all outcomes. DCB, drug-eluting balloon; DES, drug-eluting stent; M-H, Mantel-Haenszel.

was associated with fewer binary restenosis, LLL, and single MACE outcome.

Currently, several studies (21–25) have investigated the comparative efficacy and safety of DCB vs. DES for treating *de novo* lesions in small-vessel coronary disease using the meta-analytic technique. However, the meta-analyses by Li et al. (21) did not isolate diabetic patients from general populations, although authors found that DCB was non-inferior to DES, delivering a good outcome in non-fatal MI, and can be recommended as an optimal treatment strategy in patients with *de novo* small-vessel coronary artery diseases. In addition, the meta-analysis by Elgendy et al. (22) assessed the differences in reducing TLR between DCB and DES in novo small-vessel coronary artery by introducing subgroup analysis; however, this meta-analysis did not also isolate diabetic patients form general populations. Another meta-analysis by Razzack et al. (23) included eight studies first to investigate the difference in therapeutic efficacy and safety between DCB and DES in treating *de novo* lesions in small-vessel coronary disease. Then, the authors evaluated the therapeutic value of DCB in diabetic patients by introducing a subgroup analysis involving 3 studies, indicating no statistical difference between DCB and DES regarding the MACE outcome [odds ratio (OR), 1.34; 95% CI, 0.73–2.46; $p = 0.34$], inconsistent with our finding.

In the present meta-analysis, we specifically evaluated the therapeutic efficacy and safety of DCB vs. DES for treating small-vessel coronary artery lesions in diabetic patients. The results of our meta-analysis provided more specific evidence-based information for practitioners dedicated to treating small coronary vessel lesions in diabetic patients compared with that meta-analyses reported by Li et al. (21) and Elgendy et al. (22). In addition, 6 eligible studies were included in our meta-analysis. Therefore, the statistical power of this meta-analysis was significantly higher than the meta-analysis by Razzack et al. (23), generating more reliable results. As a result, we can have the confidence to convince that DCB is associated with fewer MACE outcomes than DES in treating small-vessel coronary artery lesions in diabetic patients. More importantly, the present meta-analysis not only included the MACE outcome, but also considered other outcomes, including technique success rate, binary restenosis, MLD, LLL, and NLG, which benefited us to evaluate the therapeutic efficacy and safety of DCB more comprehensively for small-vessel coronary artery lesions in diabetic patients.

Although this meta-analysis included RCTs to enhance the reliability of the pooled results, we cannot ignore that it faced some limitations. First, although 6 eligible studies were included in the final analysis, not all studies reported all outcomes. Therefore, studies included for individual outcome remains limit, which may adversely impact the robustness of the pooled results. Second, the results of the risk of bias assessment suggested that the overall methodological quality of 6 included studies was low to moderate. Therefore, we cannot eliminate the negative impact of low methodological quality on the

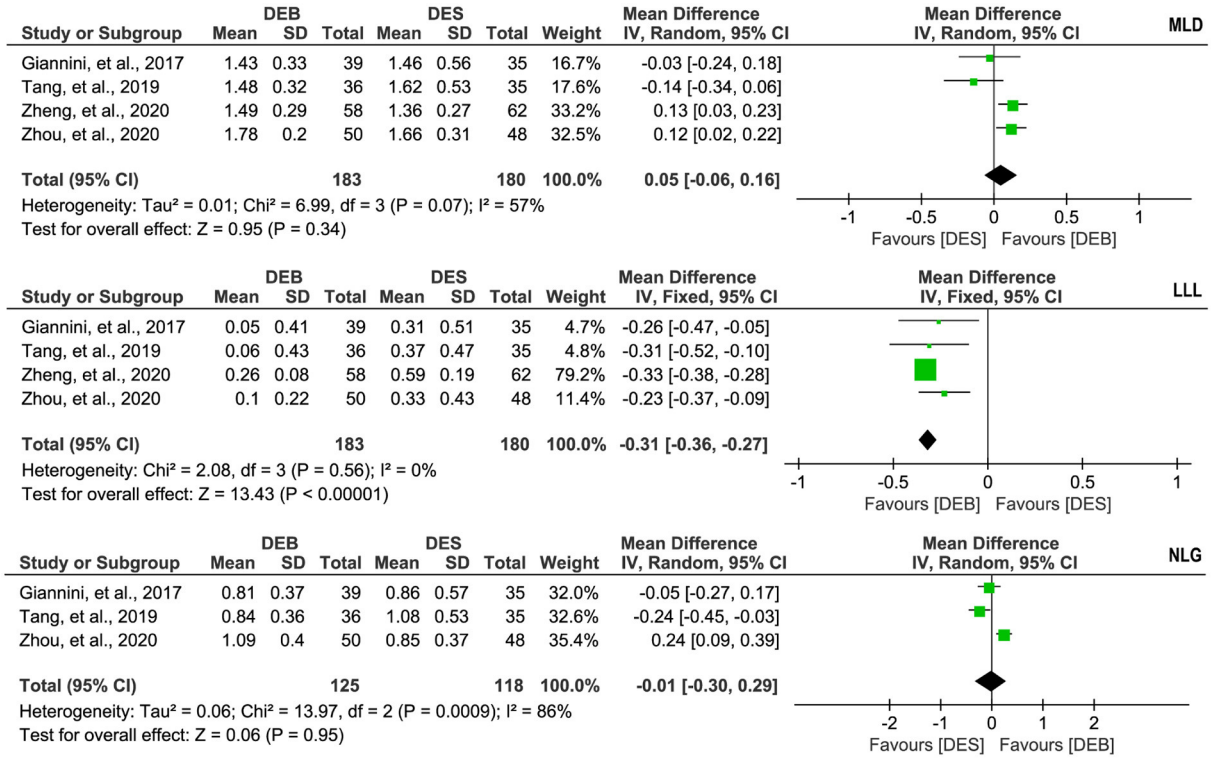


FIGURE 5
Meta-analysis of MLD, LLL, and NLG. The black diamond represents the pooled result. If the black diamonds are completely to the left of the null line ("0"), it means that DEB is better than DES in terms of MLD, LLL, and NLG; if the black diamonds are completely to the right of the null line ("0"), it means that DEB is inferior to DES in terms of all outcomes; and if the black diamonds crossed through the null line ("0"), it means that DEB is comparable to DES in terms of all outcomes. MLD, minimal lumen diameter; LLL, late lumen loss; NLG, net lumen gain; DCB, drug-eluting balloon; DES, drug-eluting stent; IV, inverse variance; SD, standard deviation.

robustness of the pooled results. Third, we detected significant statistical heterogeneity for meta-analyses of some outcomes. However, we could not conduct a sensitivity analysis to test the robustness of the results due to limited studies. As a result, we should cautiously interpret the results with significant statistical heterogeneity. Fourth, only one study reported outcomes in the long-term follow-up; therefore, we could not adequately evaluate the differences in long-term therapeutic efficacy and safety between the two techniques. Fifth, we could not assess the potential differences in treatment effect among different type of DCB because limited data are available. Sixth, our finding should be interpreted with caution because the criteria of small vessel and the follow-up duration varied slightly between included studies (as shown in Table 1). Finally, the formal protocol of this meta-analysis was not registered publicly although we conducted it in strict accordance with the process of a meta-analysis.

In conclusion, the present meta-analysis suggested that DCB is better than DES in the short-term therapeutic efficacy and safety of small-vessel coronary artery lesions in diabetic patients because DCB can significantly decrease the LLL and reduce the risk of binary restenosis, and it is also associated with fewer risk of MI, TLR, and TVR. However, all findings of this meta-analysis are generated from studies with low to moderate quality. Meanwhile, only one study evaluates the long-term therapeutic efficacy and safety. Therefore, more multi-center, large-scale, and high-quality studies are needed to validate our findings and investigate the difference between the two techniques in the long-term outcomes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

KL: conceptualization, formal analysis, and writing original draft preparation. KL and KC: methodology and validation. KC: resources and project administration. JF and XP: data curation. KL, JF, and XP: writing review and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.1036766/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Meta-analysis of the MACE outcome at 3-years follow-up. The black diamond represents the pooled result. If the black diamonds are completely to the left of the null line ("1"), it means that DEB is better than DES in terms of MACE outcome; if the black diamonds are completely to the right of the null line ("1"), it means that DEB is inferior to DES in terms of MACE outcome; and if the black diamonds crossed through the null line ("1"), it means that DEB is comparable to DES in terms of MACE outcome. MACE, major adverse cardiac events; MI, myocardial infarction; TVR, target vessel revascularization; DCB, drug-eluting balloon; DES, drug-eluting stent; M-H, Mantel-Haenszel.

SUPPLEMENTARY TABLE S1
Detailed search strategies of target databases.

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Tear function in patients with diabetes mellitus: A systematic review and meta-analysis

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Purpose: To examine tear function in patients with diabetes mellitus (DM).

Design: Systematic review and meta-analysis.

Method: We searched Embase and PubMed from database inception to March 16, 2022. We included observational studies that compared tear function between patients with and without DM. Tear function was measured using invasive tear breakup time (ITBUT) and Schirmer's 1 test. Pooled results are presented as standard mean difference (SMD) with 95% confidence interval (CI) based on random-effects models.

Results: We included 59 studies (7,234 eyes) comparing the tear function between patients with and without DM. This meta-analysis indicated that patients with DM had worse tear function than those without DM (ITBUT: SMD: −0.98, 95% CI: −1.27 to −0.69; Schirmer's 1 test: SMD: −0.45, 95% CI: −0.64 to −0.26), and the results remained consistent in patients with different types of DM (e.g., type 1 DM and type 2 DM) and from different ethnic backgrounds (e.g., Asian vs. non-Asian). Patients with DM under poor glycemic control had worse tear function than those of the non-DM group (ITBUT: SMD: −1.26, 95% CI: −1.86 to −0.66; Schirmer's 1 test: SMD: −0.25, 95% CI: −0.48 to −0.02), whereas there were no significant differences in tear function between patients with DM under optimal glycemic control and non-DM groups.

Conclusions: We found that patients with type 1 or type 2 DM had significantly reduced tear function. The level of tear function could be determined by glycemic control, and therefore, our findings suggest that glycemic control in patients with DM is critical for maintaining tear function.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero, identifier CRD42021250498.

KEYWORDS

tear function, diabetes mellitus, dry eye, keratoconjunctivitis sicca, glycemic control

Introduction

Diabetes mellitus (DM), a leading public health issue, affects more than 240 million people worldwide, and this number is expected to reach 370 million by 2030 (1). In addition to vascular complications, ocular complications of DM, such as dry eye disease (DED), diabetic retinopathy, glaucoma, and cataracts, negatively affect quality of life and may impose a huge economic burden (2). Among these ocular complications, DED occurs most frequently in patients with DM (3). For example, Seifart et al. reported that 52.8% of patients with DM suffered from DED compared with 9.3% in healthy controls (4).

Patients with DED often complain of a burning sensation, photopsia, foreign body sensation, soreness, itchiness, redness, and blurred vision. The corneal complications of DED include superficial punctate keratitis, neurotrophic keratopathy, and epithelial defects. In fact, both DM and DED are risk factors for corneal infection, scarring, perforation, and irreversible tissue damage (1). DM increases the risk of developing diabetic keratopathy, which presents as dry eye or recurrent erosions in the early or mild stage and neurotrophic ulcers with secondary infection in the advanced stage (5). In patients with DM, decreased lacrimal tear production results from being neurotrophic with loss of corneal sensation because of injury to the corneal receptors, which may further develop into a dry eye vicious cycle (6, 7).

A previous systematic review and meta-analysis by Lv et al. indicated that tear function is worse in patients with DM than in individuals without the disease, but we recommend that more detailed subgroup analyses should be considered to deal with the impacts of the clinical heterogeneity within the included studies, especially as regard different types of DM (8). For example, Kan et al. recently found no negative effects on tear function in patients with gestational DM (GDM) (9). This implies that different types of DM may cause varying pathophysiologies of DM-related DED. Furthermore, previous studies have reported that the corneal conditions in patients with DM may be determined by glycemic control, age, and ethnicity (10–12), but there is insufficient evidence to explore these factors in patients with DM-related DED.

In this study, we aimed to systematically examine the evidence on tear function in patients with DM; specifically, we evaluated tear function in these patients by conducting different subgroup analyses, including type of DM, age, ethnicity, and glycemic control status.

Methods

This systematic review and meta-analysis followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (Supplemental Table S2) (13). The study protocol has been registered on PROSPERO (CRD42021250498) (14).

Search strategy and study selection

We searched Embase and PubMed for relevant records from the inception of these databases to March 16, 2022. The search strategy is presented in Supplemental Table S3. We also examined reference lists from previously published material and included studies from the lists to obtain further eligible studies. After potential records were identified from the abovementioned databases, two investigators (YKK and ETL) independently screened the study titles and abstracts. The same investigators selected studies by reviewing the full text based on our inclusion and exclusion criteria. Any disagreement about the study selection was resolved through full discussions with the third investigator (CCS).

Eligibility criteria for study selection

Inclusion criteria for studies were as follows: (a) study groups included participants with DM, including type 1 DM, type 2 DM, GDM, and unclassified DM, and the control groups included participants without DM; (b) study outcome assessments used common tests to assess DED severity (15); and (c) study designs were cohort, case–control, or cross-sectional. We excluded studies in which (a) study or control groups focused on non-human participants; (b) study participants had Graves' disease, connective tissue disorders, chronic kidney disease, or other autoimmune diseases (as autoimmune diseases disturb lacrimal secretion and dialysis alters tear quality) (16, 17); (c) study participants had a medical history of corneal disease, glaucoma, contact lens wearing, current use of ocular medication, or previous intraocular surgery (as structural damage to the cornea and eye drops interrupt tear secretion); (d) the literature was gray (e.g., conference abstracts) without detailed information on participants' baseline characteristics, risk of bias evaluation, or results extraction; (e) data reports were duplicated (from the same source population); and (f) the language of publication was not English.

Study outcomes

We included two tests for DED severity as study outcomes (18). First, Schirmer's test is typically used to detect the amount of secretion of the aqueous layer of the tear film, and Schirmer's 1 test measures total tear secretion function without topical anesthesia. Second, tear breakup time is used to determine the

stability of the tear film, whereas invasive tear breakup time (ITBUT) is performed using strips soaked in fluorescein.

To evaluate differences in tear function within different subgroups of DM types, age, ethnicity, and DM control status, we further compared (1) tear function in the pertinent DM group with type 1 DM versus type 2 DM versus GDM versus unclassified DM; (2) mean participants' age as those <65 versus >65 years old; (3) participants' ethnicity as Asian versus non-Asian; and (4) mean glycosylated hemoglobin (HbA1C) levels as <7% versus >7%.

Data extraction and quality assessment

Two investigators (YKK and ETL) independently extracted data regarding the study region, inclusion period, trial design, subgroups, sample size, mean age, sex ratio (male/female), DM duration, and HbA1C levels from the included studies. The outcome data for the meta-analyses included Schirmer's 1 test and ITBUT. Because the outcomes of study interest were continuous data, we first extracted the mean and standard deviation (SD) from the included studies for the meta-analyses. If the included studies only reported the standard error (SE) or interquartile range (IQR), we calculated the SD using the formula $SE = SD/\sqrt{N}$ and IQR/1.35, respectively (19, 20). If the included studies only reported the maximum and minimum values, we calculated the SD using the formula reported by Hozo et al. (21). If the studies only presented the subgroup data, we pooled them together into one group for the final meta-analysis.

Two investigators (YKK and ETL) independently assessed the study quality using an adapted form of the Newcastle–Ottawa Quality Assessment Scale for observational studies (22). This scale includes three major domains (selection, comparability, and outcome), with a total of 10 points. We defined studies with 7–10, 5–6, and 0–4 points as good, moderate, and low study quality, respectively (22). Any disagreement about the study quality assessments was resolved through full discussion with the third investigator (CCS).

Statistical analysis

We conducted quantitative syntheses using meta-analysis to present the mean difference with a 95% confidence interval (CI) based on the random-effects model. We used Review Manager 5.4 software provided by the Cochrane Collaboration Network for the meta-analysis (23). We calculated the standard mean difference (SMD) to adjust for various measurement units from different measurement tools used among the included studies. We calculated I^2 values to measure statistical heterogeneity among the studies. Furthermore, we performed subgroup analyses to evaluate the differences in tear function in patients with different types of DM, age, ethnicity, and HbA1C levels. We considered absolute SMDs of <0.2, 0.2–0.5, and >0.8 as small, medium, and large differences in DED severity, respectively, between the DM and control groups (24). Results with two-sided $p < 0.05$ were considered to be statistically significant.

Results

We initially identified 466 records from Embase (n = 329), PubMed (n = 134), and three additional studies from the reference lists of previous literature. After applying our inclusion and exclusion criteria, we included 60 reports from 59 studies in this systematic review and meta-analysis (Figure 1). Specifically, the Zou et al. study presented two separate reports on adults (type 2 DM) and on children (either type 1 or 2 DM) (25). Hence, the meta-analysis included 60 reports from 59 studies.

Characteristics and quality of included studies

The 59 studies contributed 7,234 eyes of participants with and without DM from China (20 studies, 2,780 eyes) (25–44), Turkey (8 studies, 891 eyes) (45–52), the United States (4 studies, 272 eyes) (9, 53–55), Japan (5 studies, 722 eyes) (56–60), Brazil (4 studies, 373 eyes) (61–64), the United Kingdom (2 studies, 117 eyes) (65, 66), India (3 studies, 569 eyes) (67–69), Korea (2 studies, 330 eyes) (70, 71), and other countries (11 studies, 1,180 eyes) (71–81). There were 1,210 (16.7%) eyes in the type 1 DM group, 4,345 (60.0%) in the type 2 DM group, 1,597 (22.1%) in the unclassified DM group, and 82 (1.1%) in the GDM group. Participants' ages ranged from 10.1 ± 2.5 to 73.7 ± 5.7 years. The other study characteristics are listed in Supplemental Table S1.

Methodological quality of included studies

Details of the risk of bias assessment are presented in Supplemental Table S4. All studies were assessed as having a low risk of bias, except for the domains of comparability. In general, the quality of the included studies was good.

Main outcome

In this meta-analysis, 59 studies of DM evaluated severity of DED (Figures 2 and 3) (9, 25–82). Compared with the control group, we found that participants with DM had a lower ITBUT (41 studies, SMD: −0.98, 95% CI: −1.27 to −0.69, I^2: 95%) and

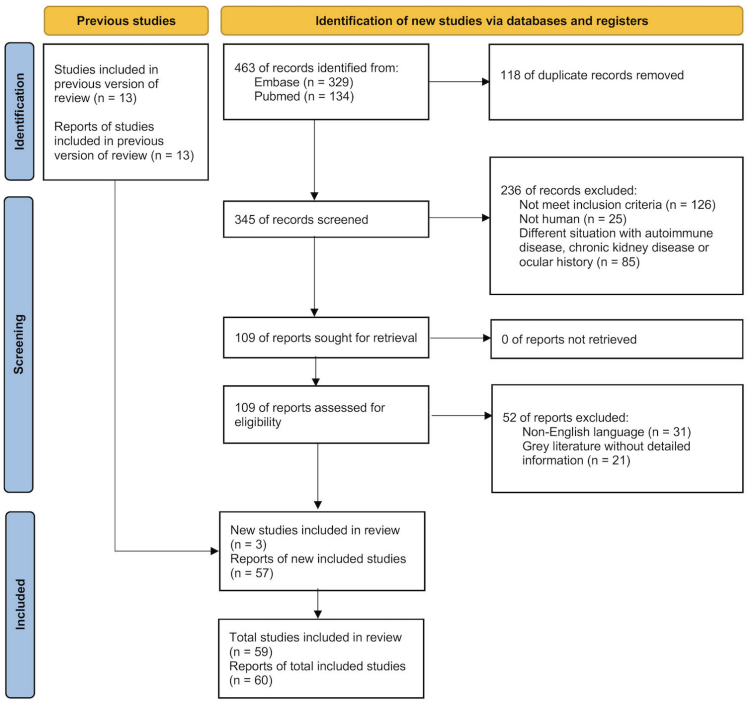
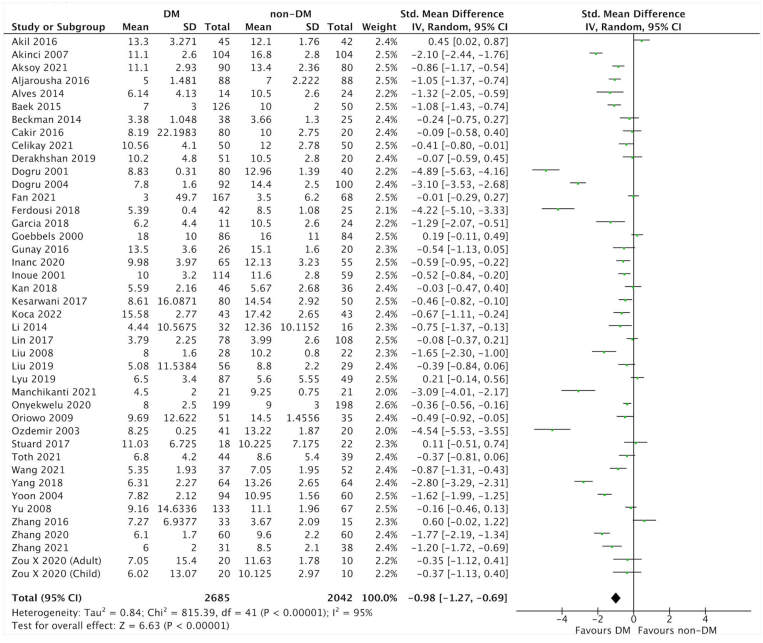


FIGURE 1
Flowchart of the systematic review with meta-analysis of the included studies.



Study or Subgroup	DM Mean	DM SD	DM Total	non-DM Mean	non-DM SD	non-DM Total	Weight	Std. Mean Difference IV, Random, 95% CI
Akil 2016	13.3	3.271	45	12.1	1.76	42	2.4%	0.45 [0.02, 0.87]
Akinci 2007	11.1	2.6	104	16.8	2.8	104	2.5%	−2.10 [−2.44, −1.76]
Aksoy 2021	11.1	2.93	90	13.4	2.36	80	2.5%	−0.86 [−1.17, −0.54]
Aljarousha 2016	5	1.481	88	7	2.222	88	2.5%	−1.05 [−1.37, −0.74]
Alves 2014	6.14	4.13	14	10.5	2.6	24	2.2%	−1.32 [−2.05, −0.59]
Baek 2015	7	3	126	10	2	50	2.5%	−1.08 [−1.43, −0.74]
Beckman 2014	3.38	1.048	38	3.66	1.3	25	2.4%	−0.24 [−0.75, 0.27]
Cakir 2016	8.19	22.1983	80	10	2.75	20	2.4%	−0.09 [−0.58, 0.40]
Celikay 2021	10.56	4.1	50	12	2.78	50	2.5%	−0.41 [−0.80, −0.01]
Derakhshan 2019	10.2	4.8	51	10.5	2.8	20	2.4%	−0.07 [−0.59, 0.45]
Dogru 2001	8.83	0.31	80	12.96	1.39	40	2.2%	−4.89 [−5.63, −4.16]
Dogru 2004	7.8	1.6	92	14.4	2.5	100	2.4%	−3.10 [−3.53, −2.68]
Fan 2021	3	49.7	167	3.5	6.2	68	2.5%	−0.01 [−0.29, 0.27]
Ferdousi 2018	5.39	0.4	42	8.5	1.08	25	2.1%	−4.22 [−5.10, −3.33]
Garcia 2018	6.2	4.4	11	10.5	2.6	24	2.2%	−1.29 [−2.07, −0.51]
Goebbels 2000	18	10	86	16	11	84	2.5%	0.19 [−0.11, 0.49]
Gunay 2016	13.5	3.6	26	15.1	1.6	20	2.3%	−0.54 [−1.13, 0.05]
Inanc 2020	9.98	3.97	65	12.13	3.23	55	2.5%	−0.59 [−0.95, −0.22]
Inoue 2001	10	3.2	114	11.6	2.8	59	2.5%	−0.52 [−0.84, −0.20]
Kan 2018	5.59	2.16	46	5.67	2.68	36	2.4%	−0.03 [−0.47, 0.40]
Kesarwani 2017	8.61	16.0871	80	14.54	2.92	50	2.5%	−0.46 [−0.82, −0.10]
Koca 2022	15.58	2.77	43	17.42	2.65	43	2.4%	−0.67 [−1.11, −0.24]
Li 2014	4.44	10.5675	32	12.36	10.1152	16	2.3%	−0.75 [−1.37, −0.13]
Lin 2017	3.79	2.25	78	3.99	2.6	108	2.5%	−0.08 [−0.37, 0.21]
Liu 2008	8	1.6	28	10.2	0.8	22	2.3%	−1.65 [−2.30, −1.00]
Liu 2019	5.08	11.5384	56	8.8	2.2	29	2.4%	−0.39 [−0.84, 0.06]
Lyu 2019	6.5	3.4	87	5.6	5.55	49	2.5%	0.21 [−0.14, 0.56]
Manchikanti 2021	4.5	2	21	9.25	0.75	21	2.0%	−3.09 [−4.01, −2.17]
Onyekwelu 2020	8	2.5	199	9	3	198	2.6%	−0.36 [−0.56, −0.16]
Oriowo 2009	9.69	12.622	51	14.5	1.4556	35	2.4%	−0.49 [−0.92, −0.05]
Ozdemir 2003	8.25	0.25	41	13.22	1.87	20	2.0%	−4.54 [−5.53, −3.55]
Stuard 2017	11.03	6.725	18	10.225	7.175	22	2.3%	0.11 [−0.51, 0.74]
Toth 2021	6.8	4.2	44	8.6	5.4	39	2.4%	−0.37 [−0.81, 0.06]
Wang 2021	5.35	1.93	37	7.05	1.95	52	2.4%	−0.87 [−1.31, −0.43]
Yang 2018	6.31	2.27	64	13.26	2.65	64	2.4%	−2.80 [−3.29, −2.31]
Yoon 2004	7.82	2.12	94	10.95	1.56	60	2.5%	−1.62 [−1.99, −1.25]
Yu 2008	9.16	14.6336	133	11.1	1.96	67	2.5%	−0.16 [−0.46, 0.13]
Zhang 2016	7.27	6.9377	33	3.67	2.09	15	2.3%	0.60 [−0.02, 1.22]
Zhang 2020	6.1	1.7	60	9.6	2.2	60	2.4%	−1.77 [−2.19, −1.34]
Zhang 2021	6	2	31	8.5	2.1	38	2.4%	−1.20 [−1.72, −0.69]
Zou X 2020 (Adult)	7.05	15.4	20	11.63	1.78	10	2.2%	−0.35 [−1.12, 0.41]
Zou X 2020 (Child)	6.02	13.07	20	10.125	2.97	10	2.2%	−0.37 [−1.13, 0.40]
Total (95% CI)			**2685**			**2042**	**100.0%**	**−0.98 [−1.27, −0.69]**

Heterogeneity: Tau² = 0.84; Chi² = 815.39, df = 41 (P < 0.00001); I² = 95%
Test for overall effect: Z = 6.63 (P < 0.00001)

FIGURE 2
Comparison of the severity of dry eye disease (DED) between diabetes mellitus (DM) and non-DM based on invasive tear breakup time (ITBUT).

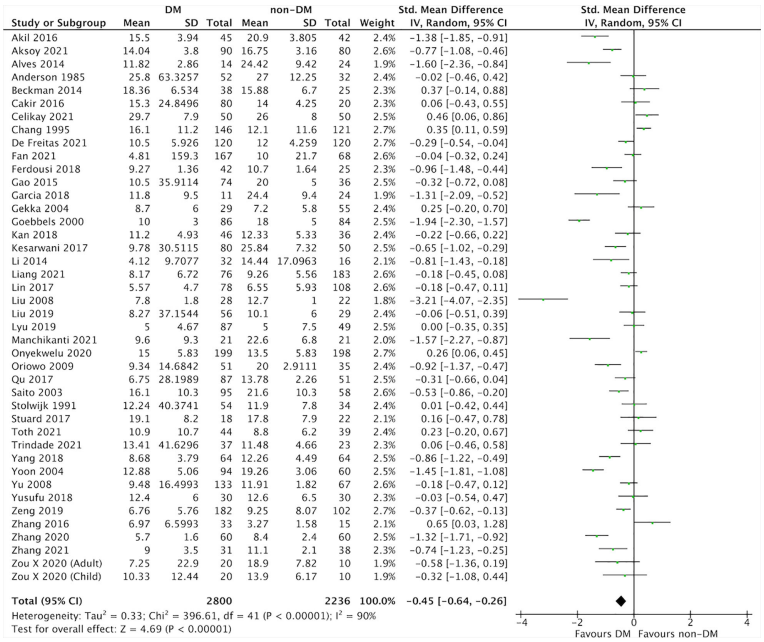


FIGURE 3
Comparison of the severity of dry eye disease (DED) between diabetes mellitus (DM) and non-DM based on Schirmer's 1 test.

Schirmer's 1 test result (41 studies, SMD: −0.45, 95% CI: −0.64 to −0.26, I^2: 90%).

Subgroup analysis stratified by different types of DM

This meta-analysis included 41 studies that evaluated ITBUT in relation to DM type (Supplemental Figure S1A) (9, 25, 28–32, 34, 35, 39–43, 45–57, 60, 62, 64, 65, 67–71, 73, 74, 79, 80, 82). Compared with the control group, we found lower ITBUTs in participants with type 1 DM (8 studies, SMD: −0.98, 95% CI: −1.70 to −0.26, I^2: 96%), type 2 DM (21 studies, SMD: −1.26, 95% CI: −1.76 to −0.76, I^2: 96%), and unclassified DM (12 studies, SMD: −0.59, 95% CI: −0.86 to −0.32, I^2: 77%). No statistical differences in ITBUT were found in participants with GDM (one study, SMD: −0.03, 95% CI: −0.47 to 0.40, I^2: not applicable).

There were 41 studies of DM where Schirmer's 1 test results were evaluated (Supplemental Figure S1B) (9, 25, 26, 28–35, 37–39, 41–44, 47, 48, 51, 53–55, 58, 59, 61–65, 67–69, 71–75, 77, 82). Compared with the control group, we found lower Schirmer's 1 test results in participants with type 1 DM (7 studies, SMD: −0.86, 95% CI: −1.39 to −0.33, I^2: 91%) and type 2 DM (25 studies, SMD: −0.41, 95% CI: −0.63 to −0.18, I^2: 88%). However, no statistical differences were found in participants with unclassified DM (nine studies, SMD: −0.24, 95% CI: −0.63 to 0.14, I^2: 86%) or GDM (one study, SMD: −0.22, 95% CI: −0.66 to 0.22, I^2: not applicable).

Subgroup analysis stratified by age

Supplemental Figure S2A presents the 35 studies where ITBUT was evaluated in relation to age (9, 25, 28–32, 34, 39–43, 45–54, 56, 57, 60, 65, 67–71, 79, 80, 82). Compared with the control group, we found younger participants had a lower ITBUT (30 studies, SMD: −1.19, 95% CI: −1.55 to −0.83, I^2: 96%), whereas no statistical differences in ITBUT were found among elderly participants (five studies, SMD: −0.27, 95% CI: −0.83 to 0.28, I^2: 89%).

The 36 studies of DM where Schirmer's 1 test results were evaluated in relation to age are shown in Supplemental Figure S2B (9, 25, 26, 28–34, 38, 39, 41–44, 47, 48, 51, 53, 54, 58, 59, 61, 63, 65, 67–69, 71–75, 77, 82). Compared with the control group, we found lower Schirmer's 1 test results in younger participants (29 studies, SMD: −0.51, 95% CI: −0.76 to −0.26, I^2: 92%) and elderly participants (7 studies, SMD: −0.23, 95% CI: −0.46 to −0.01, I^2: 67%).

Subgroup analysis stratified by ethnicity

There were 41 studies of DM that evaluated ITBUT together with ethnicity (Supplemental Figure S3A) (9, 25, 28–32, 34, 35, 39–43, 45, 47–57, 60, 62, 64, 65, 67–71, 73, 74, 79, 80, 82). Compared with the control group, we found a lower ITBUT in Asian (26 studies, SMD: −1.01, 95% CI: −1.35 to −0.66, I^2: 95%)

and non-Asian patients with DM (15 studies, SMD: −0.94, 95% CI: −1.49 to −0.39, I^2: 95%).

With regard to Schirmer's 1 test results, 41 studies included ethnicity (Supplemental Figure S3B) (9, 25, 26, 28–35, 37–39, 41–44, 47, 48, 51, 53–55, 58, 59, 61–65, 67–69, 71–75, 77, 82). Compared with the control group, we found lower Schirmer's 1 test results in Asian (26 studies, SMD: −0.47, 95% CI: −0.69 to −0.26, I^2: 89%) and non-Asian patients with DM (15 studies, SMD: −0.41, 95% CI: −0.80 to −0.02, I^2: 91%).

Subgroup analysis stratified by HbA1C levels

Twenty-one studies of DM investigated ITBUT in relation to HbA1C levels (Supplemental Figure S4A) (9, 30, 32, 34, 42, 45–51, 53, 54, 56, 60, 65, 69, 70, 80, 82). Compared with the control group, we found a lower ITBUT in participants with both poor control of DM (15 studies, SMD: −1.26, 95% CI: −1.86 to −0.66, I^2: 97%) and good control of DM (6 studies, SMD: −0.47, 95% CI: −0.87 to −0.07, I^2: 84%).

Twenty studies evaluated Schirmer's 1 test results with HbA1C levels (Supplemental Figure S4B) (9, 30, 32–34, 38, 42, 47, 48, 51, 53, 54, 58, 59, 61, 63, 65, 69, 75, 82). Compared with the control group, we found lower Schirmer's 1 test results in participants with poor control of DM (15 studies, SMD: −0.25, 95% CI: −0.48 to −0.02, I^2: 83%), but no statistical differences were found in participants with good control of DM (5 studies, SMD: −0.25, 95% CI: −0.72 to 0.22, I^2: 86%).

Discussion

Based on the meta-analyses of ITBUT and Schirmer's 1 tests, this study indicated that patients with DM presented with worse tear function than those without DM. More importantly, our findings could be the first summarized evidence on tear function within different DM subgroups. For example, unlike types 1 and 2 DM, we found patients with GDM had similar tear function to control groups. Moreover, patients with DM with good glycemic control had similar tear function to those without DM. However, tear function was similar in Asian and non-Asian patients with DM.

The influence of chronic hyperglycemia, such as in type 1 and type 2 DM, on DED has been elucidated by several mechanisms, including microvascular changes of the lacrimal gland, a reduced lipid layer in tear film composition, a high grade of conjunctival squamous metaplasia, an increased inflammatory process, and a low goblet cell density (83, 84). However, our subgroup analysis showed no significant difference between the tear function of patients with GDM and healthy pregnant women. A possible explanation could be the short duration of DM with a low degree of hyperglycemia in patients with GDM (9), so the clinical impacts from GDM on tear function may be relatively minor. Our finding may provide the fundamental evidence for further studies to confirm this proposed hypothesis.

Previous evidence regarding the role of ethnicity in tear function suggested that Asian populations were associated with higher risk of DED (12, 85, 86). However, in this systematic review and meta-analysis, we observed similar tear function in Asian and non-Asian patients with DM. Our findings may support the previous study from Butovich et al. indicating that minimal differences in meibogenesis and the process of lipid secretion from meibomian glands among different ethnicities were unlikely to differentially affect tear function between Asians and Caucasians (87).

Early studies reported age as a significant risk factor for decline of tear function, because it is associated with lacrimal gland atrophy with lymphocyte infiltration, eyelid laxity, and meibomian gland dysfunction (88–93). In this presented meta-analysis, we found elderly patients with DM may have better tear function than younger patients, contrary to previous reports. However, the impact of glycemic control on tear function in this subgroup analysis could not be ignored, because elderly patients usually have better glycemic control compared with younger patients (32, 42). Among five and seven included studies with elderly patients reporting ITBUT and Schirmer's 1 test in our meta-analysis, respectively, only two studies reported the mean baseline HbA1C levels. We found both included studies had a mean HbA1C of less than 7%, whereas there were no differences in ITBUT (two studies, SMD: −0.48, 95% CI: −1.87 to 0.90, I^2: 95%; Supplemental Figure S5A) and Schirmer's 1 test results between this population and the controls (two studies, SMD: −0.35, 95% CI: −1.07 to 0.37, I^2: 83%; Supplemental Figure S5B). Taking together all our results, we suggest that, under the optimal glycemic controls in elderly patients with DM could maintain the tear function as the control group. In addition, more studies on the tear function form elderly patients with inadequate glycemic controls should be determined.

Compared with previous systematic review with meta-analysis (8), this presented work included 46 more recent studies from China, Turkey, the United States, and other countries, which makes our findings more generalizable to clinical practice. However, some limitations should be noted before the interpretations of our study findings. First, we conducted various subgroup analyses (e.g., types of DM, age, ethnicity, and glycemic controls) with random-effects analyses to address the substantial clinical heterogeneity among the included studies. For example, some included studies were not based on well-matched designs to compare tear function between DM- and non-DM groups, so potential impacts from possible confounders could not be totally excluded. Second, result inconsistency among the studies were found, even after the subgroup stratifications with the random-effects analyses. Third, not every included study reported the mean with SD data

for our meta-analysis; however, using different published approaches, we were able to convert SE, IQR, or maximum and minimum values. Finally, because this study mainly focused on type 1 or type 2 DM, our findings may not apply to prediabetic patients whose tear function may be substantially different from type 1 or type 2 DM patients (94). Regularly updated meta-analyses with future studies are required to replicate our findings.

In conclusion, this systematic review and meta-analysis found that patients with type 1 or type 2 DM had worse tear function compared with the non-DM groups. The level of tear function could be determined by glycemic control. Our findings suggest that glycemic control in patients with DM is critical for maintaining tear function.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

Y-KK contributed to study planning, performed the systematic review search, and wrote the manuscript. S-CS performed study conception and meta-analysis and reviewed/edited the manuscript. E-TL contributed to study planning and implementation of supplemental analyses and reviewed/edited the manuscript. L-YP contributed to study planning and reviewed/edited the manuscript. LY contributed to study planning, wrote the methods, and reviewed/edited the manuscript. C-CS contributed to study conception and planning and reviewed/edited the manuscript. C-CS is the study guarantor. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1036002/full#supplementary-material

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Correlation of Baba's diabetic neuropathy classification with various diabetes-related complications

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It is known that Baba's diabetic neuropathy classification (BDC) is useful in quantitative evaluation of Diabetic polyneuropathy (DPN). In this study, we aimed to investigate the possible association between BDC and various diabetic microvascular and macrovascular complications in patients whose neuropathy was evaluated with BDC. As the results, BDC was significantly correlated with the severity of diabetic retinopathy and nephropathy. BDC was also significantly correlated with history of myocardial infarction or cerebral infarction, carotid IMT, and ABI. These data suggest that BDC may be useful in predicting the presence of various diabetic microvascular and macrovascular complications. The data also support the idea that we should perform further investigation of other diabetes-related complications in patients with severe DPN.

KEYWORDS

diabetes mellitus, diabetic polyneuropathy (DPN), complication, nerve conduction test, intima and media thickness

Introduction

Diabetic polyneuropathy (DPN), a microvascular complication of diabetes, usually occurs in a relatively early stage of diabetes compared to other microvascular complications (1). DPN is characterized by polyneuropathy and autonomic neuropathy, with negative (e.g., hypoesthesia) and/or positive (e.g., pain) symptoms

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that often reduce patients' quality of life (2) (3). The diagnosis is often delayed and clinically problematic in cases with only negative symptoms or mild impairment compared to those with positive symptoms. The golden standard in the diagnosis of diabetic polyneuropathy is nerve conduction study (NCS) (4). Baba's diabetic neuropathy classification (BDC) has been proposed to evaluate nerve conduction velocity in subjects with DPN and is useful for quantitative evaluation of neuropathy (5). Indeed, it was reported that cardiovascular prognosis was significantly worsen in patients with moderate to severe DPN assessed with this classification (6).

We use this method when diabetic patients are admitted to our hospital to accurately assess neuropathy, and although it has been suggested that DPN is associated with pathological progression of atherosclerotic lesions, there are few reports on the association between BDC and microvascular and macrovascular complications of diabetes mellitus. In this study, we aimed to investigate the possible association between BDC and various diabetic microvascular and macrovascular complications in patients whose neuropathy was evaluated with BDC.

Materials and methods

Study population and patient preparation

This was a single-center, retrospective, cross-sectional study of patients with type 2 diabetes. A total of 357 patients eligible for the present study who were admitted to our hospital for treatment of type 2 diabetes at Kawasaki Medical School Hospital from April 1st, 2018, to March 31st, 2021. The Institutional Review Board of Kawasaki Medical School (No. 5594-00) approved the study protocol, including opt-out informed consent. This study was conducted by the principles of the Declaration of Helsinki. The flow of participants in this study is shown in Figure 1. Among the 357 eligible adults with type 2 diabetes mellitus, 30 participants with malignancy and 6 participants who were taking corticosteroids and/or immunosuppressive drugs were excluded from the study. Subsequently, 33 participants whose nerve conduction velocity was not assessed and 91 participants who were regular alcohol drinkers were excluded. Finally, 197 type 2 diabetics whose nerve conduction velocity was assessed using BDC were included. The definition of BDC used in this study is shown in Figure 2: sensory nerve conduction velocity (SCV) and sensory nerve action potential (SNAP) in the sural nerve and motor nerve conduction velocity (MCV) and compound muscle action potential (CMAP) in the tibial nerve were evaluated (5, 6). Based on the results, the patients were grouped into BDC 0 (normal), BDC 1 (mild), BDC 2 (moderate), BDC 3 (moderate to severe) and BDC 4 (severe). BDC3 and BDC4 are both clinically severely neurologically dysfunctional conditions and were analyzed as the same group in this study. Consequently, 108 patients were grouped in BDC 0, 42 in BDC 1, 33 in BDC 2 and 14 in BDC 3-4.

Method of examination

Study participants' age, duration of diabetes, history of smoking, history of alcohol consumption, and current medications were obtained at the time of admission. Cases who consumed more than 20 g/day of ethanol three or more times per week were excluded from the study. Smokers were divided into current smoker, past smoker, and never smoker. All

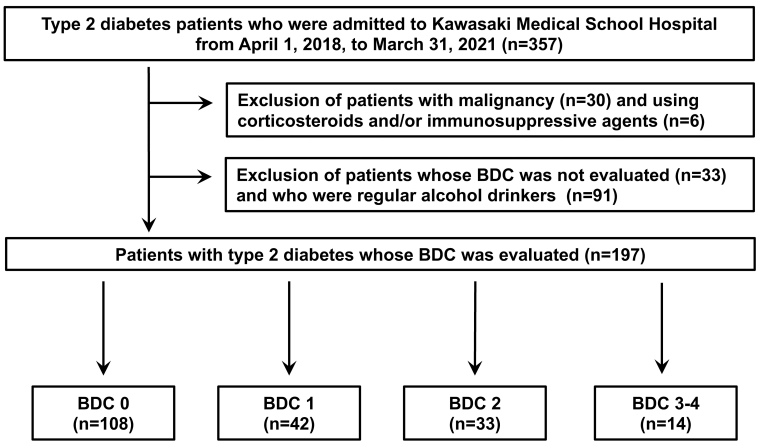


FIGURE 1
The flowchart of the participants and exclusions in this study.

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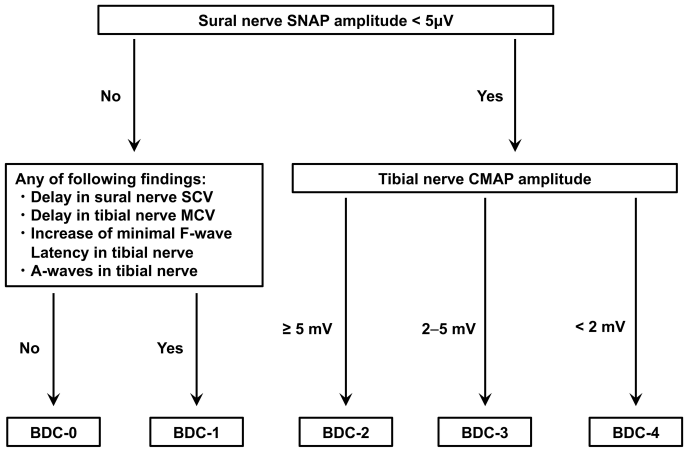


FIGURE 2
How to evaluate Baba's diabetic classification by nerve conduction studies.

patients were asked on admission by their physician whether they had an old cerebral infarction and an old myocardial infarction. Body weight, body mass index (BMI), abdominal circumference, grip strength, blood pressure, and pulse rate were measured upon admission. Blood glucose, HbA1c, lipids, and liver and kidney function data were assessed by fasting blood tests on the day following admission.

The following is a brief description of the complications of diabetes mellitus and how to test them. NCS was performed by skilled technicians using MEB-2306 or MEB-2312 (NIHON KOHDEN Corp., Tokyo). NCS was performed in the upper and lower extremities on one side and, in the presence of DPN symptoms, on the more symptomatic side. The data obtained were analyzed by a neurologist. Modified Davis staging was used to evaluate diabetic retinopathy (7), and ophthalmoscopy was used to classify into non-diabetic retinopathy (NDR), simple diabetic retinopathy (SDR), pre-proliferative retinopathy (PPDR), and proliferative diabetic retinopathy (PDR). SDR was diagnosed when retinal hemorrhage, retinal capillary varicoceles, and hard leukocoria were observed; PPDR was diagnosed when the above findings plus soft leukocoria were observed; PDR was diagnosed when neovascularization, vitreous hemorrhage, and tractional retinal detachment were observed in addition to the above findings. Diabetic nephropathy was evaluated by dividing the patients into 5 stages based on eGFR and albuminuria according to the disease stage in the Japan Diabetes Society as follows: Stage 1 was defined as eGFR >30 mL/min/1.73 m^2 and urinary albumin <30 mg/gCr; Stage 2 was defined as eGFR >30 mL/min/1.73 m^2 and urinary albumin; Stage 3: eGFR ≥ 30 mL/min/1.73 m^2 and urinary albumin ≥ 300 mg/gCr, and Stage 4: eGFR < 30 mL/min/1.73 m^2. Referring to the Current Chronic Kidney Disease (CKD) Nomenclature Used by KDIGO, participants were categorized by eGFR as follows: G1 - >90mL/min/1.73m^2; G2 - 60-89mL/min/1.73m^2; G3a - 45-59 mL/min/1.73 m^2; G3b - 30-44 mL/min/1.73 m^2; G4 - 15-29 mL/min/1.73 m^2. Participants were also categorized by creatinine-converted albuminuria as follows: A1 - <30 mg/gCr; A2 - 30-300 mg/gCr; A3 - >300 mg/gCr. Participants in this study did not include dialysis patients. CVR-R was evaluated using a twelve-lead electrocardiogram measuring device. CVR-R at rest was calculated by measuring the interval between the apexes of the R wave in 100 consecutive heartbeats in the resting supine position. CVR-R at deep breath was calculated from the interval variability of the R wave of 100 consecutive heartbeats during deep breathing. Three patients with atrial fibrillation were excluded when assessing CVR-R because it is impossible to assess CV-RR in these patients. For vibration sensation, a C128 tuning fork was applied to the endocondyle of the tibia, the vibration sensing time was measured on both sides, and the average value was calculated. Carotid IMT was assessed by an ultrasound technician with ultrasonographic equipment, Aplio series (CANON MEDICAL SYSTEMS, Tochigi, Japan). The carotid IMT was evaluated in the common carotid artery wall 10 mm centrally from the carotid sinus. Two IMT points were measured within a 10 mm area, and the mean value was defined as mean IMT. The maximum diameter in the same measurement range was defined as max IMT. Ankle brachial index (ABI) was calculated by measuring blood pressure at the bilateral upper arm and ankle in the supine position and by the ratio of the blood pressure at the higher upper arm to the blood pressure at the respective ankles on the right and left sides. In this study, the lower values of the left and right ABIs were included in the analysis.

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Statistical analysis

The primary endpoint was the correlation between BDC and atherosclerotic lesions, and secondary endpoints were diabetes-related parameters on admission and factors that could correlate with BDC. Analysis between BDC groups was performed by chi-square test and ANOVA. ANOVA adjusted for age, gender, disease duration, smoking history, HbA1c, BMI, hypercholesterolemia medication, and hypertension medication was used to evaluate mean IMT, max IMT, and ABI among BDC groups. *Post hoc* testing was performed by the Tukey method. Mean IMT, max IMT, and ABI were evaluated by natural logarithm. JMP (16.0.1) was used for analysis and EXCEL for Mac (16.58) for table creation.

Results

Clinical characteristics of study participants

The clinical characteristics of the patients in this study are summarized in Table 1. The age of all participants was 60.6 ± 15.1 years (mean ± standard deviation), HbA1c was 10.3 ± 2.3%, and duration of diabetes was 12.2 ± 11.7 years. BDC and vibration sensation, CVR-R at rest, and CVR-R at deep breathing were all significantly correlated ($p<0.005$, $p<0.05$, and $p<0.05$, respectively). Smoking history, BMI, blood pressure, and HbA1c were not significantly different in each group. Prevalence of previous myocardial infarction increased with increasing BDC grade ($p<0.05$). Compared to BDC-0, BDC-3/4 were older, had a longer history of diabetes, and were more likely to take insulin injections, alpha-glucosidase inhibitors, and medications for hypercholesterolemia and hypertension.

Correlation between BDC and diabetes microvascular complications

The correlation between BDC and diabetic retinopathy is shown in Figure 3A. the percentage of patients with retinopathy with SDR or higher in the modified Davis staging was 20.4% in BDC-0, 38.1% in BDC-1, 51.5% in BDC-2 and 57.1% in BDC-3/4. The correlation between BDC and diabetic nephropathy is shown in Figure 3B. 26.9% of BDC-0, 35.7% of BDC-1, 66.7% of BDC-2, and 78.6% of BDC-3/4 had Stage 2 or higher ($p<0.0005$, respectively). In Figure 3C, the correlation between eGFR categories and BDC was evaluated and there were no significant differences among the groups. On the other hand, BDC and severity of albuminuria categories were significantly correlated (Figure 3D).

Correlation of BDC with atherosclerosis

The correlation between BDC and carotid intima-media thickness (IMT) and ankle-brachial index (ABI), a measure of atherosclerotic lesions, was evaluated. Mean IMT was 0.84 ± 0.07 for BDC-0, 0.94 ± 0.03 for BDC-1, 0.89 ± 0.11 for BDC-2, and 1.13 ± 0.16 for BDC-3/4 (Figure 4A). Max IMT was 1.11 ± 0.10 for BDC-0, 1.20 ± 0.14 for BDC-1, 1.23 ± 0.16 for BDC-2, and 1.63 ± 0.24 for BDC-3/4 (Figure 4B). Mean and max IMT was significantly greater for BDC-3/4 than for BDC-0 ($p<0.05$, $p<0.005$, respectively). ABI was 1.18 ± 0.06 for BDC-0, 1.08 ± 0.09 for BDC-1, 1.15 ± 0.10 for BDC-2, and 1.02 ± 0.15 for BDC-3/4 (Figure 4C). ABI was significantly smaller for BDC-3/4 than for BDC-0 ($p<0.05$).

Discussion

These retrospective data indicate that BDC is useful in the evaluation of diabetic neuropathy and that the severity of various diabetes-related complications is associated with that of neuropathy. While no studies have contrasted BDC with atherosclerotic lesions, the data in this study indicate that BDC is associated with microvascular and macrovascular complications other than diabetic neuropathy. Also, the data in this study indicate that BDC may be useful in predicting the risk of diabetic microvascular and macrovascular complications.

The incidence of diabetic microvascular complications increases with hyperglycemia, while the incidence of macrovascular complications such as myocardial infarction has been reported to increase before the onset of diabetes and at relatively low HbA1c levels (5-7%) (8). In this study, BDC was significantly correlated with the prevalence of microvascular and macrovascular complications, despite no difference in HbA1c levels between groups. Vibration sensation cannot be performed in patients with amputated feet (9), and CVR-R is difficult to evaluate due to atrial fibrillation (10). The advantage of BDC is that it can be performed in such patients.

Previous studies on Baba classification showed an increased frequency of macrovascular complications such as myocardial infarction, stroke, and diabetic foot gangrene in patients with BDC 3-4, but there were concerns about bias due to other atherosclerotic factors (6). There have been no previous reports on the correlation between BDC and quantitative atherosclerotic factors such as mean IMT. In the present study, BDC 3-4 patients had an increased prevalence of both old myocardial infarction and old cerebral infarction. After adjustment for factors related to arterial stiffness (age, gender, BMI, history of diabetes, smoking, statins, fibrates, and systolic blood pressure), BDC was significantly correlated with mean IMT and max IMT. These data indicate that BDC can be used

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TABLE 1 Comparison of various parameters among patients grouped with Baba's diabetic neuropathy classification (BDC).

Parameter	BDC-0 (n = 108)	BDC-1 (n = 42)	BDC-2 (n = 33)	BDC-3/4 (n = 14)
Male/female	51/57	27/15	18/15	9/5
Age (years) *	57.9 ± 16.0	63.6 ± 13.2	62.9 ± 13.1	67.5 ± 12.6
Duration of diabetes (years) *	9.9 ± 9.6	11.5 ± 11.1	14.5 ± 9.2	26.6 ± 20.1
Body weight (kg)	70.3 ± 17.5	70.5 ± 16.9	76.5 ± 21.1	67.8 ± 16.4
BMI (kg/m^2)	27.2 ± 5.3	25.7 ± 4.7	29.3 ± 7.3	25.5 ± 4.5
Abdominal circumference (cm)	92.4 ± 13.2	91.4 ± 12.8	102.3 ± 19.3	92.9 ± 12.1
Grip strength (kg) *	26.9 ± 10.6	28.4 ± 11.2	25.5 ± 9.5	21.0 ± 8.4
Smoking history (current/past/never, %) *	42.9/28.6/28.5	24.2/12.1/63.7	34.1/19.5/46.4	26.2/11.2/62.6
History of old myocardial infarction (%) *	5.6	11.9	21.2	28.6
History of old cerebral infarction (%) *	3.8	0	0	28.6
Systolic blood pressure (mmHg)	127.8 ± 22.9	128.8 ± 20.3	127.1 ± 17.4	127.1 ± 16.6
Diastolic blood pressure (mmHg)	79.6 ± 12.9	77.3 ± 14.6	74.5 ± 12.0	74.8 ± 11.8
Pulse rate (beats/min)	85.8 ± 14.3	81.6 ± 13.3	78.3 ± 16.2	85.2 ± 13.9
Vibration sensation (sec) *	12.6 ± 0.8	11.5 ± 1.2	10.3 ± 1.2	9.3 ± 1.4
CVR-R at rest (%) *	3.13 ± 0.34	2.61 ± 0.49	3.05 ± 0.49	1.69 ± 0.57
CVR-R at deep breathing (%) *	5.04 ± 0.51	3.96 ± 0.74	4.70 ± 0.74	2.47 ± 0.87
Blood glucose (mg/dL)	164.2 ± 49.2	160.8 ± 50.8	159.1 ± 58.2	183.0 ± 32.6
HbA1c (%, NGSP)	10.1 ± 2.3	10.9 ± 2.7	10.0 ± 2.0	11.0 ± 1.8
Triglyceride (mg/dL) *	222.5 ± 287.1	160.2 ± 83.9	218.8 ± 212.1	133.89 ± 49.3
LDL-cholesterol (mg/dL) *	108.8 ± 33.3	117.1 ± 45.6	95.9 ± 28.3	86.8 ± 24.7
HDL-cholesterol (mg/dL)	42.2 ± 10.0	43.5 ± 11.2	42.1 ± 9.7	42.8 ± 5.4
AST (U/L)	34.6 ± 29.1	27.4 ± 17.2	31.9 ± 19.1	22.0 ± 4.1
ALT (U/L)	40.5 ± 32.8	29.8 ± 16.2	30.7 ± 19.8	22.8 ± 5.9
Urea nitrogen (mg/dL) *	14.8 ± 4.1	17.2 ± 4.8	16.1 ± 6.2	28.5 ± 12.2
Creatinine (mg/dL) *	0.73 ± 0.23	0.82 ± 0.35	0.84 ± 0.20	1.15 ± 0.43
Percentage of drug at time of admission				
Insulin injections *	29.6	26.2	36.4	71.4
GLP-1 receptor agonist	15.7	21.4	18.2	35.7
Sulfonylurea	5.6	19.1	12.1	14.3
Glinide	7.3	2.4	9.1	7.1
Dipeptidyl peptidase-4 inhibitor	47.2	38.1	36.4	28.6
Biguanide	50.9	50.0	39.4	42.9
Thiazolidine	12.0	9.5	18.2	0
Alpha glucosidase inhibitor *	2.8	11.9	12.1	21.4
Sodium glucose co-transporter 2 inhibitor	33.3	31.0	36.4	28.6
Hypercholesterolemia medications *	43.5	38.1	54.5	64.3
Hypertension medications *	38.9	50.0	57.6	71.4

Data are presented as mean ± standard deviation. BDC, Baba's diabetic neuropathy classification; BMI, body mass index.
*: $P < 0.05$ with chi-square test and ANOVA.

not only to evaluate nerve conduction velocity but also to predict atherosclerotic lesions.

Chronic hyperglycemia induces mitochondrial dysfunction, oxidative stress, and increased inflammation, all of which are likely associated with the development of diabetic neuropathy (11). On the other hand, small dense LDL-C induces inflammation *via* adipocytes and macrophages, and dyslipidemia is considered to be an influential factor in diabetic neuropathy (3). In the present study, more atherosclerotic lesions were observed in patients with advanced diabetic neuropathy which was compatible with previous report showing that there was a reverse correlation between CVR-R and carotid max IMT (10). Diabetic neuropathy is caused by damage in small nerve fibers (12), and both small nerve fibers and carotid IMT are affected by impaired glucose tolerance and vascular ischemia, which could explain the correlation between CVR-R and max IMT. In the present study, BDC was used as a new index of diabetic neuropathy to evaluate its correlation with atherosclerotic lesions, suggesting that macrovascular atherosclerosis and diabetic neuropathy may mutually aggravate the pathology. In the present study, BDC was strongly associated

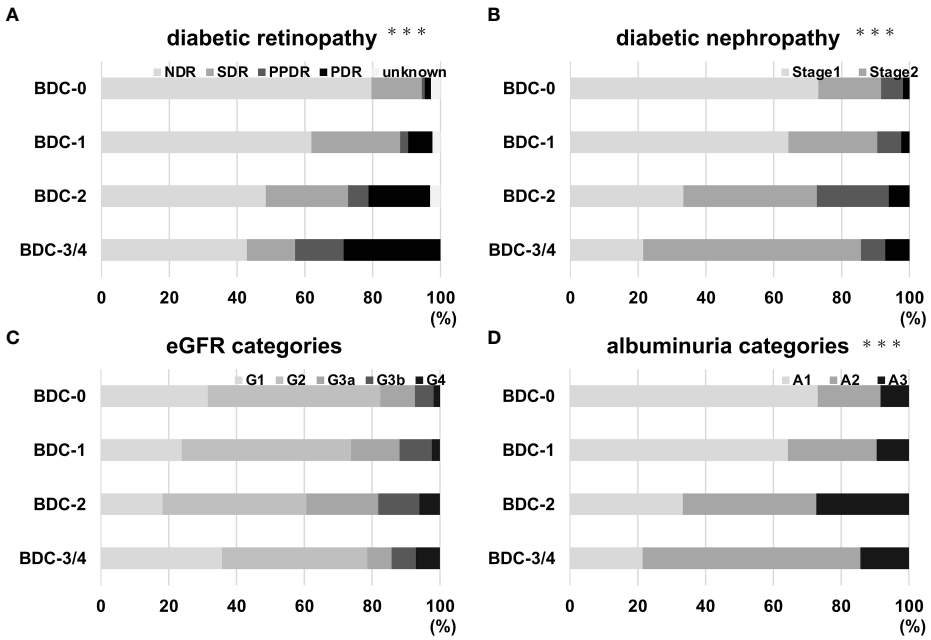


FIGURE 3
Relationship of Baba's diabetic neuropathy classification (BDC) with diabetic microvascular complications. **(A)** Correlation between BDC and severity of diabetic retinopathy. **(B)** Correlation between BDC and severity of diabetic nephropathy. **(C)** Correlation between BDC and eGFR categories. **(D)** Correlation of albuminuria categories. ***P<0.0005 with chi-square test.

with the severity of microvascular and macrovascular complications. BDC-3/4 were more likely to be on hypertension and hypercholesterolemia medications and had longer diabetes duration; HbA1c and length of disease duration may correlate with the prevalence of complications, but even after adjustment for these factors The correlation was significant. On the other hand, the IMT and ABI of BDC-1 and BDC-2 participants showed little difference after adjusting for atherosclerotic factors, indicating that the severity of BDC and macrovascular complications does not necessarily match. Further study is needed to determine whether there is a direct correlation between diabetic neuropathy and macrovascular complications, or whether it is just a parallel severity of both complications.

Even in participants with BDC-0 in this study, 20.4% of them were diagnosed with diabetic retinopathy and 26.9% with nephropathy. Diabetic neuropathy is caused by small fiber

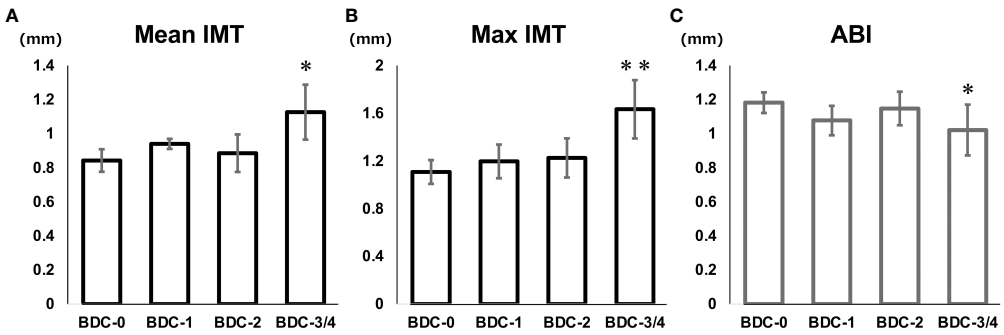


FIGURE 4
Relationship of Baba's diabetic neuropathy classification (BDC) with arteriosclerosis. **(A)** Correlation between BDC and mean IMT. **(B)** Correlation between BDC and max IMT. **(C)** Correlation between BDC and ABI. Data are presented as mean and standard deviation (SD). *P<0.05, **P<0.005 with ANOVA adjusted for age, gender, duration of disease, smoking history, HbA1c, BMI, hyperlipidemia medications, and hypertension medications.

neuropathy, and previous studies have reported that corneal confocal microscopy can detect small fiber neuropathy earlier than nerve conduction velocity. Corneal confocal microscopy has also been reported to detect abnormalities before the progression of diabetic retinopathy and diabetic nephropathy. The participants in this study were hyperglycemic participants who required hospitalization, and further studies are needed to evaluate the ability of BDC to detect early small fiber neuropathy in patients with shorter disease histories and less advanced complications.

This study has several limitations. First, this was a single-center, retrospective study. In addition, number of the patients in this study were limited, the generalizability may be limited. In addition, it is difficult to clinically differentiate alcoholic neuropathy from DPN, and participants with a history of alcohol consumption were excluded from the analysis. If patients with a history of alcohol consumption were included, the results might have differed from those of this study. Next, although the NCS was performed by a skilled examiner, obesity, edema, and examiner skill may have partially affected the results. In addition, since the NCS assesses indirect neurological function, we were not able to examine the correlation between the symptoms of DPN and BDC as reported in the patients in this study. Lastly, the participants in this study had severe hyperglycemia: HbA1c was as high as 10.3 ± 2.3%. It is possible that severe hyperglycemia could have affected the test results for BDC or complications. Future studies should be conducted to evaluate patients who are less affected by hyperglycemia.

In conclusion, BDC was significantly correlated with the progression of diabetic retinopathy and nephropathy. BDC was also significantly correlated with history of myocardial infarction or cerebral infarction and carotid IMT. These data suggest that BDC may be useful in predicting the presence of microvascular and macrovascular complications associated with diabetes. The data also support the idea that in patients with more severe diabetic neuropathy, we should perform further investigation of other diabetes-related complications.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Review Board of Kawasaki Medical School (No. 5594-00). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YI designed the study. YI, SN, TI, EN, MO, TKu, HT, HI, JS, YF, YK, TKi, FT, MS, SN, TM, and HK treated patients and collected data. YI analyzed the data. YI, SH, TKi, FT, MS, SN, TM, and HK contributed to discussion. KK supervised the project. YI wrote the manuscript. HK reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

HK has received honoraria for lectures, received scholarship grants, and received research grant from Novo Nordisk Pharma, Sanofi, Eli Lilly, Boehringer Ingelheim, Taisho Pharma, Sumitomo Dainippon Pharma, Takeda Pharma, Ono Pharma, Daiichi Sankyo, Mitsubishi Tanabe Pharma, Kissei Pharma, MSD, AstraZeneca, Astellas, Novartis, Kowa, Abbott. KK has been an advisor to, received honoraria for lectures from, and received scholarship grants from Novo Nordisk Pharma, Sanwa Kagaku, Takeda, Taisho Pharma, MSD, Kowa, Sumitomo Dainippon Pharma, Novartis, Mitsubishi Tanabe Pharma, AstraZeneca, Boehringer Ingelheim, Chugai, Daiichi Sankyo, Sanofi.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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New horizons of biomaterials in treatment of nerve damage in diabetes mellitus: A translational prospective review

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Background: Peripheral nerve injury is a serious concern that leads to loss of neuronal communication that impairs the quality of life and, in adverse conditions, causes permanent disability. The limited availability of autografts with associated demerits shifts the paradigm of researchers to use biomaterials as an alternative treatment approach to recover nerve damage.

Purpose: The purpose of this study is to explore the role of biomaterials in translational treatment approaches in diabetic neuropathy.

Study design: The present study is a prospective review study.

Methods: Published literature on the role of biomaterials in therapeutics was searched for.

Results: Biomaterials can be implemented with desired characteristics to overcome the problem of nerve regeneration. Biomaterials can be further exploited in the treatment of nerve damage especially associated with PDN. These can be modified, customized, and engineered as scaffolds with the potential of mimicking the extracellular matrix of nerve tissue along with axonal regeneration. Due to their beneficial biological deeds, they can expedite tissue repair and serve as carriers of cellular and pharmacological treatments. Therefore, the emerging research area of biomaterials-mediated treatment of nerve damage provides opportunities to explore them as translational biomedical treatment approaches.

Conclusions: Pre-clinical and clinical trials in this direction are needed to establish the effective role of several biomaterials in the treatment of other human diseases.

KEYWORDS

biomaterials, nerve damage, diabetes, diabetic neuropathy, cryogels, stem cells

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Introduction

Peripheral neuropathy (PN) is a highly complex and prevalent disease that involves the plexus of nerve tissues. In epidemiological studies from various regions in India, the overall prevalence of PN varied from 5 to 2400 per 10,000 population in various community studies (1). Neuropathy is characterized by pathologic cellular and non-cellular changes in the premises of the peripheral nerve, especially arising in type 1 diabetes (in which the pancreas does not produce insulin in sufficient quantity due to β-cell dysfunction) and type 2 diabetes mellitus (T2DM) (in which there is some insulin resistance) (2). In the United States and Europe, it is presumed that pre-diabetes is also the leading cause of peripheral neuropathy (3). In the United States, around 20 million people currently have neuropathic-related secondary complications and this number progressively doubles as the incidences of pre-diabetes and T2DM increase (4). Furthermore, the worldwide cases of pre-diabetes and diabetes are 316 million and 537 million, respectively, according to the International Diabetes Federation (IDF) (Figure 1) (4). Diabetes mellitus leads to several acute, chronic, diffuse, and focal neuropathy syndromes. Peripheral nervous system (PNS) damage, associated with diabetes includes bilateral and symmetric damage to feet nerves, having a distal-to-proximal gradient of severity also known as stocking-glove neuropathy (5). Peripheral diabetic neuropathy (PDN) has been defined as a "symmetrical, length-dependent sensorimotor polyneuropathy attributable to metabolic and micro-vessel alterations due to chronic hyperglycemia exposure and cardiovascular risk covariates" (6). PDN typically presents positive sensory symptoms in the feet that include pain, tingling, and prickling of sensations (paresthesias) along with negative symptoms such as numbness, loss of sensation (disordered sensory processing), episodes of pain in the feet region when touched (allodynia), and elevated sensitivity to noxious stimuli (hyperalgesia). The severity of PDN is associated with morbidity, lower limb fractures, ulceration, and amputations (7–10). Table 1 shows the detailed classification of PDN. There is evidence of motor nerve dysfunction associated with distal toes weakness and, in vulnerable cases, the ankles and calves. It is a matter of debate as to why the sensory axons, compared to motor axons, are vulnerable to diabetes-associated complications.

Unfortunately, despite enormous research, there are no promising treatments for PDN other than strict glycemic control and lifestyle modifications (11). A Cochrane review composed of all clinical analyses on PDN revealed that strict glycemic control in T1DM subjects can decrease the episodes of

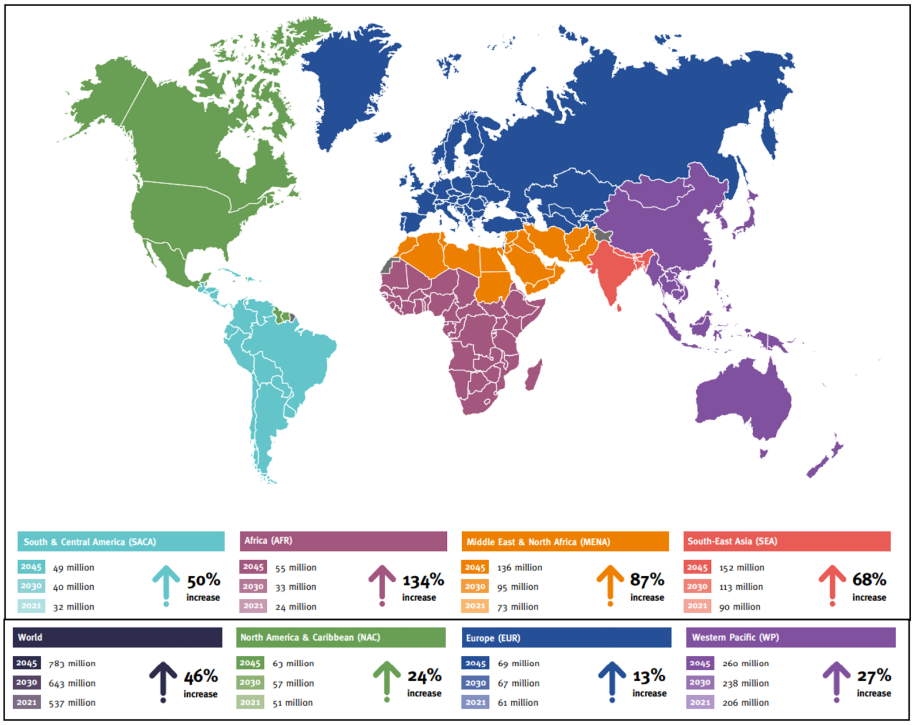


FIGURE 1
Projected number of people with diabetes worldwide and per IDF Region in 2021–2045 (20–79 years) (Source: International Diabetes Federation; 10th Atlas).

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TABLE 1 Classification of diabetic neuropathies.

A. Diffuse neuropathy
DSPN
•Primarily small-fiber neuropathy
•Primarily large-fiber neuropathy
•Mixed small- and large-fiber neuropathy (most common)
Autonomic
Cardiovascular
•Reduced HRV
•Resting tachycardia
•Orthostatic hypotension
•Sudden death (malignant arrhythmia)
Gastrointestinal
•Diabetic gastroparesis (gastropathy)
•Diabetic enteropathy (diarrhea)
•Colonic hypomotility (constipation)
Urogenital
•Diabetic cystopathy (neurogenic bladder)
•Erectile dysfunction
•Female sexual dysfunction
Sudomotor dysfunction
•Distal hypohydrosis/anhidrosis,
•Gustatory sweating
•Hypoglycemia unawareness
•Abnormal pupillary function
B. Mononeuropathy (mononeuritis multiplex) (atypical forms)
•Isolated cranial or peripheral nerve (e.g., CN III, ulnar, median, femoral, peroneal)
•Mononeuritis multiplex (if confluent may resemble polyneuropathy)
C. Radiculopathy or polyradiculopathy (atypical forms)
•Radiculoplexus neuropathy (a.k.a. lumbosacral polyradiculopathy, proximal motoramyotrophy)
•Thoracic radiculopathy
Nondiabetic neuropathies common in diabetes
•Pressure palsies
•Chronic inflammatory demyelinating polyneuropathy
•Radiculoplexus neuropathy
•Acute painful small-fiber neuropathies (treatment-induced)

PDN, but this has little effect in T2DM subjects despite more than 10 years of glycemic control (12). This difference in incidences of PDN is extensively informative and favors the ingredient that, different pathophysiological mechanisms underlie T1DM- and T2DM-associated PDN, and it is to be considered as two diseases with similar clinical presentation (12). The pharmaceutical market has left the PDN due to a lack of basic understanding of the mechanism and research, while the enormity of the complications has reached epidemic proportions (13).

The nervous system plays a crucial role in maintaining human biological events such as cognition and individual cell function. Damage to the state of the nerve may cause tremendous consequences that may be life-threatening. Peripheral nerve degeneration has increased over the past few years affecting 2.8% of trauma subjects annually (14). PDN is likely to be associated with demyelination and axonal atrophy that leads to nerve degeneration as suggested by animal experiments (15, 16). The patho-mechanistic approach leads to damage of peripheral nerve plexus in PDN including two major components. First is the impairment of endoneurial circulation following ischemia (17) and the second is abrupt axon-glia relationships followed by segmental or paranodal demyelination (18), Wallerian degeneration and neuroma (19). Despite this conundrum, PDN-related research in the last decades has focused on the therapeutic potentials of biomaterials in PDN that directly improve the severe form of complications.

A previously published study has demonstrated the repair of peripheral nerve damage with a common goal of regenerating nerve fibres at their proper place (i.e. endoneurial tubes) and maintaining functional recovery (20). The details of the conversion of a healthy neuron to a peripheral neuropathy neuron are demonstrated in Figure 2. Implantation of autografts, allografts, and xenografts from donor subjects are some of the strategic approaches available at present. The few demerits associated with these grafts primarily include loss of function or sensation at the donor nerve graft site, mismatch of the damaged nerve, and the abrupt dimension of nerve graft (20, 21). Furthermore, the allogenic and xenogenic also show disease transmission and immunological complications (21).

Tissue engineering gives new hope to regenerative medicine as an alternative to basic conventional transplantation methods that help to modulate cell behavior and tissue progression with the application of novel biomaterials. In one of the previously published studies related to regenerative tissue engineering, the researchers fabricated polymeric scaffolds seeded with nerve cells or neural origin to produce a 3D functional tissue with regenerative capability (22). Tissue engineering strategies proved to be landmark efforts to provide a therapeutic role in peripheral nerve damage induced in diabetes mellitus (i.e. PDN), spinal cord injury, and drug delivery approach (23). Nerve guidance channels (NGCs) enabled biomaterials are specially designed implants for nerve repair. A specially designed conduit with NGCs having desired topography, dimensions, stability, and biodegradability is implicated in nerve regeneration (24). Previous research has successfully demonstrated that the electrical stimulation of these conduits used for nerve cell regeneration showed better results and effective cues in stimulating the proliferation and differentiation of neural cells (25).

In this review, we discuss the pathophysiological mechanism of peripheral diabetic neuropathy with up-to-date understanding. The study also outlines the economic burden of PDN on society. The most important part of this review is focused on the therapeutic potential of conducting biomaterials-

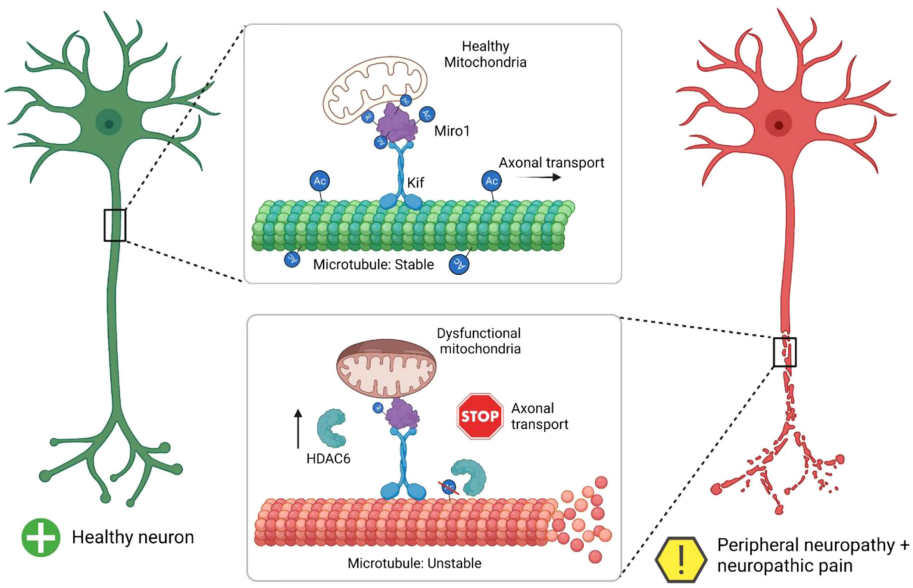


FIGURE 2
Summary of a healthy neuron undergoing neuropathy (Adopted from Ref. 20 under common creative license).

enabled scaffolds or cryogels in nerve cell regeneration. A brief evaluation of electrical stimulation approaches of conductive polymers for tissue engineering is also discussed.

Economic burden of peripheral diabetic neuropathy

Several studies published on the economic status of peripheral diabetic neuropathy showed that patients with these complications bear high costs to manage their health. One study conducted in Europe mentioned statistical data that 76% of PDN patients visited their physician at least once in 4 weeks (26). In one study, the healthcare cost of PDN patients in the UK was £2,511 of which 41% of the budget accounts for patient care (27). Drug costs involved in the treatment and management of DPN account for 30-32% of the total healthcare budget (27). Per patient, drug costs vary with the choice of effective medications. The cost of presently available generic anti-depressants is less compared to newer molecules employed in the treatment and management of PDN. In another study conducted in the UK region, it has been found that peripheral diabetic painful neuropathy (PDPN) is significantly associated with discontinuous employment and work productivity (26). It has been also reported that 35% of the patients with PDPN have some sort of disturbances in their employment due to pain associated with it (26). The situation worsens, as 59% of patients showed less productivity at a certain period of time due to PDPN (26). It is interesting to note that in this study that 14% of patients with mild pain showed work disruption, 38% of patients showed moderate pain and had an interruption to their work, and the figure was more than tripled for patients presenting with severe pain (i.e. 48%) (26).

PDPN patients with microvascular complications showed the lowest EQ-5D utility value (0.63, 0.65, 0.56 *vs* 0.41) compared to macro-vascular complications of diabetes including CAD (26, 27). Similarly, other studies have shown that utilities are lower for PDPN patients compared to other chronic diseases such as depression, asthma, and Parkinson's disease (26, 28, 29). In one of the previously published studies, Hoffman et al. (30) stated that patients presenting severe to moderate pain in PDPN had a greater mean improvement in overall health score compared to those with moderate to no/mild pain post-treatment. Data for economic burden in peripheral diabetic neuropathy was rather limited. Studies related to economic burden were done with patients in Spain (31) and the UK (26). The global burden of diabetic foot disease has been studied by Andrew et al. (2005) (32) (data shown in Figure 3).

Makeover of the peripheral nervous system

The plexus of the peripheral nervous system (PNS) comprises 12 cranial nerves and 31 pairs of spinal nerves along with neurons and glial cells (especially Schwann cells). The central nervous system (CNS) coordinates with muscles and glands *via* efferent axons. Furthermore, afferent axons coordinate between CNS and peripheral sensory receptors. A

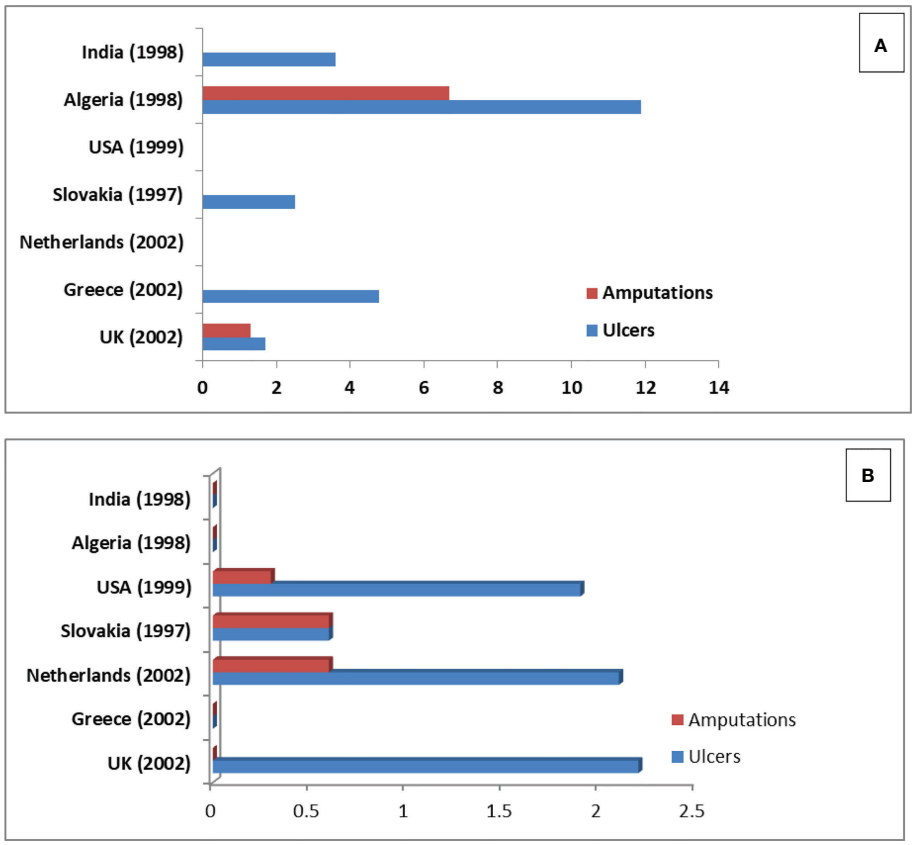


FIGURE 3
Epidemiology of foot ulceration and amputations by country **(A)** Prevalence **(B)** Incidence.

long network of axons sometimes poses a unique challenge to PNS (33). Likewise, peripheral sensory receptors are sensory neurons (i.e dorsal root neurons) located outside of the blood-nerve barrier. Moreover, motor neurons are situated in the vicinity of the ventral horn of the spinal cord spanning the blood-brain barrier (BBB). Motor neurons remain protected, enabling dorsal root ganglion neurons susceptible to systemic metabolic hypoxia-induced injuries. PNS is composed of thin (<1nm) myelination-devoid axons known as C fibers. Furthermore, several unmyelinated axons, more than myelinated axons are components of PNS. Feldman et al. (2005) reported that these C fibers are termed the "foot soldiers" of PNS and were discovered by the late Jack Griffin, a pioneer scientist in the peripheral neuropathy field (34).

The PNS is also constituted of the afferent myelinated fibers that are responsible for the transfer of information from peripheral receptors to the location of sense and touch. The mechanism of myelination of these afferent fibers by Schwann cells (SCs) is typically controlled and forms differentiated nodal domains such as; the node of Ranvier, the paranodes, and the juxtaparanodes. Vast variations in the anatomy of unmyelinated and myelinated fibers occur in diabetes and its related complications. Pre-diabetes is also among the important factors for such changes. The first case report in the field of diabetic peripheral neuropathy enabled changes in PNS was published in 1890 by Auche and their coworkers that further reviewed by Colby in the year 1965 (35). Electron microscopy also has a history associated with PDN. In the 1960s, for the first time, sural nerve biopsies (anatomical studies) from patients with diabetic neuropathies (DN) were performed by P.K Thomas, P.J Dyck, and their co-workers. The previous studies, along with current reports, mentioned changes in unmyelinated C fibers in DN subjects (36). Recent literature also reported that the degeneration and regeneration of these C fibers result in pain, hyperesthesias, and allodynia (37). A study done on pre-diabetic subjects revealed that over a long period the degeneration mechanisms override the regeneration, resulting in the loss of C- fibers (38). Axonal loss occurs due to the loss of nutrient flow from SCs to myelinated axons in DN (39). To summarize this section, there is now a clear picture of PNS

alteration in diabetic neuropathy. We will now discuss the biochemical pathways that are widely known to be dysregulated in PDN associated with diabetes mellitus.

Biochemical pathways implicated in peripheral diabetic neuropathy

Over the last few decades, research in the sector of peripheral diabetic neuropathy has focused on the detailed mechanisms and biochemical pathways associated with nervous system damage. The studies done previously have focused on enhancing the pathological relevance of polyol pathway activation, hexosamine/protein kinase C (PKC) isoform activation, accumulation of advanced glycation end products (AGEs) in the peripheral nerves plexus in a diabetic state, and oxidative stress. Each specific pathway alone is injurious to the nervous network and may cause severe complications in subjects with diabetes mellitus during prolonged and uncontrolled hyperglycemia.

(a) Polyol pathway

The polyol pathway is the most highlighted biochemical mechanism in diabetic neuropathy. Under normoglycemia, glucose undergoes glycolysis and other biochemical inlets for energy generation and storage. Moreover, during hyperglycemia, excess glucose is converted into sorbitol (alcohol) by an enzyme aldose reductase (EC No. 1.1.1.21), leading to increased sorbitol levels and osmotic imbalance. This event results in osmotic stress and compensatory efflux of myoinositol and taurine. Myoinositol is an essential component of sodium/potassium ion (Na^+/K^+) ATPase and therefore, its loss impairs nerve makeover and function.

Aldose reductase activity decreases the reservoir of nicotinamide adenine dinucleotide phosphate (NADPH) required for nitric oxide (NO) and essential antioxidant generation (i.e glutathione). The generation of cytoplasmic-reactive oxygen species (ROS) leads to intracellular injury and cellular dysfunction. This concept is known as the "metabolic flux" hypothesis, as reviewed by Oates and their co-workers (40). A pre-clinical rodent study performed on a streptozotocin (STZ) rat model with T1DM showed the relation between the polyol pathway and impairment in PNS makeover and function (41). Exploiting this premise, several AR inhibitors (ARI) have been developed and tested for pre-clinical aspects, but the clinical trials have shown the failure of promising results due to adverse effects at the clinical end-point of the studies. Currently, epalrestat (ONO2235) is the only licensed ARI in Japan as it was approved after the 3-month double-blind trials which showed promising improvement in nerve function (42). In another study conducted for ARI, it has been found that it ameliorates nerve conduction velocity (NCV) and nerve makeover in the diabetic state (43). The failure of ARI in treatment has been attributed to experimental design, inaccessibility to PNS, dose range, and secondary hepatotoxicity (44). The advent of recent and promising transgenic technology has revealed further insights into the polyol pathway. A study conducted on a galactose-fed transgenic mice model revealed that it overexpressed the human AR and developed severe diabetic neuropathy (45). Despite ample pre-clinical research, a detailed mechanism of the influence of the polyol pathway in neuropathy is still unclear.

(b) Hexosamine/PKC pathway

Increased activity of the glycolysis pathway is the response to hyperglycemia and also impairs the metabolic pathways and promotes neuronal injuries. The fructose-6-phosphate is a glycolytic intermediate that enters the hexosamine pathway and follows a series of treatments to form uridine-5-diphosphate-N-acetylglucosamine (GlcNac). GlcNac has the ability to bind serine/threonine amino acids to transcription factors, particularly Sp-1, resulting in lipid dyshomeostasis and injury to peripheral nerves along with inflammation (46). Dihydroxy-acetone phosphate (DHAP), another glycolytic intermediate converted into diacyl glycerol (DAG), is responsible for the initiation of tissue complications, especially of nerves due to activation of the neuronal protein kinase C (PKC) (47).

PKC inhibitors have also been tested for promising therapeutic results in peripheral diabetic neuropathy. In one study done on STZ-induced diabetic rats, it has been found that there is an improvement in nerve physiology and function with the use of PKC inhibitors in the treatment of diabetic neuropathy (48). In one of the landmark studies done on human subjects, the PKC-β inhibitor (ruboxistaurin) was found to have an improving role in the treatment of diabetic neuropathy but unfortunately was not effective in diabetic retinopathy and nephropathy (48). PKC is a key player in the pathogenesis of diabetic neuropathy (48). In their study, Nakamura and their co-workers did not find any significant changes in PKC activity in the tissue homogenate of whole peripheral nerve tissue in a diabetic model induced by STZ, although there was an improvement in nerve conduction velocity and blood circulation to the nerve upon using PKC-β inhibitors (49). It is presumed that using PKC-β inhibitors may prove useful in the treatment of peripheral diabetic neuropathy. In a pre-clinical study performed on animal models, PKC-β inhibitors have beneficial effects on neuropathic changes in STZ-induced diabetic rats (50). Despite successful pre-clinical animal studies, the clinical trial showed failure, in part, to the high improvement rate in the placebo group (51).

(c) Advanced glycation end-product pathways

Advanced glycation end products (AGEs) are irreversible glycation products formed *via* the Maillard reaction between glucose and amino groups of proteins. AGEs enable the cross-linking of proteins, thereby altering their structure and function. AGEs bind with the extracellular cell surface receptors, the receptors of AGEs (RAGE), which activate the nuclear factor kβ (NF- kβ) that initiate a series of complications like vasoconstriction, inflammation, and neurotrophic support loss of PNS (52). An accumulation of AGEs in the peripheral nerves was also found in patients with diabetic neuropathy in T2DM subjects (53). A previously reported study has revealed the importance of the RAGE-AGEs relationship in the pathogenesis of DN (54). Potent AGEs like di-carbonyl active species have been reported to damage sensory neurons (55).

An *in-vitro* cell line study on rat Schwann cells showed a decrease in viability in the presence of glycolaldehyde (precursor of AGEs) in a physiological environment, thus contributing to the progression of diabetic neuropathy (56). The presence of CML was reported in vascular endothelial cells, pericytes, and basement membranes of axons and Schwann cells in diabetic peripheral nerves. *In-vitro* incubation of neural cells in an AGEs-rich medium induces cell death (57). It is hypothesized that uncontrolled hyperglycemia initiates the AGEs formation and their accumulation in the peripheral nerve affects the structural and functional characteristics of proteins, thereby adversely affecting the function of the nervous system and leading to diabetic neuropathy.

There are two proposed mechanisms of diabetic neuropathy induction *via* protein AGEs. First, the advanced glycation mechanism tends to impair the biological functions and properties of the protein, thus making abnormal neuronal activity (58). Secondly, the lipid and protein AGEs bind to RAGE to initiate the signalling cascade that elevates the oxidative stress and neuronal cell injury mediated by NADP (H) oxidase (59) along with nitrosative stress (60). In one study, it has been reported that there is a structural and functional abnormality in axons, Schwann cells, and dorsal root ganglia neurons when the AGEs bind to RAGE in experimental diabetic rat and mouse models of neuropathy (61). The AGE-RAGE axis appears to mediate the prolonged cellular pro-inflammatory response in chronic diabetic complications (62). In one of the studies related to NCV, the supply of exogenous AGEs showed a delay in NCV in the diabetic neuropathy model (63). With this delay in NCV, the activity of nerve Na, and K-ATPase declined. As a result of this, the myelinated nerve fibers shrunk in size. Furthermore, preliminary clinical trials in diabetic neuropathy showed improvement with the use of benfotiamine, but there is still no molecule found that can suppress the formation of AGEs and improvement of peripheral diabetic neuropathy in humans.

(d) Oxidative stress

Enhanced glycolytic flux in hyperglycemia leads to the generation of free radicals that are primarily responsible for diabetic neuropathy (64). Numerous studies proved that oxidative stress (OS) induces peripheral nerve injury in experimental diabetes (65–67). Using this information, the studies attempted to improve neuropathic complications with antioxidants (41, 68). A previously published study used α-lipoic acid for the suppression of OS in diabetic animal models that showed improvement in NCV delay (69). A most important event of programmed cell death occurs due to the release of cytochrome-C, and caspase-3 activation (70). Oxidative stress is defined as the imbalance in the antioxidant defense mechanism. The majority of reactive oxygen species (ROS) are the products of mitochondrial respiration that cause cellular and molecular damage. In the state of hyperglycemia and excess fatty acid flux, there is excess substrate metabolized through glycolysis and the TCA cycle, respectively, enriching the dorsal root ganglion (DRG) neurons with ample NADH and $FADH_2$ electron donors. This increases the proton gradient and, hence, ROS generation. The one-electron reduction process of molecular oxygen generates a stable intermediate, known as superoxide anion ($O\cdot_2^-$) that serves as a precursor of ROS. The superoxide dismutase (EC No. 1.15.1.1) performs the dismutation of the superoxide anion that produces H_2O_2. Furthermore, the interaction of H_2O_2 and $O\cdot_2^-$ in a Haber-Weiss reaction or Fenton reaction generates a highly reactive hydroxyl radical (OH). Mitochondrial damage decreases neurotrophin-3 (NT-3) and the nerve growth factor (NGF) that disturbs neurotrophic support. It is important to note that a small amount of insulin is insufficient for lowering systemic blood glucose levels and is capable of enhancing the NCV delay (71). Moreover, nitro-oxidative stress activates poly ADP-ribose polymerase (PARP) in conjunction with sustained hyperglycemia (72), resulting in cellular dysfunction and programmed cell death that can be inhibited by using PARP inhibitors (73).

(e) Insulin physiology

Studies related to DN have evolved from the hyperglycemic viewpoint, which points out DN has a complex of secondary complications associated with metabolic and oxidative insults as described in the above sections. T1DM and T2DM exhibit different presentations of DN with different occurrence mechanisms in each disorder (74). In subjects with T1DM, DN improvement relies on different insulin shots (dose) and is suggestive of the fact that one or more insulin shots do not show strict glycemic control but have a positive impact on PNS (75). Insulin receptors (IRs) are well expressed on sensory neurons and motor neurons and showed enriched presence in SC

membranes along with nodes of Ranvier. Furthermore, the role of insulin signaling is not fully understood. Insulin binding to IRs activates the signaling cascade of intracellular mechanisms that helps in maintaining normal cell homeostasis (76). It is also studied in previous literature on T1DM with DN complications that the failure of insulin signaling leads to cell injury and promotes apoptosis (77). The improvement in PNS in DN is either due to normoglycemia or insulin-mediated neuronal signaling, but this is still a matter of debate.

How is diabetes injurious to axons?

It is speculated that SCs provide energy to axons. In the state of diabetes mellitus, the SCs not only lose their capability to provide energy to myelinated and unmyelinated axons but are also involved in the transfer of harmful lipid molecules to the point of contact of axons. The puzzle of axonal vulnerability during diabetes is difficult to decipher (78). SCs produce acylcarnitines that trigger the influx of extracellular calcium ions inside the axon which disturbs axonal mitochondrial trafficking, resulting in insufficient energy generation and mitochondrial apoptosis (78). Moreover, the diabetic rodent model showed elevated mitochondrial ROS in axons that lead to dysfunction and axonal injury (78). *In-vitro* studies on DRG neurons speculated that inhibition of mitochondrial complex 1 decreases ATP generation that in turn increased ROS generation, leading to axonal degeneration (79).

The abundant ion channel presence in axons makes them vulnerable to diabetes-associated injury. Axons are known to exhibit numerous voltage-gated sodium channels along with sodium-calcium exchanger pumps in their termini (80). Na/K ATPase pump performs an export of intra-axonal Na+ entry to generate an action potential, however, low ATP levels fail to generate this action potential and, in turn, leads to axonal degeneration. Sensory axons exhibit distinct voltage-gated sodium-calcium ion channels (Na_v1.6, 1.7, 1.8, and 1.9) compared to motor axons (Na_v 1.6) with a distinct biophysical makeover (81). The sensory neuron because of its short diameter, interplays Na+/Ca++ changes rendering them to axonal injury. In conclusion, the vulnerability of axons in diabetes mellitus-induced peripheral diabetic neuropathy is secondary in combination with energy factors and ion channel presentations.

The above sections give a focused message that there is severe to mild injury associated with the peripheral nervous system in diabetes-associated PDN. The goal of future research should be to provide a therapeutic approach for the management of neuronal injury associated with PDN. The advent of biomaterials shows promising potential for the cure of nerve injuries in PDN. Biomaterials with the appropriate property of conduction and nerve homology provide the prospects to provide relief to mankind's society. Our lab is still trying to fulfill this requirement around the clock. The next section of this review deals with biomaterials and their role in PDN.

Introduction to biomaterials

In general, biomaterials are defined as "any natural or synthetic material comprise of living component, which is capable of replacing and performing the function of essential tissue where it has been placed". Park and Lakes state that biomaterial is any synthetic material capable of replacing a part of a living system in every respect. Clemson University Advisory Board for Biomaterials state that "it is synthetically and pharmacologically inert material with a motto of implantation into the living system". Williams, in 1987, proposed that biomaterial is an implanted source intended to couple with the living system (82). Regarding tissue engineering, biomaterial serves the purpose of repairing and reconstructing the damaged and injured part of a biological system. A few examples where biomaterials can be used as a substitute include heart valves, artificial hips, dental implants, and fixing fractures. So, it can be concluded that any material with specific properties of regeneration can fit into the criteria of biomaterials. It is also stated in the previously published literature that any material with the property of biocompatibility can be considered biomaterial (83). Biomaterials can also be used to deliver molecules or essential factors required for the regeneration of tissue (84).

How many choices are there for biomaterials?

Biomaterials, as discussed in the above section, have a regeneration ability with premium characteristics of biocompatibility and similar tissue physiology. The biomaterials are broadly grouped into three choices:

Polymers

Polymeric biomaterials perform other functions apart from tissue regeneration such as fixing fractures with bone cementing materials (e.g. poly methyl methacrylate), or polyglycolic acid being used in degradable surgical sutures. Polymers in tissue engineering can be both synthetic and natural. Polymers consist of repeating units of monomers. There are a variety of synthetic polymers used in regenerative engineering and medicine. These include silicone rubber (SR), polypropylene (PP), polyethylene (PE), poly (ethylene terephthalate) (PET), polyvinyl chloride (PVC), polytetrafluoroethylene (PTFE), and polyethylene glycol

(PEG), along with others not included in the list. These polymers have a role in regenerative medicine and tissue engineering. Polymers with mucoadhesive properties can be used for mucosal drug delivery, while polyacrylics are used for dental implants. PP because of its good tensile strength can be used for degradable sutures. Moreover, poly lactic-co-glycolic acid (PLGA), a Food and Drug Administration (FDA) approved biodegradable polymer, is used in resorbable surgical sutures and drug delivery and orthopedic applications as a fixative.

Natural polymers also have applications in regenerative tissue engineering and include alginate, collagen, fibrin gels, hyaluronic acid, etc (85). Alginate biopolymer contains repeating monosaccharide units of i.e L-guluronic acid and D-mannuronic acid that are useful in the generation of three-dimensional gel. Alginate can be used for cartilage regeneration as alginate gels. Another natural polymer, collagen, has been implicated in skin regeneration, as it is an essential component of human skin. A previously published study used bilayered collagen to regenerate skin (86). Hyaluronan, also a natural polymer with certain modifications, can be used as a degradable scaffold in tissue regeneration and can be used for cartilage repair (87). Fibrin is another natural polymer derived from animal skin and is applicable for wound healing. This property can be exploited as a scaffold with the disadvantage of frequent fibrinolysis and fast degradation. To overcome this problem, a slight modification has been employed, so it can be used for a longer period (88).

Metals

Biomaterials can be used in the form of metals apart from synthetic and natural polymers. Metals can also serve a major role in tissue engineering and have a significant economic impact on tissue regeneration and biomedicine. Metallic implants like steel 316, 316L, cobalt, F75, Vitallium, silver, tantalum, and alloys of Ti, Cr+, Co, Mo, etc., can be successfully used for implants in tissue engineering. Metallic implants have associated disadvantages such as corrosion susceptibility and low biocompatibility (89). Metallic implants can be used in tooth implants, penis implants, and in facial reconstruction (90).

Ceramics

Ceramics is another category of biomaterials used in tissue engineering. Ceramics are broadly used as a component of eyeglasses, diagnostic instruments, thermometers, etc. These classes of biomaterials possess high biocompatibility, high resistance, and reduced thermal and electrical conductivity, which makes them suitable for implants (91). New bone tissue regeneration uses hydroxyapatite (HAp), a class of ceramics. Other ceramics include aluminum oxide, titanium oxides, calcium phosphate, calcium aluminates, carbon bioglass, etc. Ceramics exhibits disadvantages of low impact resistance and difficulty in fabrication. The major achievements with the use of ceramics include heart valve implants, hip implants, and knee implants as demonstrated by landmark studies in tissue engineering (92, 93). The elaborated list is depicted in Table 2.

Characteristics of biomaterials enabling its clinical application

The first and foremost characteristic of biomaterials is biocompatibility both *in-vitro* and *in-vivo*. *In-vitro* evaluation of biomaterial with an *in-vitro* cell setup is an inexpensive primary approach for testing its interaction. Moreover, the results of the *in-vitro* testing are not useful until the desired biomaterial is not able to fulfill the *in-vivo* implant motto. Cytotoxicity assessment (i.e. apoptosis, alterations in cell permeability) of biomaterials for the determination of biocompatibility is a primary test for its implant viability in regenerative medicine. Biomaterials that initiate cell apoptosis (death) or changes in cell properties may be proved toxic and are not suitable to use as an implant. If the biomaterial passed the cytotoxicity examination, then it has to qualify for the next test of biocompatibility performed on a popularly used mammalian fibroblast cell line (L-929 cells) and MTT (3(4,5dimethylthiazol2yl)2,5diphenyltetrazolium bromide) assay.

TABLE 2 State-of-the-art materials for long-term implants.

Materials	Applications
Titanium alloys	Dental implants, femoral stems, pacemaker cans, heart valves, fracture plates, spinal cages
Cobalt–chromium alloys	Bearing surfaces, heart valves, stents, pacemaker leads
Platinum group alloys	Electrodes
Nitinol	Shape memory applications
Stainless steel	Stents, orthopedic implants
Alumina	Bearing surfaces
Calcium phosphates	Bioactive surfaces, bone substitutes
Carbon	Heart valves
UHMW polyethylene	Bearing surfaces
PEEK	Spinal cages
PMMA	Bone cement, intraocular lenses
Silicones	Soft tissue augmentation, insulating leads, ophthalmological devices
Polyurethane	Pacemaker lead insulation
Expanded PTFE	Vascular grafts, heart valves
Polyester textile	Vascular grafts, heart valves
Cryogels	Nerve Implants

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Experiments on a suitable animal model system are the final step for testing the regenerative ability of the biomaterials in implants (Table 3). The biomaterial implants with desired characteristics are placed at the site of damage or defect, and further experiments on animals are performed to prove their biocompatibility and ability to regenerate. Moreover, post-implantation, the hemocompatibility and biodegradation of the implant are tested. The combo of both *in-vitro* and *in-vivo* testing is a necessary part of tissue regenerative engineering. Currently, the reforming of a damaged nerve is conducted using nerve autografts. Such treatment modalities can be replaced with engineered conduits suitable for nerve lesions and large nerve defects. Carbon nanotubes (CNTs) are considered to be a novel biomaterial with vast biomedical applications. CNTs exhibit unique characteristics that could be used for nerve repair in the form of conduits. CNTs have a cylindrical shape with a nanosized diameter along with properties like flexibility, electrical conductivity, and biocompatibility.

Unique classes of scaffolds: A boon for tissue engineering

The conventional biomaterials discussed in the previous section can be used as implants after fabricating into three-dimensional scaffolds to achieve the desired functions of growth, proliferation, and regeneration in tissue engineering. The above biomaterials can be fabricated into desired implanting scaffolds with varied fabrication technologies such as (i) solvent casting mechanism in combination with leaching, (ii) fiber building network, (iii) phase separation with freeze drying, and (iv) solid free form fabrication (94). Furthermore, with the advancement in technologies, the advent of next-generation biomaterials for tissue regeneration and repair can serve the regeneration purpose well. These next-generation implant biomaterials not only regenerate the desired tissue but also serve as factor-delivery vehicles that provide nourishment and an essential growth environment to the tissue. Below are the next-generation biomaterials that can be used as scaffold implants successfully.

TABLE 3 Animal model for *in-vivo* implants experiments.

Implant application	Animal model system
Cardiovascular	
(a) Heart Valves	Sheep
(b) Vascular graft	Dog, Pig
(c) Artificial Heart	Calf
Orthopedic/Bone	
(a) Bone regeneration	Rabbit, dog, pig, mouse, rat
(b) Total hip/knee joint replacements	Dog, goat
(c) Vertebral implants	Sheep, goat, baboon
Neurological implants	
(a) Peripheral nerve regeneration	Rat, cat, non-human primates
(b) Electric stimulation	Rat, cat, non-human primates
Ophthalmological	
(a) Contact lens	Rabbit
(b) Intraocular lens	Rabbit, monkey

Next-generation hydrogels

Hydrogels are the class of colloidal gel in which water is used as the dispersion medium. These biomaterials are used as implant scaffolds in tissue engineering, as they resemble the topography and function of native tissue. The aqueous behavior of these gels enables them to resemble the cells in the body. Moreover, due to the presence of fine porosity, nutrients and waste factors are exchanged easily. Natural and synthetic biopolymers can be used in tissue regeneration after the fabrication of these biopolymers into hydrogels (95). Synthetic hydrogels implemented in tissue regeneration include (PU), polyethylene oxide (PEO), poly (N isopropyl acrylamide) (PNIPAAm), polyvinyl alcohol (PVA), polyacrylic acid (PAA), and poly propylene furmaratecoethylene glycol [P(PFcoEG)], while the natural hydrogels include agarose, alginate, chitosan, collagen, fibrin, gelatin, and hyaluronic acid (96).

Next-generation cryogels

Cryogel application is a landmark achievement in the field of tissue regeneration. Cryogels are supermacroporous gel matrices formatted in mold-freezing conditions. Cryogels are the gel matrices prepared by crosslinking and polymerization, at sub-zero temperatures, from either a synthetic or natural source of biopolymers without involving any inorganic solvents. Cryogelation enables a scaffold to be formatted into diverse forms like disks, sheets, and monoliths with desired dimensions due to the presence of large and interconnected pores that provide several advantages over the conventional scaffold. In the cryogelation technique, there are two parts to the solvent; one part of the solvent used remains unfrozen (liquid microphase), while the other part of the solvent remains in the frozen state. This two-phase mechanism of solvent provides a platform for monomers and polymers for chemical reactions. The unfrozen liquid microphase is the best candidate for gel formation and further undergoes chemical modifications to form a porous scaffold, also known as porogens, upon thawing. This macroporous scaffold provides an undisturbed and continuous supply of growth factors, nutrients, gases, and liquids to the regenerative tissue (97–99). These cryogelation synthesized scaffolds provide an interconnected pore (200 mm) for the supply of essential factors needed for cell proliferation.

Cryogels can be synthesized from either monomeric or polymeric precursors that are uniformly dissolved in a solvent system and later subjected to a frozen environment for cryoconcentration of these precursors in an unfrozen liquid phase (100). Water is the best choice in the list of solvents due to its biocompatibility and environmental friendliness. Moreover, the application of water limits the choice to water-soluble compounds only, thus resulting in hydrophilic cryogels. It is also suggested by previously published studies that hydrophilic cryogels can be prepared by using organic solvents such as dimethylsulphoxide, dioxane, nitrobenzene, formamide, benzene, and cyclohexane (101–103). The cryogels synthesis can be performed by: (i) covalent chemical cross-linking of polymeric materials and free radicals polymerization; and (ii) non-covalently by cryogelation method, through the non-covalent structure and entrapment.

Gels and cryogels

The gels referred to as polymeric macromolecules are immobilized and form a three-dimensional network with non-fluctuating bonds. The method of gel preparation and the nature of the bonds contribute to the morphology of the gel creation along with the chemical structure of the polymers. Solvent immobilization within a three-dimensional polymer system plays an important role as it does not allow the polymer to be compact, and hence avoids the collapse of the gel system. Depending upon the nature of the intermolecular network of bonds, the gels can be classified into two categories; (i) chemical gels and (ii) physical gels (details not included). Once the chemically reactive groups in the gel matrices are exhausted, the reaction halts, leaving behind the solid gel with interconnected polymeric materials. Considering the biomedical application of cryogels, they have to be considered a candidate of increasing concern. The cryogels are formed by the cryotropic gelation process that produces the polymeric materials with the desired morphology when compared to non-frozen gels. Cryogels are formed at a sub-zero temperature that allows the freezing of solvent molecules (free from the solute part). Ice formation in water occurs when it is exposed to below-freezing temperatures. During the formation of ice crystals, only the water part remains in the ice lattice, expelling all solutes out of the crystals (104). The formed ice crystal acts as porogen leading to the formation of a supermacroporous cryogel (105). Cryogels could be of covalent, ionic, or non-covalent types. In the cryogel system, solvent freezing followed by the sublimation of solvent crystals (in an aqueous system), forms an interconnected porous network of polymeric biomaterials. Moreover, no gel formation occurs in the unfrozen microphase. The cryogels can be modified according to the desired characteristics and their use.

Statistical trend of cryogel research

This section demonstrates the current state of cryogels-related research and its statistical perspectives. Initially, cryogels were studied by a few research groups, but in the latter part of the 20th century, the advancement in cryogel applications made them famous, and more research groups were attracted to them. In the beginning, cryogels applications rose exponentially due to their wide suitability in the biomedical field. As time passes, cryogels have attracted more research groups to explore their characteristics for the welfare of human mankind. More than 40% of cryogels-based research describes in detail that it can be used in separation and molecular imprinting due to its macroporous behavior. This especial feature of cryogels attracted more research groups (106, 107).

Application of cryogels in the neural regeneration domain

Macroporous materials have attracted a lot of attention and also have been employed in wide applications in the field of biomedicine and biotechnology. A large pore size of 10-200μm, makes a cryogel suitable for transporting red blood cells (RBCs) of ~7 μm *via* liquid flow through a monolithic cryogel column (108). Human acute myeloid leukemia KG-1 cells expressing the CD34 possessing surface antigen and human blood lymphocyte fractions were assessed by using polyvinyl alcohol beads and dimethylacetamide (DMAAm) monolithic cryogel columns immobilized on protein A. This setup showed approximately 76% retention of cells (108). Furthermore, the application of cryogels in biomedical and biotechnology has been discussed in detail by previously published studies (108, 109). This review is especially focused on the use of cryogels in nerve cell regeneration and the management of peripheral diabetic neuropathy. Treatment of injured neuronal components in the peripheral nervous system needs new medical therapies. The regenerative ability of PNS can be exploited with cryogels to successfully treat nerve injuries or damage. Current strategies use nerve autograft, but donor site morbidity or topographical anomalies (compatibility with the damaged part) do not provide a promising solution for the repair mechanism. Alternatively, the use of nerve guidance conduits (NGCs) associated with the cryogels system provides a successful solution to promote nerve repair.

Synthetic NGCs become the superior choice for surgeons to repair injured nerves instead of traditional methods of autografts, allografts, and xenografts (110, 111). NGCs are biomaterial-based systems used for nerve repair. An absolute synthetic material may be readily formed for synthesizing NGCs containing cryogels with desired dimensions and properties. The physical and chemical make-over of the cryogel affects the

outcome of nerve regeneration and repair. Neurorrhaphy alone is not suitable for recovery of normal sensory and motor functions despite modern aids in microsurgical techniques. Previously published literature showed that only 50% of patients regain useful normal function post neurorrhaphy (112). Furthermore, additional additive factors are required for normal recovery in these cases. Cryogel conduits were implemented in previous studies to regenerate the peripheral nerve and supporting glial cells (113, 114). A wide range of natural and synthetic ingredients such as PLA (115), chitosan (116), and gelatin (117) have been implicated in the synthesis of cryogel nerve conduits. One of the studies conducted by Ju-Ying Chang et al. successfully demonstrated the role of the RDC/NHS-fixed gelatin nerve conduit in filling a large gap in the sciatic nerve of a rat model (117). Designing a 3D printed nerve conduit capable of delivering the essential nerve growth factors (NGFs) would serve as a promising approach for promoting the function of the sciatic nerve followed by post-end-to-end neurorrhaphy (118).

Improving make-over of next-generation cryogels in nerve regeneration

Advancement in technology improves the make-over of the cryogel. It updated cryogels with modern properties to serve better fulfilling its goal. Research strategies for improving nerve repair can be placed in two broad categories: (i) a methodology to improve axonal regeneration and (ii) a methodology to decrease immune responses (i.e inflammation). Furthermore, methods for enhancing the axonal regeneration include (a) axonal sprouting from the distal nerve stump (electrical stimulation and growth factors of proximal stump); (b) permissible environment for exchangers and nutrients essential for growth (enhanced cryogels pores, surface topography); (c) delaying of Wallerian degeneration and (d) shortening of period for denervation of muscles.

Nerve growth factors (NGFs) are spontaneously naturally releasing components during the process of nerve regeneration. NGFs release from nerve endings if a nerve has the effect of altering growth, differentiation, and surveillance (119). NGFs are present in a low concentration in healthy nerves, but during injury, their concentration rises in the distal stump of the damaged nerve and plays a vital role in the survival of the sensory neurons (119). Moreover, other factors for nerve regeneration include the Glial growth factor (GGF), glial cell-derived neurotrophic factor (GDNF), fibroblast growth factors (FGF), neurotrophin 3 (NT-3), and others (119). The factors are fixed with the cryogel conduit system to repair nerve gap injury (1-4cm). The delivery of these factors has been shown to hasten the recovery of injured nerves compared to a conduit alone without these factors (119). However, a previous study also reported a superior outcome in nerve autograft compared to NGFs seeded conduits (120). The future use of NGFs with cryogel having sustained release and other functional characteristics may be targeted towards nerve regeneration after injury or in PDN.

The prime role of the nerve is to transfer electrical signals (conduction) for creating coordination between the brain and the body. In this area, it is necessary to impart proper nerve stimulation to the injured nerve to restore the function of electrical conductivity. Limited research has been done on the implementation of the electrical fields/gradients across the peripheral nerve system for accelerating axonal regeneration. Studies performed on animals showed that post-repair electrical stimulation for one hour continuously promotes the function of target muscle reinnervation (121). Moreover, a clinical trial showed that a continuous one-hour electrical stimulation accelerates axonal regeneration after median nerve decompression in patients with tunnel syndrome and thenar atrophy (122). Conducting cryogels provides a promising tool for nerve regeneration during injury and PDN. Another study published on conducting cryogel scaffolds showed enhanced proliferation and growth of excitable cells like neuro 2a and C2C12 cells when stimulated at 100mV for 2h (123).

Besides the electrical conduction and neurotrophic arrangement, the topography also contributes to nerve regeneration. To improve the biological performance of the cryogels, numerous additive features have to be added to cryogels like topographical guidance, neurotrophic activity, and electrical activity. A previously published study showed that topography plays an essential role in nerve regeneration and repair (124).

The polymers chosen have already shown potential in the field of neural regeneration when used for 3-D scaffold synthesis in various techniques. In improving cryogels for nerve regeneration, molecular imprinting in the 3D domain imparts promising results. Molecular imprinted polymeric cryogels (MIPC) are tailor-made to offer high molecular recognition with satisfactory performances compared to non-imprinted entities. The synthesis of molecularly imprinted polymers can be done using a template-directed radical polymerization reaction. The target molecule itself acts as a molecular template, forming molecular imprints. The print molecule formed is mixed thoroughly with some functional monomers, followed by the addition of further monomers and crosslinkers preceding the polymerization reaction. The post-polymerization reaction yields the imprinted molecule after cumbersome extraction. The only associated disadvantage with molecularly imprinted polymers is compact and tight polymeric material with low pore size. Thus, a composite MIPC offers an attractive package for tissue engineering. Denizli and their co-workers published literature on composite cryogel with embedded beads of poly hydroxyethyl methacrylate formatted MIPC (125). MIPC showed the binding of human serum albumin (HSA) more

efficiently following the Langmuir isotherm (125). Another reputable study showed that 3D printing technology in the synthesis of cryogels conduits for nerve regeneration showed promising results and may be used for clinical purposes (126). Another showed the role of 3D-printed biodegradable conduits in neurorrhaphy (127). The whole section discussed above concluded that cryogel with some additive features may serve a better role in nerve regeneration.

Current therapeutic approaches to managing PDN

Prevention and management of peripheral diabetic neuropathies focus on strict glucose control and lifestyle modifications. The extensive physical, psychological, and economic burden of diabetic neuropathies underscores the urgency of casual therapies. Below are the current strategies to manage neuropathies. The management of PDN includes conventional (metabolic control and life style modifications) and recently developed methods (cytoprotective therapies and stem cell therapies).

Metabolic control

Strict glucose control in subjects with type 1 diabetes dramatically reduces the episodes of distal symmetric polyneuropathy (DSPN) by 78% (relative risk reduction) (128, 129). Contrastingly, there is a 5-9% relative risk reduction of DSPN in type 2 diabetes (130, 131). In one small trial performed on a Japanese population with type 2 diabetes, it was found that intensive insulin treatment was related to the improvement in DSPN (132). An Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial on type 2 diabetes subjects also showed a mild reduction in DSPN episodes (131). The specific glucose control approach also had some discrepancies associated with it. For example, in a Bypass Angioplasty Revascularization Investigation in Type 2 Diabetes (BARI 2D) trial male patients treated with insulin sensitizers had lower episodes of DSPN over 4 years compared to those who received sulfonylurea/insulin (133). Although, there are no randomized controlled trials of intensive insulin therapy in PDN management. Moreover, the data available showed that strict glycemic control is of greatest use in the management of PDN.

Lifestyle modification

The appropriate model for the management of PDN includes intensive lifestyle interventions as suggested by American Diabetes Association guidelines (11). The programs launched for lifestyle interventions are the Diabetes Prevention Program (DPP), the Steno-2 Study (27), the Italian supervised treadmill study, and the University of Utah type 2 diabetes study (11). All these studies suggested that lifestyle modification can reduce the incidences of PDN/DSPN. In a previously published study, it is suggested that nerve fiber regeneration occurs prominently in Type 2 diabetes mellitus patients engaged in exercise (11). This conventional approach has had a large effect on the reduction of DSPN incidences.

Cytoprotective therapies

a. Lowering cell apoptosis

There is debate on whether a neuronal loss in PDN occurs. If it is happening, then there is debate whether the cells are undergoing death/apoptosis, nonapoptotic programmed cell death, or necrosis. Previously, in-vitro studies performed on rodents and cell culture models of developed neuropathy demonstrated the phenomenon of apoptosis (134–138). Mitochondria play an important role in neuronal injury that results in the depletion of dorsal root ganglion neurons (139). In diabetic neuropathy models, the activation of caspase 3, a component of the apoptotic pathway, contributes to this mechanism (140)

b. Poly ADP-ribose polymerase inhibition

Oxidative DNA damage induces the release of a PARP factor. This enzyme is involved in DNA repair by the addition of poly (ADP-ribose) subunits to DNA strand breaks and base-excision pathway (140). It is evident from previous studies that the inhibition of PARP may delay the progression of diabetic neuropathy by inhibiting the PARP-mediated depletion of NAD + and ATP (141, 142).

c. Mesenchymal stem cells therapies

Current therapies for improving glucose control include the exogenous insulin supplement, which often produces the peaks of hypoglycemic episodes, as well as pancreatic islet replacement (143). The first pancreatic islet transplantation results were promising in that they decreased the long-term side effects and imparted better glycemic control (144). However, the two limitations that exist with this method for inhibiting the use in clinical trials are (i) the high demand for pancreatic islets for effective transplantation and (ii) the short-term life of transplanted islets (144). The study also stated that at least 12,000-16000 islets/Kg are required for achieving good glycemic control in each patient, suggesting the use of more

than one cadaveric donor (144). The use of immune-suppressive drugs prolonged the life of transplanted islets and their associated severe complications (145). In one of the published studies, the outcome of pancreatic islet transplantation can be improved by using Mesenchymal Stem Cells (MSCs) thus limiting transplant rejection (146). Moreover, MSCs have the potential to increase cell survival, including neurons, and also to release trophic factors essential for nerve regeneration (147, 148). In one of the studies, it is shown that MSCs, along with pancreatic islet transplantation, improve glycemic control and neuropathic symptoms such as NCV. This might be due to the release of trophic angiogenetic factors (149).

Cryogels in diabetic peripheral neuropathies: A future hope

There has been a breakthrough expansion in our knowledge and understanding of potential pathophysiological mechanisms, highlighting the neuropathies associated with diabetes mellitus that span the complete somatosensory peripheral nervous system initiating from afferent terminals to the sensory cortex. This has to be translated into a clear phenomenon-based approach for the treatment and management of PDN. The conventional approaches proved an effective system to manage PDN but have certain limitations. Moreover, experimental approaches developed recently also possess the potential to treat PDN but might also be associated with certain demerits, as discussed above. To overcome these limitations, a next-generation cryogel system must evolve with the potential for the most effective management and treatment of PDN. These may provide hope for regenerative medicine and tissue engineering. The advantage of cryogels use in various fields has already been discussed. Due to their vast application, these may be easily implemented in the treatment and management of peripheral diabetic neuropathy. The conducting cryogels can especially be used in the regeneration of damaged nerves due to injury. Peripheral diabetic neuropathy (PDN) is associated with nerve injuries or the altered behavior of peripheral nerves. Currently, conducting devices, including pacemakers, electrodes for stimulating the brain, stimulators of the spinal cord, etc. are in commercial use (150). In a previous study, it is demonstrated that polypyrrole-based cryogels scaffolds (conduits) implanted subcutaneously in rats reveal less immune cell infiltration as compared to FDA-approved poly lactic-co-glycolic acid. In another study, no inflammatory response was observed in polypyrrole-based tissue implantation (151). Similarly, polyaniline-based tissue implantation showed a low level of inflammation (151). The conducting-cryogel-based scaffold loaded with desired growth factors may prove an excellent candidate for nerve regeneration damage in PDN. Diabetic foot ulcer-initiated wounds (DFU), a severe complication associated with PDN, can be effectively solved (152–155). In summary, given the variety of mechanisms, which may overlap, a prime future goal is to better the understanding of conducting cryogels with a 3D system in clinical trials. There is now tentative evidence in the literature that supports the benefits of 3D-printed conducting cryogels (121). Moreover, these conducting cryogels may be changed according to the desired environment to treat the PDN. Conducting cryogels need to be tested in clinical trials, the outcome may be the eradication and prevention of PDN.

Conclusion

In summary, PDN research has evolved to study deregulated pathways such as the polyol pathway, PKC, AGEs, and oxidative stress to herald a new era of insulin physiology. The disease is increasingly prevalent due to the absence of effective treatment. Biomaterials can be implemented with desired characteristics to overcome the problem of nerve regeneration in PDN and injury. Cryogel is a priority candidate in the management of PDN. Successful implantations with cryogel scaffolds loaded with growth factors and suitable topography may prove a landmark approach for the treatment of PDN beyond other conventional approaches used. The next-generation cryogel will be an effective approach to this complication.

Author contributions

Conceptualization, AR, G-BJ, MS, RG and SA. Methodology, AR and G-BJ. Software, AR. Validation, G-BJ, SA, MS, RG, SA and SK. Formal analysis, G-BJ, SA, MS, RG and SK. Investigation, AR. Resources, SK. Data curation, AR and MS. Writing—original draft preparation, AR. Writing—review and editing, MS, RG, SA and G-BJ. Visualization, SA, MS, RG and SK. Supervision, SA, MS, RG, G-BJ and SK. project Administration, SK. All data were generated in-house, and no paper mill was used. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The prescribing pattern of sodium-glucose cotransporter-2 inhibitors and glucagon-like peptide-1 receptor agonists in patient with type two diabetes mellitus: A two-center retrospective cross-sectional study

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Background: The use of sodium-glucose cotransporter-2 inhibitors (SGLT2i) and glucagon-like peptide-1 receptor agonists (GLP-1 RA) in patients with type 2 diabetes mellitus (T2DM) remains limited, especially in those with other compelling indications. Thus, this study aimed to describe the prescribing patterns of GLP-1-RA and SGLT2i in patients with T2DM and to determine the factors that affect the prescribing of these medications.

Methods: This multicenter retrospective cross-sectional study reviewed the electronic health records of adult patients diagnosed with T2DM who received care between January and December 2020. The patients were classified according to their compelling indications into "patients who are more likely" to benefit from SGLT2i or GLP-1 RA and "patients who are less likely" to benefit from them. They were then further categorized depending on whether these medications were prescribed.

Results: A total of 1,220 patients were included; most were female (56.9%). SGLT2i or GLP-1 RA were preferably prescribed in only 19% of the patients for reasons including BMI $\geq$ 27 kg/m^2 (85.6%), uncontrolled T2DM (68.5%), high risk for ASCVD (23.9%), or established ASCVD (14%). The remaining 81.0% were

underprescribed these agents. Patients at an older age or with a history of stroke or transient ischemic attack had higher odds of being underprescribed (OR 1.02; 95% CI: 1.01–1.03 and OR 2.86; 95% CI: 1.33–6.15), respectively.

Conclusion: The results concur with those of previous studies highlighting the underutilization of GLP-1 RA and SGLT2i in patients with T2DM but also with compelling indications. To optimize the use of GLP-1 RA and SGLT2i for their additional benefits, prescribers need to assess the benefits of using these agents in patients who would likely benefit from them, regardless of DM control.

KEYWORDS

type 2 diabetes, underprescription, sodium-glucose cotransporter-2 inhibitors, glucagon-like peptide-1 receptor agonists, comorbidities

Background

Sodium-glucose cotransporter-2 inhibitors (SGLT2i) and glucagon-like peptide-1 receptor agonists (GLP-1 RA) are among the emerging classes of antidiabetic medications for the management of type 2 diabetes mellitus (T2DM). Several trials have demonstrated that SGLT2i and GLP-1 RA can reduce major adverse cardiovascular events (MACE), death from cardiovascular diseases (CVD), and heart failure (HF) hospitalization, as well as delay the progression of chronic kidney disease (CKD) in patients at high risk of or with established atherosclerotic cardiovascular disease (ASCVD) (1–8). Thus, the treatment decision for patients with T2DM should follow a patient-centered approach based on the presence of CVD and renal comorbidities, the agents' efficacy and safety, and the patient's preference (9, 10).

Since SGLT2i and GLP-1 RA exhibit cardiovascular, renal, and weight-reduction benefits independent of their blood-glucose-lowering effect (10), recent diabetes guidelines recommend using these medication classes for T2DM patients with established ASCVD, HF, or CKD, or for those at high risk of ASCVD (11, 12). Moreover, the Kidney Disease: Improving Global Outcomes (KDIGO) Foundation and the American Diabetes Association (ADA) recommend first-line treatment with metformin or SGLT2i in patients with CKD and T2DM (13, 14). The updated American Heart Association and the European Society of Cardiology guidelines recommend using SGLT2i in patients with HF with or without T2DM (15, 16). In addition, due to the significant weight-reduction effect of SGLT2i and GLP-1 RA, these agents should be considered for individuals with T2DM who are overweight or obese (9, 17).

Despite the proven cardiorenal benefits and weight-reduction effects of SGLT2i and GLP-1 RA in patients with T2DM, along with the guidelines that recommend their use, their application in practice remains limited (18–20). Several studies have reported that the rate of using SGLT2i or GLP-1 RA among patients with T2DM and at high risk of or with established ASCVD or CVD ranges from 4.1 to 19% (18, 20, 21). The underutilization of these medications may be attributed to the older age of these patients, as well as the increased number of comorbidities, costs, and formulary barriers (18, 22). Other factors that limit the use of SGLT2i and GLP-1 RA may be related to the prescriber's specialty, concerns about the safety of these medications, or the need for prior authorization (19, 23).

Most previous studies have examined the underutilization of SGLT2i and GLP-1 RA in T2DM patients with established CVD or ASCVD (18, 21, 24). However, there are limited data on the effective prescribing of SGLT2i and GLP-1 RA in patients with T2DM and other compelling indications who are more likely to benefit from using these medications. Thus, this study aimed to describe the prescribing patterns of GLP-1 RA and SGLT2i in patients with T2DM.

Abbreviations: DM, Diabetes mellitus; SA, Saudi Arabia; ASCVD, atherosclerotic cardiovascular disease; T2DM, type two diabetes mellitus; SGLT2i, sodium-glucose cotransporter-2 inhibitors; GLP-1 RA, glucagon-like peptide-1 receptor agonists; CHD, coronary heart disease; PAD, peripheral artery disease; HF, heart failure; MACE, major adverse cardiac events; CV, cardiovascular; CVD, Cardiovascular disease; BMI, body mass index; PMH, past medical history; HFrEF, heart failure with a reduced ejection fraction; TIA, transient ischemic attack; IM, internal medicine; ADA, American Diabetes Association.

Methods

Study design and patients

This multicenter retrospective cross-sectional study was conducted in two centers in Riyadh, Saudi Arabia: a secondary care hospital, King Abdullah bin Abdulaziz University Hospital, and a tertiary care center, King Abdulaziz Medical City. We included patients with T2DM who were 18 years of age or older

and who had received care in one of the two centers between January 1, 2020, and December 31, 2020. We excluded patients with other types of DM (type 1 DM or gestational DM). This study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional review board at Princess Nourah bint Abdulrahman University (log number: 21–0291) and King Abdullah International Medical Research Center (Ref. #SP20/477/R). The patients' informed consent was not required because all the data were collected from their electronic medical records after de-identification.

According to the ADA patient-centered approach to using SGLT2i or GLP-1 RA for patients with T2DM (25), the patients were split into two major groups. The first group consisted of "patients who were more likely to benefit from SGLT2i or GLP-1 RA" and the second group, "patients who were less likely to benefit from SGLT2i or GLP-1 RA." The preference criteria for using SGLT2i or GLP-1 RA were based on the ADA recommendations and are detailed in Table 1 (25). Patients who were more likely to benefit from these agents and were prescribed them were categorized as *preferably prescribed*. In contrast, patients who were more likely to benefit from either GLP-1 RA or SGLT-i but were not prescribed these medications were categorized as *underprescribed*. Patients who were less likely to benefit from GLP-1 RA or SGLT2i and were not prescribed these medications were categorized as *preferably not prescribed*, but if they were prescribed them, then they were categorized as *prescribed but unpreferred*.

TABLE 1 The definition of SGLT2i and GLP-1 RA preference criteria based on the ADA recommendations (25).

Patients' criteria to likely benefit from the use of either SGLT2i or GLP-1 RA	**SGLT2i for patients with T2DM and one of the following:** 1–Chronic kidney disease. 2–Established ASCVD. 3–High-risk of ASCVD; defined as having a 10-year ASCVD risk score ≥20%, having stenosis, or left ventricular hypertrophy. 4–BMI ≥ 27 Kg/m^2. 5–HFrEF (<EF <45%). 6–Patient is on two other antidiabetic medications*. **GLP-1 RA for patients with T2DM and one of the following:** 1–Chronic kidney disease. 2–Established ASCVD. 3–High-risk of ASCVD; defined as having a 10-year ASCVD risk score ≥20%, having stenosis, or left ventricular hypertrophy. 4–BMI ≥ 27 Kg/m^2. 5–Patient is on insulin. 6–Patient is on two other antidiabetic medications*.

SGLT2i, Sodium–glucose Cotransporter−2 Inhibitors; GLP−1 RA, Glucagon–like Peptide−1 Receptor Agonists; ASCVD, Atherosclerotic Cardiovascular Disease; T2DM, Type 2 diabetes mellitus, BMI, body mass index, HFrEF, Heart failure with reduced ejection fraction; Stenosis, Diagnosis of stenosis of the carotid, coronary, or lower extremity with > 50%. *This is used to indicate the patient has uncontrolled T2DM.

Data collection and study outcomes

The data were collected from the patients' electronic medical records and included their demographic information and past medical history (PMH) during clinic visits or upon hospital admission. In addition, information about SGLT2i and GLP-1 RA, doses, and prescribers was gathered. The primary outcome was the proportion of patients who were or were not prescribed GLP-1 RA or SGLT2i as the preferred approach according to ADA standards (25). The secondary outcomes were factors associated with the underprescribing of GLP-1 RA or SGLT2i and the distribution of prescriber specialties.

Statistical analysis

Descriptive statistics, which included means with standard deviations for continuous variables and frequencies with percentages for categorical variables, were used to summarize the study variables. The unpaired *t*-test for continuous variables and a chi-squared test for categorical variables were used to make an unadjusted comparison between the *preferably prescribed* and the *underprescribed* groups. Multivariable logistic regression analysis was used to identify the patients' characteristics associated with underprescribing SGLT2i and GLP-1 RA. The data were collected from the patients' electronic medical records using the Research Electronic Data Capture (REDCap®) software, version 7.3.6, and then analyzed using the SAS® software, version 9.4 (SAS Institute Inc., Cary, NC).

Results

Patients' clinical characteristics

The study included 1,220 patients from the two centers. The overall mean age was 59.3 ± 13.2 years, and the majority of the patients were female (56.9%). The most common PMH in our patients was dyslipidemia (69.0%), followed by hypertension (67.6%), as shown in Table 2. According to the ADA patient-centered criteria listed in Table 1; 1,167 patients were categorized as "more likely to benefit from SGLT2i or GLP-1 RA," while the remaining 53 patients were classified as less likely to benefit from either SGLT2i or GLP-1 RA (Table 2).

In the patient group, which included those who were less likely to benefit from either SGLT2i or GLP-1 RA, the commonly observed PMHs were dyslipidemia and hypertension (56.9% and 37.3%), respectively. The mean age at 54.1 ± 14.4 years and the mean BMI of 24.4 ± 2.4 kg/m^2 was much lower than those of

TABLE 2 Patients' baseline characteristics ($n = 1220$).

Characteristics	Overall	Patients more likely to benefit from the use of either SGLT2i or GLP−1 RA ($n = 1167$)		Patients less likely to benefit from the use of either SGLT2i or GLP−1 RA ($n = 53$)*
		Preferably prescribed	Underprescribed	Preferably not prescribed
	$n = 1220$	($n = 222$)	($n = 945$)	($n = 51$)
Age in years	59.3 ± 13.2	57.3 ± 11.4	60.0 ± 13.3	54.1 ± 14.4
Sex, Female	694 (56.9)	139 (62.6)	523 (55.3)	31 (60.8)
BMI (kg/m2)	31.9 ± 6.5	34.2 ± 6.9	31.7 ± 6.3	24.4 ± 2.4
Smoker or former smoker	93 (7.6)	18 (8.1)	70 (7.4)	5 (9.8)
Family history of T2DM	81 (6.8)	13 (5.9)	62 (6.8)	6 (12.2)
Past medical history				
Hypertension	825 (67.6)	144 (64.9)	661 (69.9)	19 (37.3)
Dyslipidemia	842 (69.0)	178 (80.2)	634 (67.1)	29 (56.9)
Hypothyroidism	161 (13.2)	36 (16.2)	115 (12.2)	9 (17.6)
Hyperthyroidism	13 (1.1)	2 (0.9)	11 (1.2)	0 (0.0)
Retinopathy	57 (4.7)	25 (11.3)	31 (3.3)	1 (2.0)
Neuropathy	35 (2.9)	14 (6.3)	20 (2.1)	1 (2.0)
ASCVD 10–year score category				
Low risk (<5%)	188 (24.1)	56 (33.5)	124 (21.3)	8 (26.7)
Borderline risk (5–7.5%)	103 (13.2)	20 (12.0)	76 (13.1)	6 (20.0)
Intermediate risk (≥7.5–<20%)	250 (32.1)	51 (30.5)	183 (31.5)	16 (53.3)
High risk (≥20%)	238 (30.6)	40 (24.0)	198 (34.1)	0 (0.0)

Data are presented as frequency (%) or mean ± SD. p–values are from the Chi–square test for categorical variables and the t–test for continuous variables.
SGLT2i, Sodium–glucose Cotransporter−2 Inhibitors; GLP−1 RA, Glucagon–like Peptide−1 Receptor Agonists; ASCVD, Atherosclerotic Cardiovascular Disease; T2DM, Type 2 diabetes mellitus; SD, Standard Deviation.
ASCVD includes stroke, transient ischemic attack, acute coronary syndromes (ACSs), a history of myocardial infarction, stable or unstable angina, coronary or other arterial revascularization, or peripheral arterial disease (PAD) presumed to be of atherosclerotic origin.
*Two patients received GLP−1 RA or SGLT2i while not being preferred to be prescribed these medications.

the patient group who were more likely to benefit from SGLT2i or GLP-1 RA. In the patients who were less likely to benefit from SGLT2i or GLP-1 RA, more than half had an intermediate risk of ASCVD (53.3%), while 26.7% had a low risk of ASCVD, as shown in Table 2.

The characteristics of the patients who were more likely to benefit from SGLT2i or GLP-1 RA were mostly balanced between patients who received SGLT2i or GLP-1 RA and those who did not, except for the number of female patients and the presence of dyslipidemia, retinopathy, and neuropathy, which were significantly higher among patients who were preferably prescribed SGLT2i or GLP-1 RA, as shown in Table 3. In contrast, the mean age was significantly higher among patients who were underprescribed SGLT2i or GLP-1 RA compared to those prescribed these agents (60.0 ± 13.3 vs. 57.3 ± 11.4 years, $p = 0.002$). In the underprescribed group, significantly more patients had established ASCVD, including stroke/ transient ischemic attack (TIA), compared to the preferably prescribed group (20.7% vs. 14.0%, $p = 0.0217$, 10.6% vs. 4.1%, $p = 0.0026$, respectively; Table 3).

SGLT2i or GLP-1 RA prescribing patterns

Among patients who were more likely to benefit from SGL2i or GLP-1 RA ($n = 1167$), only 19.0% ($n = 222$) received SGLT2i or GLP-1 RA, while the remaining 81.0% did not receive either. In comparison, of the patients who were less likely to benefit from either SGLT2i or GLP-1 RA ($n = 53$), only two received SGLT2i or GLP-1 RA. The main reasons for using SGLT2i or GLP-1 RA in the patients who were preferably prescribed these agents were obesity (BMI ≥ 27 kg/m^2 and T2DM) at 85.6%, uncontrolled DM indicated by using two or more other antidiabetic agents

TABLE 3 Factors associated with the under–prescribing of SGLT2i or GLP–1 RA for patients with type 2 diabetes mellitus ($N = 1167$).

Factors	Overall ($n = 1167$)	Preferably prescribed ($n = 222$)	Underprescribed ($n = 945$)	Unadjusted analysis p–value	Adjusted analysis AOR (95%CI)
Age in years	59.5 ± 13.0	57.3 ± 11.4	60.0 ± 13.3	0.0020	1.02 (1.01 –1.03)
Sex, female	662 (56.7)	139 (62.6)	523 (55.3)	0.0492	0.78 (0.56 –1.08)
Smoker or former smoker	88 (7.5)	18 (8.1)	70 (7.4)	0.7220	––
Family history of T2DM	75 (6.6)	13 (5.9)	62 (6.8)	0.6613	––
Past medical history					
Hypertension	805 (69.0)	144 (64.9)	661 (69.9)	0.1407	––
Dyslipidemia	812 (69.6)	178 (80.2)	634 (67.1)	0.0001	0.45 (0.31 –0.66)
Hypothyroidism	151 (12.9)	36 (16.2)	115 (12.2)	0.1060	––
Hyperthyroidism	13 (1.1)	2 (0.9)	11 (1.2)	0.7368	––
Retinopathy	56 (4.8)	25 (11.3)	31 (3.3)	<0.0001	0.36 (0.19 –0.67)
Neuropathy	34 (2.9)	14 (6.3)	20 (2.1)	0.0008	0.47 (0.21 –1.08)
Patients' criteria to be prescribed SGLT2i or GLP–1 RA					
Chronic kidney disease	106 (9.1)	17 (7.7)	89 (9.4)	0.4115	––
HFrEF (EF <45%)	37 (3.2)	7 (3.2)	30 (3.2)	0.9869	––
Established ASCVD	227 (19.5)	31 (14.0)	196 (20.7)	0.0217	––
Myocardial infarction	83 (7.1)	11 (5.0)	72 (7.6)	0.1636	1.86 (0.85 –4.07)
Stable angina	10 (0.9)	1 (0.5)	9 (1.0)	0.4646	––
Unstable angina	18 (1.5)	2 (0.9)	16 (1.7)	0.3879	––
Stroke/TIA	109 (9.3)	9 (4.1)	100 (10.6)	0.0026	2.86 (1.33 –6.15)
PAD	6 (0.5)	0 (0.0)	6 (0.6)	0.2339	––
Revascularization, including stent or CABG	96 (8.2)	18 (8.1)	78 (8.3)	0.9433	0.53 (0.27 –1.04)
Indicators of high risk					
ASCVD 10–year risk score ≥20%	238 (20.4)	40 (18.0)	198 (21.0)	0.3288	––
Stenosis	51 (4.4)	9 (4.1)	42 (4.4)	0.7956	––
Left ventricular hypertrophy	68 (5.8)	7 (3.2)	61 (6.5)	0.0588	2.09 (0.91 –4.80)
Obesity (BMI ≥ 27 Kg/m2)	933 (79.9)	190 (85.6)	743 (78.6)	0.0405	0.70 (0.45 –1.09)
Patient is currently on					
Two other antidiabetic medications	753 (64.5)	152 (68.5)	601 (63.6)	0.1723	––
Insulin	503 (43.1)	122 (55.0)	381 (40.3)	<0.0001	0.54 (0.40 –0.75)

Data are presented as frequency (%) or mean ± SD. p–values are from the Chi–square test for categorical variables and the t–test for continuous variables.
SGLT2i, Sodium–glucose Cotransporter–2 Inhibitors; GLP–1 RA, Glucagon–like Peptide–1 Receptor Agonists; ASCVD, Atherosclerotic Cardiovascular Disease; T2DM, Type 2 diabetes mellitus; HFrEF, Heart failure with reduced ejection fraction; SD, Standard Deviation; AOR, Adjusted odds ratio.
ASCVD: stroke, transient ischemic attack, acute coronary syndromes (ACSs), a history of myocardial infarction, stable or unstable angina, coronary or other arterial revascularization, or peripheral arterial disease presumed to be of atherosclerotic origin.

(68.5%), the presence of high risk for ASCVD (23.9%), and established ASCVD (14%), as presented in Table 4. Moreover, patients with established HF or CKD were 3.2% and 7.7%, respectively, in the preferably prescribed group. In contrast, in the underprescribed group, the most common compelling indications for the use of SGLT2i or GLP-1 RA were obesity (78.6%), uncontrolled DM (63.6%), and a high risk for ASCVD (29.5%), followed by 20.7% having established ASCVD (Table 4). Also, 3.2% and 9.4% of the underprescribed group had HF and CKD, respectively.

In the patients who received SGLT2i or GLP-1 RA for preferred reasons, 58.2% were prescribed SGLT2i, and 51.8% were prescribed GLP-1 RA. It is worth noting that 22 patients received both SGLT2i and GLP-1 RA. Dapagliflozin (90.7%) and liraglutide (71.4%) were the most commonly prescribed SGLT2i and GLP-1 RA, respectively (Table 5). In addition, the most commonly prescribed antidiabetic medication, along with SGLT2i or GLP-1 RA, was metformin (86%), followed by insulin (55%). The additional antidiabetic agents prescribed with SGLT2i or GLP-1 RA are detailed in Table 5.

TABLE 4 Patient–centered preference criteria for using either SGLT2i or GLP–1 RA ($n = 1167$).

Patient criteria	Overall $n = 1167$	Preferably prescribed ($n = 222$)	Underprescribed ($n = 945$)
Chronic kidney disease	106 (9.1)	17 (7.7)	89 (9.4)
HFrEF (EF <45%)	37 (3.2)	7 (3.2)	30 (3.2)
Established ASCVD	227 (19.5)	31 (14.0)	196 (20.7)
Myocardial infarction	83 (7.1)	11 (5.0)	72 (7.6)
Stable angina	10 (0.9)	1 (0.5)	9 (1.0)
Unstable angina	18 (1.5)	2 (0.9)	16 (1.7)
Stroke/TIA	109 (9.3)	9 (4.1)	100 (10.6)
PAD	6 (0.5)	0 (0.0)	6 (0.6)
Revascularization, including stent or CABG	96 (8.2)	18 (8.1)	78 (8.3)
Indicators of high risk	332 (28.4)	53 (23.9)	279 (29.5)
ASCVD 10–year risk score ≥20%	238 (20.4)	40 (18.0)	198 (21.0)
Stenosis	51 (4.4)	9 (4.1)	42 (4.4)
Left ventricular hypertrophy	68 (5.8)	7 (3.2)	61 (6.5)
Obesity (BMI ≥ 27 Kg/m^2)	933 (79.9)	190 (85.6)	743 (78.6)
Patient is currently on			
Two other antidiabetic medications	753 (64.5)	152 (68.5)	601 (63.6)
Insulin	503 (43.1)	122 (55.0)	381 (40.3)

Data are presented as frequency (%). p–values are from the Chi–square test.
SGLT2i, Sodium–glucose Cotransporter–2 Inhibitors; GLP–1 RA, Glucagon–like Peptide–1 Receptor Agonists; HFrEF, Heart failure with reduced ejection fraction; ASCVD, Atherosclerotic Cardiovascular Disease; TIA, transient ischemic attack; PAD, peripheral arterial disease presumed to be of atherosclerotic origin; CABG, Coronary artery bypass graft; BMI, Body mass index; Stenosis, Diagnosis of stenosis of the carotid, coronary, or lower extremity with > 50%.

Factors associated with underprescribing

Older age and a history of stroke/TIA were significantly associated with higher odds of underprescribing SGLT2i or GLP-1 RA (OR 1.02; 95% CI 1.01–1.03 and OR 2.86; 95% CI 1.33–6.15, respectively). Conversely, patients with a PMH of dyslipidemia (OR 0.45; 95% CI 0.31–0.66) or retinopathy (OR 0.36; 95% CI 0.19–0.67) had lower odds of being underprescribed either SGLT2i or GLP-1 RA. Fortunately, the odds of underprescribing were significantly lower among patients with T2DM who were receiving insulin (OR 0.54; 95% CI 0.40–0.75). The details of the logistic regression used to assess these factors are presented in Table 3.

TABLE 5 Antidiabetic medication prescribed for patients with type 2 diabetes mellitus ($N = 1167$).

Antidiabetic medication or class of medications	Overall ($n = 1167$)	Preferably prescribed ($n = 222$)	Underprescribed ($n = 945$)
SGLT2i	129 (11.1)	129 (58.1)	——
Dapagliflozin	117 (90.7)	117 (52.7)	——
Empagliflozin	12 (9.3)	12 (5.4)	——
GLP–1 RA	115 (9.8)	115 (51.8)	——
Liraglutide	81 (70.4)	81 (36.5)	——
Semglutide	33 (28.7)	33 (14.9)	——
Dulaglutide	1 (0.9)	1 (0.4)	——
Additional antidiabetic agents			
Metformin	982 (84.1)	191 (86.0)	791 (83.7)
Insulin	503 (43.1)	122 (55.0)	381 (40.3)
DPP–4 inhibitors	438 (37.5)	74 (33.3)	364 (38.5)
Sulphonylurea	381 (32.6)	67 (30.2)	314 (33.2)
Thiazolidinedione	19 (1.6)	6 (2.7)	13 (1.4)

Data are presented as frequency (%).
SGLT2i, Sodium–glucose Cotransporter–2 Inhibitors; GLP–1 RA, Glucagon–like Peptide–1 Receptor Agonists; DPP–4, Dipeptidyl peptidase–4.

Prescriber specialties

Endocrinologists were the most frequent specialty prescribing SGLT2i or GLP-1 RA (60.6%), followed by internal medicine (IM) physicians (11.4%). Cardiologists were responsible for only 9.8% of the SGLT2i or GLP-1 RA prescriptions, and nephrology for 2%. Most of the SGLT2i agents ($n = 129$) were prescribed by endocrinologists (59%), followed by IM physicians (13%) and cardiologists (10.9%). In contrast, GLP-1 RA prescriptions ($n = 115$) were also mostly prescribed by endocrinologists (62.6%), family medicine physicians (11.3%) and then IM physicians (9.6%) accounted for the other significant percentages, as depicted in Figure 1.

Discussion

This retrospective cross-sectional study evaluated the prescribing patterns of SGLT2i and GLP-1 RA in patients with T2DM. Among the patients who were more likely to benefit from SGLT2i or GLP-1 RA, only 19% were preferably

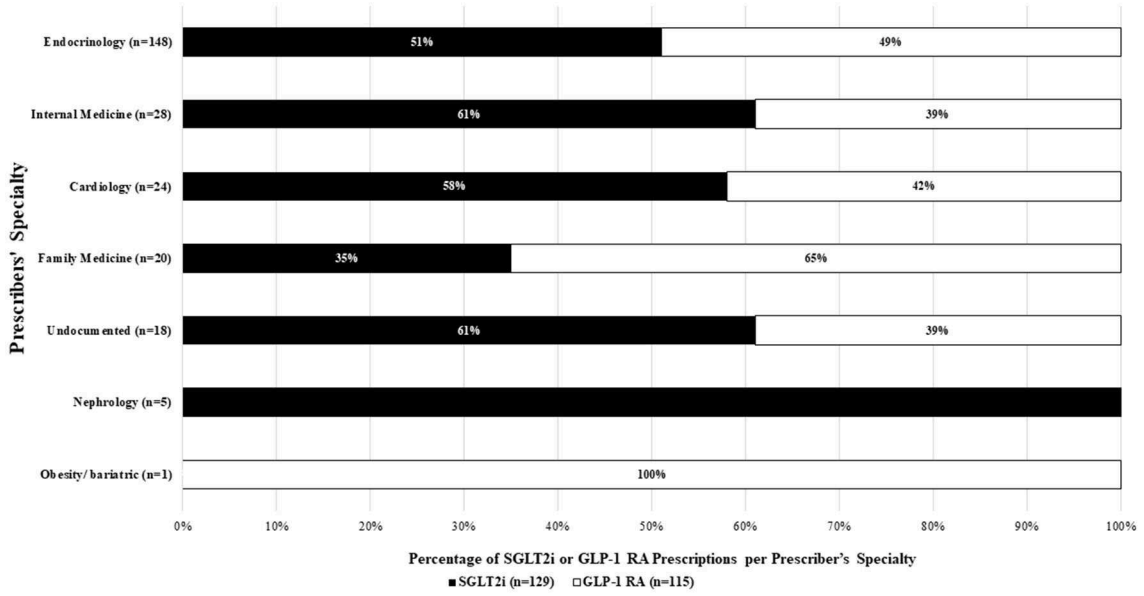


FIGURE 1
SGLT2i or GLP-1 RA prescribers' specialty ($N = 224$).

prescribed them for diabetes management or cardioprotective, renoprotective, or weight-reduction benefits. In contrast, 96% of the patients who were less likely to benefit from SGLT2i or GLP-1 RA were not prescribed these agents.

Although SGLT2i or GLP-1 RA are preferred over other antidiabetic medications for their organ protection effect in patients with T2DM (26), we still observed a low rate of prescribing SGLT2i or GLP-1 RA in patients with T2DM and established ASCVD, HF, CKD, or obesity who were likely to benefit from these medications. This low prescribing rate is similar to that found in a previous retrospective cross-sectional study that used a large set of U.S. claims data (27). That study reported that SGLT2i or GLP-1 RA was prescribed in patients with T2DM at a rate of < 12% and at an even lower rate in patients with ASCVD (< 9%) (27). It is worth noting that the data, in this case, were collected in 2015 before the first set of ADA guidelines (2017) was published, which recommended the use of SGLT2i or GLP-1 RA in patients with T2DM and ASCVD (22, 27). However, a 2020 nationwide study from U.S. Veterans Affairs, which included 537,980 patients with ASCVD and T2DM, found that the prescription of SGLT2i or GLP-1 RA remained low at 19% (21). Similarly, we found that 86% of the patients with established ASCVD and 83% of those at high risk of ASCVD (10-year score ≥ 20%) were not prescribed SGLT2i or GLP-1 RA.

In this study, we noticed a high rate (81%) of underprescribing SGLT2i or GLP-1 RA among patients who were likelier to benefit from them. Specifically, patients with increased age and a history of stroke/TIA had higher odds of being underprescribed SGLT2i or GLP-1 RA. This finding is consistent with a retrospective study that found that the mean age of patients who were not prescribed SGLT2i or GLP-1 RA was greater than that of those who had been prescribed these medications (73.0 ± 9 years vs. 69.2 ± 8 years, $p <$ 0.01 for SGLT2i; 72.8 ± 9 vs. 69.6 ± 8, $p < 0.01$ for GLP-1 RA). Moreover, they found a significantly greater proportion of patients with ischemic cerebrovascular disease among the nonusers of GLP-1 RA and SGLT2i compared to the users (27.4% vs. 23.9%, $p < 0.01$ for SGLT2i; 27.2% vs. 25.6%, $p <$ 0.01 for GLP-1 RA) (21), which is similar to what we found in our study.

The underprescription of GLP-1 RA and SGL-2i could be attributed to patient-related factors, such as a patient's preference to avoid using multiple medications for diabetes management, or the claim that they would comply with a healthy diet and regular exercise to avoid additional agents. Moreover, in the present study, the underprescribing of SGLT2i or GLP-1 RA in older patients may be attributed to lenient glycemic control goals. In addition, there is some concern about increased comorbidities, a high risk of polypharmacy and side effects such hypoglycemia specially in older adult patients. All of these reasons may prevent prescribers from initiating the use of additional agents. Another interesting observation in this study was that 91% of the patients with a history of stroke/TIA were not prescribed SGLT2i or GLP-1 RA, despite the recommendation to use these medications in patients with

established ASCVD, including cerebrovascular diseases (9). This underutilization in patients with stroke/TIA may have been influenced by the fact that most of the available evidence supports the use of SGLT2i or GLP-1 RA for primary prevention. Still, limited studies have proven their benefits for secondary stroke prevention in patients with DM (8–31).

The results also revealed that the odds of underprescribing GLP-1 RA and SGLT2i were significantly lower in patients with a history of dyslipidemia, retinopathy, or on insulin therapy. A retrospective cohort study of patients with T2DM and CVD or CV risk factors (e.g., hyperlipidemia, hypertension, obesity, smoking, or micro/macroalbuminuria) found high rates of prescribing SGLT2i (OR 3.94; 95% CI 3.90–3.99) or GLP-1 RA (OR 1.19; 95% CI 1.17–1.20) in patients at risk of CV (20). In that study, the likelihood of using SGLT2i or GLP-1 RA increased with obesity or dyslipidemia diagnosis, as in our study (20). Since high lipid levels are the main driver of ASCVD, this could be suggested as a marker for prescribers to initiate SGLT2i or GLP-1 RA in patients with established ASCVD who are at high risk for ASCVD, or even obese patients. The use of insulin therapy in patients with T2DM may indicate uncontrolled DM, since it is recommended to start with hemoglobin A1C levels above 10% (9). Insulin therapy may also be combined with GLP-1 RA to exhibit longer glycemic durability (9). However, the fear of hypoglycemia resulting from the combination of insulin with SGLT2i or GLP-1 RA may lead to the underprescription of these agents in patients using insulin.

Furthermore, this underutilization of SGLT2i and GLP-1 RA may be driven by prescriber or payor factors, such as high costs, limited prescriber awareness, and formulary restrictions (22, 31). Insurance companies may also deny coverage of these drugs and push for alternative agents (18, 22). In the present study, most of the SGLT2i and GLP-1 RA prescriptions were given by endocrinologists (60.6%), followed by IM physicians (11.4%). The prescription rates of SGLT2i and GLP-1 RA by cardiologists and nephrologists were low. At the same time, 84% of the patients with CKD, 86% with established ASCVD, and 81% with HF were not prescribed SGLT2i or GLP-1 RA. This may indicate the limited prescribers' knowledge about the effective use and the cardiovascular and renal benefits of SGLT2i and GLP-1 RA in patients with T2DM and these comorbidities. The trends in the specializations of the prescribers are similar to national and global models, where endocrinologists and primary care physicians are the most common prescribers for SGLT2i and GLP-1 RA, while cardiologists are the least common prescribers (32, 33). A cross-sectional survey in Saudi Arabia that included 103 prescribers showed that 15.5% of prescribers admitted to choosing SGLT2i as a second-line therapy, whereas 31.1% chose it as a first-line therapy for patients with CVD (34).

The behavior of underprescribing SGLT2i or GLP-1 RA is consistent with the findings of previous studies that have highlighted the issue of physicians' lack of familiarity with the effective utilization of these agents (23, 33). Since SGLT2i and GLP-1 RA are relatively new agents, providers may be reluctant to prescribe them (21, 23). Cardiologists and nephrologists, in particular, tend to be hesitant to prescribe SGLT2i or GLP-1 RA, as they view the use of antidiabetic medications to be beyond their scope (35). Moreover, they may be concerned about the adverse effects of SGLT2i, such as volume status and other safety concerns, especially in patients with CVD or CKD. The limited prescription of SGLT2i or GLP-1 RA may also be influenced by institutional limitations on the scope of SGLT2i and GLP-1 RA (21) Prescribers, including cardiologists and nephrologists, need to understand that the decision to use SGLT2i or GLP-1 RA is based on their cardiorenal protection, independent of these medications' hypoglycemic effects. SGLT2i and GLP-1 RA are currently recommended in patients, regardless of the presence of T2DM (13, 15, 16). More importantly, the latest ADA standards recommend SGLT2i or GLP-1 RA as first-line therapy in patients with an established or high risk of ASCVD, HF, or CKD (10, 14). These new recommendations emphasize the importance of prescribers assessing the advantages of using these agents in patients who would likely benefit from them, regardless of DM control.

Our study is one of several that have assessed the real-world prescribing patterns of SGLT2i and GLP-1 RA in patients with T2DM (18, 20, 21, 27). Most previous studies have focused on the rate of prescribing SGLT2i or GLP-1 RA in patients with established CVD or ASCVD (18, 21, 27). However, this study assessed all patients with T2DM, regardless of their history or risk for ASCVD, to determine if SGLT2i or GLP-1 RA are prescribed according to a personalized approach and individual comorbidities. However, the study has several limitations, such as its retrospective design and small sample size, which may limit the generalizability of its results. Specifically, the number of patients prescribed SGLT2i or GLP-1 RA was relatively small, which may have limited our ability to identify additional factors that might have affected the prescribing of these medications. Also, we cannot exclude the possibility that the use of SGLT2i or GLP-1 RA may be driven by the medications' hypersensitivity, drug-to-drug interaction, or failing other agents, which were not investigated in the present study.

Conclusions

This study concurs with previous studies highlighting the underutilization of GLP-1 RA and SGLT2i in patients with T2DM, but this time in patients with T2DM and additional compelling indications. To optimize the use of GLP-1 RA and SGLT2i for their additional benefits, prescribers need to follow the most recent guidelines in managing patients with T2DM. Thus, increasing prescribers' awareness about the appropriate use of GLP-1 RA and SGLT2i is essential. In light of the expected

increases in the use of GLP-1 RA and SGLT2i with the new recommendations, larger-scale studies are needed to investigate their effective use.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

This study was conducted following the Declaration of Helsinki and was approved by the Princess Nourah bint Abdulrahman University (PNU) Institutional Review Board (IRB) (IRB Log Number: 21–0291) and King Abdullah International Medical Research Center (Ref.#333 SP20/477/R).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ultra-widefield color fundus photography combined with high-speed ultra-widefield swept-source optical coherence tomography angiography for non-invasive detection of lesions in diabetic retinopathy

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Purpose: To compare the detection rate of diabetic retinopathy (DR) lesions and the agreement of DR severity grading using the ultra-widefield color fundus photography (UWF CFP) combined with high-speed ultra-widefield swept-source optical coherence tomography angiography (UWF SS-OCTA) or fluorescein angiography (FFA).

Methods: This prospective, observational study recruited diabetic patients who had already taken the FFA examination from November 2021 to June 2022. These patients had either no DR or any stage of DR. All participants were imaged with a 200° UWF CFP and UWF SS-OCTA using a 24 × 20 mm scan model. Images were independently evaluated for the presence or absence of DR lesions including microaneurysms (MAs), intraretinal hemorrhage (IRH), non-perfusion areas (NPAs), intraretinal microvascular abnormalities (IRMAs), venous beading (VB), neovascularization elsewhere (NVE), neovascularization of the optic disc (NVD), and vitreous or preretinal hemorrhage (VH/PRH). Agreement of DR severity grading based on UWF CFP plus UWF SS-OCTA and UWF CFP plus FFA was compared. All statistical analyses were performed using SPSS V.26.0.

Results: One hundred and fifty-three eyes of 86 participants were enrolled in the study. The combination of UWF CFP with UWF SS-OCTA showed a similar detection rate compared with UWF CFP plus FFA for all the characteristic DR lesions ($p > 0.05$), except NPAs ($p = 0.039$). Good agreement was shown for the identification of VB ($\kappa = 0.635$), and very good agreement for rest of the DR lesions between the two combination methods (κ-value ranged from 0.858

to 0.974). When comparing the grading of DR severity, very good agreement was achieved between UWF CFP plus UWF SS-OCTA and UWF CFP plusr FFA ($\kappa = 0.869$).

Conclusion: UWF CFP plus UWF SS-OCTA had a very good agreement in detecting DR lesions and determining the severity of DR compared with UWF CFP plus FFA. This modality has the potential to be used as a fast, reliable, and non-invasive method for DR screening and monitoring in the future.

KEYWORDS

ultra-widefield angiography, color fundus photography, swept-source optical coherence tomography angiography, fundus fluorescein angiography (FFA), diabetic retinopatathy

Introduction

Diabetic retinopathy (DR) is the most common complication of diabetes and the leading cause of blindness among working-age populations worldwide (1). Screening and prompt treatment of DR play an important role in blindness prevention. The Early Treatment Diabetic Retinopathy Study (ETDRS) established the current standard method for assessing the severity of DR (2, 3). It is based on seven standard retinal fields seen on stereoscopic color fundus photographs (CFPs). The small retinal field covered by conventional ETDRS seven field fundus photographs limits its ongoing clinical application.

In the past decade, retinal imaging technology has evolved rapidly. Ultra-widefield color fundus photography (UWF CFP) taken by scanning laser ophthalmoscope (SLO) could capture fundus images up to 200° and detect DR lesions located in the peripheral retina (4, 5). Previous studies have shown that UWF CFP had a substantial agreement with ETDRS 7-field photographs when determining the severity of DR (5, 6). UWF CFP is becoming increasingly common for rapid screening of diabetic retinopathy. However, the UWF CFP obtained by SLO had some shortcomings, such as pseudo-color, peripheral distortion, image magnification, and lower resolution compared with optical fundus photography (4). Moreover, UWF CFP could not recognize retinal non-perfusion areas (NPAs), and it was difficult to distinguish intraretinal microvascular abnormalities (IRMA) from retinal neovascularization (NV). Therefore, fundus fluorescein angiography (FFA) was a commonly used auxiliary diagnostic method to detect NPAs and other DR lesions that were sometimes difficult to distinguish (7, 8). Thus, UWF CFP combined with FFA was a regular backup option for ophthalmologists or retinal experts in managing DR. Nevertheless, the invasive and time-consuming nature of the procedure and the risk of severe dye-related adverse events limited the use of FFA.

In recent years, swept-source optical coherence tomography angiography (SS-OCTA), as a non-invasive, safe, and easily repeatable retinal blood flow imaging system, was developed. The feasibility of its application in the diagnosis, screening, and follow-up of DR has been evaluated by a large number of studies (9–12). The OCTA equipment used in most of these studies could only capture an area of 12 × 12 mm by a single scan. To expand the range of observation, some studies adopted image mosaic (9, 10), while others used extended field imaging (EFI) technique (13). SS-OCTA image acquired with EFI could capture only slightly wider than the 55° field. On the other hand, Cui et al. used two separate superior and inferior 15 × 9 mm images to achieve a 15 × 15 mm montage image of a 56° field of view (FOV) centered on the fovea (10), and *Khalid et al.* composed five 12 × 12 mm OCTA images to form a montage image of approximately 80° FOV (9). However, the computer built-in Montage scan protocol requires a longer acquisition time and may introduce more artifacts.

The recently developed TowardPi highspeed ultra-widefield SS-OCTA (UWF SS-OCTA) system has an A-scan rate of 400 kHz, and it can obtain a 24 × 20 mm (about 120° FOV) retinal blood flow image within 15 s by a single scan (14, 15). This scanning protocol can display DR lesions, such as microaneurysms (MAs), IRMA, NPAs, and NV. To the best of our knowledge, there is no report on this novel highspeed UWF SS-OCTA system with a 24 × 20 mm scanning area for the detection of DR lesions with a large number of DR cases. Here, we present our preliminary results using this UWF SS-OCTA system to detect DR lesions and to evaluate the idea that UWF CFP plus UWF SS-OCTA could be a fast, reliable, and non-invasive alternative to screen and monitor DR.

Materials and methods

Subjects

This prospective, observational study was conducted at the Sichuan Provincial People's Hospital (SPPH) from November

2021 to June 2022. All participants were recruited from patients who have already been scheduled to take the FFA examination (Spectralis HRA + OCT [Heidelberg Engineering, Heidelberg, Germany]) due to various fundus diseases, including DR, macular degeneration, or other retinal disorders. Inclusion criteria: patients with type 1 or type 2 diabetes. These patients may have varying degrees of diabetic retinopathy, including no DR (NDR), non-proliferative diabetic retinopathy (NPDR), and proliferative diabetic retinopathy (PDR). The exclusion criteria were history of glaucoma, concomitant retinochoroidal diseases, the opacity of refractive media affecting more than 30% of OCTA images, and signal intensity of less than 6, poor imaging quality, or serious image artifacts that affect image evaluation. This study was approved by the Institutional Review Board of SPPH and informed consent was obtained from all subjects. All procedures adhered to the tenets of the Declaration of Helsinki and Health Insurance Portability and Accountability Act regulations.

Before the FFA examination, all patients enrolled in the study completed detailed ophthalmic examinations, including best-corrected visual acuity, intraocular pressure, slit lamp anterior segment examination, and slit lamp fundus examination. And UWF SS-OCTA and UWF CPF (California, Optos, Dunfermline, UK) were taken on the same day if they had not taken these two exams within 2 weeks at SPPH.

Image acquisition protocol

OCTA images were obtained using a 400 kHz UWF SS-OCTA instrument (BM-400K BMizar, TowardPi Medical Technology, Beijing, China). It uses a swept-source vertical-cavity surface-emitting laser (VCSEL) with a wavelength of 1,060 nm and with a 400,000 A-scans speed, providing a transverse resolution of 10 μm and an axial optical resolution of 3.8 μm. This instrument has an A-scan depth of 6.0 mm in tissue (2,560 pixels). The 24 × 20 mm scan model uses 1,536 A-scans per B-scan at 1,280 B-scan positions, resulting in an A-scan and B-scan separation of 15.625 μm. Two sequential B-scans were performed at each fixed position before proceeding to the next transverse location on the retina. When centered on the fovea, the BM-400K BMizar OCTA imaging is capable of capturing images of retinal blood flow in a range of 24 × 20 mm by a single scan and generate a total FOV of up to 120 degrees (Figure 1A).

The built-in software automatically stratifies the blood vessels in the retina. Each of these segmented volumes is referred to as a slab. Manual correction of retinal segmentation was performed when automatic stratification was inaccurate. The vitreous segmentation and superficial retinal segmentation were selected to detect retinal neovascularization. The superficial retinal slab was defined as the volume between the internal limiting membrane (ILM) and the outer boundary of the inner plexiform layer (IPL). The vitreous slab was defined with an outer boundary positioned on the ILM and with no inner boundary (all the blood flow signals were collected above the ILM).

Image grading

All images were reviewed independently by two masked ophthalmology specialists. All grading was done on the same monitor, at different time points and in different orders. Disagreements between graders were open adjudicated by an independent senior retina specialist (ZL) who had more than 30 years of working experience in the diagnosis and treatment of DR. UWF CFP and FFA images were analyzed for detecting DR lesions within the 200° FOV and the *en face* UWF SS-OCTA showed about 120° FOV. UWF SS-OCTA images included structural optical coherence tomography (OCT) B-scans with overlay flow signal, and the *en face* OCTA images displayed the superficial capillary plexus and vitreoretinal interface. Based on the three types of images, the two graders identified DR lesions, including MA, intraretinal hemorrhage (IRH), NPAs, IRMA, venous beading (VB), NV of the optic disk (NVD), neovascularization elsewhere (NVE), vitreous or preretinal hemorrhage (VH/PRH), and graded the severity of DR according to the International Classification of DR severity (16), including no DR, mild to moderate NPDR, severe NPDR, and PDR.

All lesions were identified based on their characteristics on OCTA as mentioned in previous reports (9, 10, 17). MAs were defined as moderate or hyperreflective spots with various morphologic patterns, including fusiform, saccular, curved, and rarely a coiled shape (18). MAs arose primarily from the deep part of the inner retinal capillary plexus and were located mostly in the inner nuclear layer. Horizontal OCT B-scans images were used to confirm the presence of Mas, which were identified as capsular shapes and ring signs. Capillary dropouts larger or equal to one-fourth of the disc area were defined as NPAs. IRMAs were tortuous, dilated, and looped intraretinal new vessels adjacent to the areas of capillary loss. VB refers to irregular constriction and dilatation of venules in the retina. NV was observed as extraretinal vessels shown in the vitreoretinal interface slab after segmentation error corrections, and was also defined as a new vessel that has broken through the ILM confirmed by B-scan. NV located in the optic disc or within 1 disc diameter from the margin were classified as NVD, while the rest were classified as NVE. NVD and NVE were confirmed as preretinal hyperreflective material on OCT B-scans.

Combination of image evaluation and grading

To compare the detection rate of DR lesions by UWF CFP combined with the other two vascular structure

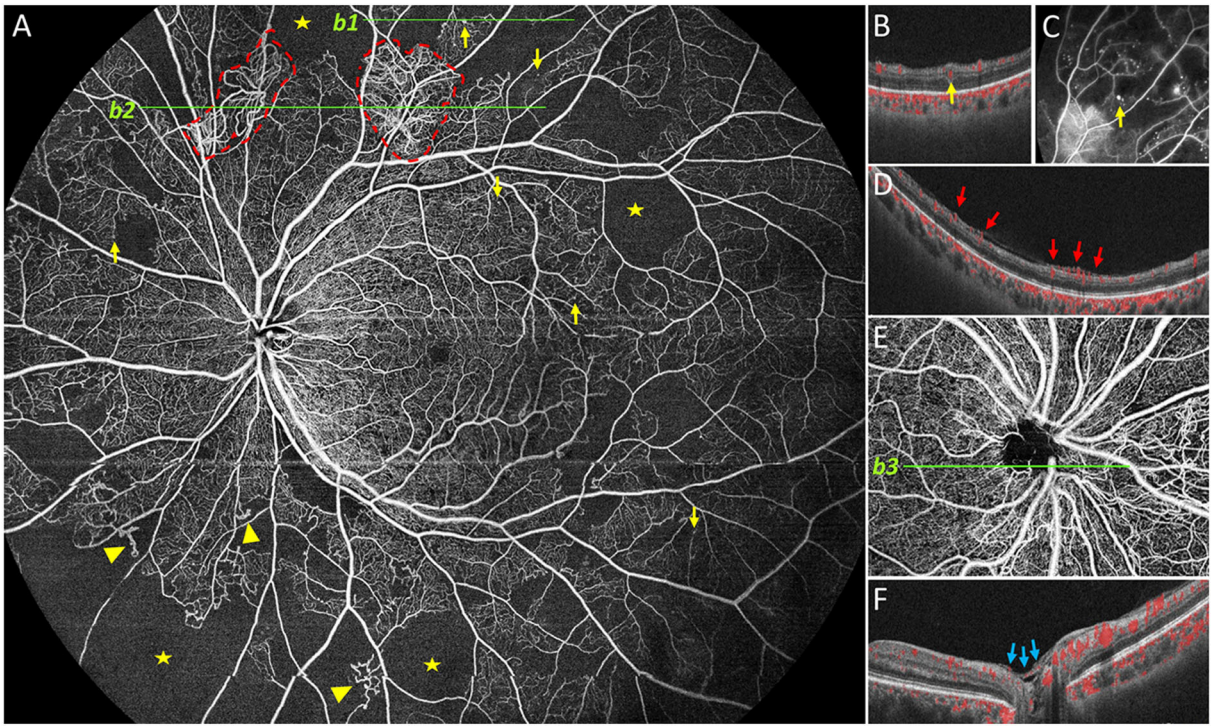


FIGURE 1
Representative 24 × 20 mm image obtained by UWF SS-OCTA. Representative DR lesions were marked with yellow arrows for microaneurysms (MA), solid triangles for intraretinal microvascular abnormalities, red dashed line for neovascularization elsewhere, pentastar for nonperfusion area, blue arrow for neovascularization of the disc (NVD). In case, neovascularization was found elsewhere on **(A**, *b2*), and corresponding B-scan is provided on **(D)**; B-scan (*b1*) of the MA near the superior edge of **(A)** is shown in **(B)**, and corresponding FFA image is provided in **(C)**. In another case, NVD was found and a corresponding B-scan is shown **(E,F)**.

imaging techniques, the evaluation results of UWF CFP were superimposed with the evaluation results of UWF SS-OCTA and FFA (non-invasive versus the other invasive), respectively (Figure 2). The principle of superposition was: if any of the two images of the same eye was positive for a certain DR lesion, the DR lesion of this eye was considered positive. For example, in UWF CFP plus UWF SS-OCTA combination group, if an MA was detected by either of the two methods in one eye, then we considered MA positive for this eye.

Data analysis

All data were tabulated and organized with Microsoft Excel (Microsoft Corp., Redmond, WA). All statistical analyses were performed using IBM SPSS Statistics 26.0 (IBM Corporation, New York, USA). Normally distributed continuous variables were presented as mean ± SD. DR lesion detection rate differences between different groups were compared using McNemar's test. Inter-rater agreement was calculated using Cohen's Kappa test, wherein a Kappa (κ) value of less than 0.2 means poor agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 good agreement, and 0.81–1.00 very good agreement. A nominal two-sided p-value of 0.05 was considered to indicate statistical significance.

Results

Demographics

A total of 153 eyes (49 eyes with PDR, 56 eyes with severe NPDR, 37 eyes with mild to moderate NPDR, and 11 eyes with diabetic patients with NDR) in 86 participants were enrolled in the study (Table 1). The average age of the participants was 56.7 ± 11.9 years old, and the average duration of diabetes was 10.5 ± 7.6 years. Most of the participants (85/86, 98.8%) had type 2 diabetes mellitus. Except that the macula in three eyes was obscured by vitreous hemorrhage, a total of 98 (65.3%) eyes had diabetic macular edema (DME), of which 67 eyes had center-involved DME.

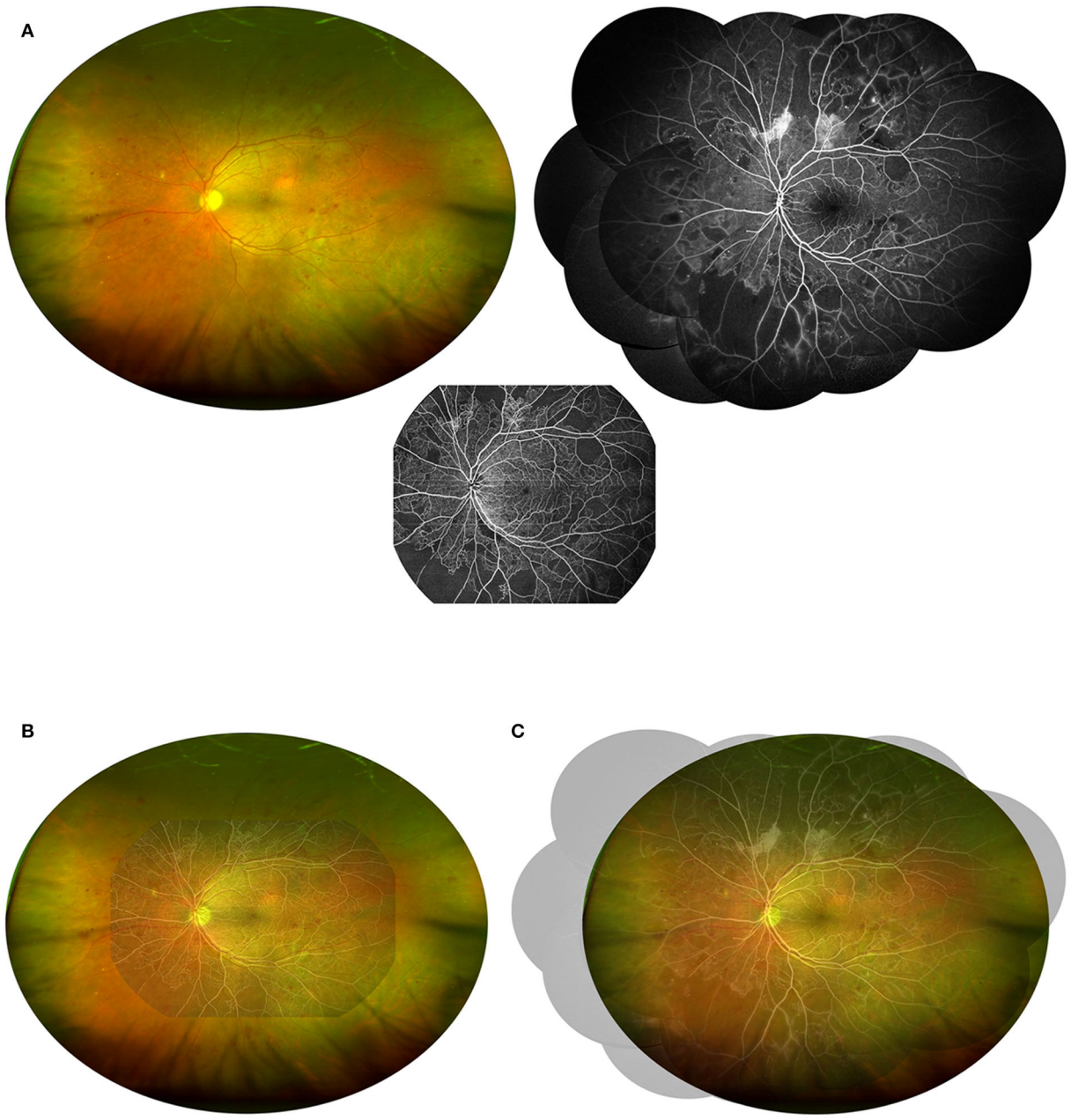


FIGURE 2
Demonstration of the combination of UWF CFP with UWF SS-OCTA or FFA. **(A)** The UWF CFP, UWF SS-OCTA, and FFA (montaged) images of the same patients shown in Figure 1 were adjusted to a 1:1 ratio and merged in pairs to visually demonstrate the field of view of the two combinations including UWF CFP plus UWF SS-OCTA **(B)** and UWF CFP plus FFA **(C)**.

Detection rate of DR lesions

UWF SS-OCTA vs. FFA

For all DR lesions except NPAs that we investigated, UWF SS-OCTA showed a comparable detection rate with FFA ($p > 0.05$), but less number in MA and VH/PRH and more number in IRMAs, VB, NVD, and NVE. UWF SS-OCTA had better performance in detecting NPAs (73 vs. 69%, $p = 0.039$). Except for VB ($\kappa = 0.279$), good or very good agreements were reached for the identification of all DR lesions between UWF SS-OCTA and FFA ($\kappa = 0.791$–0.935) (Table 2). There were two eyes with NVE and two eyes

with NVD detected by UWF SS-OCTA was defined as NPDR by FFA.

UWF CFP plus UWF SS-OCTA vs. UWF CFP plus FFA

The combination of UWF CFP with UWF SS-OCTA showed a similar detection rate compared with UWF CFP plus FFA for all the characteristic DR lesions ($p>0.05$), except NPAs ($p = 0.039$). Good agreement was shown for the identification of VB ($\kappa = 0.635$), and very good agreement for the identification of all the rest of DR lesions ($\kappa > 0.8$) (Table 3). On the evaluation of the presence and absence of DR lesion, the concordance rates of UWF CFP plus UWF SS-OCT and UWF CFP plus FFA from high to low were VH/PRH (99.3%), NVD (98.7%), MA (98.0%), NVE (98.0%), IRH (97.4%), IRMAs (94.8%), NPAs (94.1%), and VB (82.4%), respectively (Figure 3).

TABLE 1 Demographics of participants.

Participants (eyes)	153
Mean ± SD age, y	56.7 ± 11.9
Males	53
Females	33
Type of diabetes (patients)	
Type 1	1
Type 2	85
Mean ± SD duration of diabetes, y	10.5 ± 7.6
Groups, severity of DR eyes*	
No DR in DM patients	11
Mild to Moderate NPDR	37
Severe NPDR	56
PDR	49
Right eyes	77
Left eyes	76
Macular edema#	
No DME	52
Mild DME or moderate DME	31
severe DME (Center involved DME)	67

DM, diabetes mellitus; *DR*, diabetic retinopathy; *NPDR*, non-proliferative diabetic retinopathy; *PDR*, proliferative diabetic retinopathy, *DME*, diabetic macular edema. *Grading of DR severity based on WF CFP, UWF SS-OCTA, and FFA; #three eyes with macular was obscured by vitreous hemorrhage.

Grading of diabetic retinopathy

UWF SS-OCTA vs. FFA

Analysis of UWF SS-OCTA images resulted in a diagnosis of no DR in 15 eyes, mild to moderate NPDR in 35 eyes, severe NPDR in 60 eyes, and PDR in 43 eyes in comparison with 12, 42, 57, and 42 eyes, respectively, when the diagnosis was based on FFA. Of 153 eyes with both UWF SS-OCTA images and FFA images graded, 129 (84.3% [95% CI, 78.6–90.1%]) eyes had an exact agreement. Overall, agreement in grading severity of DR between UWF SS-OCTA and UWF CFP was found to be good ($\kappa = 0.778$) (Table 4).

UWF CFP plus UWF SS-OCTA vs. UWF CFP plus FFA

Analysis of UWF CFP plus UWF SS-OCTA images resulted in a diagnosis of no DR in 13 eyes, mild to moderate NPDR in 31 eyes, severe NPDR in 62 eyes, and PDR in 47 eyes in comparison with 11, 37, 58, and 47 eyes, respectively, when the diagnosis was based on UWF CFP plus FFA. Of 153 eyes graded by both combination methods, 139 (90.9% [95% CI, 86.3–95.4%]) eyes had exact agreement. The overall agreement in grading severity of DR between UWF CFP plus UWF SS-OCTA and UWF CFP plus FFA was found to be very good ($\kappa = 0.869$) (Table 5).

TABLE 2 Detection rate of DR lesions on UWF SS-OCTA vs. FFA.

	Detection rate of DR lesions on UWF SS-OCTA (24 × 20 mm) vs. FFA (eyes, %)			
DR lesions	**UWF SS-OCTA**	**FFA**	***p*-value**	**κ value**
MA	136/153 (0.89)	140/153 (0.92)	0.125	0.852
IRH	NA	128/153 (0.84)		
NPAs	112/153 (0.73)	105/153 (0.69)	0.039	0.858
IRMAs	99/153 (0.65)	93/153 (0.61)	0.070	0.888
VB	47/153 (0.31)	40/153 (0.26)	0.371	0.279
NVE	41/153 (0.27)	39/153 (0.25)	0.625	0.932
NVD	14/153 (0.09)	12/153 (0.08)	0.500	0.916
VH/PRH	14/153 (0.09)	18/153 (0.12)	0.219	0.791

DR, diabetic retinopathy; *MA*: microaneurysms; *IRH*, intraretinal hemorrhage; *NPAs*, non-perfusion areas; *IRMA*, intraretinal microvascular abnormalities; *VB*, venous beading; *NVE*, neovascularization elsewhere; *NVD*, neovascularization of the optic disc; *VH/PRH*, vitreous hemorrhage or preretinal hemorrhage.

TABLE 3 Detection rate of DR lesions on WF CFP plus UWF SS-OCTA vs. WF CFP plus FFA.

	Detection rate of DR lesions on WF CFP plus UWF SS-OCTA (24 × 20mm) vs. WF CFP plus FFA (eyes, %)			
DR lesions	**WF CFP plus UWF SS-OCTA**	**WF CFP plus FFA**	***p*-value**	***κ* value**
MA	138/153(0.90)	141/153(0.92)	0.250	0.878
IRH	128/153(0.84)	132/153(0.86)	0.125	0.898
NPAs	112/153(0.73)	105/153(0.69)	0.039	0.858
IRMAs	104/153(0.68)	98/153(0.64)	0.070	0.884
VB	67/153(0.44)	56/153(0.37)	0.052	0.635
NVE	42/153(0.27)	41/153(0.27)	1.000	0.950
NVD	14/153(0.09)	12/153(0.08)	0.500	0.916
VH/PRH	22/153(0.14)	23/153(0.15)	1.000	0.974

DR, diabetic retinopathy; *MA*, microaneurysms; *IRH*, intraretinal hemorrhage; *NPAs*, non-perfusion areas; *IRMA*, intraretinal microvascular abnormalities; *VB*, venous beading; *NVE*, neovascularization elsewhere; *NVD*, neovascularization of the optic disc; *VH/PRH*, vitreous hemorrhage or preretinal hemorrhage.

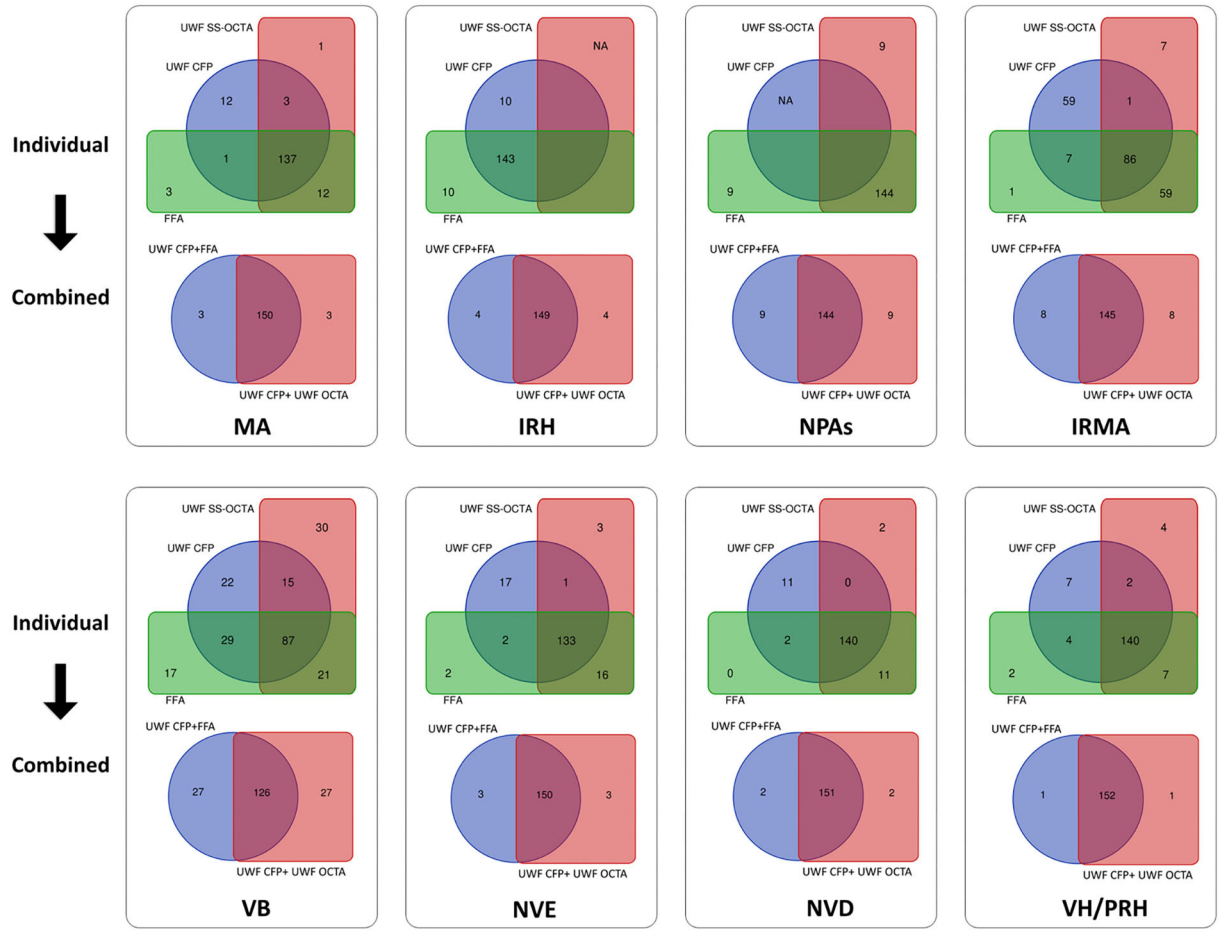


FIGURE 3
Venn diagram showing the results of two combination methods for detecting DR lesions. The first and third rows show the detection number of each DR lesion by individual modalities, including UWF CFP, UWF SS-OCTA, and FFA. The second and fourth rows show the corresponding combined results of UWF CFP plus UWF SS-OCTA and UWF CFP plus FFA. During the evaluation of the presence and absence of DR lesions, the concordance rates of the three individual modalities from high to low were NP (94.1%), IRH (93.5%), NVD (91.5%), VH/PRH (91.5%), MA (89.5%), NVE (86.9%), VB (56.9%) and IRMAs (56.2%), respectively. And the concordance rates of UWF CFP plus FFA and UWF CFP plus UWF SS-OCTA from high to low were VH/PRH (99.3%), NVD (98.7%), MA (98.0%), NVE (98.0%), IRH (97.4%), IRMAs (94.8%), NPAs (94.1%), VB (82.4%), respectively. MA: microaneurysms, IRH: intraretinal hemorrhage; NPAs: non perfusion areas; IRMA: intraretinal microvascular abnormalities; VB: venous beading; NVE: neovascularization elsewhere; NVD: neovascularization of the disc; VH/PRH: Vitreous hemorrhage or preretinal hemorrhage.

TABLE 4 Agreement in grading DR between UWF SS-OCTA versus FFA.

	FFA				
UWF SS-OCTA	**No DR**	**Mild to moderate NPDR**	**Severe NPDR**	**PDR**	**Total**
No DR	10	5	0	0	15
Mild to moderate NPDR	2	29	4	0	35
Severe NPDR	0	8	50	2	60
PDR	0	0	3	40	43
Total	12	42	57	42	153

DR, diabetic retinopathy; *NPDR*, non-proliferative diabetic retinopathy; *PDR*, proliferative diabetic retinopathy.

TABLE 5 Agreement in grading DR between UWF CFP plus UWF SS-OCTA vs. UWF CFP plus FFA.

	UWF CFP plus FFA				
UWF CFP plus UWF SS-OCTA	**No DR**	**Mild to moderate NPDR**	**Severe NPDR**	**PDR**	**total**
No DR	11	2	0	0	13
Mild to moderate NPDR	0	30	1	0	31
Severe NPDR	0	5	54	3	62
PDR	0	0	3	44	47
Total	11	37	58	47	153

Discussion

In this prospective, observational study, we found that UWF SS-OCTA was similar to FFA in detecting DR lesions and grading of DR. At the same time, we found even closer DR lesion detection rate between UW CFP plus UWF SS-OCTA and UW CFP plus FFA. Additionally, a very good agreement was achieved when determining the severity of DR between UW CFP plus UWF SS-OCTA and UW CFP plus FFA. Cui et al. have proposed similar results (10). The difference of this study is that we used a single angiography image of 24 × 12 mm instead of a 15 mm montage image, and we compared the consistency of UWF CFP combined with two angiography methods (one invasive and one non-invasive) in the grading of DR with more cases. Overall, UWF CFP combined with UWF SS-OCTA is a reliable method for the noninvasive and rapid detection of DR lesions.

There have been a large number of previous studies on UWF CFP for the detection of DR lesions. For example, Aiello et al. had revealed that UWF CFP had moderate to a substantial agreement with ETDRS 7-field when determining the severity of DR within the 7 standard fields (6). Moreover, the UWF CFP could detect more lesions outside the 7-field. Silva et al. found approximately one-third of MA, IRMA, and NVE were predominantly outside ETDRS fields and could be detected by UWF CFP (5). The additional peripheral lesions identified by UWF CFP suggested a more severe assessment of DR in 10% of eyes than that of the ETDRS fields. UWF CFP was helpful for the early diagnosis of DR and the detection of more severe lesions. Therefore, UWF CFP has gradually become more and more important in DR screening, diagnosis, and clinical research (19). However, compared with FFA or OCTA, the pseudo-color images of UWF CFP were difficult to distinguish IRMA and NV, and it was almost impossible to complete the task of identifying NPAs. Overall, compared with the two vascular imaging techniques, UWF CFP performed relatively poorly in detecting DR lesions (see Supplementary Tables 1, 2). FFA and recently developed SS-OCTA were therefore needed as a complement, as both are good ways to visualize the subtle vascular structure. In the absence of wide-field OCTA, FFA was the best complementary method to observe DR vascular abnormalities.

FFA is not very prevalent in Western countries in the management of DR, since the impactful ETDRS concluded that color stereoscopic fundus photographs alone were sufficient for DR management (7). Recently, updated guidelines for DR in the United States maintain that routine FFA was not indicated as a part of the regular examination of patients with diabetes (20, 21). However, practice patterns and healthcare delivery systems for patients with diabetes mellitus differ around the world (16). In the past decades, FFA was widely used in China and Japan to diagnose moderate to severe diabetic retinopathy and to help make treatment decisions (8). In our hospital and most other tertiary hospitals in China, outpatient departments were usually crowded with patients, we often routinely screened DR by a rapid UWF CFP and fundoscopy examination. If the patient may have severe NPDR or PDR, FFA would be advised

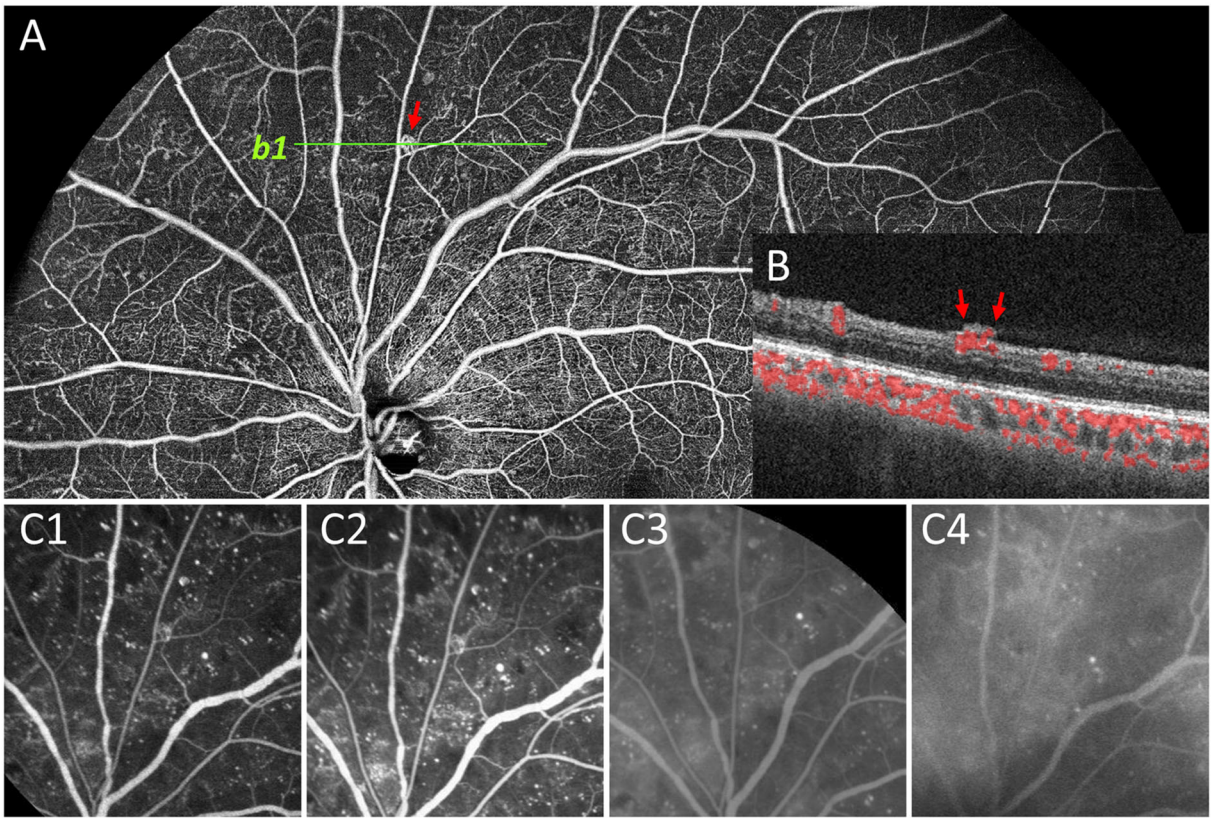


FIGURE 4
Representative image to show undetected NVE by FFA. In panel **(A)**, the NVE above the optic disc (red arrow) was confirmed by OCT B-scan that the lesion had broken through the ILM (*b1*, **B**). During the whole FFA process **(C1–C4)**, no obvious fluorescein leakage was seen in the corresponding lesion compared with the background leakage. Therefore, the lesion was documented as IRMA rather than NVE on FFA.

to assist in making the next treatment decision. However, many undeniable shortcomings of FFA including its time consuming and invasive nature, and possible adverse events limited its clinical utility. A non-invasive, rapid alternative to FFA would be ideal for these DR patients. In recent years, with the rapid development of OCTA technology, especially wide-field OCTA, OCTA montage images could observe a much wider range than before. Therefore, a great amount of enthusiasm and resources have been devoted to research in detecting DR lesions using OCTA (17, 22, 23). These previous clinical research results have expanded our understanding of DR, but there were limitations. First, the single scanning range of most previous used OCTA systems was only 12 × 12 mm, which can only cover the posterior 56° of FOV. Second, to get a wider FOV, multiple scanning and montage image were required, which was very time consuming and requires high cooperation from patients (10, 17). Additionally, the EFI technique was introduced to expand the scanning area of OCTA. The mean extension ratio of EFI-OCTA compared to OCTA without EFI was 1.51–1.98, which was reported by different studies (13, 24, 25). However, the EFI-OCTA technique also had several limitations, including a long learning curve, introducing variation in magnification between eyes, and decreasing image resolution.

In this study, we used the latest commercially available high-speed ultra-widefield SS OCTA for the non-invasive detection of DR lesions. The special lens of the TowardPi OCT system is not like the additional lens used in the EFI technique; it is an optical design within a complete set of the system. With a group of large-diameter lenses (ocular lenses more than 60 mm across), the FOV of the system reaches up to 120°, while maintaining a lateral resolution of 10 um (14, 15). The retinal vascular image in the range of 24 × 20 mm can be obtained by a single scan, and the acquisition time is only 15 s at the minimum. It allows technicians to quickly and non-invasively obtain wider and more complete images of retinal angiography image than previous UWF SS-OCTA systems. This undoubtedly improves the efficiency of the examination and makes it easier for patients to cooperate.

For the first time, we used this latest developed UWF SS-OCTA to detect DR lesions in a large number of diabetic patients and compared its practicability with FFA. Our preliminary results showed that there was no significant difference in the

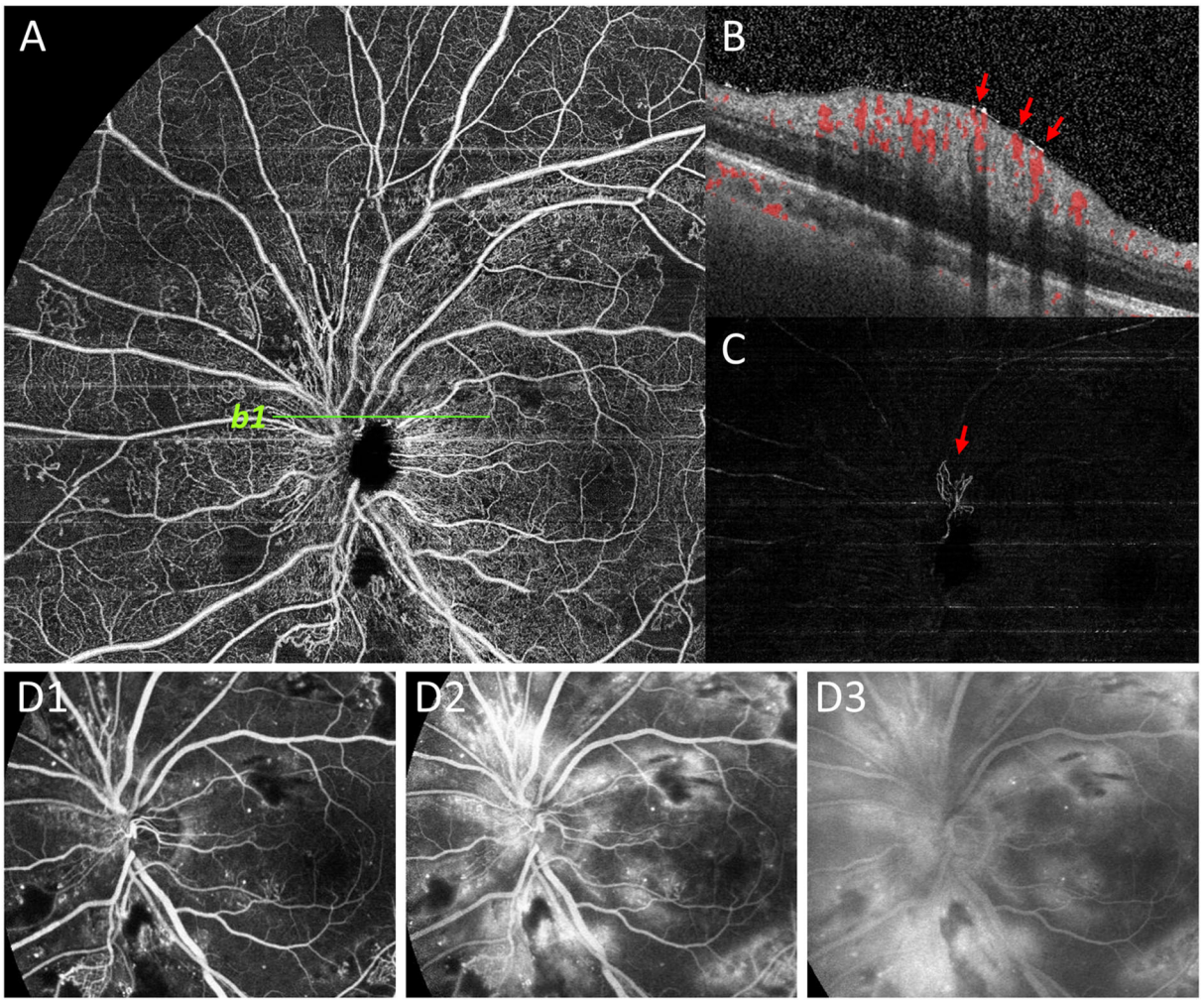


FIGURE 5
Representative image to show undetected NVD by FFA. NVD was not clearly shown on superficial retinal segmentation of OCTA **(A)**. However, the NVD (red arrow) on the upper edge of the optic disc was clearly shown on the vitreous segmentation **(C)**, and the presence of NVD was also confirmed by B-scan (*b1*, **B)**. On the contrary, due to the large amount of fluorescein leakage around the optic disc area by dilated capillaries on FFA images, the background hyperfluorescence affected the observation of NVD (D1-D3).

detection rate of most DR lesions between UWF SS-OCTA and FFA, and the former had better performance in detecting the rate of NPAs (Table 2). The same result was found when comparing UWF CFP plus UWF SS-OCTA with UWF CFP plus FFA for DR lesion detection (Table 3). During the evaluation of the presence and absence of the DR lesions, the combination methods reached a very high concordance rates in detecting VH/PRH (99.3%), NVD (98.7%), MA (98.0%), NVE (98.0%), IRH (97.4%), IRMAs (94.8%), and NPAs (94.1%) (Figure 3). In addition, there were four eyes with small proliferative lesions (NVE or NVD), which were undetected by FFA and were caught by UWF SS-OCTA (Figures 4, 5). These results indicated that the 24 × 20 mm UWF SS-OCTA was a reliable modality to identify DR lesions, which was similar to previous studies (10, 12). Meanwhile, according to international diabetic retinopathy disease severity scales (DSS), agreement in grading severity of DR between UWF SS-OCTA and UWF CFP was found to be good ($\kappa = 0.778$; Table 4). Furthermore, when combined with UWF CFP, UWF CFP plus UWF SS-OCTA and UWF CFP plus FFA had even better performance in DR grading (very good agreement achieved, $\kappa = 0.869$; Table 5).

UWF CFP and FFA have been validated by numerous studies in the diagnosis of DR (5–8, 26). Since UWF CFP cannot detect NPAs and the identification of NV and IRMA is often subjective and ambiguous, FFA was used as a complement to color fundus photo to assist DR diagnosis. However, the clinical utility of FFA was limited mainly due to its invasive nature and some other disadvantages. Previous studies and our study showed that compared with FFA, UWF SS-OCTA could also clearly display DR vascular lesions. Moreover, compared

with FFA, UWF SS-OCTA had many apparent advantages, such as being fast, non-invasive, repeatable, and inexpensive, which were shared by UWF CFP. Our study confirmed that the grading of DR severity by UWF CFP combined with UWF SS-OCTA was the same as that obtained by UWF CFP plus FFA. Therefore, we suggest that this combination modality might be an optimal choice in the management of DR.

Why do we suggest that UWF SS-OCTA should be used in combination rather than alone for DR screening and follow-up? That's because we have found many shortcomings when we review the UWF SS-OCTA images. For example, the identification of MA and intraretinal hemorrhage on OCTA *en face* images was difficult and sometimes ambiguous. Searching for corresponding lesions on B-scan images was often needed to assist in diagnosis, which was very time consuming. This task would be much easier with the help of UWF CFP images. Furthermore, although 24 × 20 mm is currently one of the widest scanning ranges of commercialized OCTA equipment, many DR lesions are located in the peripheral areas (Figure 2) (27). Therefore, if OCTA, which is more sensitive to NPAs, IRMA, and NV, can be combined with UW CFP, which can more easily identify MA, IRH, and peripheral DR lesions, will undoubtedly improve the reliability of DR diagnosis and grading. Compared with the individual method, the combined methods had a higher concordance rate of DR lesion detection (Figure 3). At the same time, we believe that with the progress of OCTA technology, a combination of both non-invasive imaging techniques has the potential value to gradually replace the majority of FFA examination, and become the first-line tool for fast and large-scale DR screening, diagnosis, and follow-up in the future. A new OCTA-based DR classification consensus would be discussed by international healthcare communities in the near future.

There are limitations to our study. First, the FFA in this study was performed using the 55° field frames instead of an ultra-widefield FFA, which would have lost some clinically significant DR lesions. However, due to the uneven distribution of medical and financial resources, China and many other regions have not yet employed UWF FFA facilities. In addition, the result of our current single-center study needs longitudinal and multi-center studies for further evaluation. Patients with refractive media opacity might have affected the accuracy of OCTA data collection. Compared to FFA, worse contrast sensitivity and motion artifact increased the difficulty to identify MA and VB by OCTA image. In addition, compared to OCTA, we should not ignore the advantages of FFA, which can reveal the destruction of the blood-retinal barrier, and may evaluate iris neovascularization at the same time. Finally, in this Study, we only focused on the evaluation of the presence or absence of DR lesions by the two combination methods, but not studied the number of DR lesions and their implied clinical significance.

In general, UWF CFP combined with UWF SS-OCTA achieved almost the same DR lesion detecting and grading ability as UWF CFP plus FFA. This combination method could be a fast, reliable, and non-invasive method to diagnose DR at all stages, which has been applied in our daily clinical practice and may become a routine choice for DR management in the Future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by institutional review board of Sichuan Provincial People's Hospital (SPPH). The patients/participants provided their written informed consent to participate in this study.

Author contributions

JZ and JL had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. JZ, JL, and DW concept and designed the research. SL, MLi, DW, LC, WD, MLiu, HL, YW, XN, and YL acquired and sorted the data. MM and MLi reviewed and graded the images. JL, FL, and DW wrote the draft of the manuscript. JZ critically reviewed and extensively revised the manuscript. MM, SL, and MLi performed the statistical analyses. JL, SL, FL, and JZ acquired funding. JZ and WD supervised the study. All authors reviewed the manuscript and provided final approval for submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.1047608/full#supplementary-material

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Application of urine C-peptide creatinine ratio in type 2 diabetic patients with different levels of renal function

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Objective: This study aims to investigate the effect of single urine C peptide/creatinine (UCPCR) in assessing the islet β Cell function of type 2 diabetes mellitus (T2DM) patients with different renal function.

Methods: A total of 85 T2DM patients were recruited in this study, all the patients were assigned to one group with estimated glomerular filtration rate (eGFR)≤60 ml·min^{-1}·1.73 m^{-2} and another group complicated with eGFR>60 ml·min^{-1}·1.73 m^{-2}. Serum creatinine, urine creatinine, serum fasting C-peptide (FCP), fasting blood glucose (FBG), glycosylated hemoglobin (HbA1C) and 24-hour urinary C-peptide (24hUCP) were measured. The modified homeostasis model assessment-islet β cell function [HOMA-islet (CP-DM)], the modified homeostasis model assessment-insulin resistance [HOMA-IR(CP)] and UCPCR were calculated.

Results: When compared with group eGFR ≤60 ml·min^{-1}·1.73 m^{-2}, the levels of UCPCR, FCP, the modified HOMA-IR(CP) and HOMA-islet (CP-DM) were promoted and the concentrations of HbA1C, FPG, creatinine were decreased in the patients of eGFR>60 ml·min^{-1}·1.73 m^{-2} (P<0.05); FCP was uncorrelated with 24hUCP while associated with UCPCR in the patients of eGFR ≤ 60 ml·min^{-1}·1.73 m^{-2}; UCPCR was positively correlated with FCP and HOMA-IR(CP) in the T2DM patients with different levels of renal function; the cut-off (UCPCR ≤ 1.13 nmol/g) had 88.37% sensitivity and 95.24% specificity [95% confidence interval (CI):0.919-0.997] for identifying severe insulin deficiency in T2DM patients[area under the curve (AUC) 0.978].

Conclusion: UCPCR can be used to evaluate islets β Cell function in T2DM patients with different renal function status.

KEYWORDS

diabete, urinary C peptide/creatinine ratio, 24-hour urinary C-peptide, EGFR, C peptide

1 Introduction

Diabetes is a chronic metabolic disease, and its prevalence is increasing at an alarming rate along with the population aging and lifestyle changes. The number of diabetes patients is projected to rise to about 592 million globally including about 143 million in China by 2035, which will bring a large economic burden on society (1). Type 2 diabetes mellitus (T2DM) with islet β-cell secretory dysfunction and insulin resistance as the major characteristics, accounts for around 95% of all cases of diabetes (2). Therefore, the evaluation of islet function in T2DM patients has gained clinical significance.

C-peptide (CP) is a peptide hormone containing 31 amino acids and is formed during the cleavage of insulin from proinsulin secreted by islet β-cells. CP can be measured in blood and urine and is not affected by exogenous insulin. The blood CP can reflect the instantaneous level at the time of blood collection. 60–69% of the blood CP is filtered through the glomerulus, and 80% is reabsorbed by the renal tubules and metabolized by the kidney (3). The daily output of CP is about 5% of the insulin secretion. In patients with normal renal function, 24-h urine CP (24-h UCP) reflects the average value of blood CP produced in a day, and its stability is better than that of blood CP (4). The radioimmunoassay is the commonly used method to detect 24-h UCP and can be used to evaluate the functional state of islet β-cells. Thus, it helps in the diagnosis, classification, and prognosis of diabetes (5). 24-h UCP can also be used to predict the postoperative pancreas remanent volume and the functional gastrointestinal tract reconstruction (6). However, it is inconvenient to collect 24-h UCP samples, and the accuracy of 24-h UCP is found to be poor in diabetes patients with moderate and severe renal damage (7). Therefore, there is an urgent need for a simple and convenient approach to evaluate the function of islet β-cells, which can eliminate the impact caused by renal damage. Single urine CP creatinine ratio (UCPCR) is a detection index that uses CP/creatinine (Cr) ratio to correct urine dilution. Previous studies have shown that UCPCR can be used to determine hepatocyte nuclear factor 1alpha (HNF1α) and HNF4α for distinguishing between maturity-onset diabetes of the young (MODY) and other types of diabetes (8, 9). Recent studies have shown that UCPCR can also be used to evaluate the function of islet β-cells in patients with T1DM and T2DM (10, 11). However, no study has been carried out to assess the application of UCPCR for the evaluation of islet β-cell function in patients with T2DM till now, especially in T2DM patients with differences in renal function. In this study, estimated glomerular filtration rate (eGFR), 24-h UCP, blood CP, and UCPCR were measured in T2DM patients to investigate the potential application of UCPCR in the evaluation of islet function in T2DM patients with different levels of renal function. The study can provide a basis for the application of UCPCR in the clinical practice of diabetes.

2 Materials and methods

2.1 Subjects

Considering the inconvenience of 24-h urine sample collection, the patients recruited in this study are all inpatients. Eighty-five T2DM patients including 56 males and 29 females, with an average age of 57.72 ± 4.43 years, who were hospitalized in the Department of Endocrinology from February 2020 to December 2021 were selected.

All subjects were given oral hypoglycemic drugs or exogenous insulin therapy except insulin secretagogues before admission. T2DM was diagnosed according to the World Health Organization (WHO) diagnostic criteria 1999. Exclusion criteria of this study include diabetes ketoacidosis, hyperglycemia and hypertonic state, severe infection, stress state, and severe liver dysfunction. Based on the eGFR, the patients were divided into two groups: eGFR≤ 60 mL·min-1·1.73 m-2 and eGFR>60 mL·min-1·1.73 m-2. All the subjects signed informed consent, and this study was conducted in accordance with the tenets of the World Medical Association's Declaration of Helsinki and had been approved by the ethics committee of The First Affiliated Hospital of USTC.

2.2 Methods

2.2.1 Specimen collection

The height, weight, age, gender, course of the disease, and other general information of all the subjects were recorded. Blood samples and single urine samples were collected in the morning after a 12-hour fast. Five mL of each morning urine sample was stored at -80°C and was used for the detection of urine creatinine (UCr) and UCP. After the single urine samples were collected, the rest of urine samples over 24 h beginning from 09:00 am on that day to 09:00 am on the next day were collected to measure 24-h UCP.

2.2.2 Specimen detection

7600-120 automatic biochemical instrument (Hitachi, Tokyo, Japan) was used to detect fasting blood glucose (FPG), serum creatinine (SCr), blood urea nitrogen (BUN), and UCr. Hemoglobin A1c (HbAlc) was determined by high-pressure liquid chromatography (Primus Ultra, Ireland), whereas fasting insulin (Fins), fasting CP (FCP), 24-h UCP, and morning urine UCP were measured by chemiluminescence [The intra- and inter-

assay coefficients of variability were 1.5% and 3.5%%, respectively] on an Atellica IM analyzer (Siemens Healthcare Diagnostics, Shanghai). The lower limit of the CP assay was 0.03 ng/ml, and for this analysis, all CP concentrations<0.03 ng/ml were recorded as 0.03 ng/ml. The upper limit was 30 ng/ml, and all CP concentrations>30 ng/ml were recorded as 30 ng/ml.

2.2.3 Calculation of the indicators

eGFR was calculated by the modified form of the Modification of Diet in Renal Disease (MDRD) equation. The input parameters in the eGFR calculation software were gender, SCr value, and the age of the patient. The formula for calculating eGFR is given by eGFR ($mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$) = 30849 × SCr $(umol/L)^{-1.154}$ × Age $(years)^{-0.203}$ × Gender coefficient (male coefficient is 1, female coefficient is 0.742), and can be used to estimate eGFR of all the subjects. Instead of insulin, fasting C peptide was used to evaluate insulin resistance and islet function.

The modified homeostasis model assessment-islet β cells was calculated according to the formula [HOMA-islet (CP-DM)] = 0.27 × FCP (nmol/L)/[FPG (mmol/L) [3.5]. The insulin resistance index is assessed by the modified homeostasis model assessment-insulin resistance and expressed as, [HOMA-IR (CP)] = 1.5 + FPG (mmol/L) × FCP (nmol/L)/2800 (12). UCPCR is given by UCP/UCr and the body mass index (BMI) is calculated as weight (kg)/height ($m^{<xsp>2</xsp>}$).

2.3 Statistical treatment

Statistical Product and Service Solutions (SPSS) 22.0 software was used to perform a statistical analysis of the data. The measured data were expressed as mean ± standard deviation (± s). The independent sample t-test was used for the comparison between groups. Multivariable logistic regression and pearson analysis was used for the correlation analysis. Receiver operating characteristic (ROC) curve were used to identify cut-off values of UCPCR for distinguishing islet cell function in T2DM patients. Statistical significance was accepted at $p < 0.05$.

3 Results

3.1 Comparison of clinical characteristics between the two groups

The average value of eGFR ≤ 60 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$ group was observed to be 48.79 ± 6.44 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$, while that of eGFR>60 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$ group was observed to be 90.26 ± 16.8 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$. Compared to T2DM patients with eGFR ≤ 60 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$, patients with eGFR>60 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$ were younger and had a shorter course of disease, lower HbA1c, FPG, SCr, UCr and higher 24-h UCP, UCP, UCPCR, FCP, HOMA- HOMA-islet (CP-DM), HOMA-IR (CP) ($p < 0.05$) (Table 1).

3.2 Results of multiple linear regression analysis

As depicted in Table 2, FCP in T2DM patients was used as the dependent variable, while 24h UCP and UCPCR were used as the independent variables. After adjusting for gender and age, multivariable logistic regression analysis showed that FCP was associated with both UCPCR and 24h UCP in T2DM patients with eGFR > 60 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$ while it was associated with UCPCR but uncorrelated with 24hUCP in the patients of eGFR ≤ 60 $ml{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$.

3.3 Correlation analysis between UCPCR and various indicators under different eGFR conditions

When eGFR > 60 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$, UCPCR was positively correlated with FCP (r = 0.455, $p = 0.002$), 24-h UCP (r = 0.577, $p<0.001$), and HOMA-IR(CP) (r = 0.312, $p = 0.037$), while it was negatively correlated with duration (r = -0.372, $p = 0.012$). When eGFR ≤ 60 $mL{\cdot}min^{-1}{\cdot}1.73\ m^{-2}$, UCPCR was positively correlated with FCP (r = 0.698, $p<0.001$) and HOMA-IR(CP) (r = 0.345, $p = 0.029$) (Table 3 and Figure 1).

When eGFR ≤ 60 mL·min-1·1.73 m-2, UCPCR was positively correlated with FCP (a) and HOMA-IR(CP) (c); When eGFR > 60 mL·min-1·1.73 m-2, UCPCR was positively correlated with FCP (b), HOMA-IR(CP) (d) and 24-h UCP (f); UCPCR was negatively correlated with duration (e).

3.4 ROC curve analysis for UCPCR in distinguishing the function of islet β cells in T2DM patients

UCPCR was well-correlated with FCP. FCP < 0.3mmol/l are suggestive of marked insulin deficiency (13). ROC curve were used to identify the cutoff of UCPCR that provided the optimal sensitivity and sensitivity for distinguishing the function of islet β cells in T2DM patients by the software MedCalc V15.2. UCPCR cut-off ≤1.13 nmol/g had the highest Youden index for detecting FCP < 0.3mmol/l and thus identifying severe insulin deficiency, with 88.37% sensitivity and 95.24% specificity (AUC, 0.978, 95% confidence interval (CI) (0.919–0.997), $P < 0.001$) (Figure 2).

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TABLE 1 Comparison of clinical parameters between the two groups of patients.

Characteristics	eGFR ≤ 60	eGFR>60	*t*	*P*
n	40	45	/	/
Age(y)	71.48 ± 4.4	65.2 ± 4.14	6.767	<0.001
Duration (y)	9.13 ± 3.26	5.78 ± 1.85	5.908	<0.001
BMI (kg/m2)	24.83 ± 2.34	25.25 ± 2.71	-0.77	0.443
HbA1C (%)	10.13 ± 1.89	8.51 ± 2.07	3.774	<0.001
FPG (mmol/L)	8.49 ± 1.65	7.79 ± 1.23	2.230	0.028
FCP (nmol/L)	0.20 ± 0.02	0.39 ± 0.05	-20.701	<0.001
24h UCP (nmol/L)	3.46 ± 0.99	6.61 ± 1.27	-12.599	<0.001
BUN (mmol/L)	5.90 ± 1.13	6.12 ± 1.47	-0.766	0.446
Cr (umol/L)	116.8 ± 11.95	70.63 ± 9.35	19.948	<0.001
eGFR	48.79 ± 6.44	90.26 ± 16.8	-14.671	<0.001
UCr (umol/L)	16348.79 ± 2610.88	9664.73 ± 1987.66	13.364	<0.001
UCP (nmol/L)	1.61 ± 0.25	1.87 ± 0.38	-3.707	<0.001
UCPCR (nmol/g)	0.89 ± 0.21	1.67 ± 0.41	-10.740	<0.001
HOMA-islet (CP-DM)	12.29 ± 6.25	27.51 ± 12.66	-6.892	<0.001
HOMA-IR (CP)	2.10 ± 0.13	2.57 ± 0.17	-14.276	<0.001

TABLE 2 Results of multiple linear regression analysis.

Groups	B	SE	t	*P*
GFR ≤ 60				
24h UCP	-0.005	0.004	-1.188	0.243
UCPCR	0.080	0.014	5.576	<0.001
GFR>60				
24h UCP	0.019	0.006	3.485	0.001
UCPCR	0.059	0.018	3.339	0.002

TABLE 3 Correlation analysis between UCPCR and other indicators.

		Age (y)	Duration (y)	BMI (kg/m2)	HbA1C (%)	FPG (mmol/L)	FCP (nmol/L)	24h UCP (nmol/L)	HOMA-IR (CP)	HOMA-islet (CP-DM)
eGFR ≤ 60	*r*	0.154	-0.224	-0.288	0.128	-0.047	0.698	-0.052	0.345	0.290
	P	0.342	0.164	0.071	0.432	0.773	<0.001	0.750	0.029	0.069
eGFR>60	*r*	0.254	-0.372	0.148	-0.014	-0.106	0.455	0.577	0.312	0.166
	P	0.092	0.012	0.330	0.930	0.489	0.002	<0.001	0.037	0.275

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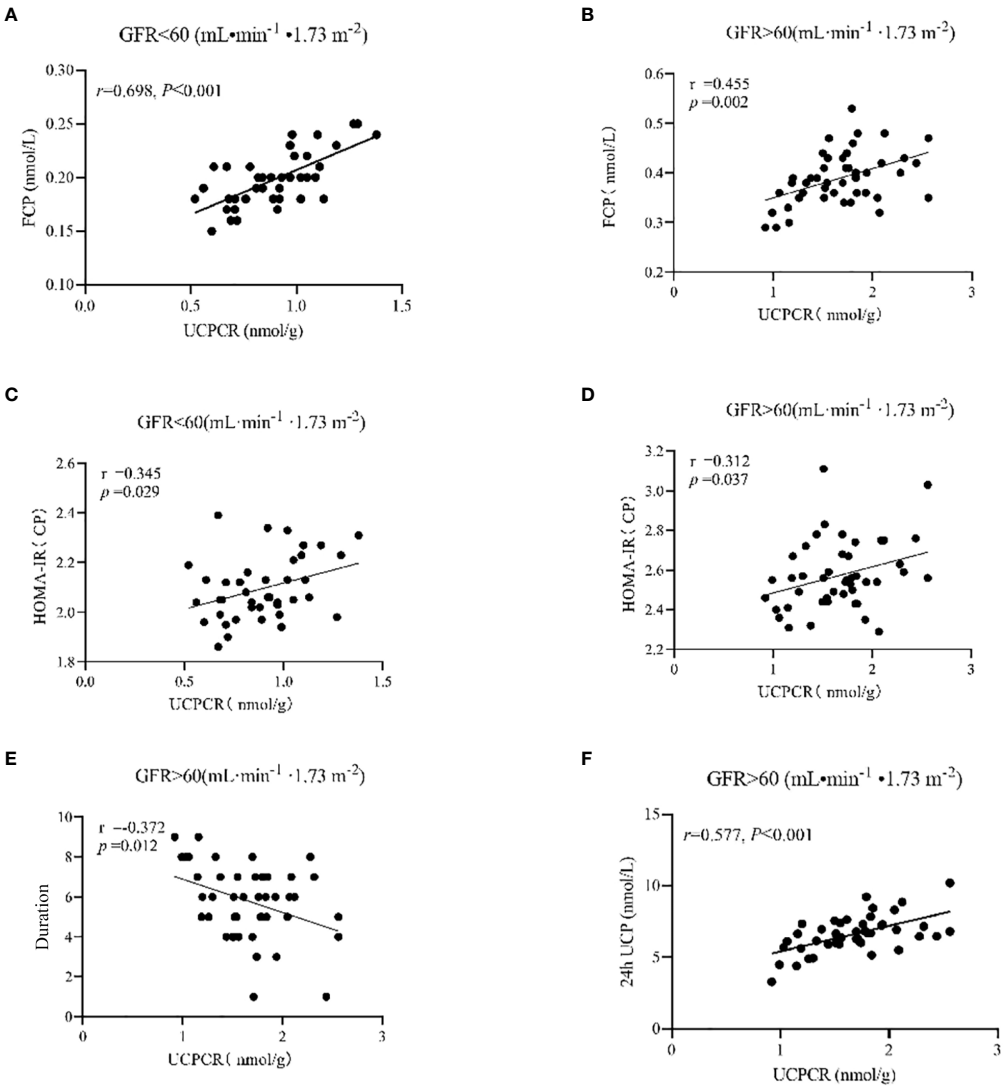


FIGURE 1
When eGFR ≤ 60 mL.min-1.1.73 m-2, UCPCR was positively correlated with FCP **(A)** and HOMA-IR (CP) **(C)**; When eGFR > 60 mL.min-1.1.73 m-2, UCPCR was positively correlated with FCP **(B)**, HOMA-IR(CP) **(D)** and 24-h UCP **(F)**; UCPCR was negatively correlated with duration **(E)**.

4 Discussion

The evaluation of islet function includes the evaluation of insulin sensitivity and the secretion ability of islet β-cells which refers to the synthesis and secretion of insulin and peptides by β-cells. There are some indicators, e.g., hyperglycemic clamp technique, insulin secretion index, insulin resistance index, the area under the C-peptid curve, that can be used to evaluate the islet function. C-peptide is often used as a simple index to clinically evaluate the function of the islet, which is produced by the enzymatic cleavage of proinsulin, secreted in equal amounts as endogenous insulin, and mainly cleared by the kidney (14). Blood CP reflects the instantaneous CP level at the time of blood collection. As CP is easily degraded by prion enzymes, the sample should be immediately centrifuged after blood collection and sent for examination on ice. The stability of 24-h UCP is better than that of blood CP and can reflect the one-day average CP level in the subjects. Recent study have shown that 24-h UCP can be used for the evaluation of islet β-cell function and can indicate the blood CP level in case of normal renal function (15). Jamal et al. (16) found that the blood and UCP levels of T1DM patients were lower than those of control group, and there was a positive correlation between blood CP and 24-h UCP in each group. The 24-h UCP values can also be used to classify diabetes. A study by Nouran et al. found that the levels of blood CP and 24-h UCP in T1DM patients were significantly lower than those in the control group and T2DM

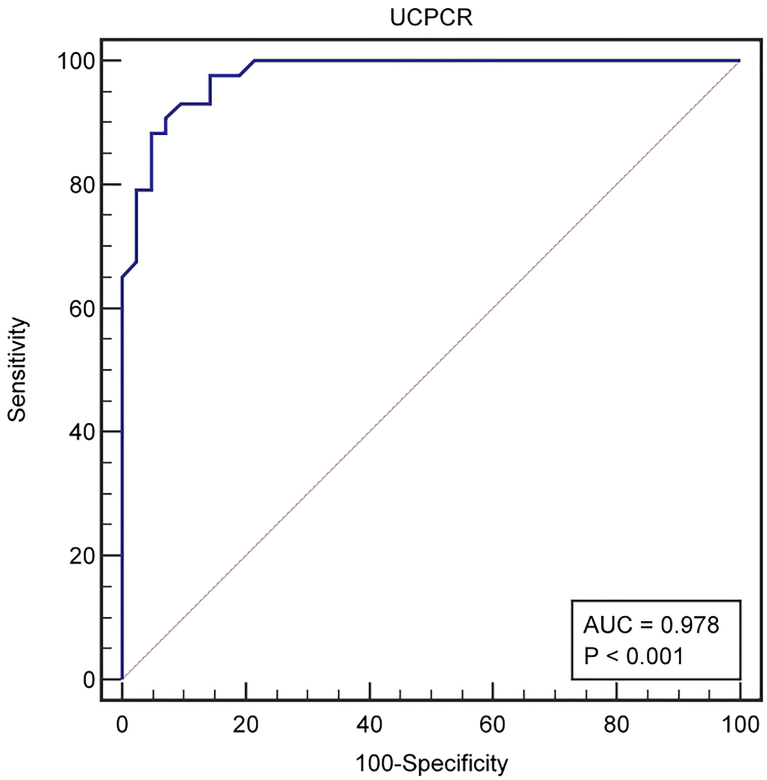


FIGURE 2
The ROC curve identified a cut-off UCPCR ≤1.13 nmol/g for discriminating poor islet function in T2DM patienets (AUC 0.978) with 88.37% sensitivity and 95.24% specificity.

group, indicating poor secretion of insulin from the islet β-cells in T1DM patients (17). There were no significant differences in 24-h UCP between patients with normal renal function and patients with mild and moderate renal insufficiency, and 24-h UCP was positively correlated with blood CP (18). However, 24-h UCP test has some limitations. For diabetes patients with moderate and severe renal damage, 24-h UCP cannot reflect the blood CP levels. Koskinen et al. (19) reported that blood CP and 24-h UCP values were significantly different in diabetes patients with moderate and severe renal damage, which indicated that the decreases of the clearance of CP by kidney result in the retention of CP when renal function is damaged. In this study, when compared with T2DM patients with mild renal damage, 24-h UCP and FCP decreased in patients with moderate and severe renal damage. The multivariable logistic regression indicated that FCP was associated with 24h UCP in T2DM patients with eGFR > 60 mL·min^{-1}·1.73 m^{-2} while it had no correlation with 24hUCP in the patients of eGFR ≤ 60 ml·min^{-1}·1.73 m^{-2}, which is in accordance with the previous research findings. In addition, 24-h urine collection is cumbersome, and is not suitable for outpatients. If urine is diluted or concentrated, it can affect CP levels, thus limiting its wide clinical application.

UCPCR is a non-invasive outpatient diagnostic tool. Previous studies have shown that UCPCR can be used to distinguish between MODY and T2DM patients affected for more than 2 years (20). Recent studies have shown that UCPCR is a useful alternative to blood CP and 24-h UCP tests, and can better reflect the function of the islet. Other tests have some limitations such as the instability of blood CP and the difficulty in 24-h UCP sample retention, but UCPCR sample can be stored for at least 3 days at room temperature with the addition of boric acid preservative (21). The experiment adopted in this paper has little interference from proteinuria and naked eye hematuria, and has stable performance and high stability. Therefore, UCPCR is especially suitable for outpatients to monitor insulin secretion. It is easy to perform and is not affected by the location of sample collection. Elzahar et al. (22) reported that UCPCR is a simple, non-invasive and reliable marker, which can be used for the diagnosis of T2DM and T1DM in children. As a non-invasive marker, UCPCR can distinguish between T1DM and T2DM or monogenic diabetes (11, 23). UCPCR ≤ 0.20 nmol/mmol reflects the severe impairment of β-cell function in T2DM patients (24). In our study, the cut-off (UCPCR ≤ 1.13 nmol/g equals 0.13 nmol/mmol) for a differential diagnosis of T2DM is also helpful to identify patients who need insulin or secretagogue therapy. A study by Ryota et al. (25) observed that UCPCR was significantly lower in the insulin-treated patients than in the insulin-untreated patients and, so it was applicable for differentiating T2DM from T1DM. In a previous

study conducted by our research group, UCPCR had a good correlation with blood CP and 24-h UCP in T2DM patients and can be used as a practical indicator for insulin secretion in T2DM patients (26).

Urine C-peptide is easily affected by urine volume, renal function, etc. As a product of muscle tissue metabolism, UCr is filtered through the glomerulus, and its daily excretion in urine is stable. When compared with 24-h UCP, UCPCR can overcome the differences caused by urine dilution by measuring UCP and the corresponding UCr in a single morning urine sample and calculating their ratio. This method can overcome the influence of renal function on the UCP value, thus avoiding the interferences caused by urinary tract infection, neurogenic bladder and other factors. It can be used to evaluate the islet β-cell function in diabetes patients with renal insufficiency. At present, only a few studies evaluate the impact of differences in renal function on UCPCR. McDonald et al. reported that UCPCR can be used to replace 24-h UCP in patients with renal insufficiency to evaluate the secretory function of islet β-cells (27). Bowan et al. (28) observed that UCPCR was positively correlated with serum CP in diabetic patients with moderate renal damage (eGFR ≤ 60 mL·min^{-1}·1.73 m^{-2}). This study observed that in T2DM patients with eGFR ≤ 60 mL·min^{-1}·1.73 m^{-2}, FCP was uncorrelated with 24-h UCP, but it was still correlated positively with UCPCR. The correlation analysis indicated that UCPCR in patients with differences in renal function was positively correlated with FCP, implying that UCPCR can be used for evaluation of islet β-cell function in T2DM patients with differences in renal function. In addition, this study observed improved insulin resistance and functional indices. Insulin can be replaced by fasting CP, which can better indicate the correlation between CP and UCPCR. In this study, UCPCR was positively correlated with HOMA-IR (CP) in patients with differences in renal function. These findings are consistent with the research results of Oram et al. (29), indicating that UCPCR also reflects the insulin resistance levels of T2DM patients with differences in renal function.

The novelty of this study is to analyze and compare UCPCR, 24-h UCP, FCP, HOMA-IR(CP) and HOMA-islet (CP-DM) in T2DM patients with differences in renal function. It is evident that UCPCR is not affected by the state of the renal function and hence can be used to detect the islet function and insulin resistance levels of diabetes patients with differences in renal function. UCPCR can better evaluate the islet β-cell function in T2DM patients. This provides a basis for the application of UCPCR in the clinical diagnosis and treatment of diabetes. However, this study also has some limitations, such as the small sample size. Therefore, there is a need to carry out prospective research with a large sample size in the future. In addition, future studies also need to determine the cut-off point value of UCPCR in identifying differences in renal function.

There were some limitations in our study. Firstly, it was a single-center and hospital-based study, the sample size was relatively small. In addition, only one urine sample was collected from each patient, while postprandial C peptide and urine C peptide had not been collected. Further studies are necessary to extend the validity of our findings.

5 Conclusion

Briefly, UCPCR has great repeatability and the samples collected for this test are easy to retain. This test can reflect the islet function in T2DM patients with differences in renal function, which can significantly increase its clinical application and popularity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

All the subjects signed informed consent, and this study was conducted in accordance with the tenets of the World Medical Association's Declaration of Helsinki and had been approved by the ethics committee of The First Affiliated Hospital of USTC. The patients/participants provided their written informed consent to participate in this study.

Author contributions

WZ designed the research, analyzed the data, and wrote the manuscript. JL and XY collected the data. HQZ and HRZ analyzed the data. SY and WW reviewed and edited the manuscript. WZ is the guarantor of this work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Case report: Successful treatment of human diabetic foot ulcer using low-intensity diagnostic ultrasound combined with microbubbles: Two cases

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Background: Diabetic foot ulcer (DFU) is one of the serious complications of diabetes, which has high disability rate and mortality. Low-intensity ultrasound combined with microbubbles in blood circulation can enhance the blood perfusion effect of local soft tissue, which has the potential to promote the healing of diabetic ulcer. Here, we report how this method was used to help the healing of two patients with chronic refractory DFUs.

Case Presentation: In case 1, a 56-year-old man with 3-years history of type 2 diabetes had a 3.0×2.0 cm ulcer which infected with staphylococcus aureus on his right calf for more than half a month. In case 2, a 70-year-old man with 10-years history of type 2 diabetes presented with an 8-month right heel ulcer that developed to 7.5×4.6 cm. And he also had hyperlipidemia, hypertension, and renal impairment. Both patients were enrolled in our study to receive treatment of low-intensity diagnostic ultrasound (LIDUS) combined with microbubbles. They were discharged after a 20-minute daily standard treatment for 7 consecutive days. The ulcers in both cases completely healed in 60 days and 150 days, respectively, and haven't recurred for more than one year of follow-up.

Conclusion: It is feasible, safe, and effective to use commercial LIDUS combined with commercial microbubbles in the treatment of diabetic lower extremity ulcers. This study may provide an innovative and non-invasive method for the treatment of DFUs.

KEYWORDS

diabetic foot ulcer, diagnostic ultrasound, microbubble, ultrasound treatment, case report

Introduction

Diabetic foot ulcer, as one of the serious complications of diabetes, has brought heavy economic and public health burden to the society due to its high incidence (15-25%) (1), high disability rate and high mortality (2) in diabetic patients.

Microcirculatory dysfunction is an important cause of DFU. On one side, hyperglycemia and hyperinsulinemia promote characteristic extensive endothelial hyperplasia, basement membrane thickening, and even calcification in arterioles, leading to ischemia-hypoxia and poor perfusion in foot soft tissue (3). On the other, hyperglycemia and oxidative stress lead to endothelial dysfunction, characterized by impaired auto-regulation of micro vessels and a blunted response to vasodilatory stimuli, thereby exacerbating functional perfusion defects in the limbs (4).

Endovascular shear force is an important means to regulate endothelium-derived vasoactive substances and control micro vasodilation (5). According to this, a series of related drugs and modified endogenous active substances have been developed to treat tissue ischemia through improving microcirculation perfusion.

Low-intensity pulse ultrasound, is a kind of ultrasonic energy mainly with mechanical effect, but not thermal effect. The shear force, micro jet and shock wave generated by ultrasonic pulse produce a series of physical and biological effects, which are widely used in the therapeutic field. Perfusion effect is one of these effects, which is to enhance local blood perfusion in tissues by setting appropriate acoustic parameters (6). The microbubbles in the circulation, as cavitation nuclei, could make the ultrasound produce very high shear force and multiply the effect of local blood flow enhancement. Therefore, when low-intensity ultrasound is combined with microbubbles, it has a very good potential for the treatment of tissue ischemic diseases.

In this report, we presented two complete healing cases of refractory DFU treated by commercial LIDUS combined with commercial microbubbles for the first time.

Materials and methods

A GE LOGIQ 9 ultrasound scanner (GE Healthcare, Waukesha, WI) equipped with a 9L Linear array probe (GE Healthcare) was used for both conventional ultrasonography and Contrast-Enhanced Ultrasonography (CEUS). In conventional ultrasonography, thyroid imaging mode was used with a frequency of 9MHz and an imaging depth of 4cm. In CEUS, "Contrast" key was clicked.

An Acuson S2000 ultrasound scanner (SIMENS Healthcare, Erlangen, Germany) equipped with a 9L4 Linear array probe was used for all treatments. Contrast pulse sequencing (CPS) mode were used to monitor microbubble perfusion and an intermittent flash of high MI impulses. The frequency of flash was set at 4 MHz, Imaging depth at 4 cm, with a frame rate of 50 frames per second and an MI of 0.86 (79% acoustic output power).

Same CEUS imaging sections of the ulcerative and surrounding soft tissue before and after treatment were used for perfusion evaluation, chartered with adjacent vessels or bony structures. All parameters of ultrasound were consistent in both patients during diagnosis and treatment.

The microbubbles used for ultrasonic diagnosis and treatment were SonoVue (Bracco Imaging Scandinavia AB, Oslo, Norway), a commercial ultrasound contrast agent. The microbubble suspension with a concentration of 11.8 mg/mL were prepared according to the manufacturer's instructions, with 59 mg sulfur hexafluoride lyophilized powder mixed with 5 mL of normal saline. A 140 ×110 ×7 mm acoustic coupling pad (Foshan SiEn Technology Co., LTD., Guangzhou, China) was used during imaging and treatment procedures for better coupling.

After routine clinical debridement of the wound, the acoustic coupling pad was placed on the ulcerated skin area. CEUS was first performed on local tissue, and 2.4 ml microbubble suspension was injected rapidly through the cubital vein, followed by flushing with 5 ml normal saline. CPS angiography combined with microbubble Flash mode was used for treatment: "Microbubble Flash → Microbubble Contrast → Microbubble Flash → Microbubble Contrast". In the first 5 minutes, the remaining circulating microbubbles from previous CEUS were used to mediate ultrasound therapy. Then another 5 ml of the prepared microbubble suspension was taken and injected slowly and continuously through the vein for 10 min. Finally, the remaining circulating microbubbles were used again to mediate ultrasound treatment for 5 min, and the total time of ultrasound treatment was 20 min. The ultrasound treatment cycle was 7 days, once a day, and the treatment process was the same for each time. Follow-up observation was conducted for 6 months.

Case presentation

Case 1

A 56-year-old man with 3-years history of type 2 diabetes fell to the ground while cycling two weeks ago, resulting in a skin ulceration on his right calf. He received basic debridement and daily dressing change at local hospital but the ulcer did not heal. Subsequently, the patient was admitted to the department of Endocrinology in our hospital. The patient was not taking any medication at admission.

Physical examination showed an ulcerated surface on the patient's medial right calf, about 3.0 ×2.0 cm in size, with a depth of about 6 mm (Figure 1A). The granulation tissue was relatively

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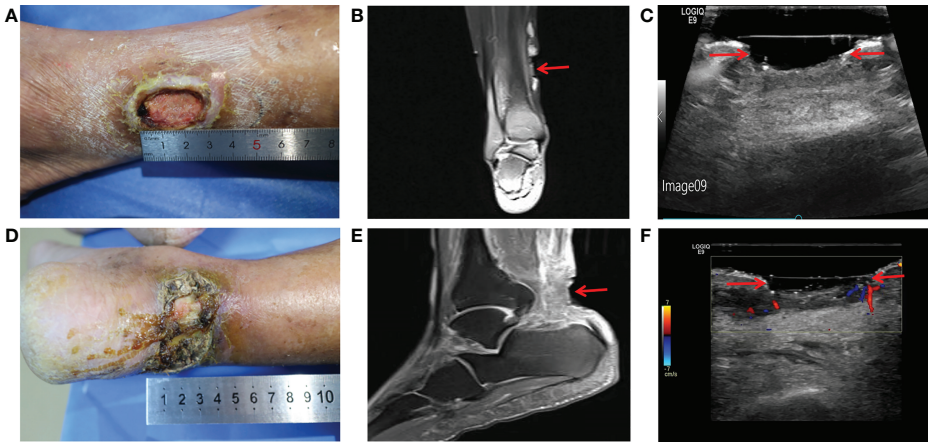


FIGURE 1
Imaging diagnosis of diabetic lower extremity ulcers before treatment for case 1 **(A-C)** and case 2 **(D-F)**. **(A)** Picture showed an ulcerated surface on the inner skin of the right calf, about 3.0×2.0 cm in size and 6 mm in depth; **(B)** MRI showed a local subcutaneous soft tissue defect at the medial margin of the right calf, with swelling in the margin and adjacent soft tissue space; **(C)** Gray-scale sonography showed a heterogeneous low echo area in the subcutaneous soft tissue of the medial side of the right calf; **(D)** Picture showed an ulcerated surface in the skin of the right heel, about 7.5 × 4.6 cm in size and 4 mm in depth, with necrosis and exudation; **(E)** MRI showed extensive swelling of the soft tissue and fascia in the lower part of the right calf with unclear and disordered layers; **(F)** CDFI showed local skin defects and discontinuity in the skin of the right heel. The blood flow signal in the low echo surface was not obvious, and a little blood flow signal could be seen in the periphery. The red arrows indicate the ulcer defects.

fresh, with a little dark red bloody exudate on the surface, and the surrounding soft tissues were red, swollen and slightly tender. Laboratory examination showed increased fasting blood glucose (16.2 mmol/L), increased HbA1c (7.10%), normal liver and renal function indexes. Secretion culture from the ulcer indicated an infection of Staphylococcus epidermidis (Table 1). Magnetic resonance imaging (MRI) examination showed that the ulcer did not involve bone tissue (Figure 1B). Color Doppler Flow Imaging (CDFI) showed no significant abnormalities in peripheral arteries (Figure 1C). Ankle-brachial index (ABI) was normal (ABI=1.20), Current perception threshold (CPT) was 0.00, and there was no abnormal sensation (Table 1). Based on these evidence, the patient preliminary diagnosed as diabetic foot ulcer (Grade 3 of Wagner classification).

After admission, the patient received antibiotic therapy for 8 days (intravenous cefotiam hydrochloride, 1g/8h, once a day). The ulcer wound dressing were changed once a day. For blood glucose control, the patient also received subcutaneous injection of recombinant human insulin (4 IU, three times a day), before each meal, subcutaneous injection of protamine human insulin (10 IU, once a day), at 22:00 every day, oral metformin hydrochloride sustained-release tablet (0.5 g, twice a day), oral sitagliptin phosphate tablet (100 mg, once a day). LIDUS combined with microbubbles therapy was performed once a day from the 5th day of admission (Figure 2A). On the 8th day of anti-infection, there was no purulent secretion in the wound, and the redness and swelling of the surrounding block were alleviated. On the 12th day of blood glucose control, the blood glucose level reduced to 6.3 mmol/L (Table 1). On the 12th day after enrolling in the LIDUS therapy, the ulcer area also decreased from 3.0 ×2.0 cm to 2.8 × 1.4 cm and its depth decreased from 6 mm to 4 mm, filled with granulation tissue, no purulent exudation was present. The patient was then discharged and continued to receive standardized blood sugar control treatment. Follow-up found the ulcer skin recovered 60 days after enrolling in LIDUS therapy (Figures 2B–F). Liver and kidney function were reexamined after ultrasound combined with microbubble treatment, and no abnormalities were found. Ankle brachial index was 0.96, slightly decreased. The CPT grade was 7.0, indicating mild hypoesthesia (Table 1). After discharge on the 12th day, the following medications were given to control glycemic for the next 15 days, subcutaneous injections of recombinant insulin lyprol (10 IU-8 IU, twice a day), before breakfast and dinner, oral metformin hydrochloride sustained-release tablet (0.5 g, twice a day), oral sitagliptin phosphate tablet (100 mg, once a day). Subsequently, the patient stopped glucose-controlling drugs by himself, and wound dressing was changed daily. To be clear, the timeline of entire treatment process of this case was presented in Figure 3.

Case 2

A 70-year-old male patient diagnosed with type 2 diabetes for more than 10 years had poor glycemic control due to

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TABLE 1 Details of blood routine, blood glucose level and other testing.

Indices	Pre-therapy	Post-therapy	Normal range
Case 1			
Total bilirubin(TBIL)	5.8 umol/L	5.6 umol/L	5-28 umol/L
Aspartate aminotransferase(AST)	13.1 IU/L	14.6 IU/L	9-60 IU/L
Alanine aminotransferase(ALT)	18.9 IU/L	22.1 IU/L	15-45 IU/L
Urea	6.95 mmol/L	5.3 mmol/L	2.9-7.2 mmol/L
Serum creatinine(SCr)	71 umol/L	70 umol/L	44-133 umol/L
Uric Acid(UA)	302 umol/L	328 umol/L	100-432 umol/L
Glucose(GLU)	16.29 mmol/L	6.3 mmol/L	3.80-6.10 mmol/L
Glycosylated hemoglobin(HbA1c)	7.10%	6.9%	4.00-6.50%
Ankle-brachial index (ABI)	1.2	0.96	0.9-1.3
Current perception threshold (CPT)	0.00	7.00	0.00
Case 2			
Total bilirubin(TBIL)	11.3 umol/L	12.6 umol/L	5-28 umol/L
Aspartate aminotransferase(AST)	22.9 IU/L	36.3 IU/L	9-60 IU/L
Alanine aminotransferase(ALT)	22.5 IU/L	22.6 IU/L	15-45 IU/L
Urea	16.7 mmol/L	33.14 mmol/L	2.9-7.2 mmol/L
Serum creatinine(SCr)	261 umol/L	292 umol/L	44-133 umol/L
Uric Acid(UA)	559 umol/L	575 umol/L	100-432 umol/L
Creatinine Clearance(CCR)	33.7 mL/min	29.6 mL/min	>80 mL/min
Glucose(GLU)	5.91	10.77	3.80-6.10 mmol/L
Glycosylated hemoglobin(HbA1c)	8.90%	–	4.00-6.50%
Ankle-brachial index (ABI)	1.12	1.32	0.9-1.3
Current perception threshold (CPT)	8.37	8.37	0.00

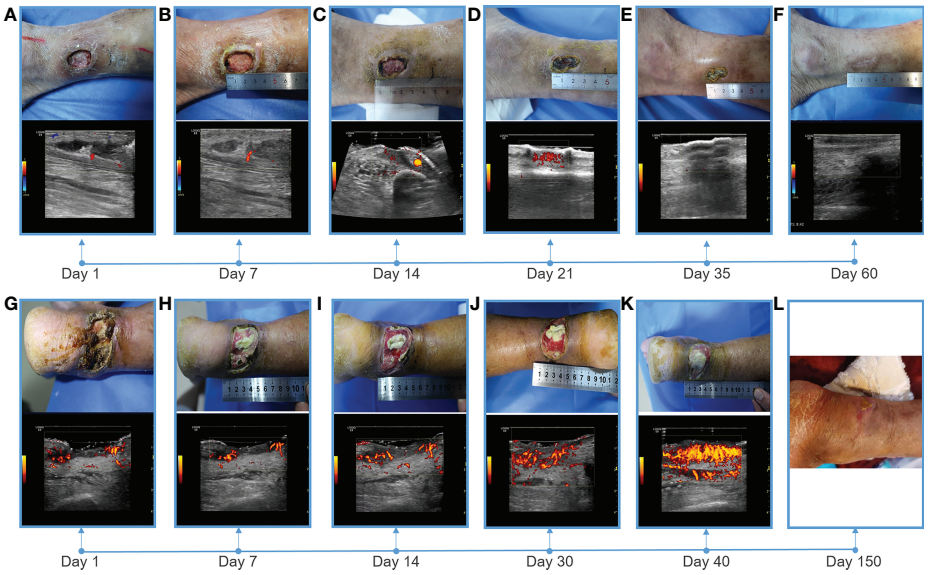


FIGURE 2
Showing of skin wounds and CDFI or PDI ((Power Doppler Imaging)) ultrasound imaging for progress in the treatment of diabetic lower extremity ulcers in two cases. Wound conditions at the **(A)** 1th, **(B)** 7th, **(C)** 14th, **(D)** 21th, **(E)** 35th, and **(F)** 60th day of post therapy showed the gradual healing of ulceration for case 1; Wound conditions at the **(G)** 1th, **(H)** 7th, **(I)** 14th, **(J)** 30th, **(K)** 40th, and **(L)** 150th day of post therapy showed the gradual healing of ulceration for case 2. PDI showed that the blood flow of the soft tissue around the ulcer gradually increased, and the blood flow was very abundant before healing.

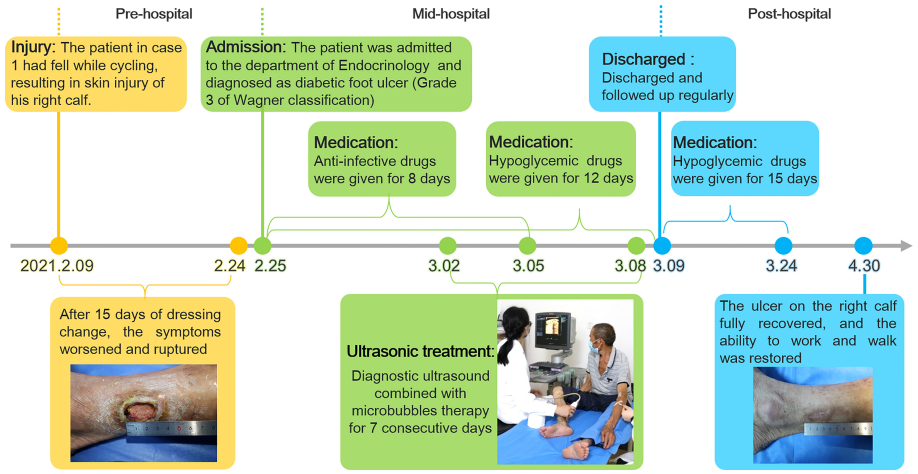


FIGURE 3
The timeline of the treatment process of case 1 from the day of injury to the day of healing.

irregular medication. The patient found an ulcer on his right heel without any known injuries eight months ago. After removing the black scab on the surface of the ulcer by himself, the ulcer was getting worse and the patient was subsequently admitted to the Endocrinology Department of our hospital. Since the onset of the ulcer, the patient had complained about a progressively deterioration in mental, physical, appetite and sleep. The patient also had a history of alcohol abuse for more than 30 years (about 500mL strong wine a day).

Physical examination revealed a skin defect about 7.5 × 4.6 cm in size and 4 mm in depth, with black crusts and yellowish exudate, surrounding skin redness and swelling, and pain when walking and pressing the wound (Figure 1D). Laboratory examination showed normal fasting blood glucose (PP 5.91 mmol/L), increased HbA1c (8.90%), normal liver function, and Renal insufficiency (blood urea (16.76 mmol/L, serum creatinine 261 umol/L, and serum uric acid 559 umol/L) (Table 1).Blood pressure test showed hypertension (160/110 mmHg). MRI examination showed that the ulcer did not involve bone tissue (Figure 1E). CDFI showed mild atherosclerosis of lower extremity arteries (Figure 1F). Ankle-brachial index was in the normal range, and the CPT grade was 8.37, suggesting moderate hypoesthesia. Based on these evidence, the patient preliminary diagnosed as type 2 diabetes, diabetic foot, hyperlipidemia, hypertension and renal impairment in the outpatient department of endocrinology of our hospital.

Heel ulcer debridement was performed on the patient first (Figure 2G). After debridement, the ulcer surface showed no obvious granulation tissue and light red color, and the Achilles tendon was partially necrotic and pale color with partial yellowness. After 7 consecutive days of ultrasound combined with microbubble therapy, the ulcer area did not change significantly (Figure 2H). On the 7th day, the granulation tissue grew obviously, and the tendon tissue grew slightly (Figure 2H). On the 14th day, the wound contracted slightly and granulation tissue grew with bright red color (Figure 2I). On the 30th day, the granulation tissue tended to fill the wound, and a little epidermal tissue grew around it (Figure 2J). On the 40th day, the wound contracted and became slightly smaller, granulation tissue protruded from the skin surface, and the tendon tissue was completely repaired with normal color (Figure 2K). During the LIDUS therapy, the patient's local pain and itching gradually increased. Subsequently, the patient was to be treated by skin grafting in the burn department. However, due to the long-term high blood glucose (about 10 mmol/L), the surgeon suggested controlling blood glucose before operating. With no other treatment, after 150 days, the wound was covered with epidermis and the ulcer was basically healed (Figure 2L). There was no significant change in liver and kidney function before and after ultrasound treatment, ankle-brachial index increased (ABI=1.32), indicating arterial stiffness, and the CPT grade was 8.37, indicating moderate hypoesthesia (Table 1). The patient was treated only in the outpatient department, and glycemic control was simply by oral metformin hydrochloride sustained-release tablets (0.5g, twice a day), which was not effective.

Discussion

The therapeutic effect in 2 patients was positive and encouraging. The size and depth of the ulcer determined the time to cure, and the ulcer completely healed in 60 to 150 days, and the patients were able to live independently. The safety of the treatment process was also verified. There was no significant

difference in liver and kidney function in two patients before and after LIDUS therapy. In addition, there was no ecchymosis on the local body surface, and no thrombosis and other adverse events occurred in local veins and arteries. What is noteworthy is patient 2, whose wound was large and blood sugar fluctuated for a long time and remained high. After careful surgical evaluation, skin grafting was finally abandoned to close the wound. Unexpectedly, after 150 days of slow growth, the skin healed on its own. Both patients were followed up for more than one year and had no recurrence of ulcers. At a recent follow-up visit, Patient 1 said that compared with other methods he had known, receiving our treatment was like undergoing ultrasound examination, which was painless, non-invasive, easy to adhere to, and had definite efficacy, and he was happy to share this treatment with other patients.

To our knowledge, this was the first human trail that used LIDUS combined with microbubbles to treat diabetic foot ulcer. Almost all previous studies on ultrasonic treatment of diabetic foot ulcer were *in vitro* or preclinical (7–9). Until now, the study of low-intensity ultrasonic cavitation in the treatment of human diabetic foot ulcer has not been reported. The only known clinical application of LIDUS plus microbubbles is in the field of tumor therapy. Kotopoulis (10) and Liuzheng (11) have respectively used this method to enhance microcirculation blood supply to pancreatic and breast cancer tumors, and improve the efficacy of chemotherapy.

Blood flow enhancement by LIDUS is the premise of this clinical experiment, which was found in our previous animal studies. When MI was set to 0.3, 5 minutes after ultrasound combined with microbubbles irradiation for VX2 tumor, the tumor blood supply was significantly increased by contrast enhanced ultrasound by a direct visualization method and TIC curve quantitative analysis (12). Similarly, in this study, after the soft tissue around DFU was treated for 20 minutes, its blood perfusion was observed increased by direct visualization, and reached a higher peak intensity (-46 to -42dB) by quantitative analysis in a shorter time and decreased more slowly after treatment. The results showed that vascular resistance of muscle tissue decreased and blood perfusion increased after treatment. *In vivo* studies have reported that low-intensity ultrasound combined with microbubbles irradiating muscle tissue for more than 10 minutes can reverse ischemia up to 24 hours (6).

As a chronic refractory wound, diabetic ulcer healing also involves cell proliferation, angiogenesis and other processes. Several studies have explored the molecular mechanism of low-intensity ultrasound combined with microbubble therapy, namely, the enhanced effect of local blood flow is related to the increased synthesis of local vasodilators Nitric oxide (NO) and prostaglandin (13), while the production of ATPase makes cell proliferation and metabolism more active (6). At the same time, this method can increase vascular endothelial growth factor (VEGF) and other growth factors and promote angiogenesis (14). Hypoxia-inducible factor-α (HIF-α) and the activation of immune pathway are also involved in this process (15, 16). In this case, the wounds of the 2 patients were gradually and slowly healed after short treatment. The timing and molecular mechanism of initiating active wound repair and continuing to heal need to be further studied.

Diagnostic low-intensity ultrasound was selected in this study primarily for the safety of human trials. Due to the strict FDA restrictions (17), the acoustic intensity of diagnostic ultrasound is constrained within an admissible range (0.05-0.5 W/cm^2) in the form of low energy. As the cavitation nuclei, the microbubbles can reduce the cavitation threshold and enhance the cavitation effect (18, 19). Moreover, the visualization advantage of diagnostic low-intensity ultrasound enables it to observe the whole process of microbubble perfusion and rupture. In the experiment, it was observed that the muscular tissue perfusion microbubbles disappeared after a flash operation. From this, it can be speculated that the existing parameter Settings caused Sonoporation in the treatment, and the physical effects such as shear waves and micro jets may promote the occurrence of healing events. High parameters of MI (0.89 and 0.86), sound power (79%) and Frame (50) were set to improve the duty cycle of treatment pulses, and Flash mode was selected to promote the occurrence of cavitation through breaking microbubbles. The possibility of cavitation induced by commercial equipment was also confirmed by Lindar (6) and Kitoplis (10).

The importance of this study may be reflected in the following aspects: firstly, it was a real world clinical study in humans, and secondly, the therapeutic equipments and microbubbles used are commercial products, which could make this technology promising for clinical promotion and could provide a new non-invasive method and idea for the treatment of diabetic ulcers. The limitations of this study mainly focus on the small sample size, the interpretation of the results needs to be cautious, and more samples need to be accumulated to further verify the treatment effect.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of The General Hospital of Western Theater Command of PLA. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JT, RW and ZC: conception and design of the work. XZ and LP: data collection. XZ, YC and RW: Image analysis and interpretation, manuscript writing, and critical revision of the article. ZC: approval of the final version of the article.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Assessment of QRISK3 as a predictor of cardiovascular disease events in type 2 diabetes mellitus

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Background: The risk of cardiovascular disease (CVD) in diabetes mellitus (DM) patients is two- to three-fold higher than in the general population. We designed a 10-year cohort trial in T2DM patients to explore the performance of QRESEARCH risk estimator version 3 (QRISK3) as a CVD risk assessment tool and compared to Framingham Risk Score (FRS).

Method: This is a single-center analysis of prospective data collected from 566 newly-diagnosed patients with type 2 DM (T2DM). The risk scores were compared to CVD development in patients with and without CVD. The risk variables of CVD were identified using univariate analysis and multivariate cox regression analysis. The number of patients classified as low risk (<10%), intermediate risk (10%-20%), and high risk (>20%) for two tools were identified and compared, as well as their sensitivity, specificity, positive and negative predictive values, and consistency (C) statistics analysis.

Results: Among the 566 individuals identified in our cohort, there were 138 (24.4%) CVD episodes. QRISK3 classified most CVD patients as high risk, with 91 (65.9%) patients. QRISK3 had a high sensitivity of 91.3% on a 10% cut-off dichotomy, but a higher specificity of 90.7% on a 20% cut-off dichotomy. With a 10% cut-off dichotomy, FRS had a higher specificity of 89.1%, but a higher sensitivity of 80.1% on a 20% cut-off dichotomy. Regardless of the cut-off dichotomy approach, the C-statistics of QRISK3 were higher than those of FRS.

Conclusion: QRISK3 comprehensively and accurately predicted the risk of CVD events in T2DM patients, superior to FRS. In the future, we need to conduct a large-scale T2DM cohort study to verify further the ability of QRISK3 to predict CVD events.

KEYWORDS

cardiovascular disease, type 2 diabetes mellitus (DM), risk score, QRESEARCH risk estimator version 3 (QRISK3), framingham risk score (FRS)

Introduction

Diabetes mellitus (DM) has become more widespread owing to its high prevalence and related disability, estimated to affect 693 million individuals by 2045 (1, 2). The most challenging aspect of treatment is controlling diabetes-related complications. Macrovascular complications (cardiovascular and cerebrovascular diseases) and microvascular illness (diabetic nephropathy) lead to a significant increase in care expense, hospitalization frequency, mortality and a decline in quality of life (1, 3, 4). The risk of cardiovascular disease (CVD) was reported to be two- to three-fold higher in people with DM (5, 6). The development of CVD in DM patients is often a complication with high mortality, which mainly manifests in the coronary system, aorta and cerebral artery (7). Uncoordinated vasoconstriction and dilation, platelet aggregation, and lipid deposition in the vessel wall resulting in hyperglycemia, hyperlipidemia, hyperviscosity, and hypertension, resulting in an exponential increase in the incidence of DM-related CVD (8). Blood glucose control and clinical risk variables by themselves are unable to anticipate the onset of vascular complications. The concern is to find alternative indicators to identify CVD patients at high risk of DM disease.

If the subject has not yet suffered a CVD event, the QRESEARCH risk estimator version 3 (QRISK3) algorithm can be used to estimate a person's probability of suffering a fatal or non-fatal heart attack or stroke within the next 10 years (9, 10). Based on QRISK2, this algorithm was jointly created in 2017 by doctors and academics working for the UK National Health Service. Developers and researchers thoroughly verified the QRISK algorithm utilizing UK primary care databases such as QResearch and other large cohorts clinical studies (9, 11). In addition to the clinical factors already included in the QRISK2 risk prediction model, the QRISK3 risk prediction model includes additional clinical factors (severe mental illness (schizophrenia, bipolar disorder, moderate/severe depression), atypical antipsychotic use, corticosteroid use, systolic blood pressure variability measurements (standard deviation of repeated measurements), migraine, systemic lupus erythematosus (SLE), and erectile dysfunction) to assist physicians in identifying patients most at high risk for CVD events, early intervention, and treatment (9). There have been several previous studies on the effectiveness of QRISK3 in predicting CVD events in SLE and inflammatory bowel disease (IBD), but few studies on the performance of cardiovascular events in T2DM (12, 13).

A popular and well-known calculator, the Framingham risk score (FRS), is recommended in the American College of Cardiology/American Heart Association (ACC/AHA) practice guideline on the prevention of CVD disorders in clinical practice (14, 15). In addition to the CVD events listed in the original FRS model, the most recent version of the FRS, created in 2008, also includes transient ischemic attack (TIA) and cerebrovascular accident (CVA) (14). The FRS model incorporated multiple risk factors, such as age, sex, hypertension treatment, diabetes status, smoking status, high-density lipoprotein (HDL), total cholesterol, and systolic blood pressure (SBP), to estimate the 10-year risk of CVD (14). However, clinical factors related to CVD such as chronic kidney disease and family history were not included in the FRS compared to QRISK3.

To our knowledge, QRISK3 has not been tested in patients with T2DM in China. We tried to assess the application of QRISK3 for assessing CVD risk in individuals with type 2 diabetes mellitus (T2DM) in this present study. Since the FRS is a widely used CVD risk calculator, we compared the prediction performance of QRISK3 and the FRS to identify the presence of subclinical atherosclerosis in individuals with T2DM (16). Therefore, we designed a cohort trial in patients with newly-diagnosed T2DM for about 10 years, aiming to explore the effectiveness of QRISK3 and FRS as cardiovascular risk assessment tools, to best predict the development of CVD related to T2DM.

Methods

Study population

This was a retrospective analysis on prospectively collected T2DM patients data from the third affiliated hospital of soochow university. We selected 1003 newly-diagnosed T2DM patients in our hospital from December 2010 to September 2014 for long-term follow-up.

Inclusion criteria (1): meet the American Diabetes Association (ADA) classification criteria for T2DM and were newly-diagnosed patients (17) (2); age 25-84 years old (met the applicable range of QRISK3 algorithm).

Exclusion criteria (1): a history of CVD before enrolment in the cohort study (2); participation in clinical trials during the study period (3); incomplete clinical records.

Abbreviations: ALB, albumin; BUN, blood urea nitrogen; CVD, cardiovascular disease; CVA, cerebrovascular accident; CHD, coronary heart disease; C-statistics, concordance statistics; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; FRS, Framingham Risk Score; FCP, fast C-peptide; FIB, fibrinogen; HDL, high-density lipoprotein; HR, hazard ratio; IBD, inflammatory bowel disease; MI, myocardial infarction; NPV, negative predictive value; QRISK3, QRESEARCH risk estimator version 3; PAD, peripheral artery disease; RA, rheumatoid arthritis; PPV, positive predictive value; SLE, systemic lupus erythematosus; SBP, systolic blood pressure; Scr, serum creatinine; T2DM, type 2 diabetes mellitus; TIA, transient ischaemic attack; TyG index, triglyceride-glucose index; WBC, white blood cell.

The Framingham study in 2008, this study defined CVD events as (1) coronary heart disease (CHD) including coronary death, myocardial infarction (MI), coronary insufficiency and angina (2); atherosclerotic CVA including ischaemic stroke, haemorrhagic stroke and TIA (3); peripheral artery disease (PAD) secondary to atherosclerosis (intermittent claudication) (4); heart failure secondary to atherosclerosis (14).

We need to collect relevant data, including baseline data and information on cardiovascular risk factors (hypertension, smoking status, family history, psychiatric history) for calculating risk scores. The first CVD events identified throughout the follow-up period classified patients as 'CVD patients', while patients without CVD events were classified as 'Non-CVD patients'.

CVD risk algorithms

Although the CVD risk algorithm tools have relatively similar components, their efficacy may vary since they utilize various risk derivation algorithms and the same components have varying weights. For example, Age accounts for a high weight in the FRS model, but lacks indicators related to cardiovascular risk factors such as family history and chronic kidney disease history. In this study, QRISK3 and FRS established three categories of risk: low risk (<10%), intermediate risk (10-20%), and high risk (>20%).

FRS: includes age, sex, treatment for hypertension, DM status, smoking status, HDL, total cholesterol and systolic blood pressure (14).

QRISK3: includes age, sex, ethnicity, smoking status (non-smoker, ex-smoker, light smoker, moderate smoker, heavy smoker), DM status, family history of CVD (angina or heart attack in a first-degree relative younger than 60), chronic kidney disease, atrial fibrillation, blood pressure treatment, migraine, rheumatoid arthritis (RA), SLE, severe mental illness (schizophrenia, bipolar disorder, moderate/severe depression), atypical antipsychotic medication, steroid tablets use, diagnosis or treatment of erectile dysfunction, Cholesterol/HDL ratio, SBP and standard deviation of repeated blood pressure, height and weight.

Data collection

The date of collection of baseline data and associated clinical data necessary to calculate the risk score was the 'baseline date'. The 'baseline date' was defined as the date of the first visit to our hospital due to T2DM. Baseline clinical data was from the electronic medical record system, while clinical records (such as drug use history and family history) required to calculate the risk score and the CVD outcome are collected through follow-up.

Statistical analysis

All statistical analysis was performed with SPSS 25.0. The median (interquartile range) [M (P25, P75)] was used to represent data with a non-normal distribution, whereas the mean ± standard deviation (SD) was used to express data with a quantitative normal distribution. The qualitative variable was selected as percentages (%). $P < 0.05$ was considered statistically significant.

The CVD risk score for each patient was calculated at the 'baseline date' using QRISK3 and FRS and assessed based on whether CVD occurred at the 10-year point. The low-risk (<10%), intermediate-risk (10%-20%) and high-risk (>20%) patient numbers of the two tools were identified and compared, and their sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV) and concordance (C) statistics were reported. Creating a proportional risk model (cox regression) to evaluate the correlation between QRISK3 and CVD. In addition, for each risk level, we estimated diagnostic hazard ratio (HR). C-statistics was used to assess the discriminative of each tool, where C-statistics was the area under the curve (AUC) of the receiver operating characteristics (ROC) curve with observed CVD as the outcome. C-statistics of 0.5 suggests no discrimination, 0.5-0.7 is considered acceptable, 0.7-0.9 excellent and greater than 0.9 outstanding (18). Kappa statistics were used to observe the similarities in total risk, low risk and high risk categories assigned by each tool. In addition, sensitivity analysis was carried out for the patients who lost to follow-up. Patients with loss to follow-up were included in the non-CVD and CVD groups, respectively, to compare whether there were differences in the conclusions.

Results

Out of the 1003 individuals in the cohort, 206 individuals were excluded due to loss of follow-up, 96 individuals were excluded due to insufficient clinical data, and 135 individuals were excluded due to ineligibility (105 individuals had CVD events prior to enrolment, and 30 individuals did not meet the age criteria). The final queue size was 566 persons (Figure 1). The average follow-up time of the T2DM clinical registration cohort was 8.68 ± 1.86years. There were 138 CVD events among the 566 individuals identified in our cohort. Of the 138 CVD events identified in our cohort, 84 (60.9%) developed coronary heart disease (see Figure 2 for classification of CVD events).

The specific demographic and clinical characteristics of 566 T2DM patients are given in Table 1. Particular variables of discrepant include CVD patients being on average older in age ($p<0.001$), having higher blood pressure ($p<0.001$), higher fast C-peptide (FCP) ($p=0.029$), higher triglyceride-glucose index

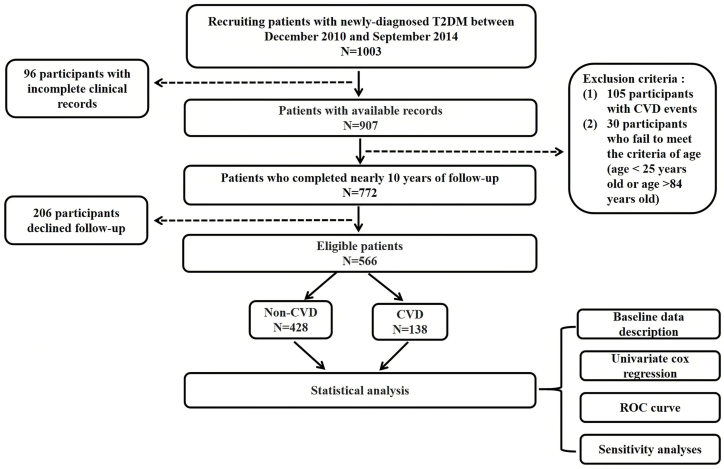


FIGURE 1
Flow chart of study. Legend: The flow chart shows the entire research process. T2DM, Type 2 diabetes mellitus; CVD, Cardiovascular disease; ROC, Receiver operating characteristics.

(TyG index) (p=0.018) and TC/HDL (p=0.023), and greater proportion with a history of smoking (p<0.001) and hypertension (p<0.001).

Risk factors for CVD

Table 2 shows risk factors for the prediction of CVD in T2DM patients. In univariate analysis, QRISK3 and FRS were significant risk factors for CVD (p<0.001). The results of cox regression analysis showed that QRISK3 (HR=5.972, p< 0.001, 95% CI 4.565 to 7.813) and FRS (HR=3.223, p< 0.001, 95% CI 2.535 to 4.097) were risk factors for CVD. In addition, age, sex (male), smoking history, hypertension history, SBP, FCP, white blood cell (WBC), fibrinogen (FIB), blood urea nitrogen (BUN), serum creatinine (Scr), uric acid (UA), TyG index and TC/HDL were also risk factors for CVD in patients with T2DM (p<0.05). While albumin (ALB) and estimated glomerular filtration rate (eGFR) were protective factors for CVD (p<0.05).

Tool evaluation

Interestingly, Table 1 shows that QRSKI3 and FRS scored higher in CVD patients compared with non-CVD patients. The

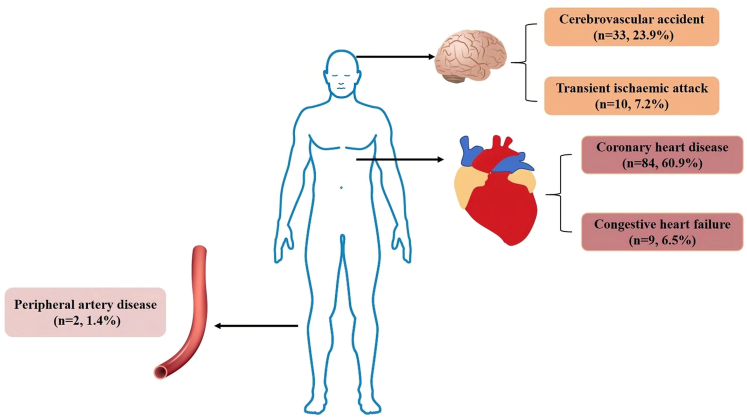


FIGURE 2
Classification of CVD events. Legend: In our cohort, there were 138 CVD events, 84 patients with coronary heart disease, 9 patients with congestive heart failure, 33 patients with cerebrovascular accident, 10 patients with transient ischaemic attack and 2 patients with peripheral arterial disease. CVD, Cardiovascular disease.

TABLE 1 Demographic and clinical characteristics of patients with Non-CVD and CVD.

Group	Non-CVD (n = 428)	CVD (n = 138)	Total (n = 566)
Age (years)	47.3 ± 11.7	59.5 ± 10.1	50.3 ± 12.5
Sex (male, %)	260.0 (60.7)	101.0 (73.2)	361.0 (63.8)
BMI(kg/m^2)	25.2 (23.2, 27.1)	25.4 (23.4, 27.2)	25.3 (23.2, 27.1)
Smoking, n (%)	152.0 (34.3)	72.0 (52.2)	224.0 (39.6)
Hypertension, n (%)	144.0 (33.6)	80.0 (58.0)	250.0 (44.2)
SBP (mmHg)	135.4 ± 16.7	141.3 ± 19.1	135.0 (125.0, 147.0)
FBG (mmol/L)	9.5 (7.9, 11.7)	9.8 (7.9, 12.4)	9.5 (7.9, 11.9)
PBG (mmol/L)	14.5 (11.7, 17.1)	14.9 ± 4.1	14.6 (11.7, 17.1)
HbA1c (%)	10.8 (9.0, 12.3)	10.6 ± 2.6	10.7 (8.9, 12.4)
FCP (ng/ml)	1.6 (1.1, 2.3)	1.9 (1.3, 2.5)	1.7 (1.2, 2.4)
WBC (10^9g/L)	6.2 (5.4, 7.4)	6.7 (5.7, 8.1)	6.4 (5.5, 7.6)
Hb (g/L)	143.0 (134.0, 152.3)	141.2 (132.0, 149.3)	143 (133, 152)
NLR	1.6 (1.2, 2.0)	1.6 (1.3, 2.1)	1.6 (1.2, 2.0)
FIB (g/L)	2.4 (2.2, 2.9)	2.7 (2.2, 3.0)	2.5 (2.2, 2.9)
D-dimer (ug/L)	130.0 (100.0, 250.0)	160.0 (100.0, 282.5)	140.0 (100.0, 260.0)
ALT (U/L)	25.0 (18.0, 43.0)	27.0 (17.0, 43.0)	26.0 (18.0, 43.0)
AST (U/L)	16.0 (12.0, 23)	15.0 (12.0, 22.0)	16.0 (12.0, 23.0)
ALB (g/L)	37.9 (35.4, 40.2)	36.4 (34.5, 39.2)	37.4 (35.0, 39.9)
Bicarbonate (mmol/L)	23.7 (22.2, 25.6)	24.3 ± 2.7	23.9 (22.2, 25.7)
BUN (mmol/L)	4.7 (3.8, 5.7)	5.5 (4.2, 6.5)	4.8 (3.9, 5.9)
SC r (μmol/L)	69.2 ± 18.2	74.5 ± 16.6	70.5 ± 15.7
UA (μmol/L)	262.8 (211.2, 320.3)	289.5 ± 85.6	269.8 (216.8, 325.6)
CysC (mmol/L)	0.7 (0.6, 0.9)	0.7 (0.6, 0.9)	0.7 (0.6, 0.9)
eGFR (ml/min/1.73 min)	109.5 (91.1, 132.6)	96.1 (79.0, 112.3)	104.8 (88.5, 129.2)
K (mmol/L)	4.3 (4.1, 4.5)	4.2 ± 0.4	4.3 (4.0, 4.5)
Ca (mmol/L)	2.4 (2.3, 2.4)	2.3 (2.3, 2.4)	2.4 (2.3, 2.4)
P (mmol/L)	1.2 (1.1, 1.3)	1.2 ± 0.2	1.2 (1.1, 1.3)
TC (mmol/L)	4.8 (4.1, 5.5)	4.8 (4.3, 5.5)	4.8 (4.2, 5.5)
TG (mmol/L)	2.5 (1.7, 3.7)	2.9 (2.0, 4.2)	2.6 (1.8, 3.8)
HDL (mmol/L)	1.0 (0.9, 1.2)	0.9 (0.8, 1.1)	1.0 (0.9, 1.1)
LDL (mmol/L)	2.3 (1.9, 2.8)	2.4 (2.0, 2.8)	2.3 (1.9, 2.8)
Apoa1 (g/L)	1.2 (1.1, 1.3)	1.2 (1.1, 1.3)	1.2 (1.1, 1.3)
Apob (g/L)	1.0 (0.9, 1.2)	1.1 (0.9, 1.3)	1.0 (0.9, 1.2)
UACR (mg/g)	9.9 (6.7, 16.7)	10.0 (6.7, 18.4)	10.0 (6.7, 16.9)

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TABLE 1 Continued

Group	Non-CVD (n = 428)	CVD (n = 138)	Total (n = 566)
TyG index	9.8 (9.4, 10.3)	10.1 (9.7, 10.4)	9.9 (9.5, 10.3)
TC/HDL	4.9 (3.9, 5.9)	5.1 (4.3, 6.2)	4.9 (4.0, 5.9)
TSH (uIU/ml)	2.0 (1.3, 2.9)	2.0 (1.3, 2.6)	2.0 (1.3, 2.8)
fT3 (pmol/L)	4.3 (3.9, 4.7)	4.2 ± 0.7	4.3 (3.8, 4.7)
fT4 (pmol/L)	16.6 (15.0, 18.3)	16.5 ± 2.2	16.6 (14.9, 18.3)
QRSKI3	7.5 (3.2, 14.4)	23.8 (16.6, 32.6)	10.6 (4.3, 19.5)
QRSIK3 (<10%), n (%)	262.0 (61.2)	12.0 (8.7)	274.0 (48.4)
QRSIK3 (10%-20%), n (%)	128.0 (29.9)	35.0 (25.4)	163.0 (28.8)
QRSIK3 (> 20%), n (%)	38.0 (8.9)	91.0 (65.9)	129.0 (22.8)
FRS	9.7 (4.7, 18.4)	25.3 (15.6, 30.0)	13.3 (5.6, 21.6)
FRS (<10%), n (%)	214.0 (50.0)	15.0 (10.9)	229.0 (40.5)
FRS (10%-20%), n (%)	129.0 (30.1)	41.0 (29.7)	170.0 (30.0)
FRS (> 20%), n (%)	85.0 (19.9)	82.0 (59.4)	167.0 (29.5)

The eGFR is calculated according to the CKD-EPI formula. CVD, cardiovascular disease; BMI, body mass index; SBP, systolic blood pressure; FBG, fasting blood glucose; PBG, postprandial blood glucose; HbA1c, haemoglobin A1c; FCP, fasting c- peptide; WBC, white blood cell; Hb, haemoglobin; NLR, neutrophil to lymphocyte ratio; FIB, fibrinogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, serum albumin; BUN, blood urea nitrogen; SCr, serum creatinine; UA, uric acid; CysC, cystatin C; eGFR, estimated glomerular filtration rate; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; Apoa1, apolipoprotein a1; Apob, apolipoprotein b; UACR, urine albumin-to-creatinine ratio; TyG index, triglyceride-glucose index; TSH, thyroid stimulating hormone; fT3, free triiodothyronine; fT4, free thyroxine; QRISK3, QRESEARCH risk estimator version 3; FRS, framingham risk score.

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TABLE 2 Cox regression analysis of CVD- related risk factors.

Variables	β-coefficient	HR (95% CI)	*P* value
Age	0.087	1.190 (1.074, 1.108)	<0.001*
Sex (female)	0.524	1.689 (1.159, 2.463)	0.006*
BMI	0.018	1.018 (0.968, 1.070)	0.492
Smoking	0.856	2.374 (1.692, 3.330)	<0.001*
Hypertension	1.52	4.571 (3.113, 6.711)	<0.001*
SBP	0.018	1.019 (1.009, 1.028)	<0.001*
FBG	0.056	1.057 (0.997, 1.121)	0.062
PBG	0.022	1.023 (0.981, 1.067)	0.297
HbA1c	-0.023	0.977 (0.910, 1.049)	0.521
FCP	0.181	1.198 (1.018, 1.410)	0.029*
WBC	0.155	1.168 (1.067, 1.278)	0.001*
Hb	-0.003	0.997 (0.987, 1.006)	0.484
NLR	-0.001	0.999 (0.988, 1.010)	0.857
FIB	0.209	1.232 (1.037, 1.456)	0.018*
ALT	-0.004	0.996 (0.989, 1.002)	0.206
AST	-0.009	0.991 (0.976, 1.005)	0.204
ALB	-0.073	0.930 (0.894, 0.968)	<0.001*
Bicarbonate	0.004	1.045 (0.987, 1.107)	0.132
BUN	0.123	1.131 (1.065, 1.201)	<0.001*
SC r	0.018	1.019 (1.008, 1.029)	0.001*
UA	0.002	1.002 (1.000, 1.004)	0.032*
CysC	0.299	1.349 (0.677, 2.687)	0.394
eGFR	-0.015	0.985 (0.979, 0.991)	<0.001*
K	-0.254	0.776 (0.546, 1.102)	0.157
Ca	-0.993	0.370 (0.170, 1.279)	0.116
P	-0.535	0.586 (0.241, 1.423)	0.238
TC	0.044	1.045 (0.913, 1.196)	0.524
TG	0.029	1.030 (0.976, 1.086)	0.284
HDL	-0.71	0.492 (0.240, 1.008)	0.053
LDL	0.062	1.064 (0.839, 1.350)	0.608
Apoa1	0.428	1.534 (0.624, 3.773)	0.352
Apob	0.356	1.428 (0.964, 2.115)	0.076
UACR	0.002	1.002 (0.998, 1.006)	0.392
TyG Index	0.277	1.320 (1.049, 1.659)	0.018*
TC/HDL	0.105	1.111 (1.014, 1.216)	0.023*
TSH	0.001	1.001 (0.901, 1.112)	0.99
fT3	-0.113	0.875 (0.723, 1.060)	0.172
fT4	-0.04	0.961 (0.903, 1.024)	0.217
QRISK3 (Grade)	1.787	5.972 (4.565, 7.813)	<0.001*
FRS (Grade)	1.17	3.223 (2.535, 4.097)	<0.001*

CVD, cardiovascular disease; BMI, body mass index; SBP, systolic blood pressure; FBG, fasting blood glucose; PBG, postprandial blood glucose; HbA1c, haemoglobin A1c; FCP, fasting c-peptide; WBC, white blood cell; Hb, haemoglobin; NLR, neutrophil to lymphocyte ratio; FIB, fibrinogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, serum albumin; BUN, blood urea nitrogen; SCr, serum creatinine; UA, uric acid; CysC, cystatin C; eGFR, estimated glomerular filtration rate; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; Apoa1, apolipoprotein a1; Apob, apolipoprotein b; UACR, urine albumin-to-creatinine ratio; TyG index, triglyceride-glucose index; TSH, thyroid stimulating hormone; fT3, free triiodothyronine; fT4, free thyroxine; QRISK3, QRESEARCH risk estimator version 3; FRS, framingham risk score.

median 10-year CVD risk scores for the QRISK3 and FRS among patients without CVD were 7.5% and 9.7%, respectively (Figure 3). However, for CVD patients, the median 10-year CVD risk scores were 23.8% and 25.3%, respectively (Figure 3).

When examining risk stratification, QRISK3 classified most non-CVD patients as low and intermediate risk, with 262 (61.2%) patients at low risk, and 128 (29.9%) patients at intermediate risk, respectively (Figure 4). Moreover, QRISK3 classified most CVD patients as high-risk, with 91 (65.9%)

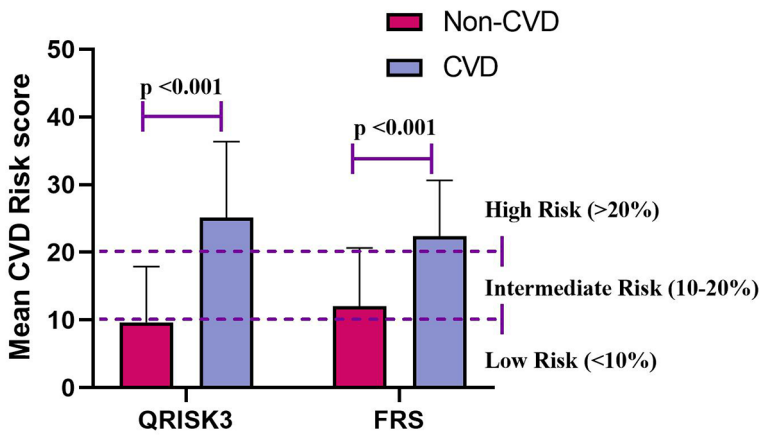


FIGURE 3
Mean CVD risk score for QRISK3 and FRS. Legend: Stratified according to patients with CVD (n=138) and patients without CVD (n=428). CVD, cardiovascular disease; QRISK3, QRESEARCH risk estimator version 3; FRS, Framingham risk score.

patients (Figure 4). However, FRS classified 85 (61.6%) non-CVD patients as high risk, and the ratio of CVD patients classified as high risk based on FRS was lower than QRISK3 (61.2% vs 59.4%) (Figure 4).

To compare the risk prediction between QRISK3 and FRS, AUC analysis was used. The AUC of QRISK3 and FRS were 0.878 and 0.805 (Figure 5). Moreover, Table 3 lists the sensitivity, specificity, PPV, NPV and C-statistics obtained by dichotomizing the risk score using the cut-off of 10% and 20% 10-year CVD for the two tools. The FRS had the best PPV of 93.4% and 86% but the lowest NPV was 36.5 and 49.1%. In addition, with a 10% cut-off dichotomy, FRS had a higher specificity of 89.1%, but a higher sensitivity of 80.1% on 20% cut-off dichotomy. Meanwhile, QRISK3 had a high sensitivity of 91.3% on a 10% cut-off dichotomy, but a higher specificity of 90.7% on a 20% cut-off dichotomy. The C-statistics of QRISK3 in the two cut-off dichotomies were the highest, which were 0.763 and 0.787, respectively (Table 3). In addition, we calculated the Kappa values of the total-risk category, low-risk category and high-risk category, which were 0.587, 0.726 and 0.645 respectively (Table 4).

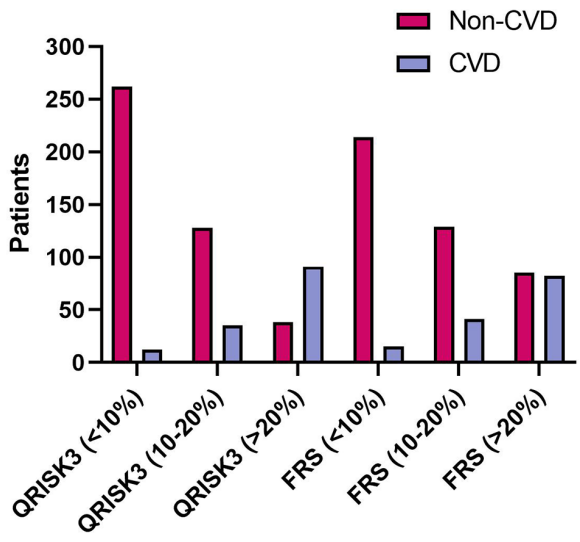


FIGURE 4
The number of patients considered low (<10%), median (10%–20%) and high (>20%) risk between patients according to QRISK3 and FRS. Legend: QRISK3 identified the majority of non-CVD patients as low or intermediate risk, with 262 (61.2%) patients classified as low risk and 128 (29.9%) patients classified as intermediate risk. Furthermore, QRISK3 identified 91 (65.9%) of CVD patients as high-risk. However, FRS classified some non-CVD patients as high risk. The number of CVD patients classified as high risk was 82 (59.4%), while the number of non-CVD classified as high risk was 85 (61.6%). CVD, cardiovascular disease; QRISK3, QRESEARCH Risk estimator version 3; FRS, Framingham risk score.

Sensitivity analyses

We conducted sensitivity analyses to assess the impact of loss to follow up on the results. The results of the number of patients who lost to follow-up were re-analyzed and compared by non-CVD and CVD, respectively. In both different endings, the AUC of QRISK3 and FRS were within the same range and

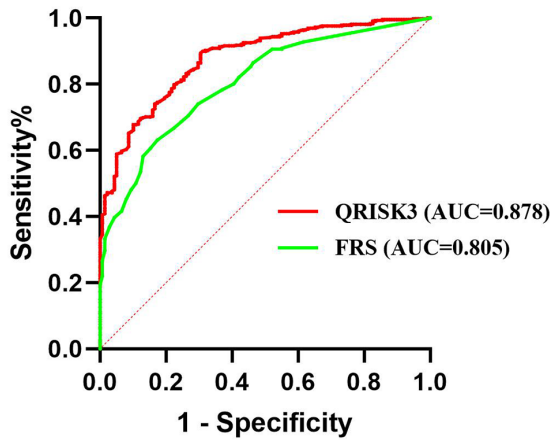


FIGURE 5
ROC for QRISK3 and FRS. Legend: ROC curves were drawn without distinguishing the risk levels, and the AUC of QRISK3 and FRS were high, which were 0.878 and 0.805, respectively. ROC, Receiver operating characteristics; AUC, area under the ROC curve; QRISK3, QRESEARCH Risk estimator version 3; FRS, Framingham risk score.

performed well (Figures 6, 7). When patients with loss to follow-up were defined as non-CVD and CVD groups, the AUC of QRISK3 was still higher than FRS. In addition, there was no significant difference in PPV, NPV and C-statistics analysis based on the analysis of the two outcomes (Tables 5, 6). Hence, the loss to the follow-up population did not have a significant impact on the performance of QRISK3 and FRS.

Discussion

Studies demonstrated that abnormal glucose metabolism can hasten the formation of atherosclerotic plaque, promote plaque rupture and thrombosis, impair normal endothelial function, and result in CVD events (19). As a result, the risk of CVD grows fast in T2DM patients, and the accompanying CVD classification and prediction tools are essential in assisting patients in risk stratification and guiding preventive therapy (20–22). Using up to 21 clinical risk indicators, QRISK3 is a novel algorithm model based on QRISK2 that forecasts 10-year CVD risk. FRS is a tool that has been widely used in the clinical prediction of 10-year CVD risk.

To our knowledge, this is the first preliminary study to assess QRISK3 in predicting CVD outcomes in the T2DM cohort in China. Our study was a single-center analysis of prospectively collected data from 566 newly-diagnosed T2DM patients, 138 of whom had a CVD event. The QRISK3 algorithm calculates a person's risk of developing a heart attack or stroke over the next 10 years. It displays the typical risk of individuals who share the same risk factors as those entered for that individual (23). In addition, QRISK3 was widely used to estimate CVD risk in SLE

TABLE 3 Sensitivity, specificity, PPV, NPV and C-statistics of QRISK3 and FRS.

Tools	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	C-statistics (95% CI)
QRISK3†	91.3	61.2	43.1	95.6	0.763 (0.721, 0.804)
FRS†	50.0	89.1	93.4	36.5	0.696 (0.650, 0.742)
QRISK3††	66.7	90.7	69.7	89.4	0.787 (0.737, 0.837)
FRS††	80.1	59.4	86.0	49.1	0.698 (0.644, 0.751)

†Dichotomised risk scores using a cut-off of 10% 10-year CVD. †† Dichotomised risk scores using a cut-off of 20% 10-year CVD. PPV, positive predictive value; NPV, negative predictive value; C-statistics, concordance statistics; QRISK3, QRESEARCH risk estimator version 3; FRS, framingham risk score.

TABLE 4 Kappa coefficient demonstrating agreement among total risk category, low risk category and high risk category of QRISK3and FRS.

Kappa	QRISK3	FRS	QRISK3†	FRS†	QRISK3††	FRS††
QRISK3	1	0.587				
FRS	0.587	1				
QRISK3†			1	0.726		
FRS†			0.726	1		
QRISK3††					1	0.645
FRS††					0.645	1

†Dichotomised risk scores using a cut-off of 10% 10-year CVD. †† Dichotomised risk scores using a cut- off of 20% 10-year CVD. QRISK3, QRESEARCH risk estimator version 3; FRS, framingham risk score.

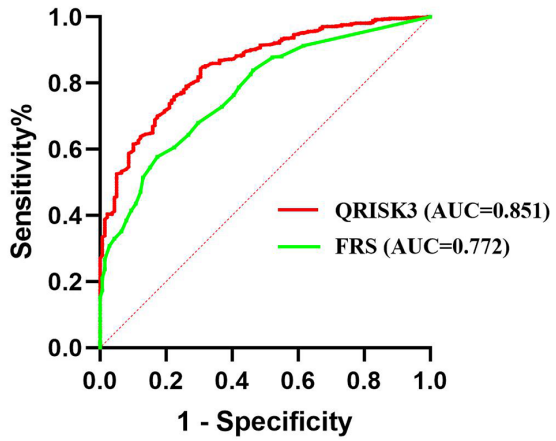


FIGURE 6
ROC for QRISK3 and FRS (patients with loss to follow-up were classified as non-CVD groups). Legend: All the patients who lost to follow-up were classified as non-CVD group. ROC, Receiver operating characteristics; AUC, area under the ROC curve; QRISK3, QRESEARCH Risk estimator version 3; FRS, Framingham risk score; CVD, cardiovascular disease.

and RA (23, 24). However, only a few studies reported the performances of QRSKI3 in calculating CVD risk related to T2DM (25, 26). In this study, we examined whether QRISK3, as CVD risk tools, can accurately predict CVD in patients with T2DM, and compared its predictive performance with FRS. The results of the univariate analysis were similar to the common CVD risk factors reported in previous literature (age, male, smoking history, hypertension history, etc.) (27). Additionally, there were several risk variables for CVD that were exclusive to DM individuals, such as FCP and TyG index. Compared with non-CVD patients, CVD patients had higher FCP and TyG index. The results were similar to previous literature (19, 28–30). FCP is a molecular peptide separated from proinsulin, which reflects the function of insulin secretion by islet cells (31). TyG index, a viable alternative indicator of insulin resistance, combines triglyceride and fasting blood glucose levels and is significantly related to insulin resistance (32). Studies revealed that a higher TyG index is linked to a higher risk of CVD, and it was shown that insulin resistance is associated with the pathophysiology of the disease (33). There was no difference in hemoglobin A1c (HbA1c) and blood glucose between CVD and non-CVD groups. It might be related to the fact that the subjects were all newly-diagnosed as T2DM patients, so the blood glucose levels in the past three months would not be very different.

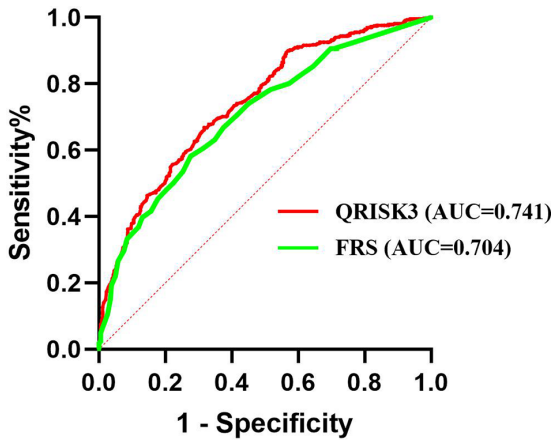


FIGURE 7
ROC for QRISK3 and FRS (patients with loss to follow-up were classified as CVD groups). Legend: All the patients who lost to follow-up were classified as CVD group. ROC, Receiver operating characteristics; AUC, area under the ROC curve; QRISK3, QRESEARCH Risk estimator version 3; FRS, Framingham risk score; CVD, cardiovascular disease.

QRISK3 and FRS were reliable tools in predicting CVD in T2DM patients, according to the results of the AUC. QRISK3 had superior overall prediction performance than FRS. First, the C-statistic of QRISK3 was the highest no matter which cut-off dichotomy method was used. Second, they performed differently, though, depending on the level of risk stratification at which the sensitivity and specificity were determined. FRS had a high specificity on a 10% cut-off dichotomy and a high sensitivity on a 20% cut-off dichotomy. Furthermore, the NPV of FRS performed poorly, but PPV performed excellently because it identified the most proportion of patients as high-risk and maximized the number of false positives for high CVD risk. However, the NPV of QRISK3 performed well at both the 10% and 20% cut-off dichotomy. QRISK3 had higher specificity based on a 20% cut-off dichotomy and higher sensitivity based on a 10% cut-off dichotomy.

We also demonstrated a discrepancy between the two tools in predicting CVD outcomes. In the low-risk and high-risk categories, QRISK3 and FRS showed higher consistency. But the consistency calculated by the total risk category only showed a ‘moderate’ level of agreement. Thus, the two tools for identifying high-risk and low-risk individuals had similar consistency. But the predictive performance of intermediate-risk people was relatively poor. Early identification of intermediate-risk people may have direct consequences for preventative treatment and cause disagreement in the risk management of physicians treating patients with T2DM. Therefore, further large sample-size studies are needed to verify the predictive power of QRISK3 in intermediate-risk population.

Limited studies explored the roles of both tools on CVD outcomes in T2DM. We conducted a comparative study of the predictive performance of QRISK3 and FRS for CVD events in a large number of newly-diagnosed T2DM patients over a follow-up period of almost 10 years in China. This study also has several

TABLE 5 Sensitivity, specificity, PPV, NPV and C-statistics of QRISK3 and FRS (patients with loss to follow-up were classified as the non-CVD group).

Tools	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	C-statistics (95% CI)
QRISK3†	54.7	91.3	96.7	30.5	0.730 (0.690, 0.770)
FRS†	43.5	89.1	94.8	25.6	0.663 (0.619, 0.708)
QRISK3††	86.1	65.9	92.1	50.8	0.760 (0.711, 0.810)
FRS††	76.3	59.4	89.6	35.3	0.679 (0.627, 0.731)

†Dichotomised risk scores using a cut-off of 10% 10-year CVD. †† Dichotomised risk scores using a cut- off of 20% 10-year CVD. PPV, positive predictive value; NPV, negative predictive value; C-statistics, concordance statistics; QRISK3, QRESEARCH risk estimator version 3; FRS, framingham risk score.

TABLE 6 Sensitivity, specificity, PPV, NPV and C-statistics of QRISK3 and FRS (patients with loss to follow-up were classified as the CVD group).

Tools	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	C-statistics (95% CI)
QRISK3†	59.4	93.6	96.7	42.6	0.665 (0.627, 0.704)
FRS†	50	77.6	73.5	55.5	0.638 (0.599, 0.677)
QRISK3††	93.5	75.0	92.1	78.8	0.661(0.621, 0.700)
FRS††	80.1	42.7	63.5	63.4	0.614 (0.574, 0.655)

†Dichotomised risk scores using a cut- off of 10% 10-year CVD. †† Dichotomised risk scores using a cut-off of 20% 10-year CVD. PPV, positive predictive value; NPV, negative predictive value; C-statistics, concordance statistics; QRISK3, QRESEARCH risk estimator version 3; FRS, framingham risk score.

limitations. First, since this is a prospectively gathered single-center retrospective cohort study, it is not as scientifically valid as a clinical trial or prospective study, and it may suffer from recollection bias and loss of follow-up. Fortunately, the results of sensitivity analysis showed that the lost population did not have a significant impact on the predictive performance of the two tools. Subjects were recruited in the third affiliated hospital of soochow university, hence it was unable to determine how representative this sample was in the overall T2DM community. Second, data on 'erectile dysfunction' were missing from the QRISK3 score. The QRISK3 score has a low weight for 'erectile dysfunction' when calculating the 10-year CVD risk, which is relatively insignificant. Finally, other limitations may emerge from potential confounders, such as the use of numerous medicines in individuals with more clinical comorbidities, side effects, or interactions that might alter our results.

Conclusion

In conclusion, this study supports the claim that QRISK3 is a good predictor of CVD events in T2DM patients during follow-up. QRISK3 has a better predictive ability than FRS in these subjects. In the future, we need to integrate large-scale T2DM cohort studies to further verify the relevant tools for predicting 10-year CVD risk, including QRISK3 and FRS. More research and optimization are needed to develop new or improved CVD risk prediction tools.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

This research study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University (2013#27). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, HZ and MY. Writing—original draft preparation, XM. Supervision, AW and HH. Funding acquisition, HZ. All authors have read and agreed to the published version of the manuscript. All authors were involved in the study conception and design, acquisition of data, and analysis and interpretation of data.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1077632/full#supplementary-material

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Re-understanding and focusing on normoalbuminuric diabetic kidney disease

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Diabetes mellitus (DM) has grown up to be an important issue of global public health because of its high incidence rate. About 25% of DM patients can develop diabetic foot/ulcers (DF/DFU). Diabetic kidney disease (DKD) is the main cause of end-stage kidney disease (ESKD). DF/DFU and DKD are serious complications of DM. Therefore, early diagnosis and timely prevention and treatment of DF/DFU and DKD are essential for the progress of DM. The clinical diagnosis and staging of DKD are mostly based on the urinary albumin excretion rate (UAER) and EGFR. However, clinically, DKD patients show normoalbuminuric diabetic kidney disease (NADKD) instead of clinical proteinuria. The old NADKD concept is no longer suitable and should be updated accordingly with the redefinition of normal proteinuria by NKF/FDA. Based on the relevant guidelines of DM and CKD and combined with the current situation of clinical research, the review described NADKD from the aspects of epidemiology, pathological mechanism, clinical characteristics, biomarkers, disease diagnosis, and the relationship with DF/DFU to arouse the new understanding of NADKD in the medical profession and pay attention to it.

KEYWORDS

diabetic kidney disease, diabetes mellitus, normoalbuminuric diabetic kidney disease, diabetic foot, diabetic foot ulcer

1 Introduction

Diabetic kidney disease (DKD), formerly known as diabetic nephropathy (DN), is the most common microvascular complication in patients with diabetes (diabetes mellitus, DM) and one of the leading causes of death in patients with diabetes. Approximately 50% of end-stage kidney disease (ESKD) originates from DKD (1, 2). About 30 to 50% of DM patients in the world will develop to DKD (2, 3), and DKD patients have a higher risk of survival and mortality compared with simple DM patients (4). Since the concept of DKD was first

proposed by the American Kidney Foundation in 2007, it has been widely recognized by the medical academic community, and related research has been deepened. Professional terms have also become more precise from overall planning, and more and more relevant markers have been discovered.

Viberti et al. found that the urinary total protein in patients with type 1 diabetes is within the reference interval, while the excretion of urinary albumin (UAlb) increases. Accordingly, they proposed the concept of microalbuminuria (previously known as albuminuria), that is, the albumin excretion rate (AER) is between 30 and 300 mg/d, which is used as a sensitive indicator of early renal damage in diabetes (5). Therefore, the process of DM complicated with chronic kidney disease (CKD) is a typical disease development with clinical manifestations of in turn increased UAlb excretion, microalbuminuria, macroalbuminuria (with reduced glomerular filtration), and ESKD. However, some DM patients show proteinuria in the normal range but were accompanied by renal insufficiency [estimated global filtration rate (EGFR) < 60 ml/(min/1.73 m^2)] (6). This phenomenon, first noticed by Lane et al. in 1992 (7), is later called normoalbuminuric diabetic kidney disease (NADKD). Subsequently, more and more references have reported that such cases are common clinically (8–13).

2 Redefining NADKD

DKD is a clinical diagnosis, and its etiology is initially thought to be proteinuria in DM patients. The definition is independent of traditional diagnostic markers of CKD, such as histological changes or decreased eGFR, and is initially limited to DM patients with macroalbuminuria. Since then, more sensitive methods for detecting urinary albumin have been developed with the innovation of medical testing technology. Compared with the early detection of macroalbuminuria, proteinuria detected by the new technology is called microalbuminuria, and diabetic patients with microalbuminuria are diagnosed with "early kidney disease. some scholars have also referred to NADKD as diabetic kidney disease without albuminuria (8), non-albuminuric renal disease (9), the non-albuminuric form of diabetes kidney disease (10), normoalbuminuric diabetic nephropathy (11), normoalbuminuric diabetes with renal insufficiency(NADRI) (12), or normoalbuminuric renal insufficiency (13) based on its clinical manifestations or characteristics in the process of clinical research due to the lack of uniform nomenclature.

Some scholars have equated urine proteins with urine albumins, which are called non-proteinuric non-proteinuric diabetic nephropathy (DKD) (14). According to clinical practice, it is inappropriate to call NADKD non-albuminuric or without albuminuria, and normal albuminuric is suitable because even healthy people will have some albumin molecules or fragments in their urine more or less. It is difficult to detect by conventional methods due to the small amount.

Traditionally, urine with UAlb excretion of 30-300 mg/d, or random urine concentration of 20-200 mg/L, or AER of 30-300 mg/d, or urinary albumin-creatinine ratio (UACR) of 30-300 mg/g is called microalbuminuria. The working group of urine albumin testing laboratory in the National Kidney Disease Education Program (NKDE) and the International Federation of Clinical Chemistry (IFCC) recommended the unification of microalbuminuria and macroalbuminuria under term urine albumin, and the abandonment of microalbumin to avoid confusion between microalbumin and small albumin molecule (15) (Table 1). The Kidney Disease: Improving Global Outcomes (KDIGO) 2012 guidelines for the management of chronic kidney disease also recommended that term microalbuminuria should no longer be used (16). Although terms microalbuminuria and urine microalbuminuria are still used in some fields, the purpose is to alert patients with diabetes and cardiovascular disease to the possibility of developing chronic kidney disease, not only DKD patients (16).

The National Kidney Foundation (NKF) and the Food and Drug Administration (FDA) redefined albuminuria in 2009 to strengthen the management of proteinuria in patients with chronic kidney disease (17). i) Those with UACR>300 mg/g are called very high albuminuria (previously called macroalbuminuria); ii) those with UACR between 30 and 300 mg/g are called high albuminuria (previously referred to as microalbuminuria); iii) those between 10 and 29 mg/g are called low albuminuria; iv) only those with UACR<10 mg/g are considered for NADKD (Table 2).

According to this definition of the NKF/FDA working group, the old name NADKD (old) includes two parts of patients with hypoalbuminemia (UACR:10-29mg/g) and normoalbuminuria (UACR<10 mg/g), and its more accurate definition should be non-high albuminuria DKD (NHDKD) (18). Based on this new definition, patients who used to be called NADKD (old) are not actually "normal albumin" DKD in the true sense.

TABLE 1 Change of terminology.

Current terms			Traditional terms
diabetic kidney disease			diabetic nephropathy
kidney disease			renal disease/nephropathy
albuminuria			normal-, micro-, macro-albuminuria
Type 1 diabetics			Type I diabetics
Type 2 diabetics			Type II diabetics
NHADKD	NADKD		NADKD
	LADKD		NADKD
	HADKD	IADKD	MiDKD
	VHDKD		MaDKD

DKD, diabetic kidney disease; NADKD, normoalbuminuric diabetic kidney disease; NHADKD, non-high albuminuria DKD; LADKD, low albuminuric DKD; HADKD, high albuminuric DKD; VHDKD, very-high albuminuric DKD; MiDKD, microalbuminuric DKD; MaDKD, macroalbuminuric DKD.

The work completes the following items to distinguish DKD patients with different levels of albuminuria, e.g., introducing a new concept according to the new definition of NKF/FDA, renaming the old name NADKD (old) to NHADKD, and defining patients with UACR between 10 and 29 mg/g as low albuminuric diabetic kidney disease (LADKD), patients with UACR < 10 mg/g as NADKD (it is a new concept unless otherwise stated in the work), DKD patients with high albuminuria as high albuminuria DKD (HADKD), and DKD patients with very high albuminuria as very-high albuminuria DKD (VADKD). According to the new definition, LADKD, HADKD, and VADKD are collectively referred to as increased albuminuria DKD (or DKD with increased albuminuria, IADKD) for description (Table 2).

Many scholars still regard microalbuminuria (ACR between 30 and 300 mg/g) as a biochemical marker for the early diagnosis of DKD (19) and identify NADKD as the early stage of albuminuria DKD because of the different division of concepts. However, some patients with DKD have been found to have normoalbuminuria in clinical practice, accompanied by decreased renal function with reduced eGFR (2). Even if the eGFR decreases to the level of stage 3 CKD, they still show normoalbuminuria. Therefore, NADKD can no longer be simply regarded as the early stage of albuminuria DKD, and it is necessary to recognize and pay attention to NADKD.

3 Prevalence of NADKD

The prevalence of NADKD in DKD varies with the method of proteinuria (mostly albumin detection) measurement, regions of the patients, inclusion criteria, and the line of judgment for proteinuria, and the results are in a wide variation (Table 1). Patients with NADKD account for only 3.6% of patients with DKD (20), whereas it is as high as 77.9% in Ref (21). The proportion of NADKD in DKD is in the range of 20-70% (22–28). The results of a recent meta-analysis show that the overall prevalence of NADKD in patients with type 2 diabetes and renal insufficiency is 45.6%. That is 24.7% in patients with DKD (albuminuria or renal insufficiency) and 8.4% in all patients with type 2 diabetes, which means that albuminuria does not occur in almost half of patients with DKD and patients with reduced eGFR (29).

The reasons for the high prevalence of NADKD might be related to its pathogenesis (see later); however, the point of the matter is that these reports all use ACR > 30 mg/g or UAE > 30 mg/24 h as a line of judgment to discriminate between NADKD and albuminuric DKD. LADKD is judged as NADKD, and it is not clear whether the proportion of LADKD in it is high or low. Therefore, the roughly reliable prevalence of NADKD can only be clarified by reinvestigation based on the latest definition.

4 Causes of NADKD generation

4.1 Disease factors: pathogenesis of NADKD

NADKD was thought to be an early stage in the classical process of DM concurrent CKD in the past, which may limit the understanding of NADKD. Still many scholars consider LADKD (10 mg/g or URL ≤ ACR < 30 mg/g) as NADKD (old) even now (Table 1). Therefore, LADKD may be the early stage of albuminuric

TABLE 2 Comparison of new and old concepts of albuminuria diabetic kidney disease.

New definition				
Albuminuria	Normoalbuminuria	Low-albuminuria	High-albuminuria	Very High-albuminuria
Albumin level	<10(URL)mg/d	10(URL)~29mg/d	30~300mg/d	>300mg/d
ACR	<10(URL)mg/g	10(URL)~29mg/g	30~300mg/g	>300mg/g
AER	<10(URL)mg/d	10(URL)~29mg/d	30~300mg/d	>300mg/d
UAlb definition	Normal-	Low-	High-	Very High-
DKD concept	NADKD	LADKD	HADKD	VADKD
			IADKD	
		NHADKD		
Traditional definition				
Albuminuria	Normoalbuminuria		Microalbuminuria	Macroalbuminuria
Albumin level	<30mg/d		30~300mg/d	>300mg/d
ACR	<30mg/g		30~300mg/g	>300mg/g
AER	<30mg/d		30~300mg/d	>300mg/d
UAlb definition	Normal-		Micro-	Macro-
DKD concept	NADKD		MiDKD	MaDKD

DKD, diabetic kidney disease; NADKD, normoalbuminuric DKD; NHADKD, non-high albuminuria DKD; LADKD, low albuminuric DKD; HADKD, high albuminuric DKD; VHDKD, very-high albuminuric DKD; MiDKD, micro albuminuric DKD; MaDKD, macro albuminuric DKD; IADKD, Increased albuminuria DKD or DKD with increased albuminuria; UAlb, urine albumin; ACR, albumin-creatinine ratio; AER, albumin excretion rate; URL, upper reference limit.

DKD. The prevalence of normal albuminuria gradually decreases with the increased duration of DM and none suffers from normal albuminuria in the 30 DM patients with renal insufficiency lasting more than 21 years (29). The results further confirm that LADKD is the early stage of albuminuric DKD. However, urinary albumin may not be detected in patients with NADKD at the later stages of renal injury (21, 29). Therefore, the occurrence of NADKD may be another form of renal pathological changes caused by DM (30–33), and its pathogenesis needs to be further confirmed and elucidated.

NADKD is a heterogeneous disease with extremely complex pathogenesis, which may involve genetic factors (34, 35), sex hormone abnormalities (36), vascular lesions (more macroangiopathy than microangiopathy) (37), glomerular lesions (38), intrarenal arteriosclerosis (39), mild forms of tubular injury (12), acute kidney injury (40), inflammation (24), metabolic syndrome (41), obesity (42), hypertension (43), hyperlipidemia (44, 45), hyperuricemia (45), and the use of renin-angiotensin-aldosterone system inhibitors (29). Therefore, when these high-risk factors are present in patients with DM, we should pay more attention to whether NADKD occurs or may occur.

4.2 Laboratory factors: improper or even false detection

UAlb in some urine samples may not be detected due to the laboratory testing methods, which leads to significantly lower test results. The results of nearly 30mg/g in HADKD patients were detected as low as LADKD or NADKD, leading to improper or even false experimental conclusions.

4.2.1 Physiological variation

Individual biological differences can lead to variations in UAlb excretion rates from 4-103% (15). Therefore, different individuals or the same individual's urine samples are collected at different times, and the results may be different or even very different, especially among different individuals.

4.2.2 Immunoreactivity of UAlb molecules

Although the two main types of methods currently used in clinical laboratories for detecting UAlb are immunological and chromatographic methods, immunological methods (antigen-antibody reactions) still predominate in clinical laboratories. Immunological methods can only detect UAlb with immunoreactivity. However, when plasma Alb flows through the kidney, only < 1% of intact Alb molecules are filtered under lysosomal enzymes, and > 99% of intact Alb molecules are biochemically modified and degraded into Alb-derived fragments of varying molecular sizes. It results in many UAlb molecules without immunoreactivity. Besides, lysosomes in the renal tubules can cause conformational changes in the Alb antigenic determinants, which causes them to form dimers or multimers and intact Alb molecules to lose their immunoreactivity. Urinary Alb molecules that have lost immunoreactivity cannot be detected by immunological methods, which reduces results (23, 24, 46).

4.2.3 Selection of standards

There are currently three main candidate reference substances for UAlb detection (47): a) ERM-DA470 and ERM-DA470K/IFCC; b) purification of human serum albumin; c) 15N-labeled recombinant human serum albumin (15N-rHSA). However, all three substances are based on human serum Alb rather than human UAlb. The urine matrix is completely different from the serum matrix. Therefore, there is no reference substance based on human UAlb, which leads to systematic errors.

4.2.4 Other

Sample collection and storage containers, preservatives, transportation methods, storage time, as well as subject position, exercise, blood pressure, protein intake, body temperature, mood, and drugs (such as penicillin, nifedipine, acetylsalicylic acid, norepinephrine) can affect the detection of UAlb.

5 Diagnosis of NADKD

No institution can formulate clear diagnostic criteria for NADKD for the clinician because of our limited understanding of NADKD and different people having different perspectives.

DKD is usually clinically diagnosed based on the presence of proteinuria and/or decreased eGFR in the absence of signs or symptoms of other major causes of kidney damage (48). Nevertheless, patients with NADKD do not have the UACR. So if we only rely on the eGFR to make a diagnosis of NADKD patients, there will be greater difficulties. The eGFR is a calculation indicator based on serum (plasma) creatinine and/or cystatin C. The level of serum creatinine is easily affected by muscle quality, dietary protein intake, hyperlipidemia, hemolysis, and the method of detection (Jaffe's method or enzymatic method). An eGFR has an obvious lack of reliability (12). Therefore, the diagnosis of NADKD is challenging.

According to relevant guidelines (16, 17, 48), the diagnosis of NADKD should meet at least the following conditions: i) diagnosis of diabetes according to the latest diagnostic criteria of the World Health Organization (WHO) or the American Diabetes Association (ADA); ii) under the following circumstances more than three months: urinary sediment abnormality, or renal tubular injury resulting in electrolytes and other abnormalities, or abnormal histological examinations, or imaging detected structural abnormalities, or

a history of kidney transplantation, or eGFR < 60ml/(min/1.73 m^2); iii) Have an examination once a month and at least twice in three months if AER<30 mg/24 h or ACR<30 mg/g is diagnosed as NADKD. It also can be diagnosed if AER < 10 mg/24h or ACR < 10 mg/g (or the range of reference values established by the local laboratory) (17, 37).

6 Biomarkers

As far as kidney damage is concerned, the DKD essence is also a chronic kidney disease (CKD). NADKD is only the case part of DKD with normal albuminuria. KDIGO defines CKD as an abnormality of the renal structure and function lasting more than 3 months, which affects human health. According to it, renal structural abnormalities can be found by histological (renal biopsy) or imaging (CT, MR, or ultrasonic) examination. The method cannot determine whether the patient has proteinuria (or albuminuria), so ACR/AER is required to distinguish DKD and NADKD. Therefore, the diagnosis of DKD and NADKD by clinicians mainly depends on the detection of ACR/AER and eGFR in the traditional renal function index (Figure 1).

Not all clinical laboratories in other countries reported eGFR except some clinical laboratories in Europe and America because of the tedious detection methods (AER), multiple influencing factors (creatinine) (16), and different degrees of popularity (cystatin C) (16) with no special requirements. The sensitivity of traditional biomarkers (such as ACR/AER and eGFR) for CKD diagnosis is poor, indicating that it is difficult to diagnose DKD/NADKD. Only relying on traditional biomarkers cannot meet the clinical application of NADKD. Therefore, some novel biomarkers emerge.

There are nearly 100 clinical markers for research, screening, or diagnosis of NADKD (Supplementary Tables 1, 2). However, only the neutrophil gelatinase-associated lipocalin, cystatin C, vascular endothelial growth factor, and liver-type fatty acid-binding protein can achieve routine testing in clinical laboratories. These projects also have the most research and clinical applications in DKD/NADKD.

6.1 Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is a small molecular protein weighing about 25kDa in the lipid carrier protein superfamily (49). NGAL, produced in ischemic proximal renal tubular cells and participates in the regeneration and repair of proximal renal tubular cells after ischemic renal injury, can reduce the apoptosis of renal tubular cells. Therefore, it can be used as an early biomarker of urine in patients with ischemic renal injury. NGAL in serum and urine may be an early predictive biomarker of acute renal injury (50). The increased level of NGAL in urine indicates that there may be an early renal

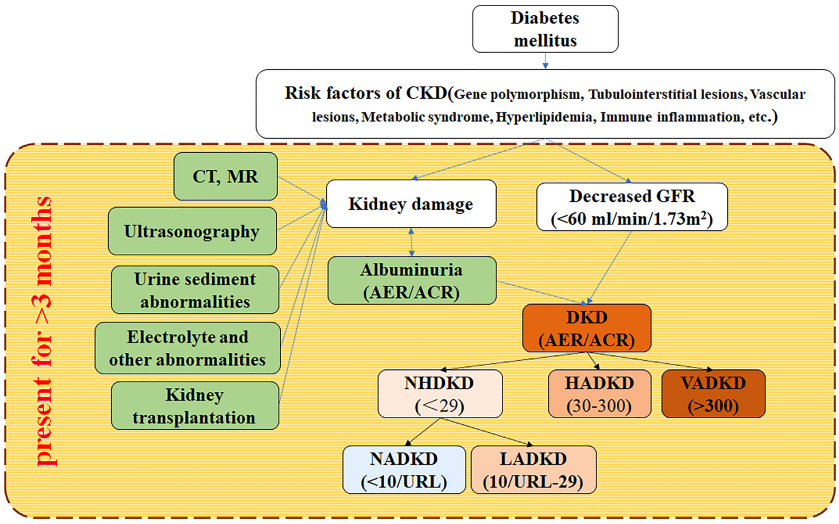


FIGURE 1
Flow for diagnosing NADKD according to KDIGO 2012 clinical practice guideline KDIGO (2012) defines CKD as abnormalities of kidney structure or function more than 3 months, which affects health. CKD is diagnosed according to markers of kidney injury and decreased GFR. DKD is a kind of CKD. CKD, chronic kidney disease; DKD, diabetic kidney disease; NADKD, normoalbuminuric DKD; NHADKD, non-high albuminuria DKD; LADKD, low albuminuric DKD; HADKD, high albuminuric DKD; VHDKD, very-high albuminuric DKD; CT, computerized tomography; MR, magnetic resonance imaging; ACR, urine albumin-creatinine ratio(unit: mg/g creatinine); AER, albumin excretion rate (unit: mg/24h); URL, upper reference limit.

tubular injury, which occurs earlier than albuminuria (51, 52). The level of urinary NGAL is positively correlated with the concentration of urinary albumin in T2DM patients with albuminuria, showing that urinary NGAL can predict disease progression in T2DM patients and is an important predictor independent of UACR and eGFR (53–57). The level of NGAL for T1DM patients will be increased before the appearance of microalbuminuria, which can evaluate the early renal involvement in the course of diabetes (11). A large number of studies have shown that the NGAL level in blood and urine is significantly higher in patients with NADKD than in healthy controls and/or in patients with DM, suggesting that NGAL in blood and urine is helpful for early diagnosis, disease stage, and prognosis evaluation of NADKD (13, 20, 39, 40, 43, 45, 52, 58–60).

6.2 Cystatin C

Cystatin C can be used to calculate eGFR and diagnose renal function damage. According to KIDIGO recommendations, when the eGFR value of patients is 45–59 ml/min/1.73 m^2 based on creatinine tests, cystatin C should be tested to diagnose or rule out CKD (16). Cystatin C is a cysteine protease inhibitor of 13-kDa, which is produced by nucleated cells at a constant rate and can be filtered freely from the glomerulus. Almost all of it (more than 99%) is reabsorbed and degraded in the proximal convoluted tubule epithelium without returning to the blood circulation as a prototype. So the cystatin C level in the blood mainly depends on eGFR (61, 62). the cystatin C level for DKD patients with albuminuria will increase before the occurrence of albuminuria. Therefore, it is an early diagnostic marker of DKD (63–65). Moreover, a large number of studies demonstrate that increased serum cystatin C in NADKD can be used for the diagnosis and prognosis of NADKD (52, 63, 64). Meanwhile, using cystatin C with/without creatinine to calculate eGFR can improve the sensitivity of eGFR diagnosis (12, 66).

6.3 Fatty acid-binding protein

Fatty acid-binding protein (FABP) is a group of low molecular proteins weighing about 14-15 kDa. At least 10 subtypes of FABP have been identified in mammals at present, of which the liver-type fatty acid-binding protein(FABP1, L-FABP), heart-type fatty acid-binding protein(FABP3, H-FABP), adipocyte fatty acid-binding protein(FABP4, A-FABP), and epidermal cell type fatty acid-binding protein(FABP5, E-FABP) are associated with DM vasculopathy (mainly L-FABP) (31). These FABPs are the key inflammatory factors associated with DM and play an important role in DM vasculopathy. The L-FABP level in the urine of patients with NADKD is significantly higher than that in normal controls, and it can reflect the severity of DKD (12, 31). Therefore, L-FABP may help diagnose NADKD. Also, FABP can be used to identify high-risk patients with proteinuria (56).

6.4 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis and the most important angiogenic factor associated with renal disease (22). it is expressed in glomerular podocytes, distal renal tubules, and collecting ducts and a small amount in proximal renal tubules in the kidney, which maintains the integrity of the glomerular filtration barrier and podocyte activity (29). Injured podocytes will release VEGF stored in the cytoplasm at the early stage of DKD, which improves the permeability of the glomerular filtration membrane. Since the glomerulus is in a state of high filtration for a long time, the VEGF level is bound to increase.

Patients with T2DM have significantly higher serum VEGF levels than controls (26, 27), and so do elevated urinary VEGF levels in children and adolescents with T1DM who are normoalbuminuric and normotensive (28). The excretion value of VEGF is higher than that of the control group and increases with increased ACR in normoalbuminuric T2DM patients (26, 27, 46). Therefore, VEGF may serve as a biomarker for diagnosing DKD occurrence and disease progression, especially in patients without albumin excretion (67). Specifically inhibiting or blocking the expression of VEGF may become a new target for treating DKD (68).

6.5 Other biomarkers

6.5.1 Biomarkers of multiple laboratory studies

Although some markers have not been used clinically, at least three or more laboratory studies have reported that they may be potential markers for diagnosing NDAKA. However, research on these markers has not been continued, and some results have no clinical values at present.

Kidney injury molecule-1(KIM-1): KIM-1 is a transmembrane protein whose expression has high tissue specificity, it is highly expressed in renal tubular epithelial cells damaged by ischemia and nephrotoxicity instead of normal renal tissues. Serum KIM-1 levels in NADKD patients are significantly higher than those in healthy controls (31), and KIM-1 levels increase with the progression of CKD (32), suggesting that KIM-1 can be used for diagnosing NADKD and disease severity.

Netrin-1: Netrin-1 belongs to an angiogenic factor whose expression is restricted to endothelial cells in normal kidneys and is rarely expressed in renal tubular epithelial cells (34). Netrin-1 is overexpressed and excreted in urine after renal injury (20, 69). Urinary netrin-1 levels in patients with NADKDK are significantly higher than those in healthy controls (21, 30–32)

and increase with increased urinary albumin levels (21); however, the changes in serum levels are not significant (32). Therefore, urinary netrin-1 can be used as a diagnostic biomarker to predict NADKD and is closely related to the progression of DKD. The excretion of netrin-1 in T2DM patients with DKD is higher than that in T1DM patients (21).

mircoRNA (miRNA): miRNAs are a class of short noncoding RNAs as well as important post-transcriptional regulators of gene expression. Compared with other organs, kidney tissues express some specific miRNAs (70). miRNA 21 and miRNA 124 are elevated in the urine of NADKD patients (40); the miRNA profiles of NADKD patients are different from those of non-DM controls, among which miRNA 145 and miRNA 130 are higher than those of controls. miRNAs contribute to the diagnosis of NADKD (Supplementary Table 1); however, these conclusions are mostly based on single-center studies. If they are used in clinical practice, they may need to be verified by large-scale multi-centers. Besides, detection requirements of miRNA are relatively high, which hinders its promotion.

Monocyte chemoattractant protein-1(MCP-1): MCP-1 is a chemokine that can be expressed by a variety of cells in the human body, but normal renal tissue cells only secrete a small amount of MCP-1. A small amount of monocyte infiltration is chemotactic to remove harmful substances and protect kidneys. DM occurrence in patients leads to disorders of various metabolic pathways. MCP-1 mRNA and proteins are highly expressed in various renal tissue cells, which aggregates macrophages and myofibroblasts and results in renal tubular damage. The renal damage degree related to serum and urine MCP-1 levels is positively correlated, indicating that MCP-1 is a marker to distinguish NADKD and IADKD at the early stage (71).

Connective tissue growth factor (CTGF): For DM patients, CTGF can affect the physiological function of renal tissue cells, cellular inflammatory responses, matrix collagen synthesis, and fibronectin synthesis, induce cell apoptosis, and promote fibroblast differentiation, which results in renal injury. Early studies on NADKD show that the serum MCP-1 level is positively correlated with the albumin excretion rate, indicating that it plays an important role in the occurrence and development of DKD (72).

Periostin: Periostin is originally isolated from a mouse osteoblast cell line and named osteoblast-specific factor 2, which can interact with a variety of cytokines and inflammatory mediators to cause inflammation and tissue fibrosis. A recent study (73) shows that serum periostin levels increase in both NADKD and IADKD and are positively correlated with UACR levels, indicating that Periostin may be a marker of early kidney injury, disease progression, and staging in DKD (38).

6.5.2 Biomarkers of single laboratory studies

Although the understanding of NADKD is unclear, clinical research on it is increasing day by day, and dozens of relevant biomarkers have been discovered and recognized (see Supplementary Table 2). However, these markers only come from a single-center laboratory and have no response and support from other laboratories. Whether they are really helpful for the diagnosis, treatment, and/or prognosis of DKD/NADKD needs more and more extensive experiments to verify and evaluate.

7 Complications of diabetes

7.1 Common complications and mechanisms of DM

Nerve damage, microvascular disease, and large vessel damage are caused by the oxidative stress reaction and pro-inflammatory response in various tissues and organs of diabetes patients due to the long-term continuous influence of hyperglycemia, which damages the heart, brain, liver, and kidney, peripheral nerve, eyes, feet, skin, and other organs damaged with complications. The common ones are diabetes nephropathy, eye disease, foot ulcers, heart disease, peripheral neuropathy, cardiomyopathy, autonomic neuropathy, retinopathy, and osteoporosis (74–76). The work only reviews diabetic foot/ulcer (DF/DFU) and diabetic nephropathy.

7.2 DF/DFU and DKD

DF/DFU is a common complication of DM caused by microvascular disease, which can seriously reduce the quality of life (QOL) and survival status of DM patients, including shortening the survival life (77). DF is defined as a structural or functional change of the foot, which may manifest as ulceration, osteomyelitis, or gangrene (78). DFU is one of the serious clinical manifestations of DF and an important cause of disability (amputation) in DM patients (78–80). The lifetime prevalence of DFU is high, ranging from 19 to 34%, and the ulcer recurrence rate after wound healing is as high as 40% (81–83).

The purpose of treating DFU is to control the disease and improve the QOL and survival of patients with the transformation of living conditions and medical models (84). The relevant studies have shown that the etiology of DFU is complex and diverse, and chronic inflammatory response and oxidative stress are important factors leading to poor wound healing and the formation of chronic open ulcers in DFU (85). Similarly, the pathogenesis of CKD (DKD/NADKD in DM patients), a common complication of DM, is extremely complex. Inflammatory reactions and oxidative stress are important pathological mechanisms of NADKD (12). However, the relationship between the risk of DF/DFU and the occurrence and severity of CKD is not well understood.

Some previous studies have found that CKD plays an important role in promoting the occurrence and development of DF/DFU. Firstly, CKD may act synergistically with diabetes to cause DF/DFU by increasing peripheral nerve damage and other pathways (86). CKD promotes more severe peripheral vascular disease by causing chronic inflammation and oxidative stress and inducing a prothrombotic state. It increases the risks of chronic inflammation, fluid retention, alterations in the renin-angiotensin system, and ischemic ulcers in DM patients, which ultimately leads to foot ulcers (87, 88).

Secondly, patients with DKD/NADKD have metabolic disorders and/or increased renal filtration proteins, and the body is prone to hypoalbuminemia, which causes poor ulcer healing. Meanwhile, hypoalbuminemia can lead to foot edema, which affects the blood supply of the affected limb and leads to peripheral circulatory disorders. It is more difficult for wound healing in patients with ischemia-based diabetic foot (89).

Thirdly, DKD/NADKD patients may lack trace elements such as zinc, copper, selenium, and nutrients (e.g., vitamins A, C, D, and E and amino acids). The nutritional status is the key factor for general wound healing, the occurrence of DFU, and the prognosis of DFU infection (81, 83, 90, 91).

Fourthly, retinopathy occurs in the process of CKD microangiopathy caused by DM patients, resulting in vision loss. The disturbance of the disease and vision loss will reduce the patient's mobility and the ability for foot self-care or examination, which increases the possibility of DF and DFU (90).

Both the International Working Group on the Diabetic Foot (IWGDF) and the National Institute for Health and Care Excellence (NICE) agree that patients with DKD are at higher risk for DFU and have a worse prognosis than other patients with diabetes (81, 92). Studies have shown that CKD is related to the severity of DFU patients. DFU patients with CKD have a higher risk of amputation (93). CKD can aggravate the condition of DFU patients and increase the probability of amputation and the risk of death (94, 95). Besides, lower limb complications are the main adverse events of CKD patients, which leads to a higher incidence of DF and a worse prognosis of DFU (96). Compared with CKD patients without DFU, CKD patients with foot ulcers have significantly lower eGFR; compared with patients with pre-existing CKD, the eGFR reduction in DFU patients with no early signs of CKD is also slowly progressive (97).

Since NADKD does not produce proteinuria, its clinical manifestations are more insidious. According to the existing diagnostic methods, it is easy to miss the diagnosis. Therefore, more attention should be paid to NADKD patients, especially some NADKD patients who do not develop proteinuria for life (98). Although patients with DKD are more likely to develop foot ulcers, they are not less likely to heal than diabetic patients with normal renal function (99). Therefore, there are sufficient clinical observations to support the feasibility of salvaging DF/DFU patients even in these high-risk groups.

8 Conclusions

DKD is a common serious complication in DM patients, which is associated with renal failure, cardiovascular disease, and premature death. The pathological mechanism of DM complicated with CKD (i.e., DKD occurs) is extremely complex. The proportion of NADKD in DM or DKD patients has gradually increased. Although this may be related to the improvement of the therapeutic effect of DM and the potential renal pathological changes of NADKD, it brings greater difficulties to the diagnosis of DKD. NADKD was judged at the early stage of typical DKD in the past, which might be related to the disease stratification of NADKD. NADKD was falsely judged as NADKD.

DF/DFU is also a serious complication of DM. If the influence of renal injury on DF/DFU disease is ignored due to the missed diagnosis of NADKD, it will certainly aggravate the disease development and lead to serious outcomes for DM patients. Therefore, early diagnosis is essential to improve the prognosis of DM patients. Besides traditional (ACR/AER and eGFR) markers, some novel markers with higher sensitivity and better specificity, such as serum cystatin C, NGAL, L-FABP, and VEGF, should be used as routine evaluation indicators in the clinical management of DM. They can be used to predict the occurrence of DM complications and disease progression through regular examination, screen NADKD patients with concealed clinical manifestations as early as possible, give personalized intervention and/or treatment in time, manage DM, and control the occurrence and development of its complications.

Author contributions

NA and J-FF contributed to the conception and design of the article. NA, B-TW, and J-FF wrote the first draft of the manuscript. Y-WY and Z-HH performed the framework, tables and the figure. NA and J-FF revised important intellectual content critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1077929/full#supplementary-material

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The impact of hyperglycaemic crisis episodes on long-term outcomes for inpatients presenting with acute organ injury: A prospective, multicentre follow-up study

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Background: The long-term clinical outcome of poor prognosis in patients with diabetic hyperglycaemic crisis episodes (HCE) remains unknown, which may be related to acute organ injury (AOI) and its continuous damage after hospital discharge. This study aimed to observe the clinical differences and relevant risk factors in HCE with or without AOI.

Methods: A total of 339 inpatients were divided into an AOI group (n=69) and a non-AOI group (n=270), and their differences and risk factors were explored. The differences in clinical outcomes and prediction models for evaluating the long-term adverse events after hospital discharge were established.

Results: The mortality among cases complicated by AOI was significantly higher than that among patients without AOI [8 (11.59%) vs. 11 (4.07%), Q = 0.034] during hospitalization. After a 2-year follow-up, the mortality was also significantly higher in patients with concomitant AOI than in patients without AOI after hospital discharge during follow-up [13 (21.31%) vs. 15 (5.8%), Q < 0.001]. The long-term adverse events in patients with concomitant AOI were significantly higher than those in patients without AOI during follow-up [15 (24.59%) vs. 31 (11.97%), Q = 0.015]. Furthermore, Blood β-hydroxybutyric acid (P = 0.003), Cystatin C (P <0.001), serum potassium levels (P = 0.001) were significantly associated with long-term adverse events after hospital discharge.

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Conclusions: The long-term prognosis of HCE patients complicated with AOI was significantly worse than that of HCE patients without AOI. The laboratory indicators were closely correlated with AOI, and future studies should explore the improvement of clinical outcome in response to timely interventions.

KEYWORDS

diabetes, hyperglycaemic crisis episode, acute organ injury, mortality, long-term prognosis

Introduction

Hyperglycaemic crisis episodes (HCE) refer to an acute diabetic complication that is most likely to cause short-term death. Diabetic ketoacidosis (DKA) and hyperosmolar hyperglycaemia syndrome have a morbidity of approximately 2.6% and 0.2% of diabetes, respectively. The morbidity of diabetes remains high, resulting in an annually increasing number of hospitalizations because of HCE, as well as high medical expenditure and severe social burden (1, 2). HCE patients are prone to acute organ injury (AOI), which involves myocardial infarction, acute kidney injury (AKI), and neurological injury. The risk of mortality will be significantly increased in HCE patients complicated with neurological changes (3, 4). In addition, the risk of organ failure and mortality after hospital discharge will also be considerably increased in HCE patients complicated with AOI (3, 5–7). The possible reason may be related to the continuous damage caused by AOI in the hospital and its extension to hospital discharge. Although HCE has been confirmed to increase the risk of dementia, malignancy, end-stage renal disease and cerebral ischaemic injury in previous studies (4, 8, 9), the impact of different AOIs on long-term outcomes in patients with HCE remains unknown. In this work, a prospective, multicentre follow-up observational study was conducted on inpatient HCE with or without AOI, and the adverse events after hospital discharge were investigated.

Methods

Patient recruitment and exclusion criteria

A total of 339 HCE patients were enrolled from 4 tertiary teaching hospitals between August 2017 and July 2020 in accordance with the principles of the Declaration of Helsinki (2001). This study was registered at chictr.org.cn (ChiCTR1800015981) and approved by the institutional review board of Chongqing University Central Hospital. The study was approved by the Committee for Research Ethics of Chongqing University Central Hospital (NO. 202262).Written informed consent was obtained from all participants.

Inpatients aged 20 or older diagnosed with HCE were enrolled in the study. All patients met the diagnostic criteria for diabetes and HCE recommended by the American Diabetes Association (ADA) in 2009 (10). Subjects with the following conditions or diseases were excluded: 1) pregnancy; 2) history of stroke/cognitive impairment, CKD, tumour, coronary heart disease/heart failure, chronic pancreatitis, or chronic hepatitis/liver failure; and 3) other major diseases, including systemic inflammatory response syndrome or advanced malignant diseases.

AOIs included the following: 1) acute stroke or brain dysfunction; 2) acute kidney injury; 3) acute cardiovascular accident/heart failure; 4) acute pancreatitis; and 5) acute liver dysfunction/failure. According to whether HCE was concomitant with AOI during admission, the patients were divided into an AOI group and a non-AOI group, and their differences and risk factors were explored. Long-term adverse events after hospital discharge were defined as follows: 1) stroke or cognitive impairment; 2) chronic kidney disease; 3) coronary heart disease or heart failure; 4) tumour; and 5) sudden death of unknown cause. The differences in clinical outcomes and a prediction model for evaluating the long-term adverse events after discharge were established (Figure 1).

Data collection

The baseline data of patients, including age, sex, body mass index (BMI), diabetes classification, predisposing factors, and AOI at admission and at discharge, were collected. BMI was calculated as the body weight/square of height [kg/m (2)]. The diabetes classification was determined by previous medical history.

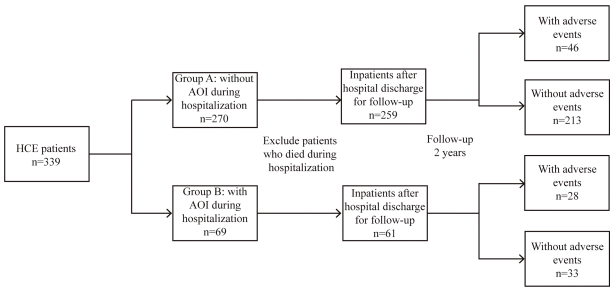


FIGURE 1
A flow chart describing the study design.

Biochemical assays

Blood glucose, blood sodium, blood potassium, C-reactive protein (CRP), procalcitonin (PCT), activated partial thromboplastin time (APTT), prothrombin activity (PTA), serum creatinine (SCR), cystatin C, creatine kinase, pH, HCO3-, base excess (BE), alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin, blood amylase, triglyceride (TG), total cholesterol (TC) and low density lipoprotein (LDL) were measured using an automatic biochemical analyser (AU5821, Beckman Coulter, USA). β-hydroxybutyrate was measured using a colorimetric enzymatic reaction (D-3-hydroxybutyrate kit; Ranbut, Randox Laboratories). White blood cells, neutrophils, lymphocytes, and platelets were determined by an automatic blood cell analyser (BC-6800, Mindray, Shenzhen, China). Troponin I was measured by chemiluminescence immunoassay using a DXI800 (Beckman Coulter, USA). Haemoglobin A1c was measured by high-performance liquid chromatography (BC-6800, Mindray, Shenzhen, China).

Statistical methods

Normally distributed data are expressed as the mean ± standard deviation and compared using unifactor ANOVA and the TAMHANE test. Nonnormally distributed data are expressed as percentiles (P25, P75 and P50) and analysed using the rank-sum test. Count data are expressed as percentages (%) and were analysed using the chi-square test. All data were corrected for *P* value by false discovery rate method. Binary logistic regression analysis was performed after selecting the relevant factors using univariate logistic regression. By calculating the variance expansion factor, we confirm that there is no unacceptable collinearity in all regression models. The ROC curves were also plotted for the related factors. The SPSS 22.2 software was used for the clinical data analysis of enrolled patients. *P* value < 0.05 or *Q* value < 0.05 were considered statistically significant.

Results

Baseline clinical and laboratory characteristics

In this study, 339 HCE patients were enrolled. Among them, 69 cases were complicated by AOI, and 270 patients were free of AOI. At admission, the age, history of diabetes, blood glucose, plasma osmotic pressure, triglycerides, blood sodium, blood chloride, SCR, urea nitrogen, cystatin C, creatine kinase, troponin I, CRP, leukocytes, HCO3-, acute infection and deaths in the AOI group were significantly higher than those in the non-AOI group (*Q* <0.05), while blood ketone and serum albumin in the AOI group were unexpectedly significantly lower than those in the non-AOI group (*Q* <0.05). Type 2 diabetes was more common in the AOI group (*Q* <0.05). As shown in Table 1 and Figure 2.

At the time of hospital discharge, the urea nitrogen, SCR, cystatin C, troponin I, and CRP levels in the AOI group were significantly higher than those in the non-AOI group (*Q* <0.05), and serum albumin was lower in the AOI group than in the non-AOI group (*Q* <0.05) (Table 1).

Comparison of clinical outcomes during hospitalization and hospital discharge

In this study, among the 69 HCE patients with AOI, 8 died during hospitalization, and among the 270 HCE patients without AOI, 11 died during hospitalization. The mortality among cases complicated by AOI was significantly higher than that among patients without AOI [8 (11.59%) vs. 11 (4.07%), *Q* =0.034] during hospitalization. Of the 61 HCE patients with

concomitant AOI who recovered and were discharged from the hospital, 13 died, and 15 developed long-term adverse events during follow-up. Of the 259 HCE patients without AOI who recovered and were discharged from the hospital, 15 died, and 31 developed long-term adverse events during follow-up. The mortality was also significantly higher in patients with concomitant AOI than in patients without AOI after hospital discharge during follow-up [13 (21.31%) vs. 15 (5.8%), Q <0.001]. The long-term adverse events in patients with concomitant AOI were significantly higher than those in patients without AOI during follow-up [15 (24.59%) vs. 31 (11.97%), Q =0.015]. The short-term and long-term prognoses

TABLE 1 Baseline characteristics of inpatients at admission or discharge.

Variables	AOI group n=69	Non-AOI group n=270	Q value
At admission			
Age	(44.50, 84.00) 65.00	(36.00, 66.00) 53.00	<0.001
Male	40/69 (57.97%)	159/270 (58.89%)	0.498
BMI	(19.60, 25.39) 22.50	(20.20, 25.10) 22.70	0.804
T2DM	66/69 (95.65%)	222/270 (82.22%)	0.005
History of diabetes, year	(0.75, 20) 8.5	(0.00, 10.00) 4.00	0.038
HbA1c (%)	(10.30, 13.90) 12.10	(10.20, 13.88) 11.85	0.791
Triglyceride, mmol/L	(1.60, 3.39) 2.37	(1.22, 2.99) 1.80	0.005
Blood Sodium, mmol/L	(133.50, 152.93) 140.40	(131.30, 139.70) 136.10	0.093
Blood Chlorine, mmol/L	(96.30, 115.63) 103.85	(96.03, 106.75) 100.50	0.024
SCR, umol/L	(92.15, 286.50) 144.20	(53.93, 113.77) 71.80	<0.001
Urea nitrogen, mmol/L	(8.96, 27.50) 13.89	(5.07, 12.70) 7.26	<0.001
Cystatin C, mg/L	(1.13, 2.80) 1.70	(0.62, 1.56) 0.85	<0.001
Lactate, mmol/L	(2.25, 4.13) 3.34	(1.83, 4.21) 2.57	0.066
Creatine kinase, U/L	(62.00, 365.00) 113.00	(54.25, 147.13) 73.00	0.040
Troponin I, ug/L	(0.00,0.11) 0.01 0.17 ± 0.61	(0.00, 0.02) 0.01 0.03 ± 0.09	0.005
Albumin, g/L	(30.10, 39.20) 36.30	(35.20, 43.93) 40.30	<0.001
CRP, mg/L	(4.61, 115.50) 22.45	(2.86, 35.58) 4.90	<0.001
White blood cells, 10 ^ 9/L	(9.62, 18.85) 12.70	(6.60, 14.40) 9.99	<0.001
pH	(7.25, 7.39) 7.31	(7.21, 7.37) 7.30	0.092
BE, mmol/L	(-13.73, -2.65) -7.25	(-17.53, -3.45) -8.90	0.308
HCO3-, mmol/L	(11.75, 21.48) 17.30	(7.80, 17.70) 14.40	0.006
Follow-up time, day	(326.50, 957.75) 604.50	(439.00, 1038.50) 728.00	0.298
Concurrent infection	51/69 (73.91%)	139/270 (51.48%)	0.001
At discharge			
Blood sodium, umol/L	(137.05, 143.4) 140.2	(136.4, 140.8) 138.9	0.093
Blood potassium, umol/L	(3.29, 4.15) 3.8	(3.55, 4.12) 3.84	0.322
Chlorine, umol/L	(101.45, 108.7) 105.1	(102.1, 107.2) 105.3	0.984
Urea nitrogen, umol/L	(4, 8.6) 5.4	(3.1, 6.83) 4.6	0.033
SCR, umol/L	(63.50, 168.05) 94.00	(49.00, 82.00) 62.50	<0.001
Cystatin C, mg/L	(1.03, 2.30) 1.50	(0.70, 1.44) 0.97	<0.001
Lactate, mmol/L	(1.72, 3.29) 2.44	(1.97, 3.06) 2.45	0.909
Creatine kinase	(48, 152) 63	(46, 132) 73	0.780
Troponin I, ug/L	(0.00, 0.07) 0.01	(0.00, 0.02) 0.00	0.017
Albumin, g/L	(28.35, 34.20) 31.20	(32.43, 38.10) 34.80	<0.001
CRP, mg/L	(6.15, 32.85) 14.15	(1.56, 31.50) 6.10	0.013
White blood cells,10 ^ 9/L	(3.69, 7.13) 4.63	(3.2, 5.32) 4.27	0.060
Blood platelets	(133.5, 329.5) 208	(152, 251.25) 200.5	0.312

AOI, Acute organ injury group; BMI, Body Mass Index; SCR, Serum creatinine; CRP, C-reactive protein; pH, Potential of hydrogen; BE, Base excess; Q < 0.05 (two-tailed) was considered statistically significant.

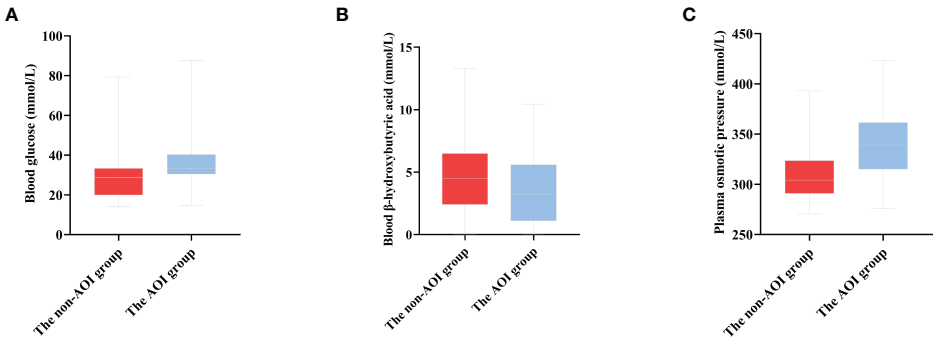


FIGURE 2
The distribution of blood glucose, blood ketone and plasma osmotic pressure in the two groups **(A)** The distribution of blood glucose in two groups [(30.43, 40.36) 33.30 vs. (19.90, 33.40) 28.70, $P < 0.001$]. **(B)** The distribution of blood β-hydroxybutyric acid in two groups [(1.10, 5.60) 3.18 vs. (2.40, 6.50) 4.50, $P = 0.031$]. **(C)** The distribution of plasma osmotic pressure in two groups [(290.83, 323.75) 304.20 vs. (315.0, 361.6) 339. 0, $P < 0.001$].

of cases complicated by AOI in the hospital were significantly worse than those of the group without AOI (Figure 3).

Logistic regression analysis of factors related to AOI

ROC analysis showed that the sensitivity and specificity were 81.8% and 81.1% in the acute brain injury model (AUC 0.864, P <0.001), 85.7% and 81.2% in the acute cardiac dysfunction model (AUC 0.822, P =0.004), 67.7% and 76.7% in the AKI model (AUC 0.726, P <0.001), respectively (Table S1).

Significant factors were included in logistic regression analysis after univariate screening and collinearity analysis. Plasma osmotic pressure [aOR 1.032; 95% CI 1.014, 1.051; $P <$ 0.001], albumin [aOR 0.908; 95% CI 0.835, 0.988; P =0.026], and HCO3- [aOR 1.115; 95% CI 1.029, 1.209; P =0.008] were independent factors of acute brain dysfunction in HCE. Plasma osmotic pressure [aOR 1.020; 95% CI 1.008, 1.032; P =0.001] and SCR [aOR 1.003; 95% CI 1.001, 1.005; P =0.005] were related to AKI. SCR [aOR 1.004; 95% CI 1.002, 1.007; P =0.002] and troponin I [aOR 4.307; 95% CI 1.091, 17.009; P =0.037] were associated with acute cardiac dysfunction (Table 2).

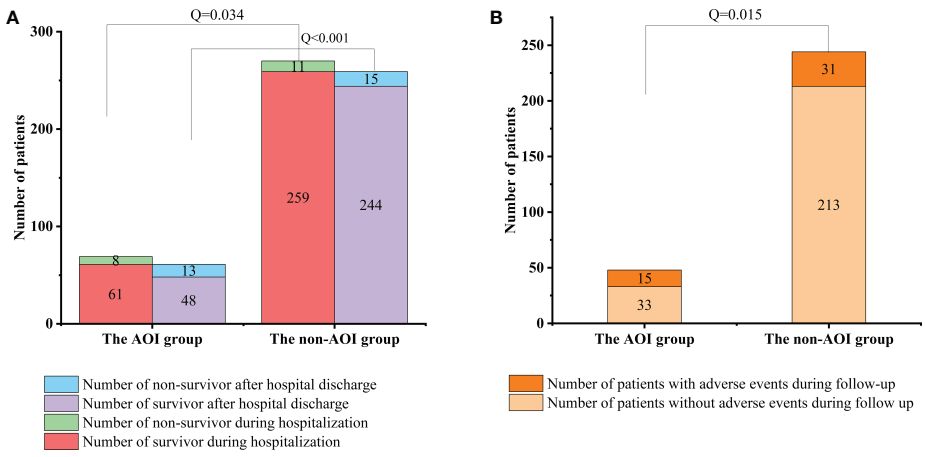


FIGURE 3
Comparison of mortality and follow-up between two groups during hospitalization and after discharge. **(A)** Comparison of mortality between the AOI group and non-AOI group during hospitalization and after discharge [8 (11.59%) vs. 11 (4.07%), $P = 0.034$; 13 (21.31%) vs. 15 (5.8%), $P <$ 0.001]. **(B)** Comparison of follow-up between the AOI group and non-AOI group after discharge [15 (24.59%) vs. 31(11.97%), $P = 0.015$].

Logistic regression analysis for factors related to long-term adverse events

Blood β-hydroxybutyrate acid [aOR 0.673; 95% CI 0.516, 0.878; *P* =0.003], Cystatin C [aOR 2.271; 95% CI 1.437, 3.589; *P* <0.001], serum potassium levels [aOR 0.120; 95% CI 0.033, 0.436; *P* =0.001] were significantly associated with long-term adverse events after hospital discharge. In addition, anion gap [aOR 1.089; 95% CI 0.998, 1.188; *P* =0.055] was potentially associated with long-term adverse events (Table 2). The ROC analysis of this model showed a sensitivity of 84.6% and a specificity of 80.3% (AUC 0.887, *P* <0.001) (Table S1).

Discussion

Our recent studies have confirmed that a high risk of mortality persisted in HCE patients with serious complications or AOI (11, 12). Age, sex, and comorbidity are regarded as important influencing factors for long-term AOI risk and mortality in HCE patients (10), which are commonly difficult to address with current clinical approaches. In addition, how to evaluate the acute and long-term AOI risk of HCE patients through laboratory indicators and how AOI affects the long-term prognosis of HCE patients remains unclear. This study demonstrated that the clinical outcomes of HCE patients with AOI were significantly worse than those of HCE patients without AOI in terms of both hospitalization and hospital discharge. Moreover, the analysis of influencing factors of AOI and long-term adverse events has laid a foundation for subsequent improved treatments. To the best of our knowledge, this model is the first in the Chinese population to predict long-term adverse events combined with AOI in HCE patients using laboratory indicators.

Acute brain injury

Plasma osmotic pressure, plasma albumin and HCO3- levels were found to be important in predicting acute brain injury in patients with HCE. Reduced body volume caused by body fluid concentration may occur in a hypertonic state, and vasoconstriction may be mediated by Rho/Rho kinase signalling (13), further leading to insufficient blood supply to support important organs. Alternatively, exposure to hypertonic saline has been confirmed in current *in vitro* experiments to potentially cause brain cell death (13), and brain cells can be directly damaged by the hypertonic environment. In addition, the academic community widely recognized that the rapid reduction in osmotic pressure may exacerbate the risk of cerebral oedema in HCE patients. A previous study confirmed that the water content of the cerebral cortex is significantly increased in animals with reduced plasma osmotic pressure (14). Therefore, we believe that a persistent hyperosmolar state and rapid reduction in plasma osmotic pressure may lead to acute neuropsychiatric symptoms in HCE patients *via* the above mechanisms.

Albumin, a highly soluble protein, can act as an indicator to roughly estimate the nutritional level of patients. Despite its crucial role in maintaining the colloid osmotic pressure of human plasma, it also possesses complex roles, including the transport of various substances, immune regulation, stabilization

TABLE 2 Logistic regression analysis of acute organ injury at hospital and adverse events after hospital discharge.

	β	*P*	OR	95%CI
Acute stroke or brain dysfunction				
Blood osmotic pressure	0.032	0.001	1.032	1.014~1.051
Albumin	-0.096	0.026	0.908	0.835~0.988
HCO3-	0.109	0.008	1.115	1.029~1.209
AKI				
Blood osmotic pressure	0.020	0.001	1.020	1.008~1.032
SCR	0.003	0.005	1.003	1.001~1.005
Acute cardiovascular accident/heart failure				
SCR	0.004	0.002	1.004	1.002~1.007
Troponin I	1.460	0.037	4.307	1.091~17.009
Adverse events				
Blood β-hydroxybutyrate acid	-0.396	0.003	0.673	0.516~0.878
Cystatin C*	0.820	< 0.001	2.271	1.437~3.589
Serum potassium ion*	-2.124	0.001	0.120	0.033~0.436
anion gap*	0.085	0.055	1.089	0.998~1.188

*Pre-discharge indicators.
AKI, Acute kidney injury; SCR, serum creatinine; P < 0.05 (two-tailed) was considered statistically significant.

of endothelial function, antioxidation, and inhibition of erythrocyte sedimentation (15). The serum albumin level has been suggested as a predictor of death, hospital stay and readmission time in acute patients. In this study, the albumin level was found to be an independent influencing factor of in-hospital acute brain dysfunction in HCE patients. Low albumin levels are partly involved in blood concentration, resulting in a further increase in plasma osmotic pressure and causing acute brain dysfunction *via* the aforementioned effects. In addition, hypoalbuminaemia may participate in acute ischaemic changes in the brain by affecting endothelial cell dysfunction and erythrocyte sedimentation (16).

In this study, HCO3-, an indicator of the degree of body acidosis, was found to be associated with acute brain dysfunction and cognitive decline in HCE patients. Hypocapnia can reduce cerebral blood flow and cerebral oxygenation (17), and insufficient cerebral blood supply and oxygen supply may be associated with vascular dementia or Alzheimer's disease. In an acidic environment, the altered adhesion and morphology of microglia and astrocytes, along with the continuous decrease in scavenging activity of astrocyte Aβ (18), are similarly involved in acute brain injury. In addition, the unrelieved acidosis state during reperfusion may cause more serious damage to the brain (19).

Acute kidney injury

Plasma osmotic pressure was found to be an independent factor affecting AKI in HCE patients. Similarly, in addition to acute brain injury, severe dehydration and blood concentration in the course of DKA were related to AKI. The kidney is highly vulnerable to plasma osmotic pressure. The reduced renal blood flow not only directly affects kidney function but also results in the reduction of renal oxygen supply and damage to renal cells. In addition, high osmotic pressure itself can also directly affect the kidney and induce cell apoptosis by affecting cell metabolism, nucleic acid stability and ion homeostasis (20), which is consistent with the fact that patients with isolated HHS or combined DKA had significantly more AOIs than patients with isolated DKA in this study. We speculated that this finding might be associated with the higher levels of plasma osmotic pressure. Therefore, this study concluded that higher plasma osmotic pressure indicates a higher morbidity of AKI for HCE patients. Therefore, active anti-infection and early active fluid rehydration therapy can play a positive role in protecting target organs from acute injury.

Creatinine is an endogenous marker for evaluating renal function, which is often influenced by infection, diet, and dehydration. Although its specificity and sensitivity are unsatisfactory, current guidelines suggest that serum creatinine remains an important marker for the diagnosis of AKI. Our study demonstrated a significant association between serum creatinine and AKI in patients with HCE. But because of the diagnosis of AKI is highly dependent on serum creatinine and the transient elevation of serum creatinine due to dehydration during HCE, we believe that there is not enough specificity in judging the risk of AKI by serum creatinine levels in the early stages of HCE. Similarly, due to severe dehydration, it is hard to judge the risk of AKI in HCE patients at an early stage based on their urine output. Current research indicates that Cystatin C, tissue inhibitor of metalloproteinases 2 (Timp-2) and insulin-like growth factor binding protein 7 (IGFBP7) are valuable markers for the diagnosis of AKI, but more studies are needed for HCE patients (21).

Acute cardiac dysfunction

Some studies have found that elevated serum creatinine levels are associated with cardiac microvascular injury, but because of the strong association between serum creatinine and AKI, it may be that endothelial dysfunction and inflammation during AKI contribute to microvascular damage (22). Although patients with heart failure may have mild to moderate elevated SCR, it does not affect patient outcomes significantly (23). Therefore, it is believed that the increase of SCR in this condition is not caused by the renal function damage. Our study found that SCR was associated with the development of acute cardiac dysfunction in patients with HCE, but we do not believe that SCR is directly associated with acute cardiac dysfunction in patients with HCE because of the multiple reasons mentioned above. However, it has some value in predicting the cardiac dysfunction of the HCE patients.

Troponin I is an important marker of myocardial cell injury. At present, it is considered as an important indicator for the diagnosis of myocardial infarction, with high sensitivity and specificity. Our study found that the level of troponin I was related to the occurrence of acute cardiac dysfunction in HCE patients. Previous studies found that troponin I was elevated in patients with acute decompensated diabetes without clinical acute coronary syndrom (24). Our study also confirmed that there are still some patients with non-AOI group whose troponin I level is close to the upper limit of the laboratory reference value. At present, studies have found that in patients with acute myocardial infarction, hyperglycemia is related to larger infarct size and higher troponin I (25). Hyperglycemia may cause myocardial injury by mediating endothelial cell disorder and activating inflammatory mediators. It has been confirmed by *in vitro* experiments that 24-48 hours of hyperglycemia exposure can stimulate the production of inflammatory mediators in myocardial cells, leading to the

elevation of markers of myocardial injury (25). Therefore, we believe that elevated troponin I is significantly related to the occurrence of acute cardiac dysfunction in HCE patients, but the elevated troponin I does not represent the occurrence of acute cardiac dysfunction in HCE patients. How to accurately determine the occurrence of acute cardiac dysfunction in HCE patients still needs further research.

Long-term adverse events after hospital discharge

In this study, the levels of blood β-hydroxybutyric acid, serum potassium concentration, anion gap and cystatin C were found to be predictors for adverse events after hospital discharge in HCE patients.

Under the condition of long-term fasting or ineffective utilization of sugar for energy supply, fat decomposition will produce ketones, including acetylacetic acid, β-hydroxybutyrate and acetone, in which β-hydroxybutyrate accounts for the most. Blood-hydroxybutyrate levels are essential for the diagnosis of diabetic ketosis, and diabetic ketosis is closely associated with AKI. The increased ketone bodies were previously considered to exacerbate the body's decompensation, resulting in an increased burden of target organs. Moreover, the increased level of blood β-hydroxybutyric acid was found to be a protective factor for HCE patients. Currently, several large-scale clinical studies have found that patients with ketosis have lower all-cause mortality during hyperglycaemic crises than those without ketosis (26, 27). Namely, HHS alone has a higher mortality than DKA alone or combined with HHS. Blood β-hydroxybutyrate can reduce renal ischemia and reperfusion injury by increasing the upstream regulator forkhead transcription factor O3 (FOXO3) and reducing caspase-1 and proinflammatory cytokines, thereby reducing cell death (28, 29). Moreover, serum β-hydroxybutyrate is also considered to be an antitumor agent (30). Exogenous ketone body intake can reduce atherosclerosis, improve heart failure and protect renal function by inhibiting inflammatory factors (31, 32) and improve cognitive function to some extent (33). Human proximal tubular cells can be directly damaged by high levels of blood β-hydroxybutyric acid (> 20 mmol/L) (34), while low concentrations of β-hydroxybutyric acid (<10 mmol/L) protect against ischaemic tissue injury. Nevertheless, blood ketones in HCE patients rarely exceed this level. Blood β-hydroxybutyrate may have no direct correlation with target organ injury in HCE patients but can reduce the risk of long-term adverse events in HCE patients. Therefore, appropriate ketogenesis can be beneficial to HCE patients. However, large-scale clinical studies are required to determine whether exogenous ketogenic supplementation is needed to improve the prognosis.

Serum potassium is involved in maintaining normal cell activity. Although malignant arrhythmia caused by severe hypokalaemia is significantly associated with the mortality of patients, studies on the relationship between hypokalaemia and the prognosis of HCE patients remain limited. Currently, studies have suggested that hypokalaemia is associated with cardiovascular events and renal adverse outcomes (35, 36), but the specific mechanism remains unclear. In this study, serum potassium concentration was found to be associated with the risk of adverse events after hospital discharge in HCE patients. Insignificant hypokalaemia is commonly associated with diuretics and poor nutritional status, and diuretics have been found to be related to long-term mortality and progression of renal disease (37). Poor nutritional status may lead to a heavy volume load and chronic inflammation by reducing plasma albumin levels, thereby resulting in higher all-cause mortality, cardiovascular events and infection-related mortality (38–40).

The anion gap is associated with coronary atherosclerotic heart disease and the risk of progression to end-stage renal disease in patients with CKD (41) and with the 30-day, 90-day, and 365-day all-cause mortality in patients with AKI (42). The anion gap mainly consists of lactic acid, phosphoric acid and ketone bodies. Lactic acid is often considered an indicator to predict the prognosis of patients with acute infection. Increased lactate levels usually indicate body hypoxia. Patients with diabetes are more likely to be infected than normal people, and aerobic glycolysis is essential for the normal immune function of cells. Conversely, lactic acid inhibits immunity in the local environment. Higher lactic acid may exacerbate the risk of reinfection or complications with diabetic foot ulcers in patients with HCE after discharge. The current study found a role for lactate in tumour occurrence and development (43), which may increase the risk of tumours in HCE patients after discharge. A previous study confirmed that compared with the normal population, DKA patients are prone to have a higher risk of developing Alzheimer's disease after discharge (44). Considering the average age of HCE patients, acidosis should be corrected more actively than in the general population to improve the cognitive function prognosis of patients.

Cystatin C, a small protein molecule produced by nucleated cells, has been proven to be a highly sensitive biomarker of renal function. Compared with serum creatinine, cystatin C can serve as a stronger predictor for AKI. Several studies have confirmed that cystatin C can be used as a predictor of all-cause mortality in the elderly population, long-term mortality in patients with severe disease and mortality in patients with chronic kidney disease (23). Our study also proposed that cystatin C can be used as a predictor of diabetic foot ulcers (45). Cystatin C not only has a stronger ability than serum creatinine to predict mortality and the development of end-stage renal disease in diabetic patients (46, 47) but also has a strong correlation with the poor healing of

diabetic foot ulcers (48) and the risk of cardiovascular disease, which seems to be associated with promoting atherosclerosis through multiple pathways (49). In this study, cystatin C was found to be not only a predictor of AKI but also an independent factor influencing adverse events after discharge in HCE patients. Although cystatin C seems to be correlated with multiple diseases, the direct involvement of cystatin C in the occurrence and development of the disease or its relationship with CKD remains unknown due to the sensitivity of cystatin C, which can be used as a diagnostic indicator of pre-CKD, and the lack of relevant clinical research.

Several limitations remain in this study. First, this work was a cross-sectional and observational study that did not include clinical intervention in HCE patients and potential improvements in clinical outcome. Second, the follow-up period was too short, and some patients could not provide detailed information, which may have led to the omission and ambiguity of a considerable portion of the positive outcomes. Therefore, we failed to conduct further analysis on the long-term outcomes and their influencing factors after discharge. Finally, we were unable to analyse DKA and HHS data due to the limited sample size and follow-up outcome.

Conclusions

In summary, we established a prediction model to observe the impact of AOI on long-term outcomes in patients with HCE. The prognosis of HCE patients with concomitant AOI was found to be significantly worse than that of HCE patients without AOI. Further study should be performed to observe whether timely intervention for AOI can improve clinical outcomes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Committee for Research Ethics of Chongqing University Central Hospital (NO. 202262). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZD, PS and WD developed the protocol and were responsible for the study design. ZD, CY, LD, YJ and FD performed the systematic literature research and extracted data. XJ, YC and WD did data analysis and interpretation. ZD and PS wrote the first draft, and GY, YM and WD revised the article for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1057089/full#supplementary-material

SUPPLEMENTARY TABLE 1
Evaluation of target organ injury model in HCE patients. AKI, Acute kidney injury.

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Bibliometric and visualization analysis of literature relating to diabetic erectile dysfunction

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Introduction: Diabetic erectile dysfunction (DMED) refers to erectile dysfunction secondary to diabetes. Erectile dysfunction is characterized by a persistent inability to achieve and maintain an erection sufficient to permit satisfactory sexual activity.

Methods: Based on the Web of Science core collection database, we firstly analyzed the quantity and quality of publications in the field of DMED, secondly profiled the publishing groups in terms of country, institution, author's publication and cooperation network, and finally sorted out and summarized the hot topics of research.

Results: From 2001 to 2022, a total of 1,403 articles relating to this topic were published in 359 journals. They represent the global research status, potential hotspots, and future research directions. The number of DMED-related publications and citations has steadily increased over the few past decades. Academic institutions from Europe and the United States have played a leading role in DMED research. The country, institution, journal, and author with the most publications were the United States (294), INHA University (39), the Journal of Sexual Medicine (156), and Ryu, Ji-Kan (29), respectively. The most common keywords were erectile dysfunction (796), men (256), diabetes (254), diabetes mellitus (239), prevalence (180), corpus cavernosum (171), dysfunction (155), mellitus (154), nitric-oxide synthase (153), and expression (140). The main keyword-based research topics and hotspots in the DMED field were oral sildenafil, smooth muscle relaxation, nitric oxide synthase, gene therapy, metabolic syndrome, cavernous nerve injury, stem cell, and penile prosthesis.

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Discussion: The terms oral sildenafil, smooth muscle relaxation, nitric oxide synthase, gene therapy, metabolic syndrome, cavernous nerve injury, stem cell, and penile prosthesis will be at the forefront of DMED-related research.

KEYWORDS

diabetic erectile dysfunction, bibliometric analysis, CiteSpace, VOSviewer, visualization

Introduction

Diabetes is a chronic, non-infectious disease caused by both genetic and environmental factors. Over the past three decades, the number of people with diabetes worldwide has more than doubled, making it one of the most important global public health challenges (1). Erectile dysfunction is defined as the persistent inability to achieve and maintain sufficient erectile capacity to permit satisfactory sexual behavior (2). Diabetic erectile dysfunction (DMED) refers to erectile dysfunction secondary to diabetes mellitus (3). The incidence of erectile dysfunction ranges from 0.1% to 18% in the normal population but is nearly three-fold higher in patients with diabetes, and affected people tend to be younger (4).

There is evidence for a link between diabetes and the development of erectile dysfunction in both animal models and humans (5). The central nervous system and pericardial nerve damage caused by a high-glucose environment is an important cause of DMED (6). In addition, age, duration of diabetes, blood glucose control, smoking, hypertension, atherosclerosis, adverse drug reactions, and psychological factors are all closely related to the occurrence of DMED. Current treatment methods for DMED can be summarized as primary disease treatment, psychological treatment, and symptomatic treatment.

The term bibliometrics was coined by Alan Pritchard in 1969 (7). Bibliometric analysis is a powerful tool that uses literature measures or indicators to quantify research performance in a given field (8, 9). CiteSpace and VOSviewer are commonly used processing tools for visualizing research impact based on co-word, co-citation, and literature-coupling analysis (10).

Based on the advantages of clustering technology and map presentation, the research trend of any given field can be analyzed and displayed as a multivariate, comprehensive, visual knowledge map (11, 12). Using such bibliometric software, literature related to DMED in recent decades can be visually displayed and analyzed. Accordingly, with Scopus as the data source, we utilized CiteSpace, VOSviewer, and Microsoft Excel to show the knowledge base, development trends, and emerging hotspots in the DMED field.

Materials and methods

Ethics statement

No approval was required from the Institutional Review Board as data were retrieved from the Web of Science (WOS) database (https://www.webofscience.com/wos/woscc/basic-search) and no human subjects were involved.

Sources and collection

The WOS is the most commonly used database in scientific and bibliometric research. It contains nearly 9,000 of the world's most prestigious high-impact journals and more than 12,000 academic conferences, thus providing a comprehensive overview of the global research results in science, technology, medicine, and other fields (13, 14).

Publication information in the DMED area was searched within one day to ensure that no data were updated. The period was January 1, 2001, through September 30, 2022, and the retrieval date was October 1, 2022. The WOS Core Collection was selected, the subject words were "Diabetic erectile dysfunction", and the article types were "article" and "review". The retrieved results were exported in "Plain text file" format, and "Full Record and Cited References" was selected for "Record content".

The search query string was described as follows: Results for "diabetic erectile dysfunction" (Topic) and Article or Review Article (Document Type) and Book Chapters (Exclude - Document Type).

Bibliometric analysis and software

CiteSpace software (Drexel University, Philadelphia, PA, USA), designed by Dr. Chen Chaomei in 2004 (11), is a freely available Java application widely used for the visualization and analysis of trends and patterns in scientific literature (15). CiteSpace (https://citespace.podia.com/download, R6.1.3) to scientometrics, data and information visualization technology as the foundation, through the analysis of the potential

knowledge of literature, regularity and distribution, present knowledge structure. In this study, CiteSpace was used for keyword clustering and salient word analysis.

VOSviewer 1.6.18 (https://www.vosviewer.com/) is a software tool for constructing and visualizing bibliometric networks (16). In this study, VOSviewer was used to visualize countries/regions, authors, institutional collaborations, citations, and keyword co-occurrence, as well as to construct density maps.

The aim was to describe all the literature characteristics, including country/institution, journals, highly cited articles, cluster network of co-cited references, and most frequently cited keywords. In particular, in addition to noun phrases extracted from article titles and abstracts, burst detection was applied to the keywords of publications in the article collection assigned to the citation extension.

Results

Time trends in publications and citations

The number of annual publications is an important parameter for evaluating the development of scientific research and can reflect the growth of knowledge in this field to a certain extent. As of September 30, 2022, a total of 1,403 publications related to DMED were published (Figure 1). The number of articles published per year is shown in Figure 2. Although the trend was upward overall, it fluctuated slightly in some years. Notably, the number of publications per year can be easily divided into two phases. In the first phase, from 2001 to 2008, the average number of publications per year was around 30; in the second phase, from 2009 to 2022, the average number of publications per year stabilized at more than 50. Overall, the amount of knowledge in the DMED research field showed a linear growth trend (R^2 = 0.6709), reflecting the increasing research interest in this field.

Analysis of the most productive countries/regions

A total of 75 countries/regions have published articles in this field. The 10 countries that made the most significant contributions to DMED-related publications were the USA (294), China (244), Italy (111), South Korea (85), England (79), Turkey (73), Egypt (45), Japan (44), Spain (40), and Germany (38) (Figure 3; Table 1).

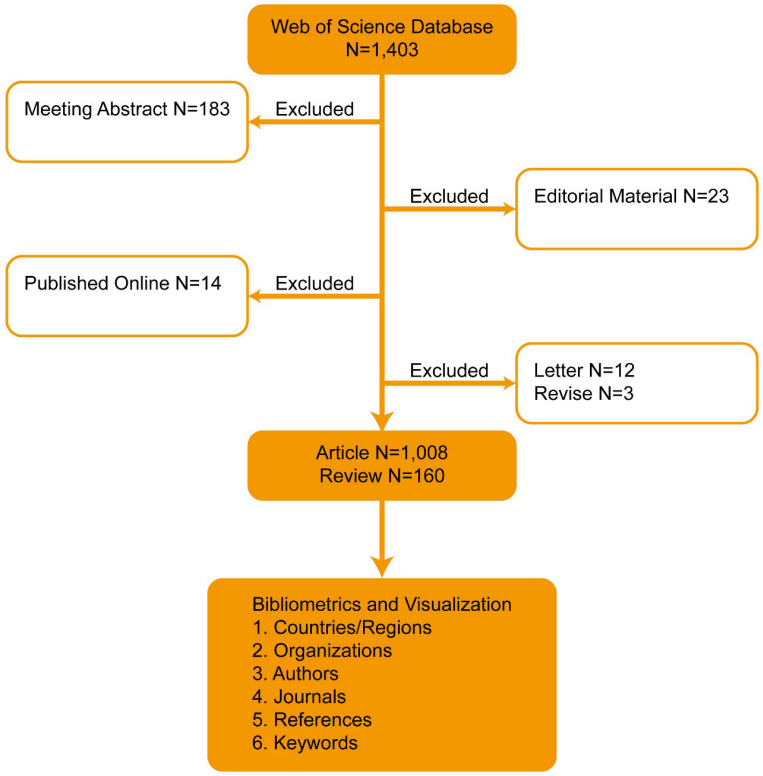


FIGURE 1
Search flowchart.

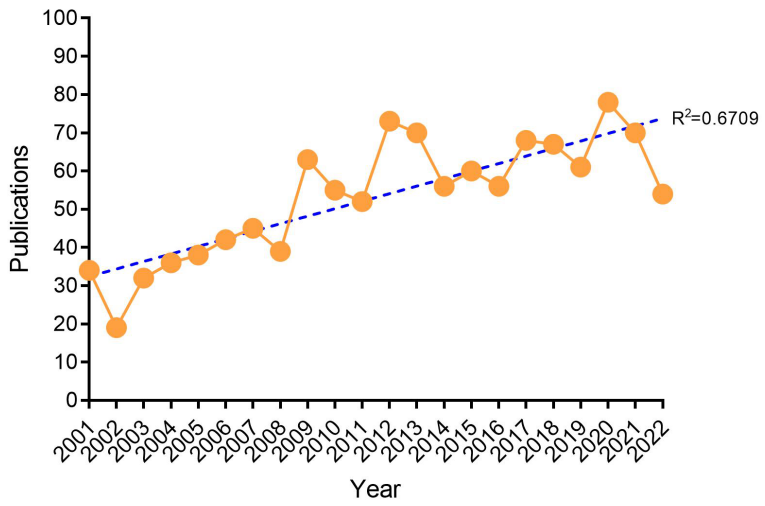


FIGURE 2
Annual trends of global publications.

The size of the nodes is determined by the number of publications (the larger the number, the larger the node). The same colors represent the same clusters. The lines between nodes represent the alignment between countries/regions (the stronger the partnership, the wider the boundaries). The total link strength reflects the combined strength between countries/regions. As shown in Table 2, the USA had the largest number of publications, the highest number of citations (15,620), and the greatest link strength (164). The USA (15,620), Italy (6,917), England (6,645), China (4,495), and Germany (2,381) were the top five countries in terms of citations. The above results indicated that these countries exerted the greatest influence on DMED-related research.

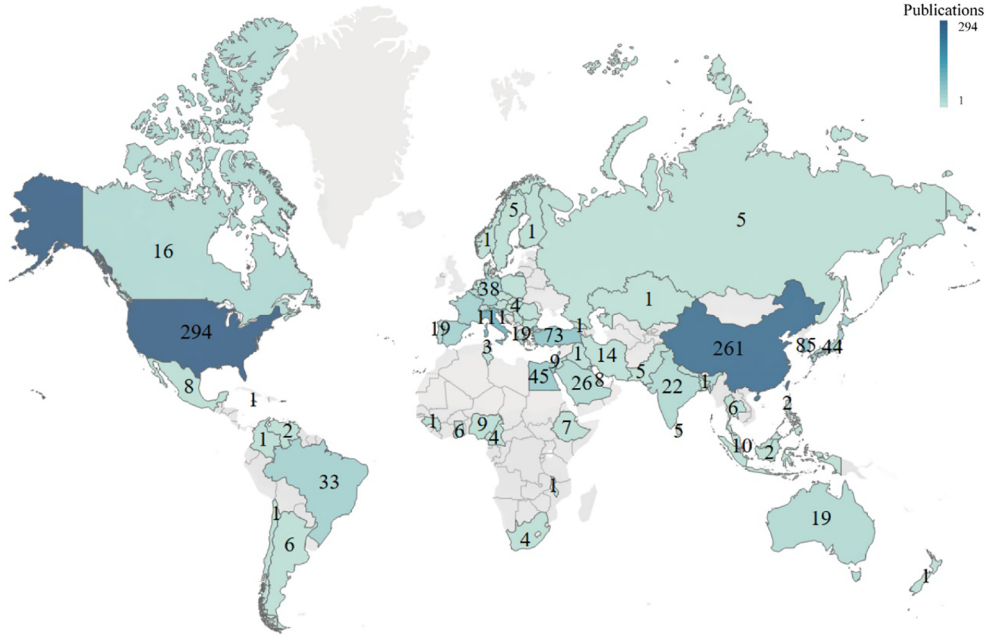


FIGURE 3
Analysis of the global research trends based on the origins of the publications.

TABLE 1 The 10 countries that contributed the most to DMED-related publications.

Rank	Country	Documents	Citations	Total link strength	H-index
1	USA	294	15620	164	51
2	China	244	4495	78	35
3	Italy	111	6917	66	40
4	South Korea	85	1510	32	24
5	England	79	6645	82	30
6	Turkey	73	844	31	15
7	Egypt	45	777	21	15
8	Japan	44	894	13	16
9	Spain	40	1280	35	17
10	Germany	38	2381	38	11

VOSviewer was used to analyze cooperation across countries, with lines between nodes indicating co-authorship between countries (the thicker the line the stronger the cooperation). China, the USA, Italy, South Korea, and England cooperated the most with other countries (Figure 4).

Contributions of the top organizations

A total of 1,465 institutions participated in the publication of papers related to DMED technology. The 10 institutions that contributed the most to DMED publications were INHA University (39), Huazhong University of Science & Technology (32), Tulane University (29), University of California San Francisco (25), Peking University (23), Seoul National University (20), Cairo University (20), University of Florence (19), Nanjing University (19), and Southern Medical University (18) (Table 3). These results demonstrated that INHA University had the greatest number of publications with the highest number of citations (663) and the greatest link strength (20). Each node represents a different institution. The size of the node is determined by the number of publications (the larger the number, the larger the node). The same colors represent the same clusters. Boundaries between nodes represent a collaboration between organizations (the stronger the partnership, the wider the boundaries). The total link strength reflects the aggregate strength between institutions. As can be seen from the map, 146 institutions actively cooperated both within and between clusters. The three institutions with the highest total link strength were Peking University (34), the University of Florence (24), and the University of California San Francisco (23). The five institutions with the greatest number of citations were Eastern Virginia Medical School (4,067), Harvard University (3,845), The University of Pavia (2,092), the University of Manchester (1,684), and the University of Rome Tor Vergata (1,558) (Table 4). Tulane University, University of Florence, University of California San Francisco, INHA University, and Huazhong University of Science & Technology were at the center of such collaborations. However, most institutions were fragmented and displayed little cooperation (Figure 5).

Analysis of authors and co-cited authors

Author co-occurrence analysis can identify the core authors in a field and the strength of collaboration among authors. Co-citation

TABLE 2 The number of citations of publications on DMED in the top 10 countries.

Rank	Country	Documents	Citations	Total link strength
1	USA	294	15620	3542
2	Italy	111	6917	1150
3	England	79	6645	1219
4	China	244	4495	2638
5	Germany	38	2381	241
6	France	34	2358	436
7	Australia	19	2057	192
8	Canada	16	2053	322
9	South Korea	85	1510	1048
10	Spain	40	1280	532

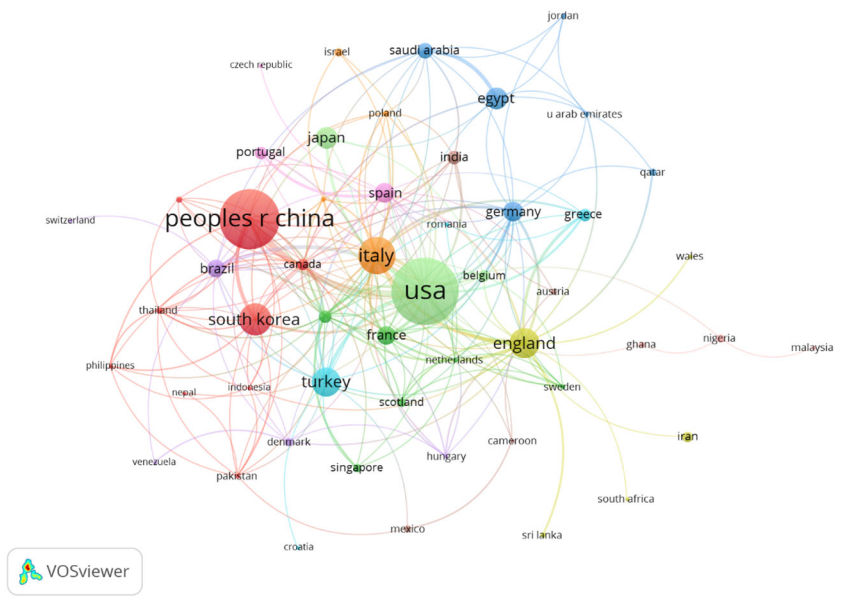


FIGURE 4
Co-occurrence map of countries/regions. The size of the nodes represents the number of articles. The thickness of the curves represents the strength of collaboration. The colors represent different collaboration groups.

means that two authors or papers are cited by a third author or paper at the same time. This study included 5,216 authors and 20,297 co-cited authors. Of these, Ji-Kan Ryu (29), Jun-Kyu Suh (29), Wang Tao (26), Yin Guo Nan (25), and Liu Jihong (20) published the most articles (Table 5). At the same time, Jihong Liu and Guiting Lin obtained the most cooperation, forming two stable author cooperation groups (Figure 6). However, there was a lack of cooperation among other authors and teams, and the research was in a relatively scattered state. The results of the co-citation relationship showed that Bivalacqua, TJ (430); Corona, G (340); Feldman, HA (259); Rosen, RC (258); and Burnett, AL (213) were the most frequently cited authors (Table 6), indicating that they play an important role in DMED research.

Journal distribution

The selected papers were published in a total of 359 journals. The 10 journals with the most publications were the Journal of Sexual Medicine (156), the International Journal of Impotence Research (77), Andrologia (36), the Asian Journal of Andrology (35), Andrology (35), BJU International (30), the Journal of Urology (29), Diabetes Care (21), Urology (20), and PLoS One (19) (Table 7; Figure 7).

A total of 4,634 journals were identified as being co-cited. The top five co-cited journals were the Journal of Sexual Medicine (3,656), the Journal of Urology (2,878), the International Journal of

TABLE 3 The 10 institutions that contributed the most publications on assisted reproduction.

Rank	Organization	Documents	Citations	Total link strength
1	INHA University	39	663	20
2	Huazhong University of Science & Technology	32	414	6
3	Tulane University	29	1048	20
4	University of California San Francisco	25	1041	23
5	Peking University	23	618	34
6	Seoul National University	20	357	19
7	Cairo University	20	346	9
8	University of Florence	19	1532	24
9	Nanjing University	19	695	15
10	Southern Medical University	18	305	14

TABLE 4 The number of citations for publications on DMED by the top 10 institutions.

Rank	Organization	Documents	Citations	Total link strength
1	Eastern Virginia Medical School	5	4067	59
2	Harvard University	6	3845	63
3	University of Pavia	12	2092	207
4	University of Manchester	8	1684	100
5	University of Rome Tor Vergata	5	1558	55
6	University of Michigan	8	1543	67
7	University of Florence	19	1532	307
8	Mayo Clinic	5	1486	33
9	University of Dusseldorf	8	1403	58
10	John Hopkins University Hospital	14	1145	375

Impotence Research (2,058), Diabetes Care (1,676), and Urology (997) (Table 8).

Analysis of highly cited and co-cited literature

A total of 1,168 references and 29,269 co-cited references were obtained. Some papers were cited over 300 times, including those by Tesfaye (2010), Boulton (2005), Vinik (2003a), Gallagher (2007), Jurenka (2008), Jones (2011), and Kapoor (2007b) (Table 9). In addition, a total of 19 references were obtained to highlight the analysis results. The three references with the highest intensities were Kouidrat Y, 2017 (doi: 10.1111/dme.13403), citation burst strength: 26.95; Malavige LS, 2009 (doi: 10.1111/j.1743-6109.2008.01168.x), citation burst strength: 21.88; and Rendell MS, 1999 (doi: 10.1001/jama.281.5.421), citation burst strength: 19.24 (Figure 8).

Keyword analysis

Changing trends in research topics over time can be identified through keyword co-occurrence and salience analysis to better grasp the development of research hotspots. A total of 3,969 keywords were obtained, the top 10 of which

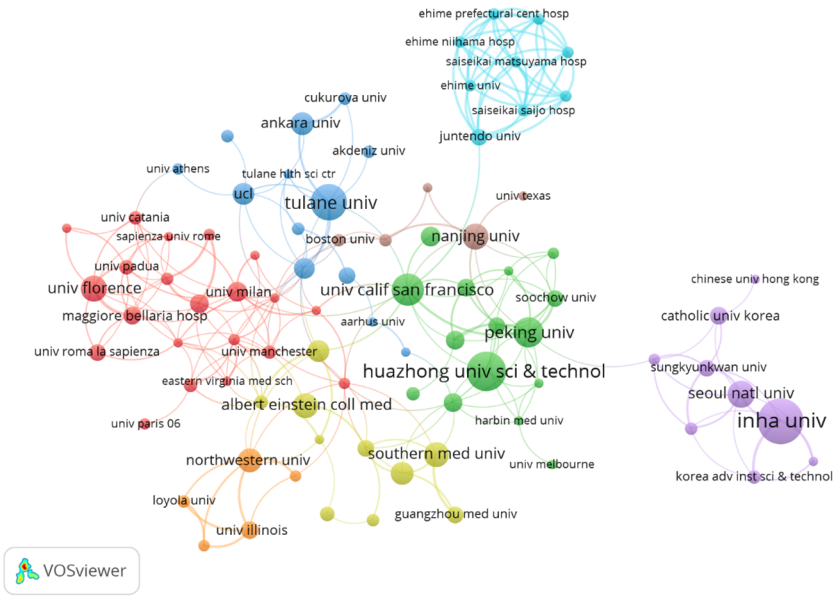


FIGURE 5
Co-occurrence map of institutions. The node size represents the number of articles; the curve thickness represents the strength of collaboration; the different collaboration groups are sorted by color.

TABLE 5 The number of articles published by the top 10 authors.

Rank	Author	Documents	Citations	Total link strength
1	Ryu, Ji-Kan	29	483	189
2	Suh, Jun-Kyu	29	483	189
3	Wang, Tao	26	294	100
4	Yin, Guo Nan	25	334	174
5	Liu, Jihong	20	213	90
6	Song, Kang-Moon	17	274	142
7	Gur, Serap	17	154	19
8	Podlasek, Carol A	15	286	46
9	Hellstrom, Wayne J	15	309	13
10	Kwon, Mi-Hye	14	257	119

were erectile dysfunction (796), men (256), diabetes (254), diabetes mellitus (239), prevalence (180), corpus cavernosum (171), dysfunction (155), mellitus (154), nitric oxide synthase (153), and expression (140) (Table 10; Figure 9).

After clustering using CiteSpace software, a total of 18 categories of keywords were obtained. From 2001 to 2011, keyword-based research hotspots in the DMED field mainly included impotence, oral sildenafil, smooth muscle relaxation, nitric oxide synthase, epidemiology, relaxation, gene therapy, *in vivo*, type 2 diabetes, metabolic syndrome, and sexual function. From 2014 to 2022, meanwhile, the research hotspots (by keyword) included cavernous nerve injury, diabetic rat, pathway, radical prostatectomy, stem cell, apoptosis, model, pathophysiology, and penile prosthesis, among others (Figure 10).

Discussion

Comprehensively and systematically summarizing the research topics, research trends, and global research status relating to DMED allows a rapid preliminary understanding of the research status of this condition. With the advent of big data, researchers need to fully understand the developments in their research field. Different from systematic review or meta-analysis, bibliometric analysis uses visualization software, such as VOSviewer and CiteSpace, to comprehensively analyze the existing literature and obtain an intuitive understanding of the research trends, as well as to predict research hotspots (16). This study is the first to summarize the research status relating to DMED in the past 20 years through bibliometric analysis.

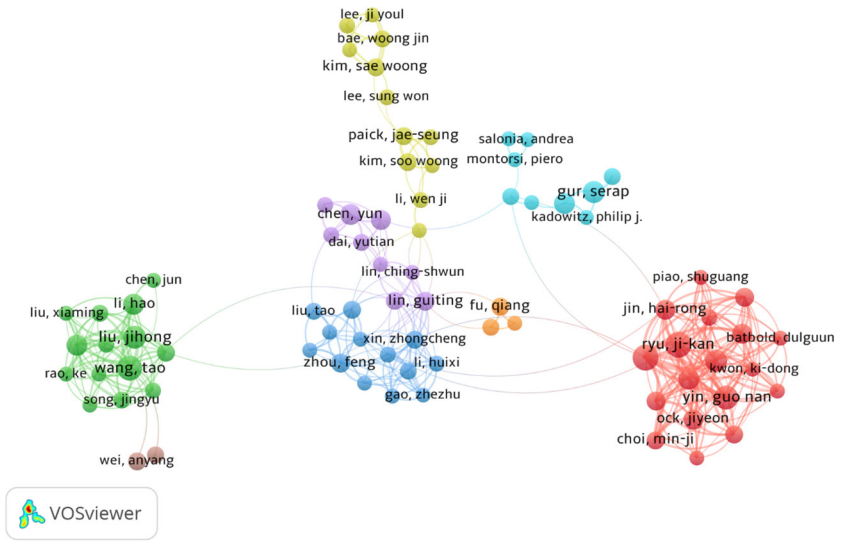


FIGURE 6
CiteSpace visualization map of authors involved in publications related to assisted reproduction. Nodes represent authors (the larger the circle, the greater the number of publications). The lines between nodes represent the cooperation between two authors of the same article (the wider the line, the more frequent the cooperation). The node color represents the year.

TABLE 6 The number of co-citations of the top 10 authors.

Rank	Author	Citations	Total link strength
1	Bivalacqua, TJ	430	6879
2	Corona, G	340	3315
3	Feldman, HA	259	2611
4	Rosen, RC	258	2373
5	Burnett, AL	213	3175
6	Musicki, B	202	2633
7	Cellek, S	167	2646
8	Angulo, J	165	2022
9	Goldstein, I	163	2161
10	Andersson, KE	163	2127

General information about the literature on diabetic erectile dysfunction

Over the past 20 years, the number of DMED-related articles published in journals showed a linear upward trend (R^2 = 0.6709).

From the perspective of countries/regions and institutions, the number of publications originating from the United States as well as the number of associated citations far exceeded that of other countries. Although the number of articles published in China ranked second, the number of citations was low, ranking only fourth. This indicates that, although the number of papers from Chinese institutions is increasing yearly, there is still a lack of high-quality articles. This may be partly explained by a lack of cooperation with internationally renowned researchers as well as language barriers. Among the 10 institutions with the most published papers, only Huazhong University of Science & Technology, Peking University, and Nanjing University were from China—with the remainder being mainly from Europe and the USA—and these institutions had low levels of research cooperation. These observations highlight the need to strengthen communication and cooperation with global research teams, especially those from countries and institutions in the Asian region.

Regarding authors and references, Ryu, Ji-Kan and Suh, Jun-Kyu had the greatest production efficiency, while Bivalacqua, TJ had the most co-citations. Corona, G; Feldman, HA; Rosen, RC; and Burnett, AL also made important contributions to DMED research. The research undertaken by Ryu, Ji-Kan and Suh, Jun-Kyu is mostly mechanism-related. The former has mainly focused on identifying potential target genes associated with DMED in mouse cavernous pericytes (17). Meanwhile, Suh, Jun-Kyu found that pericellular-derived extracellular vesicles mimicking nanovesicles can promote neurovascular regeneration and erectile function recovery under diabetic conditions through a lipid chain enzyme 2 (LCn2)-dependent mechanism (18). Both authors reported that the preservation of damaged cavernous neurovasculature through the inhibition of the proNGF/p75NTR pathway may represent a novel therapeutic strategy for radical prostatectomy-induced erectile dysfunction (19).

The Journal of Sexual Medicine published the most DMED-related papers and the 10 most cited and co-cited journals were

TABLE 7 The 10 most productive journals.

Rank	Source	Documents	Citations	IF/JCR (2022)	Total link strength
1	Journal of Sexual Medicine	156	5048	3.937/Q3	1051
2	International Journal of Impotence Research	77	1893	2.408/Q3	549
3	Andrologia	36	356	2.532/Q3	178
4	Asian Journal of Andrology	35	624	3.054/Q1	323
5	Andrology	35	410	4.674/Q1	288
6	BJU International	30	1000	5.969/Q1	179
7	Journal of Urology	29	1277	7.600/Q1	274
8	Diabetes Care	21	5982	17.152/Q1	215
9	Urology	20	351	2.633/Q3	89
10	PLoS One	19	494	3.752/Q2	173

IF, impact factor; JCR, journal citation report.

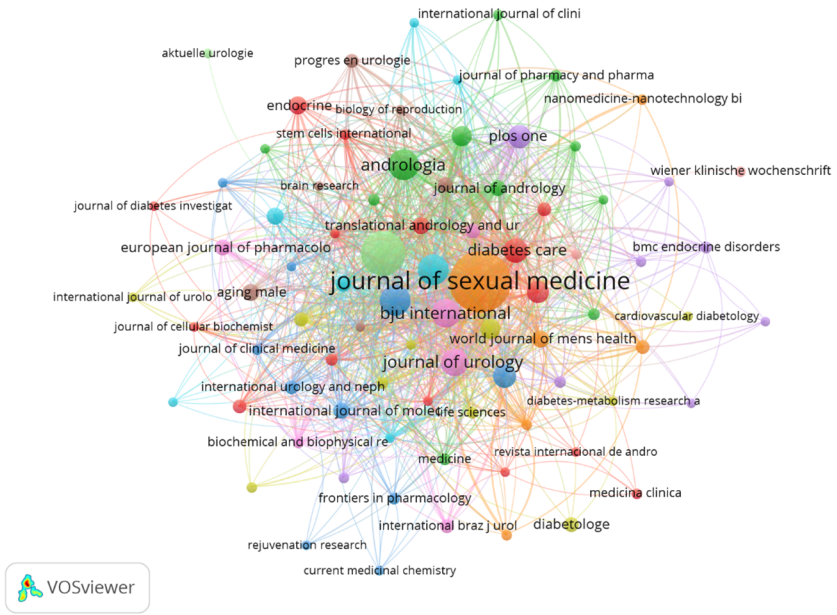


FIGURE 7
Analysis of cited journals.

mainly in Q1 and Q2. This indicates that most papers published in this field were high-quality scientific research achievements.

Hotspots and frontiers

Our analysis further demonstrated that the most influential authors and references were mostly associated with review articles and clinical guidelines from internationally renowned institutions and journals. Combining keyword co-occurrence, clustering, and salience analysis, we identified oral sildenafil, smooth muscle relaxation, acute oxide synthase, gene therapy, metabolic syndrome, cavernous nerve injury, stem cell, and penile prosthesis as the main research topics and hotspots in the field of DMED.

The main treatment for erectile dysfunction is drug therapy, including phosphodiesterase-5 (PDE5) inhibitors, androgen therapy, and vasoactive drugs (20–22). PDE5 inhibitors, such as sildenafil, tadalafil, and vardenafil, are the first-line oral drugs recommended by the World Health Organization (WHO) for the treatment of erectile dysfunction, including that associated with diabetes (23–25). These drugs increase the concentration of cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells by inhibiting PDE5 expression in the corpus cavernosa, reduce the concentration of intracellular calcium, cause smooth muscle relaxation, increase blood flow to the

TABLE 8 The 10 most co-cited journals.

Rank	Source	Citations	Total link strength
1	Journal of Sexual Medicine	3656	119103
2	Journal of Urology	2878	96586
3	International Journal of Impotence Research	2058	76294
4	Diabetes Care	1676	60565
5	Urology	997	37548
6	BJU International	970	39096
7	European Urology	956	34676
8	Diabetes	765	37672
9	Diabetologia	748	35252
10	New England Journal of Medicine	711	27729

TABLE 9 The number of citations for the top 10 references.

Rank	Document	Citations	Links
1	Tesfaye (2010)	1389	7
2	Boulton (2005)	1207	7
3	Vinik (2003a)	1157	7
4	Gallagher (2007)	510	3
5	Jurenka (2008)	395	0
6	Jones (2011)	387	2
7	Kapoor (2007b)	342	5
8	Bivalacqua (2004)	266	21
9	Malavige (2009)	256	21
10	Gandaglia (2014)	250	3

corpus cavernosa, and improve erection. Treating DMED with a PDE5 inhibitor can improve the International Index of Erectile Function-5 (IIEF-5) score and sexual success in a significant number of patients (26, 27).

Nevertheless, PDE5 inhibitors are ineffective in patients with severe erectile dysfunction (28) and are occasionally associated with side effects such as headaches, flushing, indigestion, nasal congestion, vision abnormalities, and diarrhea. The prerequisite

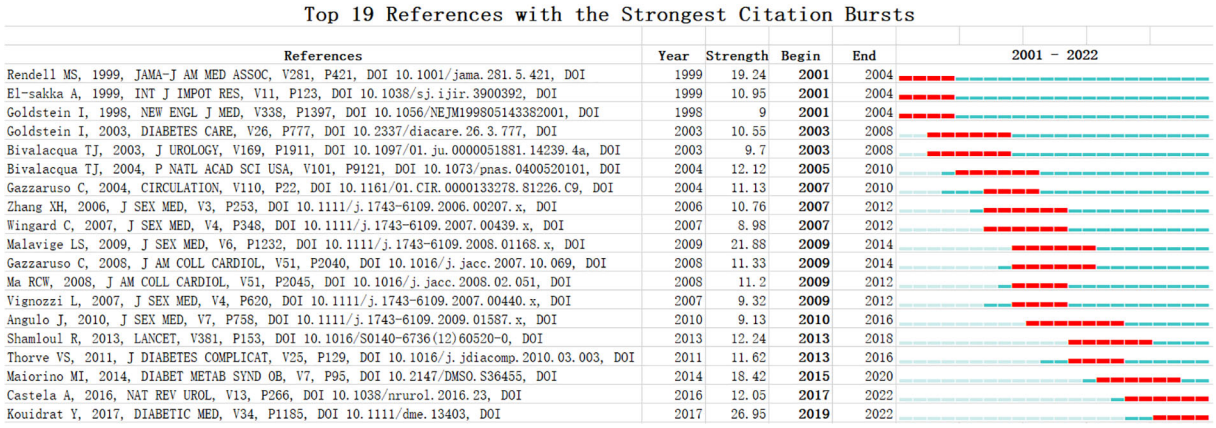


FIGURE 8
The 19 references with the strongest citation bursts.

TABLE 10 Top 10 keywords related to the field of assisted reproduction.

Rank	Keyword	Occurrences	Total link strength
1	Erectile dysfunction	796	4150
2	Men	256	1471
3	Diabetes	254	1503
4	Diabetes mellitus	239	1392
5	Prevalence	180	1004
6	Corpus cavernosum	171	1136
7	Dysfunction	155	930
8	Mellitus	154	961
9	Nitric oxide synthase	153	951
10	Expression	140	892

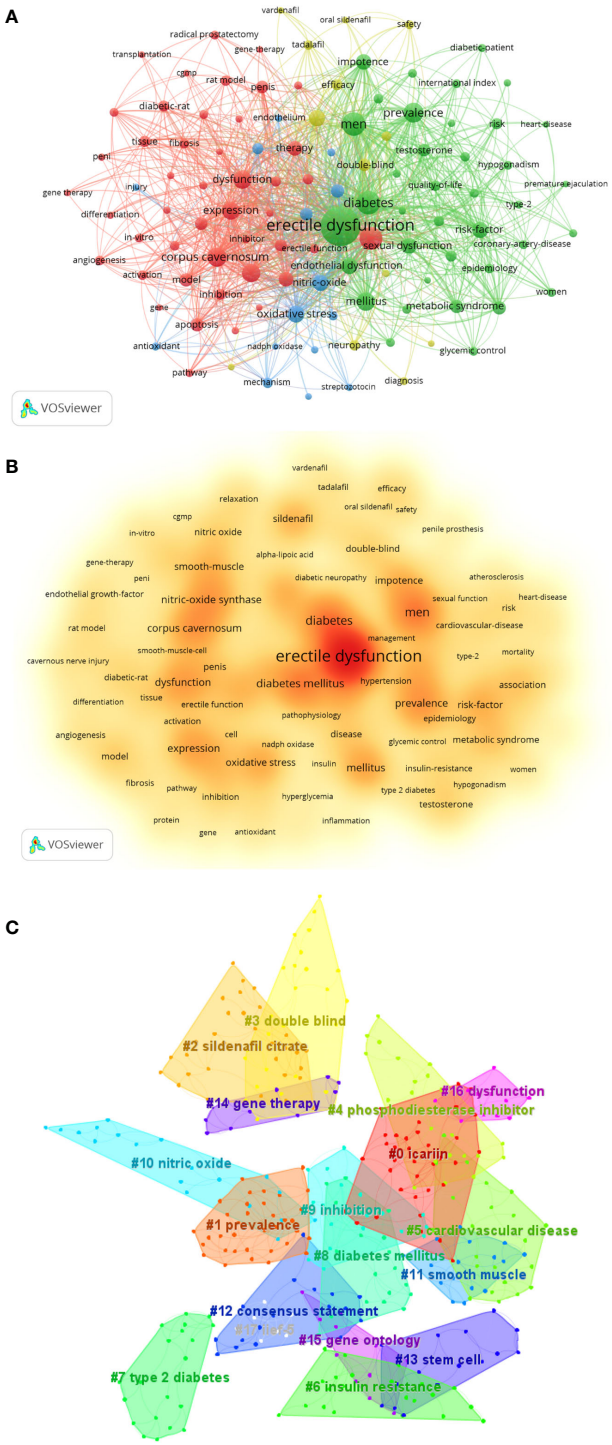


FIGURE 9
Keyword analysis. **(A)** Map of the keyword co-occurrence analysis obtained using VOSviewer. The size of the nodes represents the number of occurrences; the thickness of the curve represents the strength of collaboration; the different colors represent the different clusters. **(B)** Keyword density visualization analysis. The redder the node color, the higher the frequency of the keyword. **(C)** Keyword clustering map analysis using CiteSpace. A total of 16 categories of keywords were obtained; the different color blocks represent different keyword clusters.

Keywords	Year	Strength	Begin	End	2001 – 2022
impotence	2001	22.11	2001	2006	
oral sildenafil	2001	5.37	2001	2007	
smooth muscle relaxation	2001	6.68	2003	2009	
nitric oxide synthase	2001	5.17	2003	2008	
epidemiology	2001	4.79	2003	2006	
relaxation	2001	7.68	2004	2008	
gene therapy	2001	5.04	2004	2005	
in vivo	2001	7.41	2007	2010	
type 2 diabete	2001	6.72	2008	2010	
metabolic syndrome	2001	7.8	2009	2011	
sexual function	2001	5.78	2009	2011	
cavernous nerve injury	2001	7.22	2014	2018	
diabetic rat	2001	9.57	2015	2018	
pathway	2001	7.91	2015	2018	
radical prostatectomy	2001	6.77	2016	2022	
stem cell	2001	6.43	2017	2022	
model	2001	4.99	2017	2022	
pathophysiology	2001	4.72	2017	2020	
apoptosis	2001	7.17	2019	2022	
penile prosthesis	2001	4.88	2020	2022	

FIGURE 10
Keyword burst analysis using CiteSpace.

for PDE5 inhibitor to function is that ED patients must have intact molecular and neurological pathways and some degree of sexual stimulation. Therefore, PDE5-Is has poor efficacy in patients lacking upstream nitric oxide (NO) pathway (29) in some disease states, such as diabetes with peripheral neuropathy (30) and prostate cancer (31). In addition to drugs, other alternative strategies are available, such as vacuum contractile devices and penile prosthesis implantation The three-piece inflatable penile prosthesis (IPP) is the most common implant used in penile surgery (32).The satisfaction rate of patients is 90% ~ 100%, which varies with different prosthesis devices (33, 34).However, IPP may only be offered to patients who fail due to the high cost of conservative treatment, its invasive nature, and myriad potential complications. Thus, novel therapeutic approaches are urgently required to overcome these disadvantages in DMED treatment.

Stem cell therapy has attracted increasing attention owing to its ability to promote functional recovery and tissue structural repair in patients with diabetes. Stem cell mainly include bone marrow mesenchymal stem cells (BMSCs) (35), adipose-derived stem cells (ADSCs) (36), neural derived stem cells (37), mesenchymal stem cells (MSCs) (38), umbilical cord derived stem cells (39) and urine-derived stem cells (USCs) (40).Mesenchymal stem cells (MSCs) are adult stem cells derived from mesoderm with a high potential for proliferation, self-renewal, and multidirectional differentiation (41). The culture of bone marrow-derived MSCs is relatively simple; accordingly, they are widely used in animal studies and clinical trials (42). Given their abundant autologous availability, MSCs are one of the most promising candidates for the treatment of DMED (43). Importantly, allogeneic MSCs can also be successfully transplanted owing to the low immunogenicity (44, 45). In animals, bone marrow-derived MSC transplantation can significantly improve the recovery of erectile function by inhibiting apoptosis (46). For DMED, MSC-based treatment has also attained encouraging therapeutic effects (47). The restoration of erectile function is mainly attributed to the increased endothelial cell and smooth muscle content in the corpus cavernosum (48, 49). Various strategies have recently emerged to improve the therapeutic effect of MSCs on DMED (50, 51).In a single-blind study, 6 out of 7 ED patients developed morning erections 3 months after treatment with umbilical cord derived stem cells (39).Chen S et al. found that adipose mesenchymal stem cells were more effective in treating diabetes-related erectile dysfunction than bone marrow mesenchymal stem cells (52).

Erectile dysfunction has been associated with the upregulation of Toll-like receptor 4 (TLR4) expression. It has been reported that TLR4 expression is significantly increased in the corpus cavernosum of diabetic rats compared with that of controls (53). The overexpression of inducible nitric oxide synthase (iNOS) may also be associated with penile microvascular dysfunction in diabetes mellitus, while endotoxemia has been linked with iNOS upregulation (54). Studies on conducting vessels have shown that iNOS overexpression in endotoxemia contributes to endothelial dysfunction by reducing the activity of endothelial nitric oxide synthase (eNOS) (55). iNOS inhibitors can alleviate DMED-related injury by moderating eNOS phosphorylation and chronic iNOS overexpression as well as improving microvascular fibrosis (56).

Trabecular smooth muscle relaxation during sexual stimulation and dilatation of penile resistance arteries in the cavernous body are essential for penile erection (57). Nitric oxide (NO) is a key factor in both processes. NO can be released from nerve endings or endothelial cells to stimulate cGMP production in penile smooth muscle cells, relaxing them and increasing blood flow to the corpus cavernosum (58, 59). Defects in the NO/cGMP pathway at any level will lead to inadequate penile smooth muscle relaxation, which affects erectile function (60).

In vitro low-intensity shockwave therapy (LI-SWT) is a potential treatment option for ED. The micro-damage of Li-SWT to the spongy tissue may stimulate neovascularization and up-regulate some factors related to tissue healing and remodeling (61).A prospective, randomized, sham controlled study reported a clinically significant improvement in erectile function in 40.5% of the treatment group based on the minimum clinically significant difference (MCID) criteria (62).Another randomized clinical trial evaluated changes in penile hemodynamics and IIEF-EF scores in patients with vascular ED. In the IIEF-EF score, 56.7% of the treatment group achieved MCID at 1 month and 75% achieved MCID at 12 months (63).

Through keyword co-occurrence and salience analysis, we also found that there is a regional imbalance in the development of DMED research and influential authors and institutions are mainly concentrated in Europe and Asia, which may be due to greater research interest. Additionally, despite the numerous studies undertaken on DMED, the underlying mechanism remains incompletely understood and represents a key future research direction.

Limitations and prospects

To the best of our knowledge, this is the first bibliometric analysis of DMED-related studies undertaken in the last 20 years. However, our study still had some limitations. First, high-quality articles published in recent years may not have yet reached the ideal citation threshold, which may have introduced research bias. Second, there may be a delay in exploring the frontiers of research. Finally, we only included literature written in English from the WOS, which may have resulted in the omission of important documents in other languages.

Conclusions

Overall, our bibliometric analysis provides comprehensive information on DMED-related publications. Our findings suggested that the field of DMED is thriving and has stimulated great interest in the research community worldwide. Although DMED is a relatively common disease, much remains unknown about both the underlying mechanisms and how to treat it. Likely, the terms oral sildenafil, smooth muscle relaxation, nitric oxide synthase, gene therapy, metabolic syndrome, cavernous nerve injury, stem cell, and penile prosthesis will be at the forefront of DMED-related research.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

FM and HL (10th author) designed the study. XL, HC, LW, MZ, HL (7th author), GG and DL conducted the literature search. FM, XL and LW analyzed the data and wrote the paper. FM and JW approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Correlation analysis of serum IGF-1 and IL-6 and urinary albumin/creatinine ratio in patients with type 2 diabetic kidney disease

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Objectives: Diabetic kidney disease (DKD) is one of the most common chronic complications in diabetic patients, and there are major limitations in its pathological diagnosis. This study's objectives were to examine the changes in serum insulin-like growth factor-1 (IGF-1) and interleukin-6 (IL-6) levels in DKD patients with various urinary albumin/creatinine ratio (ACR) and to evaluate the utility of these two biological markers in the clinical diagnosis of the condition.

Methods: We chose 80 type 2 diabetic patients as the experimental group and 20 healthy normal participants as the control group. The experimental group was split into three groups based on the ACR range: diabetes without nephropathy group (ACR < 30 mg/g), microalbuminuric group (30 < ACR < 300 mg/g), and macroalbuminuric group (ACR > 300 mg/g). The levels of serum IL-6 and IGF-1 were assessed in each trial participant.

Results: Serum IGF-1 was higher in the experimental group than in the control group ($P < 0.01$), and serum IL-6 levels were also higher than in the control group ($P < 0.001$). In DKD patients, serum levels of IL-6 and IGF-1 tended to rise when ACR levels rose. By Pearson correlation analysis, serum IGF-1 and IL-6 were positively correlated with ACR ($r = 0.765$ and $r = 0.651$, all $P < 0.001$) and negatively correlated with eGFR ($r = -0.389$ and $r = -0.364$, all $P < 0.01$). Additionally, the receiver operating characteristic (ROC) characteristic curve showed that the area under the curve (AUC) values for serum IGF-1 and IL-6 were 0.9056 and 0.7850, respectively, while the AUR value for both combined was 0.9367.

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Conclusion: Serum IGF-1 and IL-6 levels can be used to diagnose DKD, and the combined analysis of these two indicators can improve the sensitivity and specificity of the disease diagnosis.

KEYWORDS

insulin-like growth factor-1, IL-6, diabetic kidney disease, diabetes mellitus, urinary albumin/creatinine ratio

Introduction

Currently, chronic complications of diabetes mellitus are in serious form worldwide. According to reports, 30–40% of diabetic patients have DKD (1), which can eventually develop end-stage renal disease (ESRD) and is one of the leading causes of death in diabetic patients. Moreover, diabetic kidney disease accounts for 30%-50% of all patients with chronic renal failure requiring hemodialysis (2). The typical pathological changes in DKD include glomerular basement membrane thickening, glomerulosclerosis, tubulointerstitial fibrosis, interstitial inflammatory infiltration and podocyte injury (3). Patients in the early stages do not have overt clinical symptoms. The clinical symptoms of early DKD patients are not obvious, and the disease has commonly progressed to the middle and advanced stages by the time the diagnosis is made. Studies have shown that prognosis of patients with DKD can be improved by aggressive interventions (4, 5). Therefore, early diagnosis and timely treatment are crucial for patients with DKD. In recent years, the role of serum IGF-1 and serum IL-6 in the development of DKD has received considerable attention from scholars.

IGF-1 is a peptide substance with a function and structure particularly similar to insulin (6). IGF-1 plays an essential role in the regulation of cell development and growth and in increasing substance metabolism and other physiological processes. Early studies have shown that elevated serum levels of IGF-1 can influence the early pathological processes of diabetic kidney disease, such as glomerular enlargement, renal hyperfiltration and renal hypertrophy (7). In addition, IGF-1R inhibitors reduced tubulointerstitial fibrosis and renal inflammatory cell infiltration in DKD mice (8).

IL-6 is a member of the chemokine family that is commonly produced by fibroblasts and T Lymphocytes. Its primary biological role is to modulate the immune response and inflammation responses (9). It was found that the expression level of IL-6 mRNA was increased in mesangial cells and tubular cells of DKD patients (10). IL-6 can be closely associated with specific miRNAs, renal podocyte injury and involved in the pathogenesis of DKD (11). Additionally, IL-6 can also directly promote the production of extracellular matrix and proliferation of mesenchymal cells, thereby promoting the development of renal fibrosis. Our study investigated the diagnostic significance of each biological index and the combination of both in DKD by looking at serum IGF-1 and serum IL-6 levels in DKD patients with different ACR.

Materials and methods

Eighty cases of type 2 diabetic patients (the diagnostic criteria of diabetes mellitus referred to the diagnostic criteria of diabetes mellitus published by WHO expert committee in 1999) who attended the inpatient department of endocrinology in Jingzhou Central Hospital from September 2021 to February 2022 were selected as the experimental group, and 20 cases of normal people who visited the health check-up center of the hospital during the same period were selected as the control group. According to the Mogensen staging and Kidney Disease Improving Global Outcomes (KDIGO) guidelines, the experimental group was divided into three groups: diabetes without nephropathy group (20 cases, ACR < 30 mg/g), microalbuminuric group (30 cases, 30 mg/g < ACR < 300 mg/g) and macroalbuminuric group (30 cases, ACR > 300 mg/g). The exclusion criteria for the DKD were as follows: patients with kidney disease caused by other diseases; patients who may affect the ACR level after treatment with drugs such as ACEI or ARB; factors that may affect IGF-1 level, such as tumors, tuberculosis, surgery, trauma, etc., are excluded, factors that may cause the increase in ACR were excluded, such as strenuous exercise in a short period of time, infection, and chronic heart failure.

Experimental methods

Measurement of general indicators

The sex, age and duration of diabetes mellitus were counted in all study subjects, height and weight were measured using a

uniform tool, and body mass index (BMI) was calculated according to the BMI formula (BMI = weight/height ^{2}, kg/m^2). After 8h fast, blood samples were taken from patients in the morning and brought to our lab in less than two hours. Serum creatinine (SCr), total cholesterol (TC), IL-6, triglycerides (TG), albumin (ALB), and total protein (TP) levels from serum were measured using an automated biochemical analyzer. Fasting plasma glucose (FPG) measured by glucose oxidation method. Calculation of estimated glomerular filtration rat (eGFR) was performed using the modified Chinese MDRD formula. Serum IGF-1 was detected by chemiluminescence immuno-sandwich assay and detected by a MAGLUI series chemiluminescence analyzer produced by Shenzhen New Industry Biomedical Engineering Co., LTD. In addition, ACR from morning urine was detected by immunoturbidimetric assay.

Statistical treatment

SPSS22.0 statistical software was used for statistical analysis of the data. For comparisons between two groups, all measures were expressed as mean ± SD when they conformed to normal distribution and variance homogeneity, and t-test was used to compare differences between groups; otherwise, the data were analyzed using the nonparametric Mann-Whitney test. For comparison of multiple data groups, one-way ANOVA and multiple comparisons were used, and for data that did not obey a normal distribution, the nonparametric Kruskal-Wallis test was used for analysis. Correlation analysis was performed using Pearson's method or non-parametric Spearman. A $p < 0.05$ was considered statistically significant.

Results

Clinical indices of the healthy control group and the experimental group.

The general data of all experimental and control groups in Table 1 were analyzed, and there was no statistical significance for age, gender, BMI, TG, TC, TP and ALB in the four groups ($P > 0.05$). Diabetes without nephropathy group, microalbuminuria group and macroalbuminuria group had higher levels of HbA1c and FPG than the healthy control group ($P < 0.01$, Table 1). In particular, we found that serum IGF-1 and IL-6 levels were significantly lower in the control group than in the diabetes without nephropathy group, the macroalbuminuria group and the microalbuminuria group ($P < 0.05$) (Figure 1).

Serum IGF-1 and IL-6 levels in diabetic patients with different ACR.

In this study, serum IGF-1 and IL-6 levels were significantly higher in the macroalbuminuria group (195.49 ± 42.25, 5.00 ± 1.25, $P < 0.0001$, Table 1, Figure 2) than in the microalbumin group (132.48 ± 29.03, 3.85 ± 0.81, $P < 0.0001$, Table 1, Figure 2).

TABLE 1 Clinical data comparison between the control group and the experimental group.

Group	Control	Diabetes without nephropathy	Microalbuminuria	Macroalbuminuria
Number	20	20	30	30
Age (years)mean +/- SE	57.45 ± 12.38	59.10 ± 8.53	57.60 ± 10.45	59.53 ± 10.22
Gender (female/male)	11/9	12/8	18/12	22/8
Course of disease (years) (interquartile)	–	6.0 (1-15)	9.0 (1-26)[a]	9.0 (1-31)[a]
BMI (kg/m2)mean +/- SE	24.63 ± 2.50	26.54 ± 3.60	25.15 ± 3.32	24.60 ± 3.22
eGFR (ml/min)mean +/- SE	–	107.87 ± 27.98	86.66 ± 28.40[d]	67.70 ± 24.51[df]
TP (g/L)mean +/- SE	69.90 ± 4.55	69.89 ± 4.55	71.08 ± 6.24	70.06 ± 6.13
ALB (g/L)mean +/- SE	41.76 ± 3.80	42.19 ± 3.21	43.73 ± 3.37	42.59 ± 4.46
TC (mmol/L)mean +/- SE	4.34 ± 0.94	4.63 ± 1.20	4.46 ± 1.05	4.63 ± 0.88
TG (mmol/L)mean +/- SE	1.52 ± 0.56	1.54 ± 0.58	1.72 ± 0.74	1.74 ± 0.84
FPG (mmol/L)mean +/- SE	5.25 ± 0.41	8.49 ± 1.25[b]	8.01 ± 1.56[b]	7.76 ± 1.49[b]
IL-6 (pg/mL)mean +/- SE	1.64 ± 0.49	2.68 ± 0.75[b]	3.85 ± 0.81[bd]	5.00 ± 1.25[bdf]
IGF-1 (ng/ml)	79.65 ± 13.23	105.21 ± 17.80[b]	132.48 ± 29.03[bd]	195.49 ± 42.25[bdf]
ACR (mg/g)	–	9.28 ± 4.72	148.94 ± 71.13[d]	556.66 ± 197.51[df]
HbA1c (%)	5.20 ± 0.27	7.62 ± 1.60[b]	8.10 ± 1.67[b]	8.30 ± 1.96[b]
DR (yes/no)	–	3/17	30/30[d]	30/30[d]

Data are means ± SD for Gaussian variables and median (interquartile) for non-Gaussian variables.
Diabetes without nephropathy, Microalbuminuric and Macroalbuminuric versus Control, [a]$P < 0.05$, [b]$P < 0.01$; Macroalbuminuric and Microalbuminuric versus diabetes without nephropathy, [c]$P < 0.05$, [d]$P < 0.01$; Microalbuminuric versus Macroalbuminuric, [e]$P < 0.05$, [f]$P < 0.01$.
BMI, body mass index; DR, diabetic retinopath; eGFR, estimated glomerular filtration rat; TP, total protein; ALB, albumin; TC, total cholesterol; FPG, fasting plasma glucose; TG, triacylglycerol; IL-6, interleukin 6; IGF-1, insulin-like growth factor-1; HbA1c, glycated hemoglobin; ACR, urinary albumin/creatinine ratio.

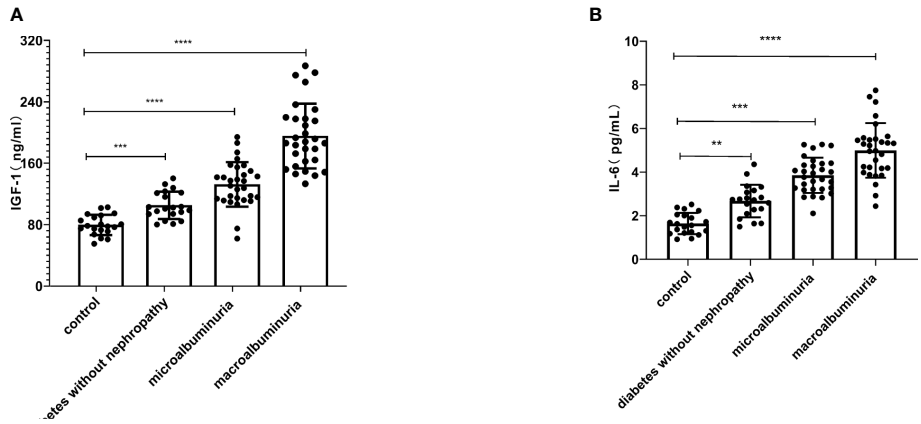


FIGURE 1
Serum insulin-like growth factor-1 (IGF-1) **(A)** and interleukin-6 (IL-6) **(B)** were higher in the diabetes without nephropathy, microalbuminuria and macroalbuminuria groups than in the control group (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Moreover, both groups were higher than the group with diabetes without nephropathy (105.21 ± 17.80, 2.68 ± 0.75, $P < 0.0001$, Table 1, Figure 2). This indicates that serum IGF-1 and IL-6 levels increased with the increase of ACR in DKD patients. And significantly more patients in the microalbuminuria and macroalbuminuria groups had diabetic retinopathy (DR) than diabetic patients without nephropathy (Figure 2).

Correlation analysis of serum IGF-1, serum IL-6 and other clinical indicators in DKD patients

Pearson correlation analysis was used to explore the close degree of serum IGF-1 and clinical indicators in patients with diabetic kidney disease. The results showed that serum IGF-1 was positively correlated with serum IL-6 (r = 0.425, $P < 0.01$) (Figure 3A) and ACR (r = 0.765, $P < 0.001$) (Figure 3B) to varying degrees and negatively correlated with eGFR (r = - 0.389, $P < 0.01$) (Figure 3C). There was no significant correlation with duration of diabetes, albumin, total protein and fasting blood glucose ($P > 0.05$) (Table 1, Figure 3D).

Similarly, serum IL-6 was positively correlated with ACR (r = 0.651, $P < 0.001$) (Figure 4B), IGF-1 (r = 0.425, $P < 0.01$) (Figure 3A) and negatively correlated with eGFR (r = - 0.364, $P < 0.01$) (Figure 4A), but not significantly correlated with FPG ($P = 0.577$, Figure 4C).

Logistic regression analysis

Logistic regression analysis was performed to correlate the progression of DKD with serum IL-6, serum IGF-1, TG, TC, FPG and HbA1c levels. The results showed that the progression of DKD was correlated with serum IGF-1 and IL-6 ($P < 0.05$, Table 2), independent of other indicators ($P > 0.05$, Table 2).

ROC curves for the separation and combination of serum IGF-1 and serum IL-6

On the basis of examination of logistic regression analysis, we additionally established ROC curves. In this study, the area under the ROC curve was used to indicate the accuracy of biological indicators to predict disease. As shown, AUC values for serum IGF-1 and IL-6 were 0.9056 and 0.7850 (Figure 5), respectively, and AUC value for both combined was 0. 9367 (Figure 5).

Moreover, the sensitivity and specificity of the serum IGF-1 index were 96.7% and 70.0% (Table 3), respectively, and the sensitivity and specificity of serum IL-6 were 66.3% and 83.0% (Table 3), respectively. The sensitivity and specificity of the combination of the two indicators were 90.0% and 83.3% (Table 3), respectively. This indicates not only that serum IGF-1 and IL-6 can be used to predict DKD progression separately, but also that the combination of the two has higher sensitivity and specificity.

Discussion

DKD is one of the most serious chronic consequences in people with diabetes and is the second most deadly condition for people with type 2 diabetes after cardiovascular disease. Histopathologic diagnoses are the gold standard for the

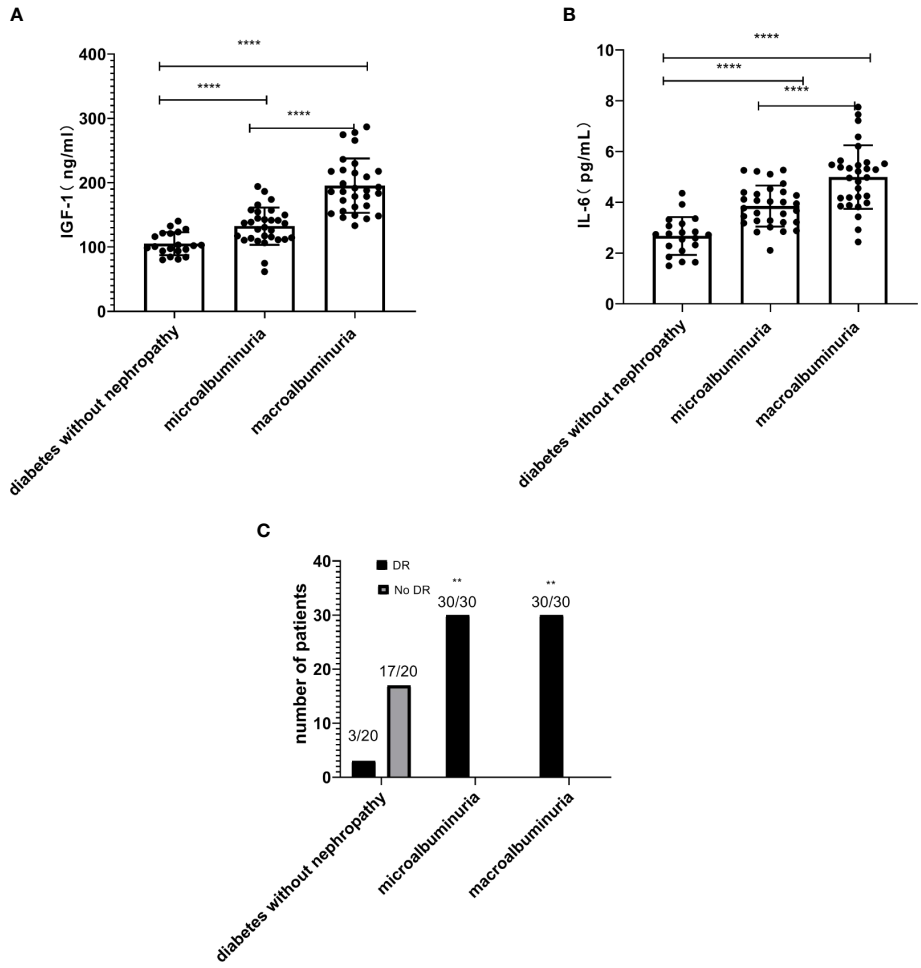


FIGURE 2
The levels of insulin-like growth factor-1 (IGF-1) **(A)** and interleukin-6 (IL-6) **(B)** increased with the increase of urinary albumin/creatinine ratio (ACR) in diabetic kidney disease (DKD) patients (****$P < 0.0001$). **(C)** The number of DKD patients with DR is higher than the diabetes without nephropathy group (**$P < 0.01$).

diagnosis of DKD, but this test is a traumatic examination with limitations and low patient willingness to be tested. Therefore, it poses difficulties for clinical diagnosis. ACR is frequently used to assist in the diagnosis and staging of DKD, however it can be interfered with by other factors. Previous studies have demonstrated an association between serum IGF-1 and the development of DKD (7). This is consistent with the results of our study, where serum IGF-1 levels were higher in the group of patients with DKD than in the control group. Moreover, serum IGF-1 levels continued to rise as the ACR index increased. As an insulin-like analogue, IGF-1 has a role in promoting cell proliferation, differentiation and accelerating substance metabolism (12). Previous reports have shown that the expression of IGF-1, IGF-1 mRNA and IGFBP1 mRNA was upregulated in the kidneys of rats with DKD compared with normal controls (13, 14). Levin et al. showed that increased IGF-1 levels were associated with early pathological changes in STZ-induced diabetic rats, including renal hypertrophy and hyperfiltration (7). In addition, elevated levels of IGF-1 activate the Akt signaling pathway, which expresses Snail1 and results in renal fibrosis (8, 15). Kong et al. showed that the activation of IGF-1/IGF-1R pathway can promote renal mesangial cells to produce type IV collagen and connexin, which are the main components of extracellular matrix and can promote the proliferation of mesangial cells and stromal thickening, thus leading to glomerular sclerosis (16). In addition, elevated IGF-1 expression levels were associated with other renal histopathological alterations, including mesangial expansion, infiltration of inflammatory factors, and renal cell proliferation (17, 18). Our study also found that IL-6 expression level was significantly elevated in DKD patients and associated with ACR. Inflammatory response is one of the pathogenic mechanisms of

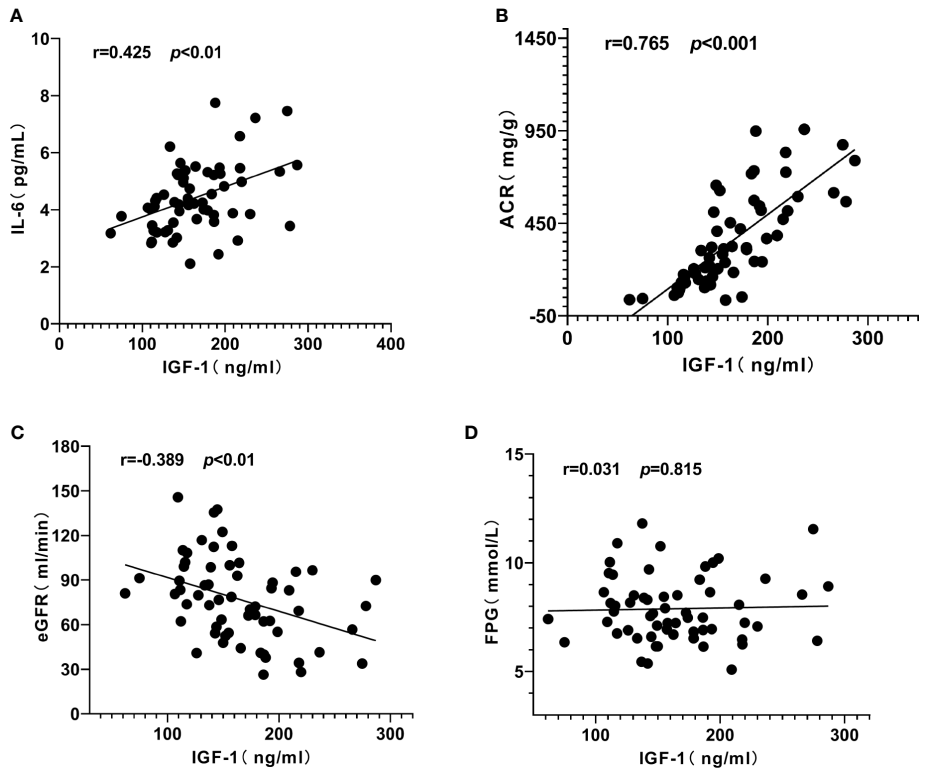


FIGURE 3
Correlation analysis of serum insulin-like growth factor-1 (IGF-1) with interleukin-6 (IL-6), urinary albumin/creatinine ratio (ACR) and estimated glomerular filtration rat (eGFR) in patients with diabetic kidney disease (DKD). **(A)** IGF-1 was positively correlated with IL-6. **(B)** IGF-1 was positively correlated with ACR. **(C)** IGF-1 was negatively correlated with eGFR. **(D)** IGF-1 had no significant correlation with fasting plasma glucose (FPG).

DKD (19). Studies have shown that gene polymorphisms of IL-6 are closely related to the development of DKD and its elevated expression level can increase the probability of the disease (20). IL-6 can not only directly stimulate mesangial cells proliferation, damage islet cells and disrupt islet function, but also damage renal vascular endothelial cells and promote renal interstitial fibrosis. IL-6 receptor blockers have been reported to inhibit the inflammatory response and reduce insulin resistance in DKD mice (21). In addition, by inhibiting the IL-6R/JAK2/STAT3 pathway in renal cells was able to protect the kidney in diabetic rats (22).

In this study, we confirmed the promoting effect of serum IGF-1 and serum IL-6 expression levels on DKD, based on which logistic regression analysis was performed and ROC curves were constructed. The results showed that serum IGF-1 and IL-6 contributed to the diagnosis of DKD, with AUR values of 0.9056 and 0.7850 (Table 3), respectively. Moreover, the sensitivity and specificity of the two indicators combined to diagnose the disease was higher, with an AUR value of 0.9367 (Table 3). The majority of the IGF-1 in the organism exists in bound form. IGF-1 binding to IGF-1R activates the downstream MAPK pathway, increases the expression of fibrosis-related genes and stromal Has2 genes, and accelerates the process of mesangial expansion and proteinuria in streptozotocin-induced diabetic mice (23). Through oxidative stress and inflammatory reactions, IL-6 may contribute to DKD (24, 25). Studies have shown that by inhibiting the p38-MAPK signaling pathway, the level of the inflammatory factor IL-6 can be attenuated, thereby reducing renal inflammatory infiltration (26). Both serum IGF-1 and IL-6 are involved in the development of DKD, and we speculate that this may be related to the coordinated action of both signaling pathways, which together promote the progression of DKD.

Correlation analysis showed that serum IGF-1 and IL-6 were negatively correlated with eGFR and positively correlated with ACR. It is hypothesized that serum IGF-1 and IL-6 exacerbate renal injury by affecting glomerular filtration rate as well as urinary albumin. Additionally, we discovered that the number of

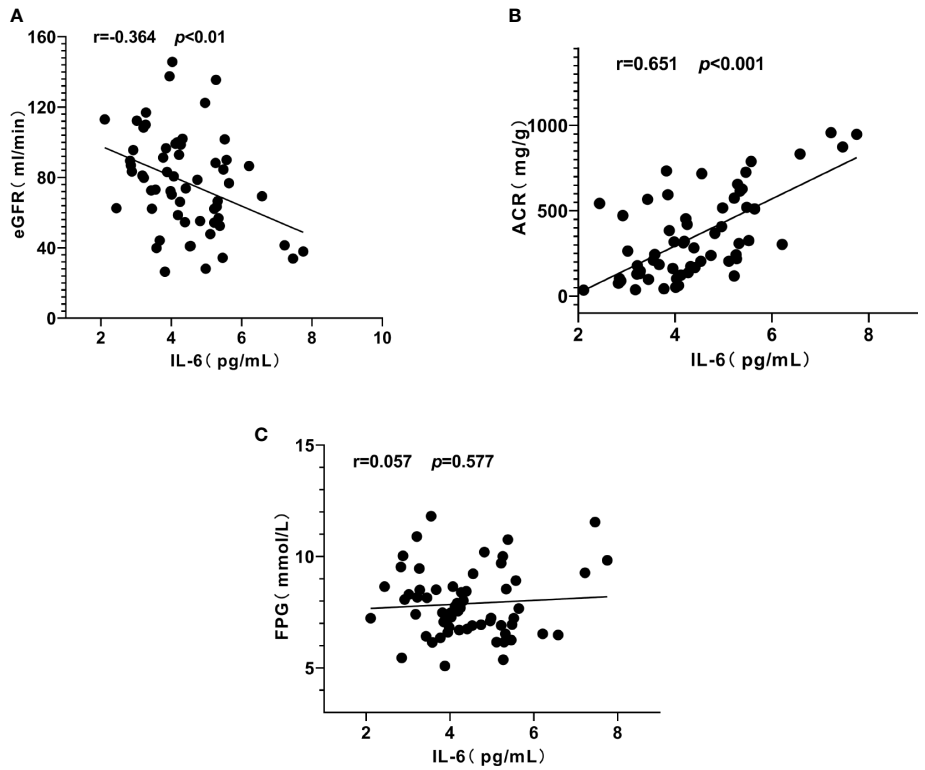


FIGURE 4
Correlation between interleukin-6 (IL-6), urinary albumin/creatinine ratio (ACR) and estimated glomerular filtration rat (eGFR) in patients with diabetic kidney disease (DKD). **(A)** IL-6 was negatively correlated with eGFR. **(B)** IL-6 was positively correlated with ACR. **(C)** IL-6 had no significant correlation with fasting plasma glucose (FPG).

TABLE 2 Logistic regression analysis of clinical biological indicators and the progression of DKD.

	B	SE	Wald	Sig.	Exp(B)	95%Cl	
IL-6	2.230	0.879	6.438	0.011	9.304	1.661	52.109
IGF-1	0.089	0.030	8.609	0.003	1.093	1.030	1.160
FPG	-0.552	0.672	0.676	0.411	0.576	0.154	2.147
TC	0.619	0.772	0.643	0.423	1.858	0.140	4.801
TG	-0.199	0.902	0.049	0.825	0.819	0.140	4.801
HbA1c	0.344	0.505	0.463	0.496	1.410	0.524	3.794

individuals with DR was greatly lower in the diabetes without nephropathy group than in the microalbuminuric and macroalbuminuric groups (Figure 2C). We hypothesize that this may be because serum IGF-1 and IL-6 are involved in the development of retinopathy. Increased IGF-1 levels were reported to hasten the onset of DR (27). IL-6 was found to be positively linked with the development of DR. Inhibiting the activation of the IL-6/STAT3 signaling pathway and altering oxidative stress and inflammatory response allowed IL-6 to play a protective role in the retina of diabetic rats (28). The relationship between serum IGF-1 and IL-6 expression levels and the severity of DR is unclear, and more studies are feasible for us to follow.

In conclusion, serum IGF-1 and IL-6 can be used as biochemical indicators for diagnosing and judging the progression of DKD, which is closely related to the kidney damage of the

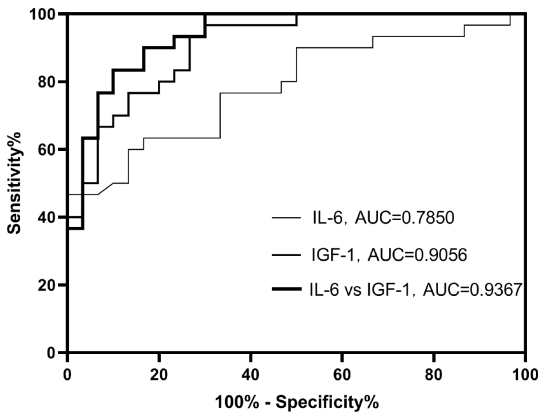


FIGURE 5
ROC curves for the separation and combination of serum IGF-1 and serum IL-6.

TABLE 3 Analysis of ROC curve results.

	AUC	p-Value	95%Cl	Specificity, %	Sensitivity, %
IGF-1	0.9056	<0.0001	(0.833, 0.978)	70.0	96.7
IL-6	0.7850	<0.0001	(0.669, 0.901)	83.0	66.3
combined	0.9367	<0.0001	(0.878, 0.995)	83.3	90.0

disease, and the combination of the two can improve the sensitivity and specificity of the assay, which has guiding significance for the subsequent diagnosis and treatment of DKD. However, there are still some limitations in this study, and the sample size can be expanded in the future to further explore the changes of serum IGF-1 and serum IL-6 levels in DKD of different severity.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Jingzhou Cental Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

The study was designed by J-EZ, and data extraction and analysis were collected by G-WL. The initial manuscript was written by G-WL and finally revised by L-FL and J-EZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1082492/full#supplementary-material

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Development and validation of a nomogram to predict the risk of peripheral artery disease in patients with type 2 diabetes mellitus

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Objective: To develop and validate a nomogram for predicting the risk of peripheral artery disease (PAD) in patients with type 2 diabetes mellitus (T2DM) and assess its clinical application value.

Methods: Clinical data were retrospectively collected from 474 patients with T2DM at the Air Force Medical Center between January 2019 and April 2022. The patients were divided into training and validation sets using the random number table method in a ratio of 7:3. Multivariate logistic regression analysis was performed to identify the independent risk factors for PAD in patients with T2DM. A nomogram prediction model was developed based on the independent risk factors. The predictive efficacy of the prediction model was evaluated using the consistency index (C-index), area under the curve (AUC), receiver operating characteristic (ROC) curve, Hosmer-Lemeshow (HL) test, and calibration curve analysis. Additionally, decision curve analysis (DCA) was performed to evaluate the prediction model's performance during clinical application.

Results: Age, disease duration, blood urea nitrogen (BUN), and hemoglobin ($P<0.05$) were observed as independent risk factors for PAD in patients with T2DM. The C-index and the AUC were 0.765 (95% CI: 0.711-0.819) and 0.716 (95% CI: 0.619-0.813) for the training and validation sets, respectively, indicating that the model had good discriminatory power. The calibration curves showed good agreement between the predicted and actual probabilities for both the training and validation sets. In addition, the *P*-values of the HL test for the training and validation sets were 0.205 and 0.414, respectively, indicating that the model was well-calibrated. Finally, the DCA curve indicated that the model had good clinical utility.

Conclusion: A simple nomogram based on three independent factors–duration of diabetes, BUN, and hemoglobin levels–may help clinicians predict the risk of developing PAD in patients with T2DM.

KEYWORDS

type 2 diabetes mellitus (T2DM), peripheral artery disease (PAD), nomogram, risk factor, predictive model

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia, the prevalence of which is increasing worldwide. In 2019, the ninth edition of the Diabetes Atlas of the International Diabetes Federation (IDF) indicated that there were approximately 463 million patients with diabetes worldwide, which is expected to increase to 700 million by 2045 (1).

Type 2 DM (T2DM) is a non-insulin-dependent DM that accounts for 90–95% of all DM cases. The main feature of T2DM is a progressive decrease in insulin secretion by beta cells (2). Peripheral artery disease (PAD) is a general term for all vascular diseases that result in functional and structural abnormalities of the aorta, its branches, and lower extremity arteries, secondary to atherosclerosis- and thromboembolism-related pathophysiology (3). PAD is characterized by occlusion of the lower-extremity arteries (4), most commonly the femoral, popliteal, tibial, and peroneal arteries, leading to a higher risk of lower-extremity amputation (5).

PAD in patients with T2DM presents a wide range of clinical features and consequences and is known as one of the major macrovascular complications of T2DM (6). According to reports, T2DM significantly increases the incidence of PAD, the risk of ischemic events and amputations (7), and disease progression and severity (8). The resulting severe disability and cardiovascular risk in patients with diabetes lead to a sharp decline in their quality of life and a rapid increase in socioeconomic burden (9).

However, in individuals with diabetes, PAD awareness is still suboptimal; this is partly related to the atypical clinical presentation of PAD in some cases. Therefore, regular and appropriate PAD screening is recommended for patients with T2DM. Currently, PAD diagnosis mainly relies on imaging examinations, such as B-ultrasound and magnetic resonance imaging (MRI), and the related risk factors have received little attention. Hence, further research is required to improve our understanding of the PAD risk factors.

Nomograms are developed using individual risk factors identified by multiple logistic regression analyses. They can graphically represent the numerical relationship between specific diseases and their risk factors and intuitively predict the incidence of adverse events through a scoring system without any complicated calculation formulas (10). In addition, nomograms can provide accurate and personalized risk predictions for each individual, allowing clinicians to effectively screen high-risk patients and provide timely interventions. Therefore, this study aimed to develop a nomogram prediction model for the risk of peripheral vascular disease in patients with T2DM to screen and identify high-risk patients early and provide a reliable reference for early clinical intervention.

Material and methods

Patients selection

Information was collected from patients with T2DM in the Air Force Medical Center's hospital database from January 2019 to April 2022 (Figure 1). The diagnostic criteria for T2DM were based on the diagnostic criteria and classification of the American Diabetes Association (ADA) (11). PAD diagnosis was determined according to the current guidelines in conjunction with ancillary tests. The tests included ankle-brachial index (ABI), Doppler ultrasound of extremity vessels, computed tomography angiography, vascular MRI, or arteriography. The inclusion criteria were as follows (1): patients with confirmed T2DM and (2) patients aged >18 years old. The exclusion criteria were as follows: patients with (1) type 1 diabetes (T1DM) or secondary diabetes; (2) diabetes during pregnancy and lactation; (3) severely impaired consciousness or poor general condition; (4) combined malignancy and cardiac, hepatic, and renal failure; and (5) incomplete clinical follow-up information.

Observation variables

Clinical variables observed included: age, sex, height, body weight, body mass index (BMI), diastolic blood pressure (DBP), systolic blood pressure (SBP), duration of T2DM, history of smoking, history of drinking, coronary heart disease (CHD), high blood pressure (HBP), hyperlipidemia, glycated hemoglobin (HbA1c), fasting blood glucose (FBG), blood urea nitrogen (BUN), serum creatinine (Scr), blood uric acid (BUA), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), white blood cell count (WBC), hemoglobin (Hb), and red blood cell-specific volume (HCT). Data were collected from blood samples taken after 8 h of fasting on the day following the patient's admission to the hospital. Furthermore, the authors checked the above data individually to determine the completeness of the data.

Building and validating the nomogram

Data from hospitalized patients with T2DM were randomized into training and validation sets at a 7:3 ratio. A nomogram was created using independent risk factors to predict the probability of PAD in patients with T2DM. The consistency index (C-index), area under the curve (AUC), receiver operating characteristic (ROC) curve, and calibration curve were used to evaluate the predictive reliability and predictive ability of the

nomogram, respectively. The Hosmer-Lemeshow (HL) test was used to verify that the model was well-calibrated. The nomogram was validated using the bootstrap method with 1000 resamples and a validation set to further evaluate the model's applicability. In addition, the clinical usability of the nomogram was estimated using decision curve analysis (DCA).

Statistical analysis

Categorical variables were expressed as numbers and percentages (%), and continuous variables were expressed as mean (SD). Categorical variables were analyzed using the Chi-square test, while continuous variables were compared using the student's t-test or Mann-Whitney U test. Multivariate logistic regression was used to identify the independent risk factors for PVD in patients with T2DM. All analyses were performed using R v.4.4.1, and statistical software (http://www.R-project.org, The R Foundation, Vienna, Austria) was used to comprehensively analyze the collected data. A two-tailed *P*-value <0.05 was considered statistically significant.

Results

Basic characteristics of the population

This study ultimately included 474 patients, with the training and validation sets comprising 332 and 142 patients, respectively (Figure 1; Table 1). In the training set, 212 patients developed peripheral vascular lesions with a prevalence of 63.9%. For all variables included in the analysis, there was no statistically significant difference between the training and validation sets (P<0.05), indicating comparability between the two groups.

Risk factors screening

Univariate logistic regression analysis showed that age, BMI, DBP, duration of T2DM, CHD, HBP, BUN, Scr, TC, TG, HDL-C, LDL-C, AST, ALB, and Hb were significantly different risk factors in the training set (P<0.05, Table 2). These risk factors were included in the multivariate logistic regression analysis, which showed that the independent risk factors for a diabetic foot in patients with T2DM were disease duration, BUN, and Hb (P<0.05, Table 2).

Creation and validation of the nomogram

A nomogram prediction model based on the independent risk factors (Figure 2) was developed to predict the risk of concomitant PAD in patients with T2DM. ROC curves of the training and validation sets were drawn. The AUC were 0.765 (95% CI: 0.711–0.819), and 0.716 (95% CI: 0.619–0.813) for the training and validation sets, respectively (Figures 3A, B). The C-index of the nomogram in the training and validation sets were >0.70, indicating that the model had a good discriminative ability.

The calibration curves of the nomogram in the training and validation sets showed a favorable consistency between the predicted and actual probabilities (Figure 4). In addition, the results of the HL test of the nomogram in the training and validation sets were χ^2 = 10.940 (P=0.205) and χ^2 = 8.197 (P=0.414), respectively. The *P*-value of the HL test was

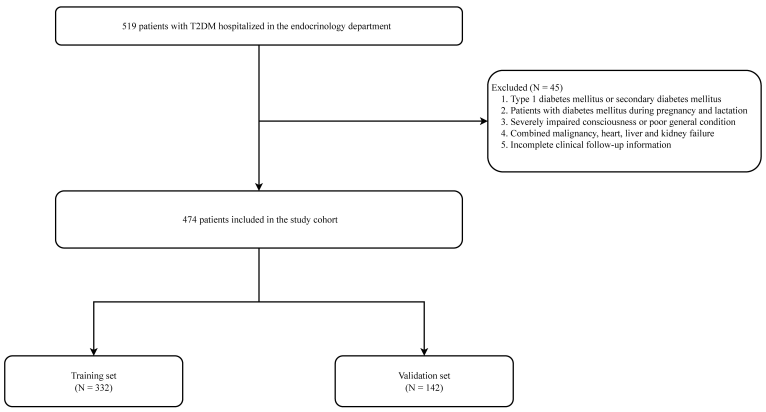


FIGURE 1
Flow chart for peripheral artery disease in patients with type 2 diabetes mellitus patients.

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TABLE 1 Baseline characteristics of patients in the training set and validation set.

Variables	Total (n = 474)	Training set (n = 332)	Validation set (n = 142)	*P* value
Age (years)	64.0 ± 10.6	63.9 ± 10.9	64.4 ± 9.9	0.643
Sex, n (%)				0.469
Male	302 (63.7)	215 (64.8)	87 (61.3)	
Female	172 (36.3)	117 (35.2)	55 (38.7)	
Height (cm)	168.2 ± 8.1	168.2 ± 8.2	168.1 ± 7.6	0.859
Body weight (kg)	70.1 ± 11.9	70.6 ± 12.3	68.9 ± 11.0	0.174
BMI, n (%)	24.7 ± 3.7	24.9 ± 4.0	24.3 ± 2.9	0.111
DBP (mmol/L)	78.0 ± 11.4	78.4 ± 10.6	77.2 ± 13.1	0.313
SBP (mmol/L)	133.0 ± 17.8	133.6 ± 17.5	131.6 ± 18.5	0.264
Duration of T2MD (months)	15.9 ± 9.2	15.6 ± 9.4	16.7 ± 8.6	0.225
Smoking, n (%)				0.583
No	286 (60.3)	203 (61.1)	83 (58.5)	
Yes	188 (39.7)	129 (38.9)	59 (41.5)	
Drinking, n (%)				0.452
No	309 (65.2)	220 (66.3)	89 (62.7)	
Yes	165 (34.8)	112 (33.7)	53 (37.3)	
CHD, n (%)				0.557
No	336 (70.9)	238 (71.7)	98 (69)	
Yes	138 (29.1)	94 (28.3)	44 (31)	
HBP, n (%)				0.127
No	140 (29.5)	105 (31.6)	35 (24.6)	
Yes	334 (70.5)	227 (68.4)	107 (75.4)	
Hyperlipidemia, n (%)				0.733
No	236 (49.8)	167 (50.3)	69 (48.6)	
Yes	238 (50.2)	165 (49.7)	73 (51.4)	
HbA1c (%)	8.8 ± 2.0	8.8 ± 2.0	8.7 ± 1.8	0.789
FBG (mmol/L)	8.6 ± 3.2	8.6 ± 3.3	8.7 ± 2.8	0.763
BUN (mmol/L)	7.0 ± 2.9	7.1 ± 3.0	6.8 ± 2.6	0.309
Scr (μmol/L)	84.4 ± 78.2	85.4 ± 83.1	82.3 ± 65.3	0.693
BUA (μmol/L)	321.9 ± 89.8	320.1 ± 89.3	326.0 ± 90.9	0.512
TC (mmol/L)	4.0 ± 1.1	4.0 ± 1.0	3.9 ± 1.1	0.403
TG (mmol/L)	1.7 ± 1.0	1.7 ± 1.0	1.6 ± 0.9	0.647
HDL-C (mmol/L)	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	0.932
LDL-C (mmol/L)	2.2 ± 0.8	2.3 ± 0.8	2.2 ± 0.8	0.608
ALT (U/L)	18.5 ± 18.7	17.8 ± 11.6	20.2 ± 29.2	0.184
AST (U/L)	18.1 ± 8.2	18.3 ± 7.8	17.9 ± 9.0	0.626
ALB (g/L)	40.0 ± 5.1	39.8 ± 5.1	40.3 ± 5.0	0.426
WBC (×10^9/L)	6.8 ± 2.1	6.7 ± 2.0	6.9 ± 2.2	0.350
Hb	122.0 ± 22.0	122.4 ± 21.3	121.0 ± 23.6	0.519
HCT (%)	0.4 (0.3, 0.4)	0.4 (0.3, 0.4)	0.4 (0.3, 0.4)	0.940

BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; CHD, coronary heart disease; HBP, high blood pressure; HbA1c, glycated hemoglobin; FBG, fasting blood glucose; BUN, blood urea nitrogen; Scr, serum creatinine; BUA, blood uric acid; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; WBC, white blood cell count; Hb, hemoglobin; HCT, red blood cell specific volume.

insignificant for the model in both the training and validation sets; this indicated that the nomogram fit well.

The DCA results for the training and validation sets are shown in Figure 5. The net benefit for patients was higher than those of the other two curves when the threshold probabilities were 40– 80% and 50– 80%, respectively. The horizontal line indicates that none of the patients developed peripheral vascular lesions, all were untreated, and no intervention benefit was generated. The diagonal line indicates that all patients developed peripheral vascular lesions and the benefit after treatment. Within these ranges, the nomogram prediction model had good clinical utility.

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TABLE 2 Univariate and multivariate logistic regression analyses for patients with T2DM.

Variable	OR 95%CI	*P* value	OR 95%CI	*P* value
Age (years)	1.06 (1.04~1.08)	<0.001	1.01 (0.99~1.04)	0.396
Sex, n (%)				
Male				
Female	0.76 (0.51~1.12)	0.161		
Height (cm)	1.01 (0.99~1.04)	0.268		
Body weight (kg)	0.99 (0.98~1.01)	0.297		
BMI, n (%)	0.94 (0.89~0.99)	0.028	0.97 (0.91~1.04)	0.437
DBP (mmol/L)	1.01 (1~1.02)	0.143		
SBP (mmol/L)	0.97 (0.95~0.98)	<0.001	0.99 (0.97~1.02)	0.600
Duration of T2MD (months)	1.08 (1.06~1.11)	<0.001	1.05 (1.02~1.07)	0.002
Smoking, n (%)				
No				
Yes	1.1 (0.75~1.63)	0.625		
Drinking, n (%)				
No				
Yes	1.17 (0.78~1.75)	0.451		
CHD, n (%)				
No				
Yes	2.73 (1.7~4.39)	<0.001	1.56 (0.91~2.68)	0.106
HBP, n (%)				
No				
Yes	1.75 (1.17~2.64)	0.007	1.15 (0.7~1.89)	0.586
Hyperlipidemia, n (%)				
No				
Yes	0.69 (0.47~1.02)	0.061		
HbA1c (%)	0.98 (0.89~1.08)	0.690		
FBG (mmol/L)	1.05 (0.98~1.11)	0.159		
BUN (mmol/L)	1.25 (1.13~1.37)	<0.001	1.16 (1.02~1.31)	0.020
Scr (μmol/L)	1.01 (1~1.02)	0.001	1 (0.99~1.01)	0.718
BUA (μmol/L)	1 (1~1)	0.161		
TC (mmol/L)	0.75 (0.63~0.9)	0.002	1.19 (0.58~2.47)	0.634
TG (mmol/L)	0.82 (0.67~0.99)	0.036	0.79 (0.54~1.18)	0.250
HDL-C (mmol/L)	0.36 (0.18~0.71)	0.003	0.31 (0.1~1)	0.050
LDL-C (mmol/L)	0.72 (0.57~0.92)	0.008	0.81 (0.36~1.81)	0.611
ALT (U/L)	0.99 (0.97~1)	0.057		
AST (U/L)	0.96 (0.94~0.98)	0.001	0.97 (0.95~1)	0.050
ALB (g/L)	0.93 (0.89~0.97)	<0.001	1.02 (0.97~1.08)	0.447
WBC (×10^9/L)	1.06 (0.97~1.16)	0.221		
Hb	0.97 (0.96~0.98)	<0.001	0.98 (0.97~1)	0.019
HCT (%)	1.03 (0.93~1.14)	0.537		

BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; CHD, coronary heart disease; HBP, high blood pressure; HbA1c, glycated hemoglobin; FBG, fasting blood glucose; BUN, blood urea nitrogen; Scr, serum creatinine; BUA, blood uric acid; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; WBC, white blood cell count; Hb, hemoglobin; HCT, red blood cell specific volume.

Clinical application of the nomogram

As an example, the basic information of a male patient with a 9-year history of T2DM had a test measurement of urea nitrogen of 8.9 mmol/L and a Hb value of 139 g/L. Combined with the legend, the total score was approximately 66. The corresponding risk of peripheral vascular disease was approximately 60–61% (Figure 6). Hence, it is recommended that this patient be

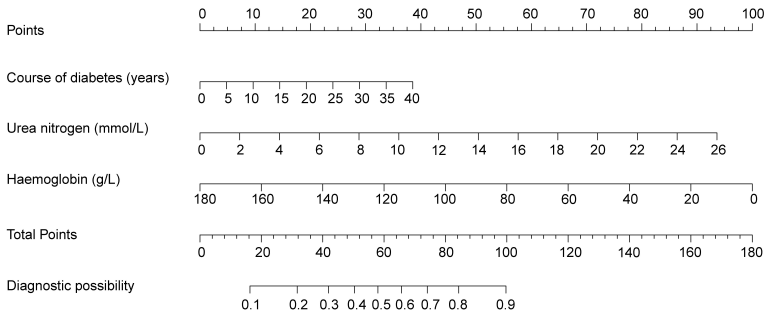


FIGURE 2
Nomogram predicting peripheral artery disease possibility of type 2 diabetes mellitus patients.

examined for peripheral vascularity to detect the onset of lesions and be administered aggressive intervention to prevent PAD.

Discussion

This retrospective study was based on predicting the risk of PAD in patients with T2DM in the Beijing area. The duration of T2DM and Hb and BUN levels were independent risk factors for PAD. The duration of T2DM and BUN were positively correlated with PAD, and Hb was negatively correlated with PAD. The original data were randomly divided into development (n=332) and validation (n=142) groups. The verification results show that both have good risk-prediction abilities. The calibration plot showed that the nomogram was accurate in predicting T2DM risk, and the DCA also demonstrated the clinical utility of the nomogram.

As a common diabetes-related complication, PAD affects about 10–20% of people with diabetes (12), and in developed countries, about 50%. PAD increases with age in patients with diabetes, and arterial occlusion often occurs in distal limbs that cannot be revascularized (13). It is estimated that PAD is present in up to 50% of diabetic foot ulcers and is an independent risk factor for its development (14, 15). Furthermore, diabetes is associated with a significant risk of diabetes-related foot disease (DFD), with a lifetime incidence of foot ulcers as high as 34%. Moreover, diabetes is the leading cause of amputation (16). When associated with diabetes, PAD has a more severe disease process, a higher likelihood of distal ischemic ulceration and extensive tissue loss, and an increased risk of amputation (17). Early PAD diagnosis and treatment are critical in patients with

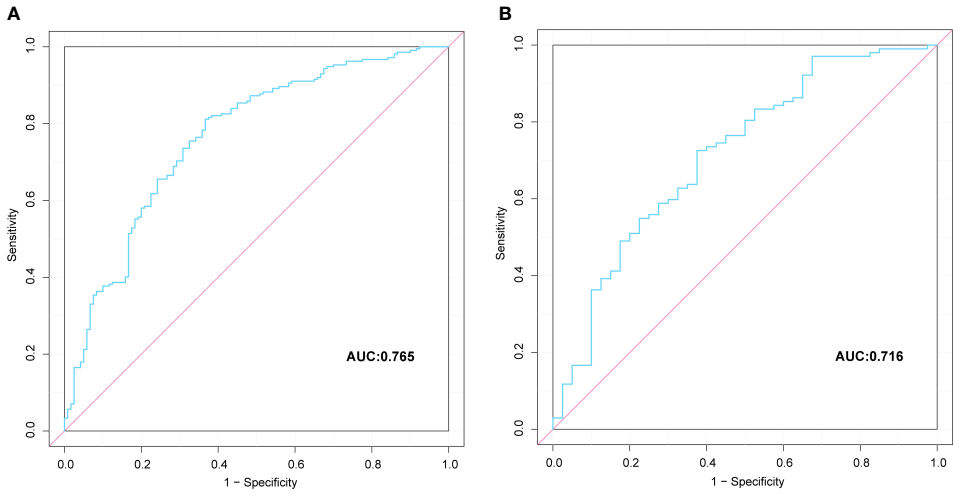


FIGURE 3
Comparison of the ROC curves of the nomogram for peripheral artery disease possibility prediction in the training set **(A)**, and in the verification set **(B)**.

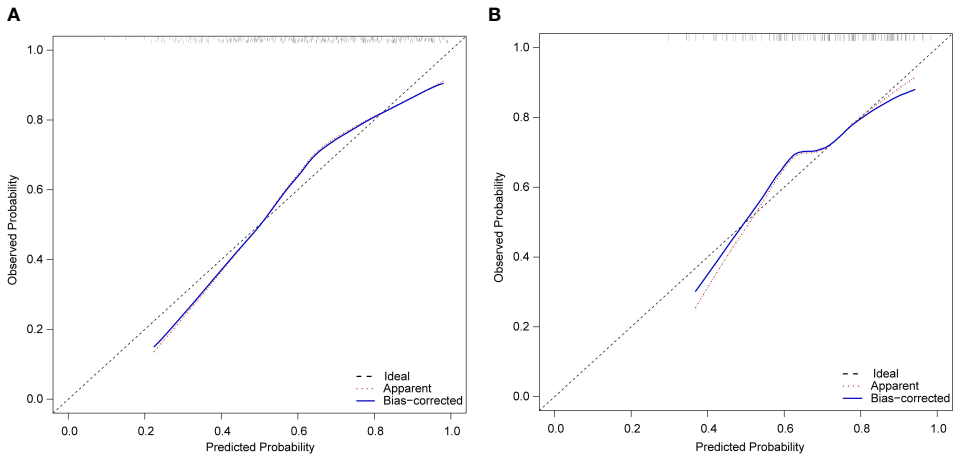


FIGURE 4
Calibration plots of the nomogram for peripheral artery disease prediction of the training set **(A)** and verification set **(B)**.

DM because of the increased risk of nonunion, infection, and amputation of the lower extremities, increased incidence of cardiovascular complications such as myocardial infarction and stroke, and a 5-year mortality rate of >50% (18–20).

In this study, PAD prevalence in patients with diabetes was 63.9%, higher than that in past reports and >10% higher than that in developed countries. We speculate that the high prevalence of PAD in our study population may be due to several factors. First, although our research unit was a specialized hospital, primary medical institutions referred most admitted patients, and their condition was relatively serious. Moreover, the hospital is well-equipped for examinations and can perform more comprehensive examinations. Second, most of this study's patients were from northern China, which may have been related to their diet and living habits. In addition, PAD prevalence varies with the diagnostic method used (the presence of intermittent claudication, palpation of vessels, or ABI) (21), which is a factor that cannot be ignored. However, foot examination has a limited role in diagnosing PAD. Moreover, many patients with diabetes and PAD have sensitive neuropathy and markedly reduced or completely blocked perception of ischemic pain (22). Hence, many potential patients with PAD have not received attention. Globally, total healthcare expenditures associated with PAD and DFD are very expensive, placing enormous pressure on patient families and national healthcare systems. Therefore,

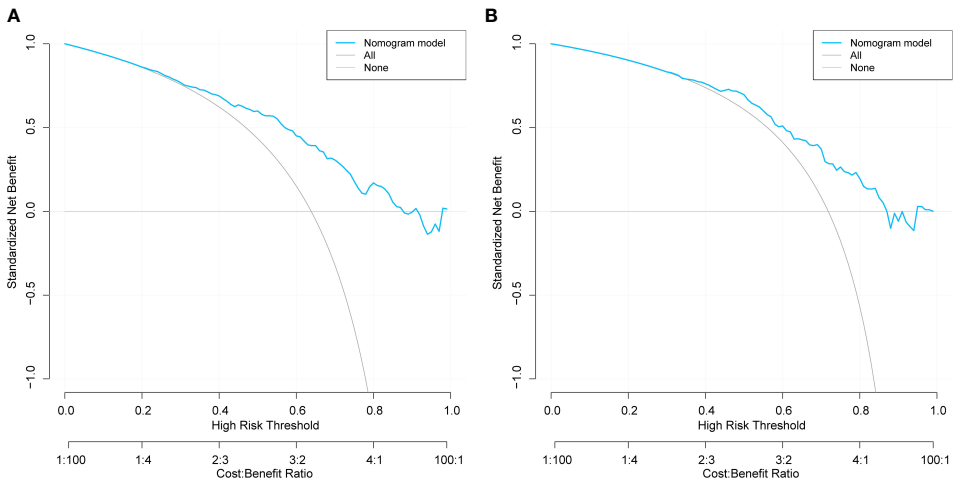


FIGURE 5
Decision curve analysis of training set **(A)** and validation set **(B)** for the risk of peripheral artery disease in patients with type 2 diabetes mellitus..

given the adverse consequences of PAD in T2DM patients, it is important to predict its risk. This study aimed to construct a risk-scoring model that could be used in clinical settings to predict the risk of PAD in patients with T2DM. Furthermore, the developed nomogram model can help clinicians identify the risk factors for PAD early, develop appropriate treatment plans, and take targeted measures to prevent morbidity and mortality.

DM is associated with many complications, of which chronic vascular complications have the most complex and important consequences. Furthermore, about 50–80% of people with diabetes die from cardiovascular disease (including coronary artery disease, stroke, peripheral vascular disease, and other vascular diseases). This has a major impact on health care, making cardiovascular diseases a leading cause of morbidity and mortality in people with diabetes (23). Previous studies have also confirmed that age, diabetes duration, and peripheral neuropathy are associated with an increased risk of PAD in patients with pre-existing DM (24–26). The present study showed that the duration of diabetes could be used to predict the risk of PAD, which is consistent with the studies mentioned above.

T2DM causes various macrovascular complications, including hyperglycemia and insulin resistance, *via* different pathogenic pathways (27). Hyperglycemia may regulate vascular inflammation, cytokines, macrophage activation, and growth factor gene expression. Furthermore, it may interfere with normal angiogenesis, collateral artery formation, and muscle repair and affect local regenerative function (28, 29). Insulin resistance causes persistent hyperglycemia and accelerates disease progression. With the prolongation of the course of diabetes, a series of chronic complications occur, such as diabetic peripheral neuropathy (DPN), nephropathy, and retinopathy. DPN and PAD are closely related among these complications, and DPN may usually precede PAD. However, arterial stenosis aggravates DPN (30). Thus, the relationship appears bidirectional. The strong correlation between them suggests that more attention should be paid to patients with PAD in clinical settings, especially those with a long history of diabetes. Furthermore, careful inquiries should be made about their neurological symptoms, and detailed examinations should be conducted to achieve early detection and treatment.

BUN is the main end-product of human protein metabolism and is one of the main indicators of renal function. Studies on BUN in patients with coronary heart disease have shown a strong association between long-term mortality and acute ST-elevation myocardial infarction (STEMI). Researchers believe that BUN is an alternative marker for the kidneys' response to systemic hemodynamic changes associated with the pathophysiological mechanisms of heart failure (31). Extensive research in this field has established BUN as an independent predictor of mortality in patients with heart failure (32). An elevated BUN level is thought to be a predictor of short-term (within 1 year) and long-term (5.3 years) survival in these patients, even better than creatinine and other parameters that reflect renal insufficiency (33, 34). A cross-sectional study showed a significant association between BUN and critical limb ischemia (CLI) in patients with peripheral arterial occlusive disease (PAOD). It also showed that BUN could be used to identify patients with atherosclerosis at a high risk of CLI (35). This study further confirmed that the BUN level is an independent risk factor for PAD. However, owing to sclerosis of the arteries, the ABI might be unreliable, especially in older patients and those with diabetes. Therefore, BUN is an easily identifiable, widely available, and inexpensive marker that can identify patients at high risk for vascular endpoints, such as PAD.

Circulating Hb plays a role in various physiological processes and pathophysiological pathways, including oxygen-carrying capacity, inflammatory processes, oxidative stress, and blood viscosity. The Hb level is also an important indicator for diagnosing anemia. The incidence of comorbid anemia was higher in patients with DM and PAD. A previous study noted that people with diabetes and anemia (compared with non-anemic diabetes) had more comorbidities (mostly hypertension

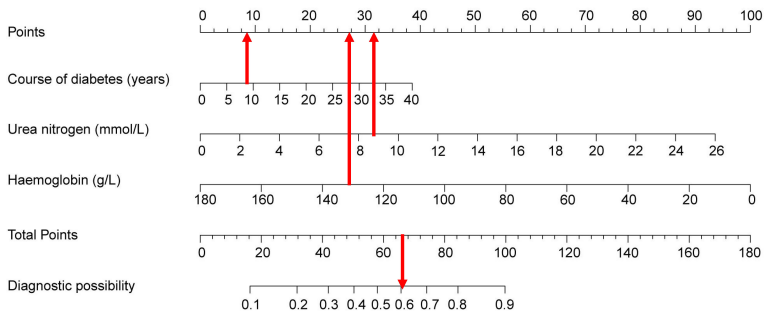


FIGURE 6
Visualization of nomogram to predict the risk of concomitant peripheral artery disease in patients with type 2 diabetes mellitus patients.

and PAD), more frequent hospitalizations, and a higher risk of death (36). Furthermore, decreased Hb levels in patients with diabetes increase the risk of hospitalization and death, and anemia appears to be a risk factor for all-cause mortality (37). In addition, Desormais et al. showed that anemia is a strong and independent predictor of death and limb loss in patients hospitalized with PAD (38). The association was gradual, with an increased risk of amputation as the severity of anemia increased (38). Thus, there appear to be multiple adverse effects of anemia in patients with DM and PAD. The present study demonstrated that Hb is an independent risk factor for patients with T2DM and PAD and suggests that adequate attention should be given.

This study's analysis shows that the nomogram is well-developed and has an accurate value for PAD prediction, as assessed by its discriminative ability. Therefore, the C-index of the training and validation sets were 0.765 and 0.765, and 0.716 and 0.716, respectively. The C-index of the nomogram in both the training and validation sets was >0.70, indicating that the model had good discrimination. A good fit was obtained by generating 1000 bootstrap samples to replace the original samples and repeating the entire modeling process to test and calibrate the calibration curve. Furthermore, the DCA curves of the training and validation sets showed that the nomogram model was more practical and accurate when the risk threshold was between 2.5% and 90% or between 3.5% and 88%. In conclusion, this study's nomogram model shows a more accurate value for PAD prediction in patients with T2DM. Furthermore, its construction greatly improves clinical practicality, thereby significantly improving the precision of treatment selection in clinical practice.

To the best of our knowledge, this is the first study to identify the risk factors for PAD in patients with diabetes using a newly proposed nomogram with excellent diagnostic accuracy. However, this study had some limitations. First, all patient data were obtained from the same hospital. Although the nomogram model achieved good accuracy, there is still room for further prospective multicenter validation to confirm and improve the reliability of the nomogram and further increase its clinical utility. Second, this was a retrospective study. Hence, not all clinicopathological data were included in the study due to limited data availability, and additional potential risk factors need to be included. Third, only internal validation was performed in this nomogram prediction model. Hence, external validation is required to improve the predictive value of the nomogram model in the future.

In conclusion, after reviewing many studies and evaluating multiple variables, this study demonstrated that diabetes duration, BUN, and Hb levels are independent risk factors for PAD in patients with T2DM. In addition, this study established and validated a nomogram for PAD risk in patients with T2DM based on these findings. The model has high accuracy and good discriminative power for PAD prediction, which helps clinicians make targeted and active treatment plans for medical intervention promptly.

Conclusions

In summary, this study found that the independent risk factors for PAD in patients with T2DM were the duration of diabetes and BUN and Hb levels. In addition, this study established a visual nomogram prediction model with good discriminatory power, calibration, and clinical utility to facilitate personalized clinical applications. It also facilitates the early prediction and identification of potentially high-risk patients with PAD in T2DM.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study involving human participants was reviewed and approved by the Medical Ethics Committee of the Air Force Medical Center. Since this is a study strictly based on a clinical case registry, individual informed consent is not required.

Author contributions

JL and SX conceived and designed the study. Data collection and data analysis were performed by JL JS,TS and LZ, JL, JS and SX drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Is the level of serum lactate dehydrogenase a potential biomarker for glucose monitoring with type 2 diabetes mellitus?

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Introduction: Type 2 diabetes mellitus (T2DM) is a metabolic disorder due to defects in insulin secretion or insulin resistance leading to the dysfunction and damage of various organs. To improve the clinical evaluation of short-term blood glycemic variability monitoring, it is critical to identify another blood cell status and nutritional status biomarker that is less susceptible to interference. This study identifies the significance of serum lactate dehydrogenase (LDH) level among T2DM patients treated in outpatient clinics and investigates the relationship of LDH level with other variables.

Methods: This study comprised 72 outpatients with T2DM over 20 years of age. Blood samples were collected followed by a hematological analysis of serum glycated albumin (GA), LDH, fasting blood glucose, glycosylated hemoglobin, C-peptide, and insulin antibodies (insulin Ab).

Results: Serum LDH level was significantly correlated with GA ($p < 0.001$), C-peptide ($p = 0.04$), insulin Ab ($p = 0.03$), and thyroid-stimulating hormone (TSH) levels ($p = 0.04$). Hence, we performed a linear regression analysis of hematological markers. GA ($p < 0.001$, $r^2 = 0.45$) and insulin Ab ($p < 0.001$, $r^2 = 0.40$) were significantly associated with LDH level. Then, we classified patients into low (<200 U/L) and high (≥200 U/L) serum LDH level groups, respectively. GA ($p < 0.001$), C-peptide ($p = 0.001$), and TSH ($p = 0.03$) showed significant differences in patients with high LDH levels compared with those in patients with low LDH levels.

Conclusion: In conclusion, we suggested that LDH level was independent of long-term but associated with short-term blood glucose monitoring. The results indicated that changes in serum GA induced cell damage and the

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abnormal elevation of the serum level of LDH may occur simultaneously with glycemic variability. It has been reported that many biomarkers are being used to observe glucose variability in T2DM. However, LDH could provide a more convenient and faster evaluation of glycemic variability in T2DM.

KEYWORDS

glycemic variability, glucose monitoring, lactate dehydrogenase (LDH), glycated albumin (GA), type 2 diabetes mellitus

1 Introduction

Type 2 diabetes mellitus (T2DM) is a metabolic disorder due to defects in insulin secretion or insulin resistance leading to the dysfunction and damage of various organs. To date, many hematological markers can be used to monitor the disease progression of T2DM, such as serum glycated albumin (GA), glucose AC (fasting blood glucose), glycosylated hemoglobin (HbA1c), connecting peptide (C-peptide), and insulin antibodies (insulin Ab). HbA1c is the most widely used indicator to determine the average glucose plasma concentration over the past 2 to 3 months prior to checkup in poorly controlled diabetes mellitus (DM). However, the HbA1c level in patients is significantly influenced by changes in the lifespan of erythrocytes (1). Clinicians may be falsely comforted by relatively low HbA1c values despite a high risk for other complications.

Serum GA is not affected by changes in the lifespan of erythrocytes in patients with T2DM with hemoglobinopathy. Serum GA measures the glycation of serum albumin, which is a protein with a half-life of approximately 14 days, and is an intermediate measure between HbA1c and serum blood glucose (2). GA level has been regarded as a specific inflammatory mediator related to the expression of inflammatory cytokines in muscle cells (3) and bovine retinal capillary pericytes (4), airway epithelial cells (5), and pancreatic β cells (6). Pu et al. observed that serum GA and C-reactive protein (CRP) levels were significantly elevated and correlated in patients with T2DM and cardiac disease (7). Although GA level is not influenced by disorders of hemoglobin metabolism, it is affected by disorders of albumin metabolism and nutritional status.

Clinically, it is important to monitor the patient's glycemic variability in T2DM. However, a single-spot blood glucose level is easily affected by food intake. GA is not routinely tested because it will be affected by albumin, and HbA1c is also easy to be influenced due to anemia or chronic disease history. Hence, to improve the clinical evaluation of short-term blood glycemic variability, it is critical to identify another biomarker that is less susceptible to interference.

Lactate dehydrogenase (LDH) is a critical marker in the metabolic pathway and also an intermediate metabolite that affects metabolism. It is often used to diagnose myocardial infarction, vessel damage, tissue injury, and certain types of malignant tumors (8). LDH may play a role in catalyzing pyruvate conversion into lactic acid through glycolysis. In DM, insulin is released according to glucose level, and it controls the metabolism of glucose through glycolysis followed by the oxidation of pyruvate in the mitochondria (9). Dmour et al. also observed that serum LDH level increased in association with serum glucose level (10). Although serum LDH level is reportedly elevated in patients with DM, the physiological effects remain unclear. This study identifies the significance of serum LDH level among T2DM patients treated in outpatient clinics and investigates the relationships of LDH level with other variables. The findings will aid in the formulation of treatment and preventive strategies for diabetes.

2 Materials and methods

2.1 Patients

All participants were Taiwanese and were followed at the Taipei Medical University Hospital. The study protocol was approved by the Ethics Committee of the Institutional Review Board of Taipei Medical University. The procedures accorded with the ethical standards of the responsible committee on human experimentation (institutional and national) and the Helsinki Declaration. All participants provided informed consent to participate in the study. The study group comprised 72 participants (Figure 1) with T2DM (ICD-10-CM E11.9) over 20 years of age. Women who were pregnant, those under 20 years, and patients with neoplasms (ICD-10-CM C00-D49), cardiac disease (ICD-10-CM I00-I99), and chronic

Abbreviations: LDH, lactate dehydrogenase; GA, glycated albumin; glucose AC, fasting blood glucose, HbA1c, glycosylated hemoglobin; C-peptide, connecting peptide; HOMA-IR, homeostasis model assessment of insulin resistance index; insulin Ab, insulin antibodies; T3, triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone.

hepatitis or liver disease (ICD-10-CM K70-K77) were excluded from the study. The other exclusion criteria were diabetic complications (diabetic ketoacidosis or hyperglycemic hyperosmolar state), creatinine clearance <30 ml/min, age under 20 years, and pregnancy. For further analysis, patients were divided into low LDH level (<200 U/L, n = 44) and high LDH level (≥200 U/L, n = 28) groups and were compared using an independent t-test.

2.2 Hematological analysis

Blood samples were collected from the participants after an 8-h fast. Hematological analysis of the level of serum glycated albumin (GA), lactate dehydrogenase (LDH), glucose AC (fasting blood glucose), glycosylated hemoglobin (HbA1c), connecting peptide (C-peptide), insulin antibodies (insulin Ab), triiodothyronine (T3), free thyroxine (FT4), and thyroid-stimulating hormone (TSH) was conducted. The hemoglobin level was determined using an automatic analyzer (XN-9000, Sysmex, Japan). The serum GA and LDH levels were analyzed by enzymatic methods by an automatic analyzer (ADVIA Chemistry XPT, Siemens, Germany), and shaking should be avoided while drawing blood. Glucose AC level was analyzed using the hexokinase method, and HbA1c level was determined through high-performance liquid chromatography using automatic analyzers (ADVIA Chemistry XPT and Variant II Turbo 2.0 System, Bio-Rad, USA). C-peptide level was detected by the method of chemiluminescence enzyme immunoassay using an automatic analyzer (ADVIA Chemistry XPT, Siemens, Germany). Serum insulin Ab level was measured by immunoradiometric binding assay in an automatic analyzer (PerkinElmer Cisbio, USA). The thyroid function profile (including T3, FT4, and TSH) was measured by the radioimmunoassay technique using an automatic analyzer (Atellica IM 1300 analyzer, Siemens, Germany).

2.3 Homeostasis model assessment of insulin resistance index

The modified homeostasis model assessment of insulin resistance (HOMA-IR) index is a simple and effective method for evaluating insulin sensitivity. The modified HOMA-IR was calculated using the following equation (11): 1.5 + (fasting blood glucose) × (fasting C-peptide)/2,800.

2.4 Statistical analysis

Results are presented as the value of mean and median. The independent t-test was used for continuous variables and the chi-square test for categorical variables. Pearson correlational analysis was applied to assess the correlations of serum LDH levels with other diabetes-related hematological markers. In addition, linear regression analysis was also performed to demonstrate the predictor of serum LDH levels. Statistical significance was indicated at p <0.05. Statistical analysis was conducted using IBM SPSS Statistics 22 (IBM Corporation, Somers, NY, USA) and MedCalc 20.113 (Med Calc Software Ltd., Ostend, Belgium).

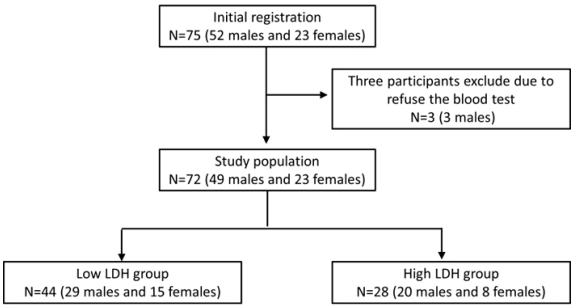


FIGURE 1
Flowchart showing the inclusion of the study population.

3 Results

3.1 Participants and characteristics

As shown in Table 1, the demographic or metabolic characteristics did not differ between the two groups, including the number of patients using insulin and those taking oral medications. The duration of T2DM was 65.7 and 68.0 months, which also did not differ between the two groups. The use of antihyperglycemic agents and insulin in the two groups at baseline was similar.

3.2 The correlation of serum LDH levels with other diabetes-related hematological markers

Table 2 presents the statistically significant correlations of serum LDH level with hematological markers. Serum LDH level was significantly correlated with GA (p < 0.001), C-peptide (p = 0.04), insulin Ab (p = 0.03), and TSH (p = 0.04) levels. However, serum LDH level was not significantly correlated with glucose AC (p = 0.10), HbA1c (p = 0.64), and HOMA-IR (p = 0.45) levels.

3.3 Regression analysis of the revealed markers and serum LDH association

To exclude the effects of liver disease, serum glutamate-pyruvate transaminase (GPT) level was also analyzed and found to be normal in all patients (mean: 26.5 U/L, data not shown). Next, we evaluated factors suspected to affect LDH levels. We performed a linear regression analysis of markers that were significantly correlated with serum LDH level. GA ($p < 0.001$, $r^2 = 0.45$, Figure 2A) and insulin Ab ($p < 0.001$, $r^2 = 0.40$, Figure 2B) were significantly associated with LDH level. Although C-peptide ($p = 0.039$, $r^2 = 0.06$, Figure 2C) and TSH ($p = 0.045$, $r^2 = 0.25$, Figure 2D) showed significant difference, their predictive ability was relatively insufficient.

3.4 The association of related markers between low levels and high levels of serum LDH

We divided participants into low and high serum LDH level groups, where low and high LDH levels were defined as normal (<200 U/L) and abnormal (≥200 U/L), respectively. The levels of GA ($p < 0.001$), C-peptide ($p = 0.04$), and TSH ($p = 0.03$) showed significant difference in patients with high LDH levels compared with those in patients with low LDH levels. Other markers were not significantly correlated with LDH level between the two groups (Table 3).

3.5 Regression analysis of related markers in different serum levels of the LDH groups

To further check the correlation between different serum levels of the LDH groups, we further performed a linear regression analysis of markers that were significantly correlated with serum LDH level. In the high LDH group, GA ($p < 0.001$, $r^2 = 0.45$, Figure 3A) and insulin Ab ($p < 0.001$, $r^2 = 0.71$, Figure 3B) were significantly associated with LDH level. Although C-peptide ($p = 0.037$, $r^2 = 0.018$, Figure 3C) showed significant difference, its predictive ability was relatively insufficient. TSH ($p = 0.210$, $r^2 = 0.18$, Figure 3D) showed no significant difference. In the low LDH group, all of the biomarkers showed no significant difference in GA ($p = 0.901$, $r^2 = 0.03$, Figure 4A), insulin Ab ($p = 0.650$, $r^2 = 0.02$, Figure 4B), C-peptide ($p = 0.499$, $r^2 = 0.01$, Figure 4C), and TSH ($p = 0.590$, $r^2 = 0.02$, Figure 4D), and their predictive ability was relatively insufficient. These results might indicate that the correlation between LDH and GA as well as insulin Ab only exists when LDH is abnormal.

TABLE 1 Clinical characteristics of the patients with T2DM. Data points are expressed in terms of the mean.

	Total	Low LDH	High LDH	*p*
BMI [Mean(Median)]	30 (27.9)	30.1 (27.6)	29.5 (27.7)	0.79
Gender [Male N (%)]	49 (68)	29 (65)	20 (71)	0.08
Age [Mean(Median)]	55.4 (15)	58.7 (58)	48.8 (48)	0.09
Duration [Mean Month (Median)]	65.9 (30.5)	65.7 (74.6)	68.0 (72.9)	0.76
Antihypertensive agents [N(%)]				
Valsartan	3 (4.2)	1 (1.4)	2 (2.8)	0.70
beta-blocker	2 (2.8)	1 (1.4)	1 (1.4)	0.60
Amlodipine	3 (4.2)	2 (2.8)	1 (1.4)	0.54
Antiplatelet agents [N(%)]				
Aspirin	12 (16.6)	6 (8.3)	6 (8.3)	0.22
Antihyperglycemic agents [N(%)]				
Metformin	69 (95.8)	32(44.4)	37(51.4)	0.11
Glimepride	11 (15.3)	6 (8.3)	5 (6.9)	0.42
Linagliptin	18 (25)	9 (12.5)	9 (12.5)	0.54
Dapagliflozin	11 (15.3)	6 (8.3)	5 (6.9)	0.54
Saxagliptin and dapagliflozin	5 (6.9)	3 (4.2)	2 (2.8)	0.38
Empagliflozin and Linagliptin	3 (4.2)	2 (2.8)	1 (1.4)	0.12
Dulaglutide	4 (5.6)	2 (2.8)	2 (2.8)	0.60
Insulin [N(%)]				
Short-acting insulin	4 (5.6)	2 (2.8)	2 (2.8)	0.70
Insulin degludec	8 (13.2)	4 (5.6)	4 (5.6)	0.21
Insulin glargine	4 (5.6)	2 (2.8)	2 (2.8)	0.24

TABLE 2 The correlation of serum LDH levels with other diabetes-related hematological markers.

		LDH	Glucose AC	GA	HbA1c	C-peptide	HOMA-IR	Insulin Ab	T3	FT4	TSH
LDH	Correlation	–	0.12	0.63**	0.06	−0.26	−0.10	0.27*	0.18	−0.03	−0.25
	p	–	0.31	>0.001***	0.64	0.04*	0.45	0.03*	0.14	0.98	0.04*
Glucose AC	Correlation	0.12	–	0.52**	0.48**	0.07	0.63**	−0.05	−0.01	0.07	−0.04
	p	0.31	–	>0.001***	>0.001***	0.59	>0.001***	0.69	0.93	0.54	0.72
GA	Correlation	0.63**	0.52**	–	0.44**	−0.35	0.08	0.38**	−0.03	−0.08	0.05
	p	>0.001***	>0.001***	–	>0.001***	0.01*	0.57	0.01*	0.82	0.54	0.71
HbA1c	Correlation	0.06	0.48**	0.44**	–	−0.16	0.10	0.02	−0.116	−0.08	0.14
	p	0.64	>0.001***	>0.001***	–	0.24	0.44	0.86	0.36	0.51	0.27
C-peptide	Correlation	−0.26	0.07	−0.35	−0.16	–	0.77**		−0.021	−0.12	−0.11
	p	0.04*	0.59	0.01*	0.24	–	>0.001***		0.87	0.35	0.37
HOMA-IR	Correlation	-0.10	0.63**	0.08	0.10	0.77**	–	−0.01	0.03	0.12	−0.14
	p	0.45	>0.001***	0.57	0.44	>0.001***	–	0.94	0.82	0.34	0.27
Insulin Ab	Correlation	0.27*	−0.05	0.38**	0.02	0.01	−0.01	–	−0.17	−0.13	0.08
	p	0.03*	0.69	0.01*	0.86	0.97	0.94	–	0.18	0.32	0.52
T3	Correlation	0.18	−0.010	−0.03	−0.12	−0.02	0.03	−0.18	–	−0.011	−0.04
	p	0.14	0.93	0.82	0.36	0.87	0.82	0.18	–	0.93	0.77
T4	Correlation	−0.03	0.07	−0.08	−0.08	−0.12	0.12	−0.131	−0.01	–	−0.11
	p	0.98	0.54	0.54	0.51	0.35	0.34	0.32	0.93	–	0.39
TSH	Correlation	0.25*	−0.04	0.05	0.14	−0.11	−0.14	0.08	−0.04	−0.11	–
	p	0.040*	0.72	0.71	0.27	0.37	0.27	0.52	0.77	0.39	–

LDH, lactate dehydrogenase; GA, glycated albumin; glucose AC, fasting blood glucose, HbA1c, glycosylated hemoglobin; C-peptide, connecting peptide; HOMA-IR, homeostasis model assessment of insulin resistance index; insulin Ab, insulin antibodies; T3, triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone.
*p < 0.05; **p < 0.01; ***p < 0.001.

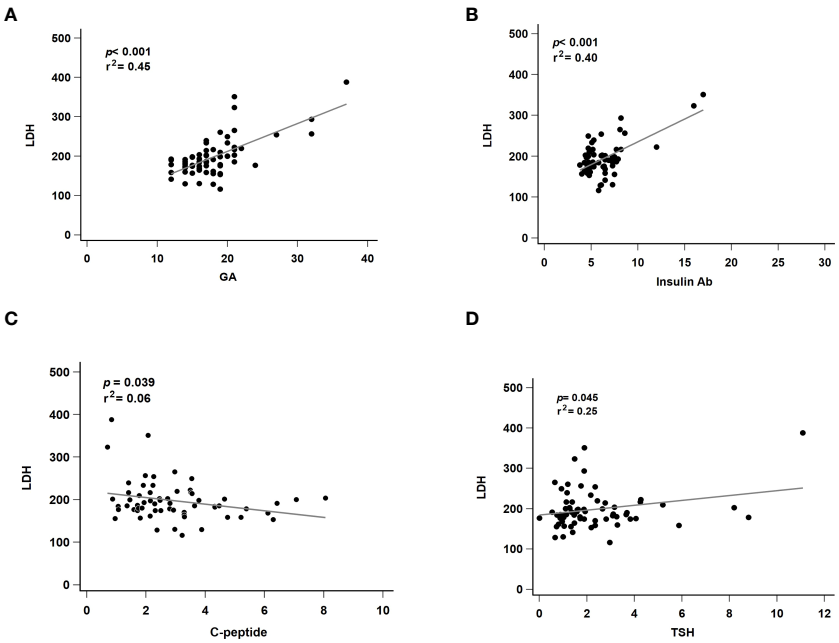


FIGURE 2
Regression analysis of revealed markers and serum LDH association. LDH, lactate dehydrogenase; GA, glycated albumin; C-peptide, connecting peptide; insulin Ab, insulin antibodies; TSH, thyroid-stimulating hormone. **(A)** The correlated with serum LDH and GA level. **(B)** The correlated with serum LDH and insulin Ab level. **(C)** The correlated with serum LDH and C-peptide level. **(D)** The correlated with serum LDH and TSH level. *p*, p-value; r^2: r-squared.

TABLE 3 The association of related markers between low (<200 U/L, *n* = 44) and high (≥200 U/L, *n* = 28) levels of serum LDH.

	Reference range	Total	Low LDH	High LDH	*p*
LDH	98–200 U/L	197.5 (186.0)	171.9 (176.0)	242.6 (222.0)	>0.001***
Glucose AC	<100 mg/dl	161.6 (144.5)	165.0 (147.0)	156.9 (134.0)	0.69
GA	11%–16%	19.8 (18.0)	17.9 (17.5)	21.9 (19.0)	0.001**
HbA1c	<5.5%	7.3 (6.8)	7.3 (6.9)	7.4 (6.9)	0.64
C-peptide	0.8–3.8 ng/ml	3.1 (2.6)	3.0 (2.8)	2.8 (2.3)	0.04*
HOMA-IR	<1.9	1.7 (1.6)	1.7 (1.6)	1.7 (1.6)	0.98
Insulin Ab	<7.5%	7.3 (6.0)	6.5 (5.8)	8.7 (6.1)	0.15
T3	60–181 ng/dl	95.2 (94.5)	94.1 (92.0)	97.0 (100.5)	0.05
FT4	0.74–1.47 ng/dl	1.2 (1.2)	1.2 (1.2)	1.4 (1.3)	0.80
TSH	0.5–4.7 μIU/ml	2.5 (1.6)	2.5 (1.9)	2.2 (1.4)	0.03*

LDH, lactate dehydrogenase; GA, glycated albumin; glucose AC, fasting blood glucose; HbA1c, glycosylated hemoglobin; C-peptide, connecting peptide; HOMA-IR, homeostasis model assessment of insulin resistance index; insulin Ab, insulin antibodies; T3, triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone.
*p < 0.05; **p < 0.01; ***p < 0.001.

4 Discussion

Serum LDH has been demonstrated to indicate many diseases or disorders. According to the results of this study, serum LDH level might also be an indicator of uncontrollable short-term glucose monitoring and unstable glycemic variability (Figure 5). Here, we demonstrated that a diabetic hematological marker, specifically abnormal elevated LDH, is associated with serum GA and insulin Ab levels.

Elevated LDH levels are observed in conditions such as tissue injury, necrosis, hypoxia, hemolysis, and severe inflammation (12). The overexpression of LDH activity has been known to interfere with normal glucose metabolism and insulin secretion due to increases in mitochondrial membrane

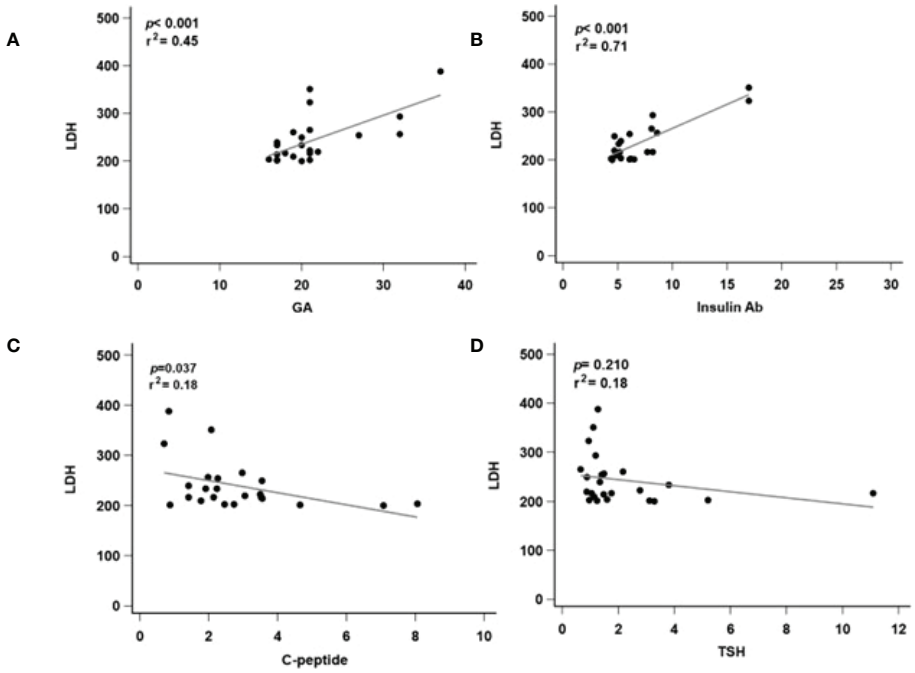


FIGURE 3
Regression analysis of related markers in high serum level of LDH groups. LDH, lactate dehydrogenase; GA, glycated albumin; C-peptide, connecting peptide; insulin Ab, insulin antibodies; TSH, thyroid-stimulating hormone. **(A)** The correlated with serum LDH and GA level. **(B)** The correlated with serum LDH and insulin Ab level. **(C)** The correlated with serum LDH and C-peptide level. **(D)** The correlated with serum LDH and TSH level. *p*, p-value; r^2: r-squared.

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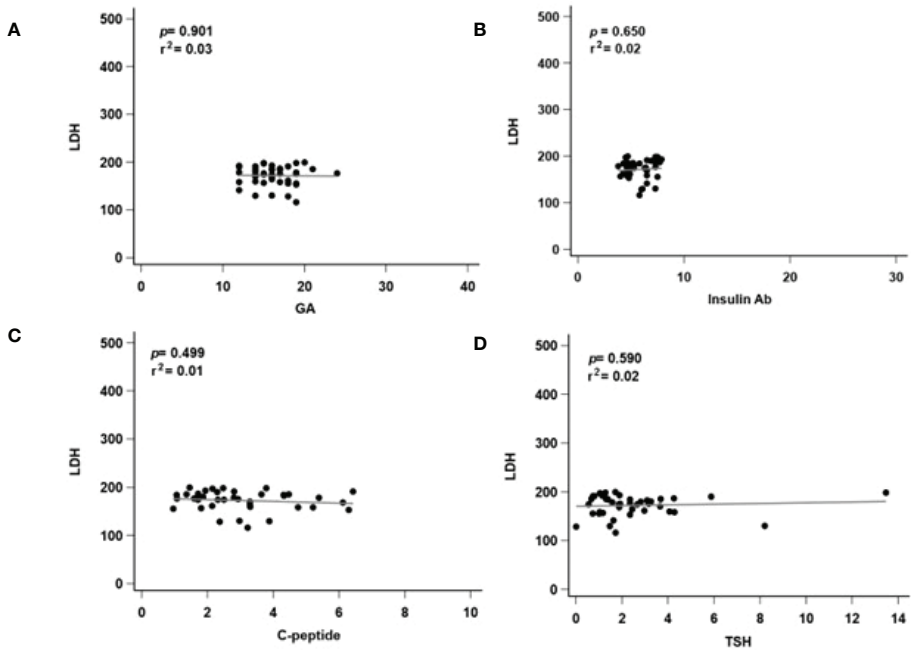


FIGURE 4
Regression analysis of related markers in low serum level of LDH groups. LDH, lactate dehydrogenase; GA, glycated albumin; C-peptide, connecting peptide; insulin Ab, insulin antibodies; TSH, thyroid-stimulating hormone. **(A)** The correlated with serum LDH and GA level. **(B)** The correlated with serum LDH and insulin Ab level. **(C)** The correlated with serum LDH and C-peptide level. **(D)** The correlated with serum LDH and TSH level. *p*, p-value; *r2*: r-squared.

potential, cytosolic free ATP, and cytosolic free Ca^{2+} in the islet beta cells (13). Oxidative modifications of albumin have been observed when the protein is glycated by pathophysiological concentrations of glucose in adipocytes. The glycation of albumin can be sufficient to oxidatively jeopardize adipocyte physiology and increase LDH activity in patients with diabetes (13). In a previous *in-vitro* study, LDH levels were demonstrated to increase in high-glucose treatments of INS-1 rat pancreatic beta cells (14). Another previous study in a population with coronavirus disease 2019 (COVID-19) also reported that fasting glucose at admission was positively associated with serum LDH level (15). In our results, serum LDH level was positively correlated with GA level, which may be due to oxidative modifications of albumin or expression of inflammatory cytokines in patients with T2DM. Therefore, we suggest that both LDH and GA might be affected simultaneously by hyperglycemia-related inflammation or oxidative stress.

To further evaluate the association between LDH and GA, we separated participants by serum LDH levels into two groups. Serum GA level was significantly elevated in patients in the high serum LDH level group than in patients in the low serum LDH level group. This suggested that serum LDH level might serve as a reference marker for short-term blood glucose monitoring, which is further supported by *in-vitro* findings (14).

Lactate metabolism and albumin levels may influence serum LDH and GA levels due to liver dysfunction; thus, we analyzed serum GPT levels to verify the measurements for all the patients who did not have liver dysfunction. In addition, diabetes and thyroid disorders have been reported to affect each other and to be associated with other conditions, and the thyroid hormone could also influence glycosylated HbA1c levels (16). In addition, serum glucose stabilization is also affected by thyroid function. Excess thyroid hormone stimulates lipolysis and the secretion of glucagon, followed by deterioration of glucose metabolism, which can cause glucose intolerance and insulin resistance in T2DM (17). To exclude variables that might have influenced the findings, we also examined thyroid function. However, in our study, T3, T4, or even TSH did not show a correlation or predictive ability with LDH. A possible reason could be that none of the patients in this study were diagnosed with thyroid disease; thus, the correlation with markers may be difficult to accurately observe under normal thyroid function status.

The administration of exogenous insulin for treatment usually induces insulin Ab to occur in the serum. The antibody might affect glycemic control in T2DM patients because of the binding insulin (18). A previous study indicated that patients with a high level of insulin Ab may develop severe clinical consequences, such as insulin resistance or hyperglycemia (19). An investigation by Zhu et al.

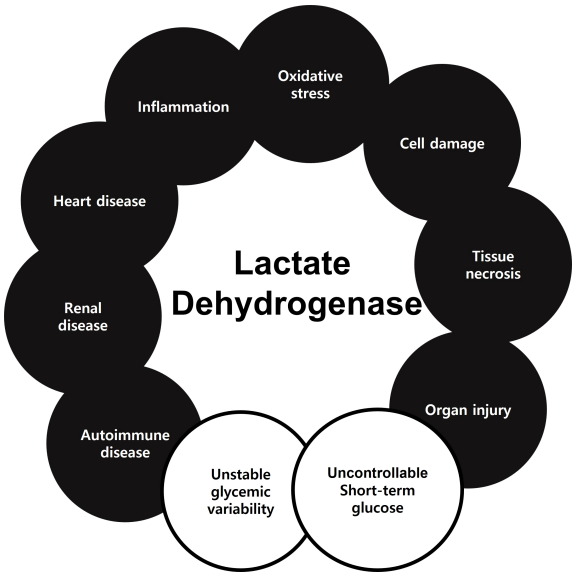


FIGURE 5
Previously studies showed that LDH could be a biomarker of oxidative stress, cell damage, tissue necrosis, organ injury, inflammation, heart disease, renal disease, and autoimmune disease. In the present study, LDH could serve as a biomarker of uncontrollable short-term glucose monitoring and unstable glycemic variability.

shows that a higher level of insulin Ab was associated with increased daily glycemic variability in T2DM patients, indicating that patients with elevated insulin Ab should undergo glycemic monitoring for assessment (20). Another clinical study indicated that patients with a high serum level of insulin Ab have a longer duration of diabetes, a higher level of BMI, and a lower level of C-peptide (21). In our result, the higher level of LDH showed a correlation with insulin Ab. This might indicate that changes in serum markers may occur simultaneously with glycemic variability, followed by induction of cell injury and elevation of serum LDH level.

This study has several limitations. First, serum albumin level was not analyzed, which would have provided indications of nutritional status; it should be analyzed in future studies. Second, the study population was small in size. Although only a few patients were included in this study, the subjects were stringently selected to increase the reliability of the results. Third, although LDH levels were not related to the result of spot fasting glucose in our study, further examination of the relationship between LDH and continuous glucose monitoring still seems necessary. Fourth, because LDH levels were not related to the results of HbA1c and glucose AC, similar data might presumably be obtained from subjects without diabetes. Finally, we did not measure other inflammatory markers such as CRP that may have affected serum LDH levels. Therefore, the present study only demonstrated a significant correlation with LDH, GA, and insulin Ab levels, and a causal relationship should be explored in further studies.

5 Conclusions

In conclusion, we suggested that an elevated serum LDH level in patients with T2DM was associated with serum levels of GA and insulin Ab but not HbA1c. LDH level was independent of long-term but associated with short-term blood glucose monitoring. The result indicates that changes in serum GA elevation induced cell damage, and an abnormal serum LDH level may occur simultaneously with glycemic variability.

In clinical practice, LDH could be a low-cost, rapid, and easily measurable serum biomarker to evaluate short-term glycemic variability. It has been reported that many biomarkers are being used to observe glucose variability in T2DM. However, LDH could provide a more convenient and faster evaluation, especially as a biomarker for short-term glycemic variability monitoring. Further clinical and laboratory study is necessary to elucidate the underlying effect of inflammatory status on serum LDH and GA levels. The findings will aid in the formulation of treatment and preventive strategies for diabetes.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of Taipei Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: Y-SH and M-CY. Methodology: Y-SH. Software: Y-SH. Validation: M-CY, Y-YL and S-FW. Formal analysis: M-CY. Investigation: Y-SH. Resources: Y-SH. Data curation: M-CY. Writing—original draft preparation: Y-SH. Writing—review and editing: Y-SH and Y-YL. Visualization: Y-SH, M-CY, Y-YL, S-FW, C-HH, C-LH, Y-PL, and A-YH. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Evaluation of the effect of a gel made with amniotic fluid formulation on the healing of diabetic foot ulcers: A triple-blind clinical trial

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Aim: The aim of this study was to evaluate the effect of a gel made with amniotic fluid (AF) formulation on wound healing in diabetic foot ulcers.

Methods: This clinical trial was performed on 92 type 2 diabetic patients referring to the Diabetes Clinic of Golestan Hospital of Ahvaz, southwest of Iran in 2019–2020. Patients were randomly divided into three groups of intervention and one placebo group. The wounds of the three intervention groups were dressed with gauze impregnated with an AF formulation gel while wounds of the control group were dressed with plain gauze without any topical agent. Chi-square tests and generalized estimating equations (GEE) with a significance level of 0.05 were used to analyze the data.

Results: At the end of the eighth week of intervention, there was a statistically significant difference among the four groups in terms of wound grade, wound color, condition of the tissues surrounding the wound, the overall condition of the wound, and the duration of wound healing ($P < 0.05$).

Conclusions: Based on our experience with the patients in the present study, we believe that AF represents a useful and safe option for the treatment of chronic diabetic foot ulcers.

Clinical trial registration: https://en.irct.ir/trial/51551, Identifier: IRCT20201010048985N1.

KEYWORDS

diabetic foot ulcer, wound healing, amniotic fluid, nursing care, Iran

Introduction

Diabetes is one of the health problems in the world today and its prevalence in adults is currently 6.4% which is estimated to reach 17.7% in 2030 (1). Common diabetes comorbidities including peripheral neuropathy, ischemia, callus formation, deformity, edema and peripheral arterial disease (PAD) are among the well-established risk factors for diabetic foot ulcers (2). Diabetic foot ulcers are the most common cause of hospitalization of diabetic patients (1, 3). Diabetic foot ulcers are more common in men than women, and type 2 diabetic foot ulcers are more likely to develop than those caused by type 1 diabetes. According to the literature, the global prevalence of diabetic foot ulcers is 6.6%. It has also been reported that the prevalence of diabetic foot ulcers in Asia is 5.5% (4). In Iran, this prevalence has been reported to be between 4–10%, and the rate of lower limb amputation in diabetic patients is 15 times higher than that in otherwise normal people (5).

In addition to long-term hospitalization, foot ulcers and amputations increase treatment costs, reduce quality of life and self-esteem increase patient dependence, and can even lead to patient mortality (6). Therefore, prevention of foot ulcers and amputations and identification of diagnostic methods and timely and effective treatment can increase the quality of life of these patients and reduce their treatment costs (7). Common treatments for diabetic foot ulcers include proper glycemic control, appropriate antibiotic therapy, debridement of necrotic tissue, pressure-relieving strategies restoring pulsatile blood flow, negative pressure wound therapy (8), high-pressure oxygen (9), and wound closure with a vacuum generator (10).

Regenerative medicine, a commonly used phrase in the field of chronic wound management, is the "process of replacing or regenerating human cells, tissues, or organs to restore or establish normal function" (11). Recent studies show that amniotic fluid (AF) contains a considerable quantity of multipotent mesenchymal, hematopoietic, neural, epithelial, and endothelial stem cells (12). AF also contains factors that are involved in wound healing. These include prostaglandins, carbohydrates, peptides, lipids, lactate, amino acids (e.g., glutamine and arginine), proteins (e.g., lactoferrin), enzymes, minerals (e.g., iron and zinc) and hormones (e.g., growth hormone and prolactin). In human amniotic fluid, transforming growth factor (TGF)-α, TGF-β1, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) seem to stimulate the cutaneous fibroblast proliferation (13, 14). Many of the substances that constitute the innate immune system have been identified in AF and have been shown to have significant antimicrobial properties, including α-defensins (human neutrophil defensins 1–3), calprotectin, secretory leukocyte protease inhibitor, psoriasin (S100A7), lactoferrin, lysozyme, bactericidal/ permeability-increasing protein, and cathelicidin (15). These potent antimicrobials have been shown to have broad-spectrum activity against bacteria, fungi, protozoa, and viruses (14). *In vitro* study showed that AF enhanced collagenase activity but inhibited activity of cathepsin, elastase, and hyaluronic acid (16). Alamouti et al., found that amniotic membrane extract heals diabetic foot ulcers (17). Also, a study by Abdo, in 2016 showed that the use of dehydrated amniotic membrane allograft is effective in healing diabetic foot ulcers (18).

AF is a biological substance that is disposable after childbirth and can be obtained for free after making arrangements with the related healthcare facilities. However, to the best of our knowledge, no study has yet examined the effect of using AF on healing of diabetic foot ulcers. Therefore, the present study was conducted to investigate the effect of AF on wound healing in diabetic foot ulcers.

Materials and methods

Design

This triple blind clinical trial was conducted in 2019–2020.

Study population

The study population included all patients with grades 1 and 2 diabetic foot ulcers referred to Diabetes Clinic of Golestan Hospital of Ahvaz and physician offices in Ahvaz, Iran. Considering recovery ratios of 73.33% and 13.33% and assuming a significance level of 0.05 a power of 90%, and an attrition rate of 10%, the final sample size was 92. The patients were assigned into four groups (A, B, C, D) using the randomized permuted block design, which included three intervention groups and one control group (23 patients in each group). The intervention of this study took 8 weeks. The participants were eligible to participate in the study if they: had grade 1 or 2 diabetic foot ulcer according to Wagner system classification, were 18 years old and older, did not smoke or use drugs, did not take medications such as corticosteroids or immunosuppressive and toxic agents that may interfere with wound healing, lack of concomitant diseases such as cancer, vasculitis, renal and hepatic failure, did not have advanced heart failure that may interfere with wound healing process, and were able to fill out the informed consent form. Patients were excluded from the study if they: had active wound infection requiring intravenous antibiotics or gangrenous ulcer requiring amputation, evidence of ischemic, venous, or traumatic lesions, malignancy in the wound area or any malignancy in the patient. Patients who either did not refer to the center for follow up, changed the dressing more than two consecutive times, participated in another research project, or had any sensitivity to AF were also excluded.

For blinding in this study, the gel with AF formulation in three percentages of 5, 10 and 15, and the placebo were produced by a pharmacologist. The dosage was determined using labels A,

B, C, D, of which only the pharmacologist was aware. The gel with AF formulation was administered to the patients by the lead researcher, and the wound healing evaluation was performed by a physician who was blind to group assignment in the diabetes clinic.

Instruments

Data collection tools in this study included a form including the patients' demographic and clinical characteristics, Wagner wound classification system and a checklist for evaluating the healing of diabetic foot ulcer. The demographic form included age, gender, occupation, marital status, educational attainment, comorbidities, type of diabetes treatment, ulcer location and body mass index.

The Wagner system assesses ulcer depth and the presence of osteomyelitis or gangrene using the following grades: grade 0 (pre-or post-ulcerative lesion), grade 1 (partial/full thickness ulcer), grade 2 (probing to tendon or capsule), grade 3 (deep with osteitis), grade 4 (partial foot gangrene), and grade 5 (whole foot gangrene) (19).

Ulcer healing assessment scale evaluates on a weekly basis 4 ulcer parameters including color, surrounding tissues, drainage, and degree, as well as overall ulcer status. Based on this scale, the maximum score for each parameter is 100, and the overall ulcer status scores range from 50 to 400. Based on this scale, higher scores represent better healing (Table 1) (20).

At the end of the eighth week, the last score (score of week 8) was compared to the first score (score of week 0) and status of the ulcer was defined as full recovery, partial recovery, no recovery, and deterioration.

Full recovery: In case that total scores of the ulcer were equal to 400 according to the checklist.

Partial recovery: In case that total scores of the ulcer were increased at least 30-fold compared to the initial score.

No recovery: In case the score did not change compared to the initial score or its increase was below 30-fold.

Worsening: In case the score decreased 10-fold compared to the initial score (20).

Intervention

In this study, access to AF was made possible after arrangements were made with Ahvaz Neurogenic Laboratory. In the neurogenic laboratory, pregnant women who were in their 14th to 20th weeks of pregnancy, after being diagnosed by a gynecologist for amniocentesis, underwent sterile amniocentesis under ultrasound guidance, and about 20–30 ml fluids were drawn in each amniocentesis. It should be noted that the AF provided to the research team was obtained from pregnant mothers whose viral test was negative for markers including HIV, HBs Ag, and HCVab. The obtained fluid was taken to Jundishapur University Diabetes Center using a cold box, and it was stored in the center's refrigerator at 1 °C. The AF was centrifuged at 1100 g for 8 min. The supernatant fluid was used to prepare the gel formulation.

2-3-1 AF gel formulation.

I order to find the most effective dose of the drug according to advice of the pharmacologist of the research team, the AF gel was prepared using 5, 10, and 15 % w/w gel base. The gel base consisted of 15% propylene glycol, 2% hydroxy propyl methyl cellulose (HPMC), and distilled water which are non-active ingredients of the formulation and have no inflammatory or immunological reactions. Therefore, the 5, 10 and 15% gels were prepared as w/w method. That is, the 5% AF gel consisted of 5 g of AF and 95 g of gel base, the 10% AF gel consisted of 10 g of AF and 90 g of gel base, and the 15% AF gel consisted of 15 g of AF and 85 g of gel base. The placebo consisted only of gel base. In sum, in the intervention groups, the gel contained AF in 5, 10 and 15 percentages, while the gel (placebo) used in the control group contained all the gel ingredients except AF.

To perform the intervention, the patients' wounds were first examined by a physician, and if necessary, wound debridement was performed. Then, in each group, the wound and the surrounding tissue were washed with 0.9% normal saline and then dried with sterile gauze. As a routine procedure for new topical drugs, to control the possible allergic effects of preparation, a skin patch test was performed on each patient as follows. We put some of the prepared formula on disk-like plates called patches and stuck the patch on the inside of the forearm or the arm. After a certain period of time, we removed the patch. Any evidence of contact dermatitis (itching, swelling, redness, induration, etc.) led to the patient's exclusion from the study population.

After washing the wound, the gel containing AF formulation was placed on the wound so that the whole wound surface was impregnated with the amniotic gel and then a sterile dressing was applied. Patients or their companions were given the amniotic gel and were taught how to use it and perform the correct dressing. It was recommended that the dressing be changed every 24 hours, and the patients were advised to refer to the diabetes clinic once a week for evaluation of wound healing (Figure 1 shows an example of wound healing).

Ethical consideration

This clinical trial was registered in the Iranian Registry of Clinical Trials (Ref. ID: IRCT20201010048985N1) and approved by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (Ref. ID: IR.AJUMS.REC.1399.516). Participants were assured that they would be given any information they need in case of any ambiguities, that they would not be charged for the treatment, and that their

TABLE 1 Diabetic foot ulcer healing assessment scale.

Total score	Ulcer parameters	Distribution of scores															
100	Degree	Stage	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		Score	100	90	90	80	75	65	65	55	50	40	40	30	25	15	15	10
100	Color	Center	Total healing			Red			Yellow			Necrotic			Necrotic + Red			
			50			40			30			20			10			
		Surroundings	Total healing			Red			Yellow			Necrotic			Necrotic + Red			
			50			40			30			20			10			
100	Surrounding tissues	Color	Normal			Red			Pale			Cyanotic			–			
			25			20			15			10			–			
		Hotness	Yes			No			–			–			–			
			0			25			–			–			–			
		Edema	Yes			No			–			–			–			
			0			25			–			–			–			
		Sense	No			Decreased			Yes			–			–			
			0			15			25			–			–			
100	Drainages	Color	Without drainages			Serosal			Bloody			Yellow			Green			
			40			30			30			20			10			
		Odor	No			Yes			–			–			–			
			20			0			–			–			–			
		Amount	Without drainages			Low			Moderate			Much			–			
			40			20			20			10			–			
Total score (total healing) = 400 (more score= more healing)																		

information would be used only for research purposes and would be kept confidential.

Data analysis

After collecting and scoring the data, data analysis was done based on GEE analysis and chi-square test using SPSS version 22. The significance level of the tests was set at $P < 0.05$.

Results

The final analysis was performed on data obtained from 83 patients, of whom 44 (53%) were female and 39 (47%) were male. Most patients (51.2%) were in the age group of 40–49 years. With regard to occupation, 48.2% were housewives, 24.1% were office workers, 8.4% were self-employed, and 19.3% were retired. Also 83.1% of the patients were married, 8.4% were widow, 7.2% were single, and 1.3% were divorced. As far as education was concerned, 26.5% of the patients were illiterate, 37.3% did not have a high school diploma, 31.4% had a high school diploma, and 4.8% had a university degree. In terms of the ethnicity of the participants, the highest frequency (55.4%) of patients were Arabs while the lowest frequency 1.2% (n = 1) were related to other ethnicities. With respect to body mass index, 51.3% of the patients had a body mass index of 25–29. According to Table 2, 29.1% of the patients' wounds in the 4 groups were on the toes while wounds on the whole foot (1.2%) were the least frequent ones. Also, most patients (51.8%) were diagnosed with grade 2 ulcers according to Wagner classification. Based on chi-square test, there was no significant difference between the 4 groups in terms of demographic information ($P > 0.05$) (Table 2).

According to the results of GEE analysis, the effect of time was significant (P-value < 0.001), with the mean scores in the placebo, Group 5% and Group 10% having an upward trend. However, this trend first increased in the Group 15% and had the highest mean in the fourth week but then decreased. Also, between-group comparison showed that Group 5% was significantly different from the control group (P-value < 0.001), but the Groups 10 and 15% were not.

Table 3 shows the mean of wound color scores of patients in the 4 groups over a period of 0–8 weeks. According to the results of GEE analysis, the effect of time was significant (P-value < 0.001), with the mean scores in the four groups having an almost upward trend, but this trend in the control group and the Group 5% was initially upward until it reached its peak at the seventh week and then it became downward in Week 8. Also,

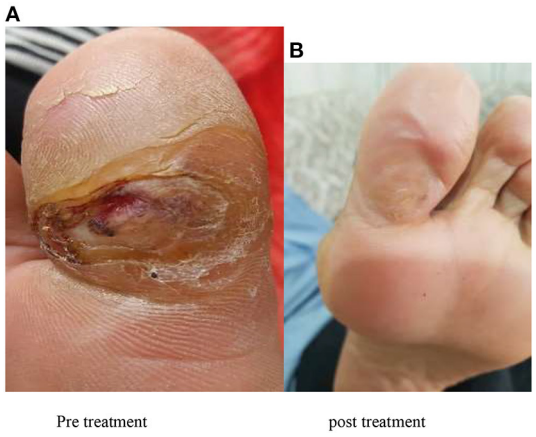


FIGURE 1
Picture shows an example of wound healing. **(A)** Pre-treatment. **(B)** Post-treatment.

between-group comparison shows that the treatment Group 5% was significantly different from the control group (P-value = 0.004), but the Groups 10 and 15% were not.

Table 3 shows the mean scores of the tissue surrounding the wound of patients in the 4 groups over a period of 0–8 weeks. According to the results of GEE analysis, the effect of time was significant (P-value < 0.001), with the mean scores in the three intervention groups having an upward trend, but this trend was initially upward in the control group until it reached its peak at the seventh week and then it became downward in Week 8. Also, between-group comparison revealed that Group 15% was significantly different from the control group (P-value = 0.038).

Table 3 shows the mean scores of wound discharge of patients in the 4 groups over a period of 0-8 weeks. According to the results of GEE analysis, the effect of time was significant (P-value < 0.001), with the mean scores in all four groups having an upward trend. Also, between-group comparison demonstrated that Group 5% was significantly different from the control group (P-value = 0.048).

Table 3 shows the mean scores of the overall wound status of patients in the 4 groups over a period of 0–8 weeks. According to the results of GEE analysis, the effect of time was significant (P-value < 0.001), and the mean scores in the four groups had an upward trend. Also, between-group comparison showed that Group 15% and Group 5% were significantly different from the control group (P-value = 0.048, P-value = 0.024, respectively).

Discussion

The aim of this study was to investigate the effect of a gel made with AF formulation on the healing of diabetic foot ulcers. Based on the findings of the present study, the most frequent wound site in the patients of the 4 groups was on the toes, while the least frequent one was on the whole foot. In studies by Nasiri et al. (6), most of foot ulcers were reported to be on the toes, which is consistent with the findings of the present study. However, the results of Aziza et al. (21) show that foot ulcers of 96% and 96% of their patients in the intervention and control groups were on the plantar surface, respectively, which is different from the results of the present study. Also in the present study, most patients had grade 2 ulcers according to Wagner classification.

One of the findings of the present study was to compare the overall healing of diabetic foot ulcers in the three treatment groups using AF gel at 5%, 10% and 15% doses (Group 5%, Group 10%, and Group 15%) and the placebo group before and after 8 weeks of intervention. The results showed that the AF gel, at different concentrations, was somewhat effective in the healing of diabetic foot ulcer, with gels at concentrations of 5% and 15% having the greatest effect on the healing process of diabetic foot ulcer compared to the 10% concentration.

On the other hand, the placebo which consisted of pharmacologically inert constituents had no effect on healing rate of wounds. In fact, like any other pharmaco-clinical study, the placebo was used to determine the real effect of test groups (AF groups). Such formula for chosen for the placebo so that it will have a non-irritating effect on tissue and be a good vehicle for AF in terms of compatibility and stability of AF gel formulation. The results of this study clearly depicted that the placebo had no real healing effect, and the wound healing effect is mainly due to the healing properties of the AF gels. Constituent of carrier gel including propylene glycol is being used as an excipient for a long time as it has subject to a report by European Medicine Agency entitled: Propylene glycol used as an excipient (22, 23). Regarding the HPMC Many techniques are available for studying the sol-gel transitions in HPMC hydrogels (24, 25). In fact this ingredient help to form a gel in association with propylene glycol. Both of them had no considerable effect on wound healing.

In line with the present finding, Alamouti et al. evaluated the safety of amniotic membrane extract in the healing of diabetic foot ulcers. Their results showed that the rate of wound healing during 4 weeks of treatment with amniotic membrane extract in the group of wounds with a size of 500 mm^2 $\geq$ was 98.9 $\pm$ 2.40%, and in wounds with a size of 500 mm^2 $\leq$, it was 92.1 $\pm$ 7.23%, which indicates the effect of amniotic membrane extract on the healing of diabetic foot ulcers (17). Also, the effect of human amniotic membrane on reducing the size of foot ulcers in diabetic patients has been repeatedly reported in clinical trial studies (17, 26, 27). In the study of Zelen et al., for example, the mean duration for complete wound healing with Epifix was 23.6 days (27). Epifix is a proprietary product for wound healing, containing dehydrated amnion and chorion membrane. It is used in wound clinics and produced by MiMedix Co, USA.

With respect to diabetic foot ulcer discharge in the four studied groups, the results indicate that amniotic fluid gel at

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TABLE 2 Frequency distribution and percentage of demographic information of the four groups of patients participating in the study.

Variable	Classification	Group 10%, *N* (%)	Group Placebo, *N* (%)	Group 5%, *N* (%)	Group 15%, *N* (%)	*P*-value*
Sex	Female	12 (57.1)	12 (57.1)	9 (42.9)	11 (55)	0.756
	Male	9 (42.9)	9 (42.9)	12 (57.1)	9 (45)	
Age (year)	18–39	3 (14.3)	1 (4.7)	0 (0)	1 (5)	0.17
	40–49	9 (42.9)	14 (66.8)	8 (38.2)	11 (5)	
	50–59	5 (23.8)	5 (23.8)	11 (52.4)	8 (40)	
	60–69	2 (9.5)	0 (0)	1 (4.7)	0 (0)	
	70>	2 (9.5)	1 (4.7)	1 (4.7)	0 (0)	
Occupation	Housewife	8 (38.1)	12 (57.1)	9 (42.9)	11 (55)	0.11
	Employed	8 (38.1)	3 (14.3)	8 (38.1)	1 (5)	
	Retired	2 (9.5)	4 (19.1)	3 (14.3)	7 (35)	
	Unemployed	3 (14.3)	2 (9.5)	1 (4.7)	1 (5)	
Marital status	Single	2 (9.5)	2 (9.5)	1 (4.8)	1 (5)	0.49
	Married	18 (85.7)	17 (81)	16 (76.2)	18 (90)	
	Divorced	0 (0)	0 (0)	0 (0)	1 (5)	
	Widow	1 (4.8)	2 (9.5)	4 (19)	0 (0)	
Education	Illiterate	4 (19)	4 (19)	9 (42.9)	5 (25)	0.27
	Primary school	6 (28.6)	12 (57.2)	5 (23.8)	8 (40)	
	High school diploma	10 (47.6)	5 (23.8)	5 (23.8)	6 (30)	
	University degree	1 (4.8)	0	2 (9.5)	1 (5)	
Body Mass Index	18–24	6 (28.6)	10 (47.6)	10 (47.6)	9 (45)	0.86
	25–29	13 (61.9)	10 (47.6)	9 (42.9)	10 (50)	
	30–35	2 (9.5)	1 (4.8)	2 (9.5)	1 (5)	
Ulcer location	Sole	5 (23.8)	5 (23.8)	7 (33.3)	5 (25)	0.63
	Heel	3 (14.3)	5 (23.8)	7 (33.3)	5 (25)	
	Toe	7 (33.3)	8 (38.2)	2 (9.5)	8 (40)	
	Foot dorsum	6 (28.6)	3 (14.2)	5 (23.8)	2 (10)	
Wound grade	Grade 1	11 (52.4)	10 (47.6)	11 (52.4)	8 (40)	0.77
	Grade 2	10 (47.6)	11 (52.4)	10 (47.6)	12 (60)	

*Chi-square test.

different concentrations was somewhat effective in the reduction of diabetic foot ulcer discharge, but gel with 5% concentration had the greatest effect in treating diabetic foot ulcer discharge compared to 10% and 15% concentrations. Consistent with the present finding, Hakim et al. (20) found a significant difference between the two groups in terms of wound discharge in 4 weeks of treatment. However, in Nasiri et al., no significant difference was reported between the experimental and control groups in terms of wound discharge after 4 weeks of intervention (6). One of the reasons for the difference between our results and those of Nasiri et al. is the different type of intervention.

As far as the tissue surrounding diabetic foot ulcer is concerned, the AF gel at 5, 10 and 15% doses was effective before and after 8 weeks of intervention compare with the placebo group. However, the gel at the concentration of 15% had the greatest effect on the discharge reduction of diabetic foot ulcers. In Hakim et al. (20) and Nasiri et al. (6), a significant difference was observed between the two groups in terms of tissues surrounding the wound in weeks 2, 3, and 4 of treatment, which is in line with the results of the present study.

Apropos of the color of diabetic foot ulcer in four studied groups, our results showed that amniotic fluid gel at different concentrations affected diabetic foot ulcer color, but gel with 5% concentration had the greatest effect on the wound color in diabetic foot ulcers. In Hakim et al. (20) and Nasiri et al. (6), there was a significant difference between the two groups in terms of wound color after 4 weeks of treatment, which is completely consistent with the results of the present study.

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TABLE 3 Comparison of mean and standard deviation of ulcer parameters and total ulcer status from week 0 to 8 in 4 group.

Ulcer parameters	Time	Group 10% Mean ± SD	Group 5% Mean ± SD	Group 15% Mean ± SD	Placebo Mean ± SD	*P** (effect of time)
Wound degree	Week 0	72.27 ± 8.96	74.31 ± 6.41	72.31 ± 4.41	71.26 ± 7.42	<0.001
	Week 1	74.77 ± 6.62	74.77 ± 6.98	73.40 ± 6.24	72.04 ± 6.66	
	Week 2	79.09 ± 7.70	80.68 ± 7.44	74.54 ± 6.52	75.45 ± 6.52	
	Week 3	83.86 ± 7.38	81.66 ± 6.95	76.56 ± 7.92	80.22 ± 6.63	
	Week 4	85.95 ± 7.51	84.00 ± 7.53	87.00 ± 8.80	83.18 ± 5.46	
	Week 5	88.94 ± 6.14	88.15 ± 7.11	77.85 ± 7.83	87.95 ± 5.49	
	Week 6	90.88 ± 4.04	89.06 ± 6.11	77.85 ± 7.51	91.50 ± 4.61	
	Week 7	94.68 ± 2.86	92.33 ± 6.77	79.04 ± 7.68	94.11 ± 6.64	
	Week 8	97.14 ± 3.23	93.18 ± 7.50	79.52 ± 7.89	95.31 ± 8.84	
P-value** (effect of treatment)		0.19	<0.001	0.54	–	
Wound color	Week 0	66.36 ± 14.65	70.68 ± 7.91	67.95 ± 11.30	77.67 ± 14.64	**<0.001**
	Week 1	69.09 ± 13.76	71.36 ± 8.47	70.00 ± 6.86	66.81 ± 13.67	
	Week 2	73.86 ± 13.96	77.27 ± 8.12	71.13 ± 9.24	70.45 ± 11.43	
	Week 3	79.77 ± 10.96	79.52 ± 7.56	72.95 ± 9.46	75.68 ± 10.94	
	Week 4	82.61 ± 11.13	83.80 ± 9.86	74.54 ± 9.37	81.13 ± 8.15	
	Week 5	85.87 ± 8.70	87.93 ± 8.39	74.52 ± 8.35	83.18 ± 17.49	
	Week 6	89.11 ± 5.22	88.12 ± 7.93	75.00 ± 7.58	90.25 ± 5.49	
	Week 7	92.80 ± 5.46	91.00 ± 8.25	79.66 ± 7.79	92.94 ± 4.69	
	Week 8	96.42 ± 4.12	90.90 ± 8.89	79.90 ± 7.98	79.18 ± 3.67	
P-value** (effect of treatment)		0.53	0.004	0.25	–	
Surrounding tissues	Week 0	80.22 ± 19.90	81.59 ± 13.57	77.27 ± 12.50	67.72 ± 16.67	<0.001
	Week 1	77.72 ± 22.92	82.27 ± 13.06	78.18 ± 11.90	70.90 ± 15.08	
	Week 2	85.68 ± 18.37	86.59 ± 12.2	79.77 ± 11.49	75.14 ± 14.14	
	Week 3	87.27 ± 13.69	87.14 ± 11.24	88.40 ± 16.15	80.00 ± 13.36	
	Week 4	89.28 ± 10.98	89.52 ± 11.05	82.04 ± 10.87	80.68 ± 20.83	
	Week 5	91.42 ± 8.36	91.31 ± 9.83	81.90 ± 11.45	87.72 ± 8.96	
	Week 6	92.42 ± 6.85	92.50 ± 8.14	81.90 ± 11.45	91.75 ± 6.54	
	Week 7	95.93 ± 5.23	96.33 ± 6.22	82.38 ± 11.57	96.11 ± 4.04	
	Week 8	98.92 ± 2.89	94.54 ± 5.68	82.61 ± 11.46	98.52 ± 2.93	
P-value** (effect of treatment)		0.057	0.54	0.038	–	
Drainages	Week 0	85.45 ± 3.70	90.15 ± 15.73	82.00 ± 24.76	80.68 ± 20.48	<0.001
	Week 1	87.50 ± 1.82	92.27 ± 10.54	85.68 ± 18.66	82.50 ± 19	
	Week 2	90.22 ± 10.05	95.22 ± 8.51	87.72 ± 16.67	84.54 ± 17.10	
	Week 3	94.54 ± 7.54	96.19 ± 7.40	88.40 ± 16.85	88.18 ± 14.92	
	Week 4	95.71 ± 6.38	69.66 ± 7.30	89.54 ± 17.03	92.72 ± 10.31	
	Week 5	97.36 ± 5.36	97.36 ± 6.31	89.52 ± 16.80	95.68 ± 8.20	
	Week 6	97.64 ± 4.37	97.18 ± 6.31	89.76 ± 16.84	89.00 ± 4.10	
	Week 7	99.06 ± 2.71	98.00 ± 5.60	89.76 ± 16.84	95.44 ± 15.79	
	Week 8	100.00 ± 0.0	98.33 ± 5.77	90.47 ± 15.56	95.52 ± 16.03	

(Continued)

TABLE 3 (Continued)

Ulcer parameters	Time	Group 10% Mean ± SD	Group 5% Mean ± SD	Group 15% Mean ± SD	Placebo Mean ± SD	*P** (effect of time)
P-value** (effect of treatment)		0.17	0.048	0.61	–	<0.001
Total ulcer status		304.31 ± 40.68	316.59 ± 33.18	300.63 ± 31.81	284.54 ± 45.77	
		309.09 ± 40.31	320.68 ± 28.96	307.27 ± 27.84	292.27 ± 39.99	
		328.86 ± 35.52	339.77 ± 26.4	313.18 ± 27.53	305.45 ± 36.93	
		345.45 ± 32.25	344.52 ± 24.43	318.86 ± 29.51	324.09 ± 33.08	
		353.57 ± 29.11	352.50 ± 28.35	324.31 ± 3.74	337.72 ± 35.86	
		363.94 ± 21.38	364.47 ± 26.18	323.80 ± 30.03	354.54 ± 23.54	
		370.58 ± 16.28	366.87 ± 24.21	324.52 ± 29.74	371.50 ± 16.86	
		382.05 ± 9.96	375.66 ± 23.66	327.85 ± 31.12	382.05 ± 9.69	
		392.50 ± 8.71	376.82 ± 25.12	367.82 ± 35.66	390.31 ± 13.84	
P-value** (effect of treatment)		0.081	0.024	0.048	–	

*Reference class–GEE analysis.
**Compared to the reference class.

Finally, the results of the present study revealed that amniotic fluid gel at different concentrations had a significant effect on the grade of diabetic foot ulcer, but the gel at 5% concentration had the greatest effect on the process of diabetic foot ulcer. In Nasiri et al., the wound grade score at the end of weeks two, three and four was significantly higher than that in the previous week, which indicates the upward trend of wound healing (6). In Hakim et al. (20), there was a significant difference between the two groups in terms of wound grade after 4 weeks of treatment.

The main limitation of the present study was that the process of wound healing is different in different people due to their genetic profile, and this might have affected the results but was beyond the control of the researchers.

Conclusion

According to the results of the present study, amniotic fluid gel at different concentrations has a significant effect on the grade of diabetic foot ulcer. Given these patients' special condition and their urgent need for various trainings in relation to their disease and the associated interventions, it is necessary that nurses and even all members of the health team attend patient training programs aimed at orienting them on how to care for and prevent diabetic foot ulcers. Also, the use of effective and available materials such as the amniotic gel used in this study should be promoted in order to treat foot ulcers of diabetic patients and thus reduce the rate of amputation and the physical, mental, social and economic complications associated with their condition.

Guarantor's statement

SM is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ahvaz Jundishapur University of Medical Sciences. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FN, SM, DB, AH, and LY researched data. FN, SM, DB, AH, LY, AS, MM, and NS wrote the manuscript and researched data. FN and SM contributed to discussion and reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Predictive value of novel inflammatory markers platelet-to-lymphocyte ratio, neutrophil-to-lymphocyte ratio, and monocyte-to-lymphocyte ratio in arterial stiffness in patients with diabetes: A propensity score–matched analysis

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Background: Increased arterial stiffness is common in patients with diabetes, and inflammation is one of the main causes of increased arterial stiffness. Platelet-to-lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR), and monocyte-to-lymphocyte ratio (MLR) are novel inflammatory markers that are reproducible, widely available, and easy to measure, and are associated with low costs. This study sought to investigate the predictive value of these novel inflammatory markers in patients with diabetes having arterial stiffness.

Methods: We retrospectively included inpatients with diabetes mellitus from the Endocrinology Department of the Chengdu Fifth People's Hospital from June 2021 to May 2022 and collected data on their general information, biochemical indicators, and brachial-ankle pulse wave velocity (baPWV). After propensity matching, the risk relationship between PLR, NLR, and MLR and arterial stiffness was assessed in the recruited patients.

Results: A total of 882 hospitalized patients with diabetes were included in this study and categorized into the low baPWV (507 cases) or high baPWV group (375 cases) based on the baPWV. After propensity matching, there were 180 patients in all in the high and low baPWV groups. Univariate and multivariate logistic regression analyses revealed that high PLR, NLR, and MLR were independently associated with an increased risk of arterial stiffness in patients with diabetes. In the receiver operating characteristic curve analysis, the NLR area under the curve (AUC) was 0.7194 (sensitivity = 84.4%, specificity = 51.1%) when distinguishing low baPWV and high baPWV in patients with diabetes,

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which was higher than that for PLR AUC (0.6477) and MLR AUC (0.6479), and the combined diagnosis for AUC.

Conclusions: NLR was superior to PLR, and MLR and combined diagnosis have certain predictive values that indicate the increase in arterial stiffness in patients with diabetes. These predictive values can help with the early identification of increased arterial stiffness in patients with diabetes.

KEYWORDS

Platelet-to-lymphocyte ratio, neutrophil-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, arterial stiffness, baPWV, diabetes

Introduction

Arterial stiffness is an important indicator of arterial elasticity and function. With an increase in age (1), the fibers degenerate and break, the content of collagen fibers increases, and the intima becomes thicker and stiffer, resulting in an increase in arterial stiffness and a decrease in compliance. Stiffness reflects changes in early vascular function and is considered to be closely related to cardiovascular and cerebrovascular events (2, 3). Patients with diabetes are more prone to increased arterial stiffness due to several complex metabolic disorders of sugars and lipids (4). Brachial-ankle pulse wave velocity (baPWV) is often used as an effective noninvasive index to assess peripheral arterial vascular stiffness owing to its reproducibility and simplicity (5), especially in patients with diabetes.

Vascular inflammation is a well-established risk factor and key pathogenic mediator in the development of endothelial dysfunction and consequent increase in arterial stiffness (6). Stimulated leukocytes may adhere and invade the vascular endothelium, resulting in capillary leukocyte arrest, vascular injury, and increased arterial stiffness (7, 8). Among leukocyte indices, the platelet-to-lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR), and monocyte-to-lymphocyte ratio (MLR) are the most suitable markers of vascular inflammation (9–11). These measurements offer distinct advantages over other established markers of inflammation owing to their excellent reproducibility, low assay costs, widespread availability, and simplicity of measurement (12). The ratio of these three distinct leukocyte subtype counts has evolved as a useful marker of vascular inflammation and has been found to correlate with arterial stiffness in many studies. However, there is still a lack of studies that have directly compared and evaluated which of PLR, NLR, MLR, and combined diagnosis is the better predictor of arterial stiffness. Therefore, in this study, we grouped patients with diabetes based on baPWV and investigated the association between PLR, NLR, and MLR and arterial stiffness.

Patients and methods

Study population

A total of 882 patients with diabetes hospitalized in the Department of Endocrinology of the Chengdu Fifth People's Hospital from June 2021 to May 2022 constituted the research subjects. The inclusion criterion was patients > 18 years of age who had a definite diagnosis of diabetes. Patients with symptoms of diabetes (polydipsia, polyuria, polyphagia, and unexplained weight loss) and fasting venous plasma glucose levels ≥ 7.0 mmol/L, random venous plasma glucose levels ≥ 11.1 mmol/L, or venous plasma glucose level ≥ 11.1 mmol/L 2 hours after glucose loading without the typical symptoms of diabetes were indicated blood glucose retesting on another day to confirm the diagnosis. The exclusion criteria were as follows: 1) patients taking hypolipidemic agents and/or antihypertensive agents in the past 6 months; 2) patients experiencing acute complications of diabetes, such as hyperosmolar hyperglycemic state, diabetic ketoacidosis, and/or lactic acidosis; 3) patients with severe blood system diseases; 4) patients with acute or chronic infections; 5) patients with hepatic insufficiency, and ALT and/or AST > 3 times the upper limit of normal; 6) patients with severe renal dysfunction, manifesting with an estimated glomerular filtration rate (eGFR) ≤ 15 mL/min × 1.73 m^2 or patients undergoing renal dialysis; 7) patients with malignant tumors; 8) pregnant women (Figure 1). This study was conducted according to the principles outlined in the Declaration of Helsinki and was approved by the Medical Ethics Committee of the Chengdu Fifth People's Hospital (No. 2022-020S-01).

Measurements

The gender, age, duration of diabetes, smoking status, and drinking status of patients were collected by consulting their medical records. Blood pressure, height, and weight of patients

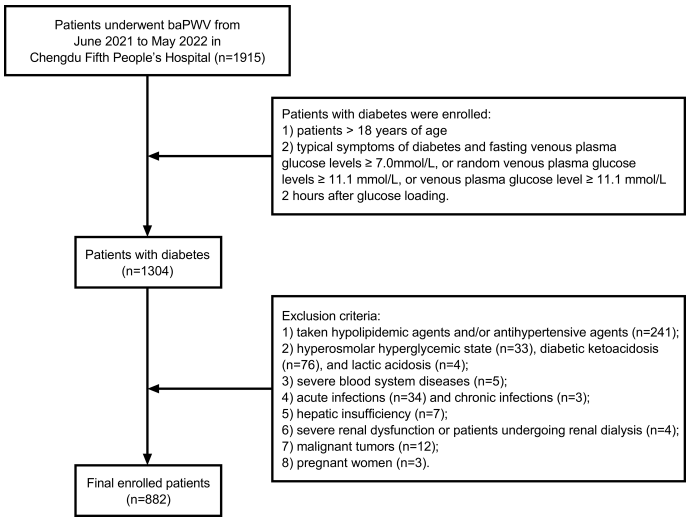


FIGURE 1
Flow chart of the study population enrollment.

were routinely measured, and general parameters, such as the body mass index (BMI), were calculated. baPWV was measured using the OMRON BP-203RPE III Arterial Stiffness Detection Device (Omron Co. LTD, Dalian, China) by professionally trained staff. Clinical data from our hospital laboratory were collected and analyzed using a Mindray BC-6800 Plus automatic blood cell analyzer (Mindray Biomedical Electronics Co., Ltd, Shenzhen, China) with reagents provided by Mindray to determine platelet, neutrophil, monocyte, and lymphocyte levels. A HITACHI LABOSPECT 008AS automatic biochemical analyzer (Hitachi Instruments Co., LTD, Shanghai, China) and reagents provided by HITACHI were used to measure fasting blood glucose (FBG), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and uric acid (UA) levels. Glycated hemoglobin (HbA1c) levels were determined using liquid chromatography. Lifotronic H9 hemoglobin detector (Lifotronic Technology Co., LTD, Shenzhen, China) reagent was provided by Lifotronic.

Grouping criteria

Pulse wave velocity measurements are meaningful only if blood circulation is not disturbed during pulse propagation; therefore, it is important to determine whether arterial circulation in the lower extremities between the ilium and tibia is normal before measuring baPWV. When obtaining baPWV, we first measured the ankle-brachial index (ABI) of the ipsilateral limb. If the unilateral ABI is ≤ 0.9, it is necessary to first determine whether the limb is affected by peripheral vascular disease. In this case, the predictive value of baPWV will be doubted, so it was excluded. ABI > 0.9 on both sides is indicative of no severe obliterative arterial stiffness, whereas baPWV ≥ 1800 ms on either side is considered a better predictor of target organ damage (13–15). Therefore, we classified patients with baPWV ≥ 1800 ms into the high baPWV group and those with baPWV < 1800 ms into the low baPWV group.

Statistical analysis

IBM SPSS 27.0 was used for data entry, processing, and statistical analysis, and GraphPad Prism 9.4.1 was used to construct calibration graphs, forest graphs, and receiver operating characteristic (ROC) curves. The baseline data of both groups were matched using a 1:1 ratio using propensity score matching, and the caliper value was set at 0.02. Independent samples *t*-test used to compare the two groups when the data were normally distributed. When the data showed skewed distribution, the Mann-Whitney U test of independent samples was used to compare both groups. Chi-square analysis was used to compare the categorical variable data between groups. The PLR, NLR, and MLR values were transformed into binary variables. Univariate and multivariate logistic regression analyses were conducted in succession. Goodness-of-fit tests were performed using the Hosmer-Lemeshow test and calibration plots were drawn. The ROC curve was used to analyze and evaluate the efficacy of PLR, NLR, MLR, and combined diagnosis for arterial stiffness. The corresponding area under the curve (AUC) was calculated, and the sensitivity and specificity corresponding to the maximum Youden's index was calculated. Normally distributed data are

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presented as the mean ± standard deviation (SD), whereas data with a skewed distribution are presented as median (upper and lower quartiles) [M (QR)]. A P-value of < 0.05 was considered statistically significant.

Results

Baseline characteristics

A total of 882 patients with diabetes were enrolled in this study. General information, including the gender, age, duration of diabetes, height, weight, BMI, blood pressure, smoking status, drinking status of patients; and the biochemical indicators, including HBA1c, FBG, TG, TC, HDL-C, LDL-C, and UA, were determined (Table 1). Patients were divided into a low baPWV group (507 cases) or a high baPWV group (375 cases) based on their baPWV. The baseline data of the patients were matched using propensity score matching. There were significant differences in gender, age, disease course, DBP, SBP, HBA1c, and UA (all P < 0.05) before propensity score matching. After propensity scores matching by gender, age, disease course, BMI, SBP, DBP, smoking status, drinking status, and HBA1c, FBG, TG, TC, HDL-C, LDL-C, and UA levels, all parameters were well matched between both groups (all P > 0.05) (Table 2). Both before and after propensity score matching, we found that all patients in the high baPWV group had higher levels of PLR, NLR, and MLR than those in the low baPWV group (Table 3).

TABLE 1 Patients' profiles.

Characteristics	All patients (n=882)
Gender (Male/Female)	507/375
Age (year)	58.69 ± 12.73
Course (year)	6.00 (1.00;10.00)
Height (cm)	161.56 ± 8.32
Weight (kg)	63.10 ± 11.78
BMI (kg/m^2)	24.07 ± 3.48
SBP (mm Hg)	128.97 ± 20.12
DBP (mm Hg)	72.16 ± 12.94
Smoking (n/%)	322 (36.5)
Drinking (n/%)	294 (33.3)
HBA1c (%)	9.51 ± 2.66
FBG (mmol/L)	9.49 ± 4.35
TG (mmol/L)	2.59 ± 2.30
TC (mmol/L)	4.91 ± 1.33
HDL-C (mmol/L)	1.12 ± 0.34
LDL-C (mmol/L)	2.78 ± 0.97
UA (μmol/L)	337.18 ± 104.98
Platelets (×10^9/L)	178.40 ± 60.50
Neutrophils (×10^9/L)	4.18 ± 1.47
Monocytes (×10^9/L)	0.36 ± 0.14
Lymphocytes(×10^9/L)	1.53 ± 0.57
PLR	129.87 ± 62.86
NLR	3.14 ± 2.07
MLR	0.26 ± 0.14

Logistic regression analysis to determine the relationship between PLR, NLR, and MLR and arterial stiffness

PLR, NLR, and MLR were converted into binary variables as independent variables, and Model 1 was constructed. Results from the univariate logistic regression analysis showed that high PLR, NLR, and MLR values were associated with the increased risk of arterial stiffness in patients with diabetes. Model 2 was constructed after adjusting for gender, age, disease course, BMI, smoking status, drinking status, SBP, and DBP. Model 3 was constructed after adjustment for HBA1c, FBG, TG, TC, HDL-C, LDL-C, and UA based on Model 2. Multivariate logistic regression analysis showed that PLR, NLR, and MLR were associated with an increased risk of arterial stiffness in patients with diabetes (Table 4). A forest plot was drawn (Figure 2).

PLR, NLR, and MLR clinically predicted arterial stiffness based on calibration diagram

Actual and predicted probabilities were calculated using the multivariate logistic regression Models 2 and 3 of PLR, NLR, and MLR using the Hosmer-Lemeshow goodness-of-fit test. A calibration chart was drawn with the actual probability on the Y-axis as the dependent variable, and the predicted probability on the X-axis as the independent variable. The predicted probabilities of Models 2 and 3 of NLR and MLR are closer to the diagonal of the calibration plot (Figure 3).

Predictive ability of PLR, NLR, and MLR in the diagnosis of arterial stiffness

The ability of PLR, NLR, and MLR in predicting arterial stiffness in patients with diabetes was further evaluated using high baPWV as a positive diagnostic result. PLR, NLR, MLR,

PLR+NLR, PLR+MLR, NLR+MLR, and PLR+NLR+MLR values were used to construct the ROC curve and calculate the AUC. The AUC for NLR was 0.7194 (sensitivity = 84.4%, specificity = 51.1%), which was higher than that for PLR (AUC: 0.6477), MLR (AUC: 0.6479), PLR+NLR (AUC: 0.7185), PLR+MLR (AUC: 0.6646), NLR+MLR (AUC: 0.7177), PLR+NLR+MLR (AUC: 0.7192) (Table 5 and Figure 4).

Discussion

In this retrospective study, we first balanced the distribution of baseline variables in the low and high baPWV groups using propensity matching. After adjustment, the consistency of the baseline data between the two groups improved, thereby enhancing the comparability of the two groups of variables. Second, the levels of PLR, NLR, and MLR in the high baPWV group were higher than those in the low baPWV group. Next, we transformed the PLR, NLR, and MLR into binary variables and constructed Model 1 for univariate logistic regression analysis. After adjusting the general information and biochemical indicators of patients, Models 2 and 3 were constructed. Multivariate logistic regression analysis of factors revealed that high PLR, NLR, and MLR were independently associated with increased arterial stiffness in patients with diabetes, but the odds ratio for NLR was the highest, indicating the strongest correlation. We further calculated the actual and predicted probabilities of Models 2 and 3 of PLR, NLR, and MLR and found that the predicted probabilities of NLR and MLR were closer to the diagonal of the calibration diagram, indicating that Models 2 and 3 of NLR and MLR predicted the effect more accurately. Lastly, ROC curve analysis showed that the NLR value for the diagnosis of increased arterial stiffness in patients with diabetes was superior to the PLR and MLR values. The combined AUCs of NLR with PLR and MLR did not significantly increase compared with the AUC of NLR alone, indicating that the combined diagnosis does not lead to a significant increase in the diagnostic power for arterial stiffness in patients with diabetes. Moreover, our comparisons revealed that NLR had the highest sensitivity in diagnosing increased arterial stiffness. Therefore, NLR is recommended as a better predictor of arterial stiffness in individuals with diabetes.

Previous studies have found that inflammatory markers may be related to arterial stiffness. The relationship between NLR and arterial stiffness, especially, is the most studied. Park et al. found that elevated NLR was independently associated with arterial stiffness in a healthy population (16). Yi et al. found a correlation between NLR and vascular stiffness in healthy males (17). Wang et al. compared healthy subjects and individuals with hypertension and found that elevated NLR was a valid predictor of hypertensive arterial stiffness (18). Li et al. found that NLR was independently related to arterial stiffness in

TABLE 2 Patients' profiles propensity score matching.

Variables	Before propensity matching			After propensity matching		
	Low baPWV n=520	High baPWV n=362	P-value	Low baPWV n=180	High baPWV n=180	P-value
Gender (Male/Female)	320/200	187/175	0.040	95/85	100/80	0.597
Age (year)	53.55 ± 11.30	66.06 ± 10.93	<0.001	61.86 ± 9.29	62.14 ± 10.87	0.790
Course (year)	4.00 (0.17;10.00)	10.00 (3.00;13.00)	<0.001	8.00 (1.00;12.00)	7.00 (2.00;10.00)	0.700
BMI (kg/m^2)	24.21 ± 3.61	23.87 ± 3.28	0.154	24.14 ± 3.72	24.40 ± 3.14	0.473
SBP (mmHg)	121.53 ± 16.20	139.66 ± 20.40	<0.001	132.09 ± 17.19	132.02 ± 18.17	0.967
DBP (mmHg)	69.77 ± 10.59	75.59 ± 15.09	<0.001	72.26 ± 13.00	73.81 ± 14.281	0.282
Smoking (n/%)	199 (38.3)	123 (34.0)	0.193	64 (35.6)	68 (37.8)	0.662
Drinking (n/%)	179 (34.4)	115 (31.8)	0.411	58 (32.2)	59 (32.8)	0.910
HBA1c (%)	9.66 ± 2.72	9.30 ± 2.57	0.047	9.21 ± 2.71	9.02 ± 2.38	0.486
FBG (mmol/L)	9.60 ± 4.33	9.34 ± 4.37	0.384	9.18 ± 4.17	9.33 ± 4.15	0.730
TG (mmol/L)	2.66 ± 2.31	2.49 ± 2.29	0.286	2.30 ± 2.07	2.32 ± 2.16	0.937
TC (mmol/L)	4.95 ± 1.23	4.86 ± 1.48	0.374	4.82 ± 1.24	4.73 ± 1.31	0.466
HDL-C (mmol/L)	1.11 ± 0.34	1.14 ± 0.34	0.197	1.15 ± 0.33	1.13 ± 0.33	0.517
LDL-C (mmol/L)	2.82 ± 0.93	2.71 ± 1.04	0.101	2.69 ± 0.90	2.67 ± 1.01	0.792
UA (μmol/L)	325.80 ± 95.59	353.54 ± 115.35	<0.001	331.57 ± 92.10	338.14 ± 107.07	0.533

TABLE 3 Comparison of PLR, NLR, and MLR propensity scores before and after matching.

Variables	Before propensity matching			After propensity matching		
	Low baPWV n=520	High baPWV n=362	P-value	Low baPWV n=180	High baPWV n=180	P-value
PLR	119.55 ± 49.47	144.71 ± 75.84	<0.001	114.30 ± 51.99	150.69 ± 88.37	<0.001
NLR	2.66 ± 1.29	3.83 ± 2.70	<0.001	2.55 ± 1.59	3.93 ± 3.30	<0.001
PLR	0.23 ± 0.11	0.30 ± 0.16	<0.001	0.24 ± 0.12	0.30 ± 0.15	<0.001

TABLE 4 Logistic regression analysis of PLR, NLR, and MLR and the risk of arterial stiffness.

	Model 1				Model 2				Model 3			
Variables	B	S.E.	Wald	P-value	B	S.E.	Wald	P-value	B	S.E.	Wald	P-value
PLR	0.765	0.215	12.689	<0.001	0.785	0.218	12.950	<0.001	0.800	0.222	13.022	<0.001
NLR	1.238	0.221	31.385	<0.001	1.339	0.234	32.885	<0.001	1.361	0.238	32.686	<0.001
MLR	0.719	0.214	11.256	<0.001	0.756	0.225	11.329	<0.001	0.779	0.230	11.413	<0.001

Model 1: unadjusted.
Model 2: adjustment for gender, age, disease course, BMI, smoking, drinking, SBP, and DBP.
Model 3: adjustment for gender, age, disease course, BMI, smoking, drinking, SBP, DBP, HBA1c, FBG, TG, TC, HDL-C, LDL-C, and UA.

patients with acute coronary syndrome (19). Cai et al. found an independent association between NLR and arterial stiffness while studying patients who underwent peritoneal dialysis (20). Yu et al. found that the NLR in individuals with osteoporosis was higher and positively linked with baPWV (21). Some studies have reported the relationship between PLR and MLR and arterial stiffness. In a study of patients with peritoneal dialysis, Chen et al. found an independent association between high PLR and arterial stiffness (22). Wang et al. reported that MLR may be a potential predictor of arterial stiffness, but they used the cardio-ankle vascular index as a measure of arterial stiffness (23). However, these studies lacked a direct comparison of the three leukocyte subtype ratios in predicting the pros and cons of arterial stiffness. To the best of our knowledge, our study is the first to directly compare PLR, NLR, MLR, and the combined diagnosis to determine the one that was superior as a predictive value in evaluating arterial stiffness. In addition, the above studies are all retrospective in nature; thus, there will inevitably be uneven baselines during comparisons between the experimental and control groups. Our study was the first to adopt the method of tendency matching to reduce bias and ensure that the baseline of the data was as even as possible.

We found that high values of PLR, NLR, and MLR were independent predictors of increased arterial stiffness in patients with diabetes. Neutrophils, monocytes, and lymphocytes are representative leukocyte subtypes, and the mechanism of their involvement in increased arterial stiffness may be related to vascular adhesion protein (VAP)-1, which not only functions as an adhesion molecule but also as an amine oxidase that is involved in the production of aldehydes and hydrogen peroxide. Owing to its semicarbazide-sensitive amine oxidase activity, it is implicated in vascular injury, as the breakdown of primary amines releases formaldehyde and methylglyoxal, which cause direct cytotoxic damage to endothelial cells (24). The primary relationship between VAP-1 and arterial stiffness is inflammation, which causes an imbalance in the production and degradation of collagen and elastin fibers (25). VAP-1 functions as an adhesion

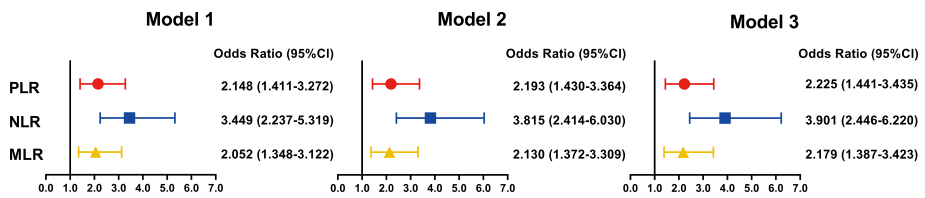


FIGURE 2
Univariate and multivariate logistic regression forest map of adjusted factors and the risk for arterial stiffness.

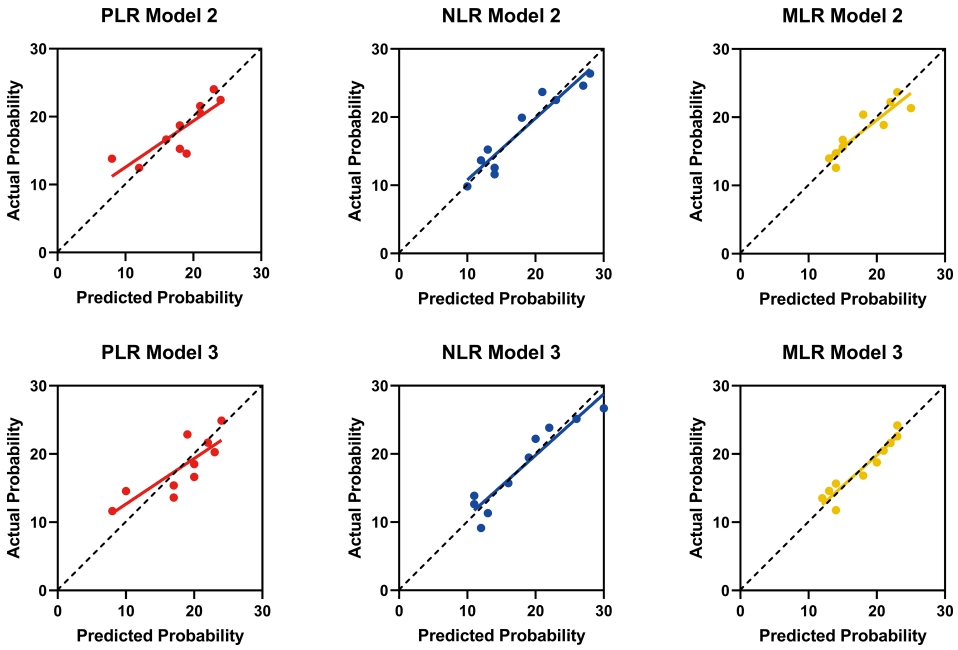


FIGURE 3
Calibration Diagram Model 2 and Model 3 of PLR, NLR, and MLR.

molecule in the transfer of lymphocytes, granulocytes, and monocytes from the blood and participates in blood vessel–wall rolling, adhesion, and migration (26). It is also linked to endothelial dysfunction and AGE synthesis, both of which contribute to arterial stiffness. Our findings further strengthen the current evidence supporting the potential use of lymphocyte biomarkers to assess cardiovascular and cerebrovascular risk in subjects with diabetes. These biomarkers are superior to other conventional markers owing to their low cost, ease of analysis, and rapid results. Single blood parameters are easily disturbed by concentration, overhydration, and blood sample–processing methods among other factors. As PLR, NLR, and PLR values are ratios, their values are relatively more stable compared with single blood parameters (27). Arterial stiffness is a chronic condition that lasts for several years. However, it is clinically silent and difficult to detect in its initial stages; thus, it is typically detected at a later stage. Therefore, this simple and effective approach could help in the early identification of arterial stiffness in patients with diabetes.

In this study, we adopted baPWV as a method to measure arterial stiffness in inpatients with diabetes. BaPWV is based on oscillometry and widely used in clinical practice, especially in Asian countries, owing to its advantages of simplicity, noninvasiveness, and reproducibility (5). At the same time, arterial stiffness can also be reflected by measuring the PWV of other segments, such as the carotid-femoral PWV (cfPWV), carotid-radial PWV (crPWV), and aortic PWV (aPWV). However, baPWV and cfPWV are most widely used in clinical practice. Although cfPWV is the gold standard for measuring

TABLE 5 ROC analysis of MLR, NLR, PLR, and combined diagnosis for arterial stiffness.

Variables	AUC	95% CI	Youden's index	Sensibility	Specificity	P-value
PLR	0.6477	0.591-0.704	0.239	66.7%	57.2%	<0.001
NLR	0.7194	0.667-0.772	0.355	84.4%	51.1%	<0.001
MLR	0.6479	0.591-0.704	0.262	80.6%	45.6%	<0.001
PLR+NLR	0.7185	0.666-0.771	0.338	77.4%	59.4%	<0.001
PLR+MLR	0.6646	0.609-0.720	0.245	42.8%	81.7%	<0.001
NLR+MLR	0.7177	0.665-0.770	0.361	77.8%	58.3%	<0.001
PLR+NLR+MLR	0.7192	0.667-0.772	0.356	75.6%	60.0%	<0.001

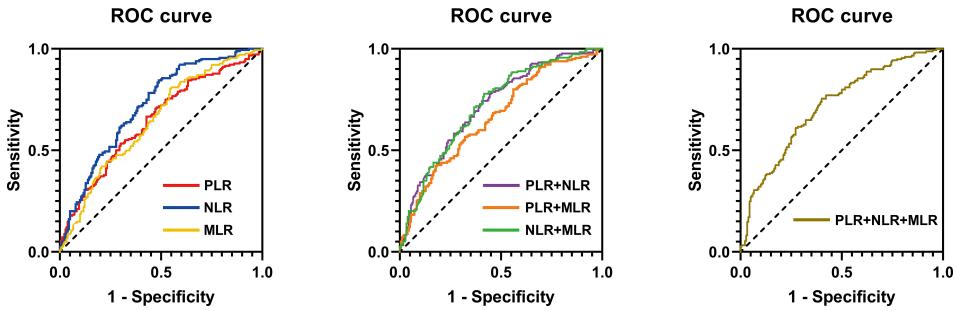


FIGURE 4
ROC curve for MLR, NLR, PLR, and combined diagnosis for predicting arterial stiffness.

arterial stiffness based on arterial tonometry, they have a different focus than baPWV; cfPWV mainly reflects aortic stiffness, whereas baPWV mainly reflects peripheral arterial stiffness, which is not negligible in patients with diabetes. Meanwhile, baPWV is easier to operate compared with cfPWV. The blood pressure cuff needs to be bound to the limb of the patient without exposing the groin area. BaPWV is more suitable for epidemiological studies involving large populations and its reliability has been clinically verified (28). In addition to arterial tonometry and oscillometry, the other modalities for PWV measurement include ultrasound imaging (29), bioimpedance plethysmography (30), photoplethysmography (31), and magnetic resonance imaging (32). However, these methods are rarely used in clinical practice owing to limitations such as high price, complex operation protocols, and limited results. As this was a retrospective study, only baPWV was used to measure arterial stiffness. We intend to carry out prospective studies to increase our knowledge on cfPWV and other related indicators of arterial stiffness as a means to further explore the relationship between arterial stiffness and NLR, PLR, and MLR.

Our study has some limitations. This single-center study only included patients of Asian/Chinese descent. Whether the results of the study can be extrapolated to other races should be further determined. We used baPWV instead of the gold standard cfPWV as a measure of arterial stiffness. Although the American Heart Association recommends baPWV as a common measure of arterial stiffness (5), relative to cfPWV, baPWV may be limited by the inclusion of long arterial muscle segments. This may lead to misclassification of the assessment of arterial stiffness (33). As this was a retrospective study, we could not collect adequate data on inflammatory factors, such as hsCRP, IL-6, and TNF-α, which are associated with other chronic metabolic diseases. Unfortunately, these factors were not compared with PLR, NLR, and MLR.

Conclusions

As a novel, convenient, and inexpensive inflammatory marker, NLR is superior to PLR, MLR, and combined diagnosis, and has a certain predictive value to identify the increase in arterial stiffness in patients with diabetes. Thus, this biomarker can be considered in a clinical setting to help identify the increase in arterial stiffness in individuals with diabetes as an approach to disease prevention or early intervention.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of the Chengdu Fifth People's Hospital(No. 2022-020S-01). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

PN and HC conceived and designed the study. JY, YT, and YO collected and cleared the data. PN and FY analyzed and interpreted the data. PN, JK, JZ, HW, and HC drafted and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1039700/full#supplementary-material

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Metabolic remodeling of glycerophospholipids acts as a signature of dulaglutide and liraglutide treatment in recent-onset type 2 diabetes mellitus

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Aims: As metabolic remodeling is a pathological characteristic in type 2 diabetes (T2D), we investigate the roles of newly developed long-acting glucagon-like peptide-1 receptor agonists (GLP-1RAs) such as dulaglutide and liraglutide on metabolic remodeling in patients with recent-onset T2D.

Methods: We recruited 52 cases of T2D and 28 control cases in this study. In the patient with T2D, 39 cases received treatment with dulaglutide and 13 cases received treatment with liraglutide. Using untargeted metabolomics analysis with broad-spectrum LC-MS, we tracked serum metabolic changes of the patients from the beginning to the end of follow-up (12^{th} week).

Results: We identified 198 metabolites that were differentially expressed in the patients with T2D, compared to the control group, in which 23 metabolites were significantly associated with fasting plasma glucose. Compared to pre-treatment, a total of 46 and 45 differentially regulated metabolites were identified after treatments with dulaglutide and liraglutide, respectively, in which the most differentially regulated metabolites belong to glycerophospholipids. Furthermore, a longitudinal integration analysis concurrent with diabetes case-control status revealed that metabolic pathways, such as the insulin resistance pathway and type 2 diabetes mellitus, were enriched after dulaglutide and liraglutide treatments. Proteins such as GLP-1R, GNAS, and GCG were speculated as potential targets of dulaglutide and liraglutide.

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Conclusions: In total, a metabolic change in lipids existed in the early stage of T2D was ameliorated after the treatments of GLP-1RAs. In addition to similar effects on improving glycemic control, remodeling of glycerophospholipid metabolism was identified as a signature of dulaglutide and liraglutide treatments.

KEYWORDS

type 2 diabetes mellitus, metabolic remodeling, dulaglutide and liraglutide, Glycerophospholipids, LC-MS

Introduction

The rapid rise of type 2 diabetes mellitus (T2D) poses one of the greatest threats to global health. Diabetes was diagnosed in approximately 463 million adults aged 20 to 79 in 2019, according to the International Diabetes Federation. Furthermore, it is estimated that in 2045, the number of individuals with T2D will reach 700.2 million (1, 2). Obesity is generally considered to be a strong risk factor for diabetes (3). Overall, about 80% of people with T2D are overweight or obese. In the obese state, excessive accumulation of visceral fat causes adipose tissue dysfunction that strongly contributes to the development of obesity-related comorbidities (3, 4).It is one of the most important public health challenges in the world to tackle the dual epidemic of T2D and obesity, as both are associated with increased mortality and morbidity (5). Glucagon-like peptide-1 receptor agonists (GLP-1RAs) have been used to treat T2D and obesity for the past 10 years. A single GLP-RA, liraglutide, has been approved for treating obesity in people with type 2 diabetes because it reduces body weight (6). Furthermore, the cardiovascular benefits of GLP-1RAs have led to significant progress in the management of diabetes (7). A greater effect on fasting glucose has been observed with long-acting GLP-1RAs, such as dulaglutide and liraglutide, compared with short-acting GLP-1RAs, mediated through insulinotropic and glucagonostatic actions. A head-to-head clinical trial found that once-weekly dulaglutide had a similar safety and tolerability profile to once-daily liraglutide for the reduction of HbA1c (6, 8). Despite these successes, less is known about the benefits of metabolic remodeling with the treatment of GLP-1RAs, in addition to improved glycemic control and weight loss.

Both humans and animal models have been examined for metabolic changes associated with T2D and obesity (9–11). Obese and diabetic individuals have both been found to have high levels of branched-chain amino acids (BCAAs) (leucine, isoleucine, and valine), as well as some of their tissue metabolites, while glutamine and glycine levels are decreased (12, 13). Other non-protein nitrogen compounds, such as uridine and uric acid, were significantly correlated with insulin resistance in ob. Insulin resistance was significantly associated with other nitrogen compounds other than protein, such as nucleotides and nucleosides, as well as their metabolites, namely uridine and uric acid in obese subjects (14, 15). Given the changes in serum metabolites that exist before the onset of metabolic disease, longitudinal studies suggest that BCAA and hyocholic acid species may serve as novel biomarkers for the early detection and differential diagnosis of metabolic syndrome (16, 17). Metabolomics has been identified as a useful tool for evaluating the metabolic effects of glucoregulatory and weight loss-promoting drugs, including GLP-1RAs. Despite the non-inferiority of a once-weekly GLP-1RA dulaglutide agonist to once-daily liraglutide, knowledge of the metabolic differences in response to different long-acting GLP-1RAs is still sparse, as previous study only reported the modulation of gut microbiome following the treatment with GLP-1RAs in the animal model (18). A high-resolution landscape of metabolites during GLP-1RAs treatment is still poorly understood. Therefore, to investigate the metabolic remodeling in relation to GLP-1RAs, we were motivated to perform the metabolomics analysis in recent-onset T2D individuals with GLP-1RAs treatment.

Here, 52 recent-onset T2D cases and 28 control cases were recruited. We first identified a few metabolites related to T2D and metabolic pathways that offer a comprehensive view of the metabolite changes in T2D. In patients with T2D, 39 cases received dulaglutide treatment (once-weekly, 1.5 mg, DU group) and 13 cases received liraglutide treatment (once-daily, 1.8 mg, LIGA group), respectively. We then determined the effects of pharmacologically stimulated GLP-1 receptor function on metabolic remodeling, and the differences in improved metabolism during 12-week treatment by longitudinal analysis. Lastly, we integrated the longitudinal analysis with the case-control status of diabetes to uncover metabolic pathways and potential targets associated with GLP-1RAs.

Materials and methods

Study design and participants

The specialist inpatients were adults (≥ 18 years) with BMI ≥ 24 kg/m^2, and newly diagnosed with T2D (known duration of diabetes less than 1 month). The diagnosis of T2D was based on WHO criteria. All subjects went through three periods: intensive insulin therapy phase (1 week), switching to GLP-1RAs (at baseline), and follow-up (12 weeks). Participants in the study were interviewed between September 15, 2021, and February 30, 2021. The criteria for short-term continuous subcutaneous insulin infusion (CSII) included HbA1c ≥ 9.0% or fasting blood glucose ≥ 11.1 mmol/L or patients with obvious symptoms of hyperglycemia. We assigned participants to DU or LIGA group according to the patient's personal choice. Exclusion criteria included the use of other anti-hyperglycemic drugs, the clearance of creatinine less than 90 mL/min, or a history of pancreatitis or gastrointestinal surgery.

Twenty-eight lean, metabolically healthy controls (CTL, 17 male, 11 female; age 45.18 ± 11.48 SD; BMI 22.78 ± 1.31 SD) were enrolled in the clinics of Tongren Hospital. Criteria for "metabolically healthy" are as follows: no previous history of hypertension and systolic blood pressure (SBP)/diastolic blood pressure (DBP) < 140/90 mmHg; fasting blood glucose (FBG) < 6.1 mmol/L, OGTT (2 h) <7.8 mmol/L and no previous history of diabetes; no previous history of high cholesterol (total cholesterol (TC) <5.18 mmol/L) and fasting serum high-density lipoproteincholesterol (HDL-c) ≥ 0.9 mmol/L (men) or ≥1.0 mmol/L (women) and fasting plasma TG <1.7 mmol/L; no history of cardiovascular or endocrine disease.

the Internal Review and Ethics Boards of Tongren Hospital, which is affiliated with Shanghai Jiao Tong University, has approved this clinical study (No. 2021-059-01); informed consent was obtained from all patients. Good clinical practices and country-specific requirements were followed according to the Declaration of Helsinki.

Clinical measurements

We assessed the following characteristics of the participants: sex, region, age, body weight, height, waist circumference, blood pressure, and body mass index (BMI). Plasma samples were collected from participants after a fast of 8 to 12 hours. An automatic biochemical analyzer was used to determine biochemical indexes, including FBG, HbA1c, alanine aminotransferase (ALT), aspartate aminotransferase (AST), TC, TG, low-density lipoprotein cholesterol (LDL-C), HDL-C and creatinine (CR) (AU5800 clinical chemistry analyzer; Beckman Coulter Inc., Brea, CA, USA), Serum insulin and c-peptide were measured by radioimmunoassay. Based on all analyses, there was 6% intra-assay variation and 10% inter-assay variation. The Homeostasis Model Assessment (HOMA2) Estimates steady state beta cell function (%β) and insulin resistance index (IR), as measured by the HOMA2 Calculator (https://www.dtu.ox.ac.uk/homacalculator/).

Sample collection and preparation

The fasting serum samples were collected from 80 participants before treatment (52 newly diagnosed T2D cases and 28 controls). Thirty-one of them (25 cases out of 39 samples in the DU group and 8 cases out of 13 samples in the LIGA group) provided serum samples for metabolomics after 12-week treatment. The fasting serum samples were remained after clinical indexes determination and immediately stored at -80 ° C. The serum was vortexed for 2 minutes after being added to 300 μl of methanol (containing a standard of 5 g/ml L-2-chlorophenylalanine). A 200-μl supernatant was obtained after centrifugation at 13,000 rpm at 4 °C for 10 minutes. In order to prepare the quality control samples, the same volume of serum was taken from all samples and mixed evenly.

Untargeted metabolomics by LC–MS

A UPLC system equipped with an electrospray ionization source was used for non-targeted metabolomics profiling of serum samples. A data dependent (dd-MS2, Top N=10) MS/MS mode was used with a full scan mass resolution of 17,000 at an m/z of 200. A range of 100 to 1,500 was scanned. A brief description of the chromatographic conditions is given below: the injection volume was 2 μl, the column temperature was 25 ° C, the flow rate was 0.35 ml/min, and the mobile phase was liquid aqueous solution containing 0.1% formic acid, and liquid b-acetonitrile containing 0.1% formic acid. Optimal chromatographic gradients were: 0-2 min, 5% in liquid B; 2-10 min, 5-95% in liquid B; 10-15 min, 95% in liquid B; 15-18 min, 5% in liquid B. Thermo Xcalibur 2.2 software was used to acquire the data (Thermo Scientific, San Jose, USA).

Compound Discoverer software (Thermo Fisher Scientific) was used to align and extract peaks. After obtaining the retention time, m/z, and peak area information, we obtained a data table. In order to analyze the data, we used Simca-P software version 13.0 (Umetrics, Umea, Sweden) to perform a principal component analysis (PCA) and an analysis of partial least squares discrimination (PLSDA). An unsupervised PCA analysis was used to evaluate the overall trend in segregation between these samples. We normalized and Pareto-scaled the ion peaks. A quality assessment was performed using R2Y(cum)

and Q2(cum) parameters for the PLS-DA models generated. Significant differences in metabolites between the T2D and control groups were detected using a supervised PLSDA analysis model. Variables with variable importance in the projection (VIP) values >1.0 were selected based on the PLS-DA model, and a two-tailed Student's t-test by SPSS Statistics 18.0.0 was used to calculate p value, and p value < 0.05 was considered statistically significant. Using partial correlation analysis, metabolic traits of the disease (age, BMI, blood pressure, glucose/insulin/HOMA-%β) were determined that exhibit the strongest association with metabolites showing significantly different levels between the diseases. A standard metabolite library was used to identify metabolites, matching their exact mass, fragment ion mass, and retention time. The online processing tool of metabolomics data was used to analyze metabolic pathways to further predict the molecular mechanism of dulaglutide and liraglutide for the treatment of GLP-1RAs. MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/) and Genecards (https://www.genecards.org/) were used to identify and beneficiate affected metabolic pathways. An interaction network between compound-protein-pathway was developed to explain the functional mechanism. The enriched metabolic pathways were merged with Genecards above by Pharm mapper to produce a virtual predictive target through MetaboAnalyst 4.0 (http://www.lilab-ecust.cn/pharmmapper/). In order to estimate the importance of each node, we used two well-established measures, degree and betweenness centrality, and STRING exported all these parameters (https://www.string-db.org/).

Statistical analysis

A statistical analysis was conducted using IBM SPSS 25 and Thermo Xcalibur 2.2 software. (Thermo Scientific, San Jose, USA). A normal distribution was ensured by log-transforming or taking the square root of skewed variables. As appropriate, comparisons were conducted using t-tests, Wilcoxon-Mann-Whitney tests, and one-way ANOVAs. Significance was defined as $p \leq 0.05$. For comparing ordinal and non-normal variables, non-parametric tests were used.

Results

The characteristics of study subjects and the research design

The study recruited 52 recent-onset T2D cases and 28 control cases. In total, 39 incident T2D cases treated with once-weekly dulaglutide 1.5 mg (DU group) and 13 cases treated with once-daily liraglutide 1.8 mg (LIGA group), and completed a 12-week treatment (Figure 1). The detailed information used for the demographic and clinical analysis is listed in Figure 2. Serial blood samples from pre-treatment, 4-week and 12-week post-treatment were collected and profiled for metabolomics. The recent-onset T2D group was made up of individuals with an age range of 28 to 65 (the median was 47, the interquartile range was 37 to 58) years old, a BMI of 28.78 ± 3.92 kg/m^2. Among them, 63.50% were male. Neither age nor race/ethnicity were significantly different (Table S1 and Figure 3A). The detailed demographic information and the baseline metabolic characteristics of the participants were listed in Table S1. In the T2D group, the BMI ($p = 0.001$), FBG ($p = 0.001$), and HbA1c ($p = 0.001$) were higher than in the CTL group (Table S1 and Figures 3B, C), whereas HOMA2-%β was significantly decreased in T2D patients (Figure 3D). Individuals with T2D had higher blood pressure ($p= 0.001$), TG ($p = 0.003$), TC ($p = 0.003$), and LDL-c ($p = 0.001$) in comparison to controls (Table S1).

A total of 1,147 metabolites were included in the final dataset after data curation and annotation. The dataset was used to i) characterize the differentiation of metabolite profiles and the alteration of metabolite groups between the recent-onset T2D cases and metabolically healthy controls, ii) shape and present a trajectory of metabolic remodeling during GLP-1RA treatment by tracking metabolic changes across individuals, and determined the universal and differential response to dulaglutide and liraglutide by longitudinal analysis, iii) identify metabolic pathways and potential targets associated with GLP-1RAs in follow-up samples concurrent with diabetes case-control status.

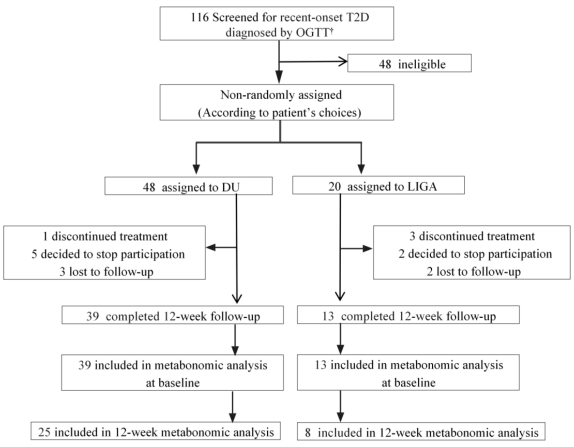


FIGURE 1
The trial profile of this study. † Data were collected from September 15, 2021, to November 10, 2021. T2D, type 2 diabetes; OGTT, oral glucose tolerance test; DU, dulaglutide; LIGA, liraglutide.

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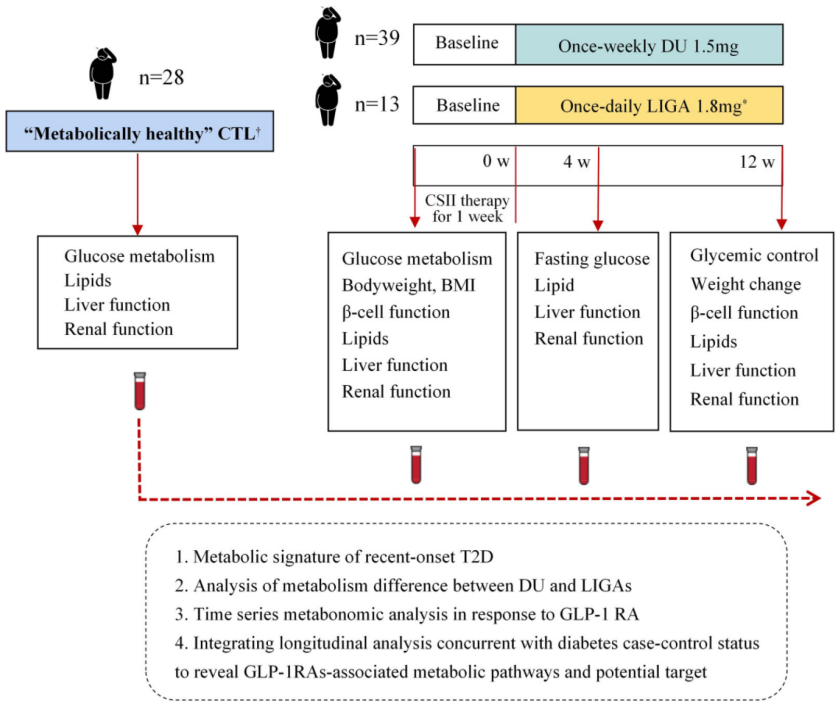


FIGURE 2
Study design and comparative analysis of metabolic profiles. *Liraglutide was up titrated from 0.6 mg/day in the first week, to 1.2 mg/day in the second week, and then to 1.8 mg/day in the third week. † Criteria for "metabolically healthy" are as follows: no previous history of high blood pressure and systolic blood pressure (SBP)/diastolic blood pressure (DBP) < 140/90 mmHg; no previous history of diabetes and fasting blood glucose (FBG) < 6.1 mmol/L, OGTT (2 h) < 7.8 mmol/L and; no previous history of high cholesterol (total cholesterol (TC) < 5.18 mmol/L) and fasting plasma TG < 1.7 mmol/L and fasting serum high-density lipoproteincholesterol (HDL-c) ≥ 0.9 mmol/L (men) or ≥ 1.0 mmol/L (women); no history of cardiovascular or endocrine disease. DU, dulaglutide; LIGA, liraglutide; CTL: control; CSII, continuous-subcutaneous insulin infusion; BMI, body mass index; CGMS, continuous glucose monitoring system; GLP-1 RA, glucagon-like peptide-1 receptor agonist.

The modulation of metabolic signatures and metabolites in the early stage of diabetes development

The metabolic profiles of participants with recent-onset T2D and those of CTLs at baseline are well separated by partial least squares-discriminant analysis (PLS-DA), suggesting significant metabolic changes in T2D compared to participants without T2D (Figures S2A–D). The differences in metabolite expression between T2D cases and controls were statistically significant (p value less than 0.05) for 198 metabolites, including 82 up-regulated and 116 down-regulated metabolites (the top 50 differentially regulated metabolites are shown in Table S2). The differential metabolites were visualized in the volcano plot (Figure 3E). The Spearman correlation analysis presented in the heat map showed that there was a significant correlation of all differential metabolites in the DU or LIGA treatment group, compared to the CTL group (Figure 3F). More than half of 15 most upregulated metabolites in individuals with recent-onset T2D belong to glycerophospholipids (p value < 0.05), while the downregulated metabolites (N = 15) were mainly lipids or lipid-like molecules, such as lysophosphatidylcholines (lysoPC) and fatty acyls (Figure 3H).

The classes of 198 differentially regulated metabolites included carboxylic acids and derivatives (BCAAs, glycine and organic acids), lipids (phospholipids and sphingolipids, fatty acyls), and organooxygen compounds (Figure 3H), which were previously reported in the T2D cohorts (general adult population). Particularly, 94 glycerophospholipids showed differential regulation between the T2D and CTL groups, in which the majority of lysoPC (except for LysoPC a 16:1) were elevated in the T2D group, as well as the PC aa C group (except for PC aa C11:1 and C22:4) (Figure 3G and Table S2). It is interesting to note that amino acids (AAs) were detected both in up-regulated and down-regulated sets. Eight AAs (glutamine, alanine, glycine, isoleucine, ornithine, phenylalanine, norleucine and lysine) were up-regulated, with acetylglutamine, 4-Methylene-L-glutamine, isoglutamine, histidine being down-regulated. (Figure 3G and Table S2). Our analysis identified 23 metabolites that were significantly associated with FBG between

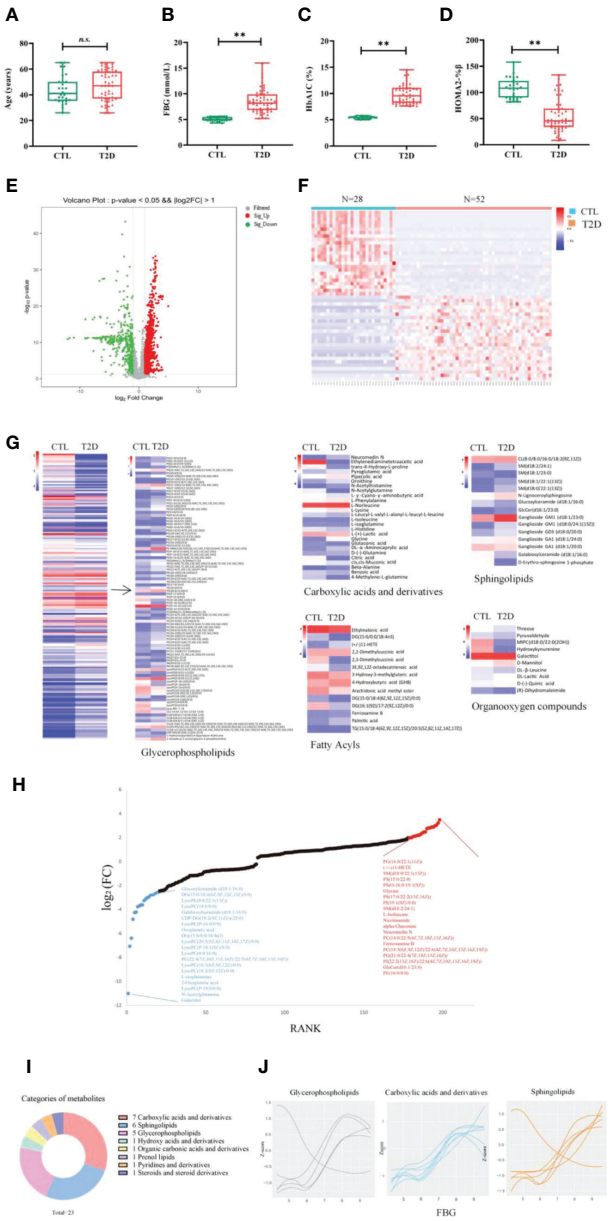


FIGURE 3
Change in metabolic signatures and metabolite groups at the early stage of diabetes development. The levels of age **(A)**, FBG **(B)**, HbA1c **(C)** and HOMA2%β **(D)** in controls (n = 28) and in patients with T2D at baseline (n = 52). Differences between the T2D group and the CTL group were tested by independent sample t test (normally distributed variables) **p < 0.01. n.s. the difference is not statistically significant. **(E)** Volcano plot showing -$\log_{10}$ p-value against $\log_2$-fold change of 198 metabolites in T2D cases versus controls. **(F)** Hierarchical cluster analysis of differential metabolites in T2D at baseline, compared to the CTL group. **(G)** Comparison of the differential metabolites between the T2D and CTL groups. Fold changes are log transformed and indicated by color scale in matrix. **(H)** Plot shows the top 15 increased (red) and decreased (blue) metabolites in T2D. **(I)** The categories of the 23 metabolites significantly associated with FBG (correlation coefficient >0.7). **(J)** The values of three main categories of FBG-associated metabolites are plotted with a Loess curve against the FBG. FBG, fasting blood glucose; HbA1c, glycated hemoglobin; HOMA2%β, the Homeostasis Model Assessment (HOMA) estimates steady state beta cell function (%β).

the two cohorts. This set of 23 metabolites associated with FBG were primarily carboxylic acids and derivatives (n = 7), sphingolipids (n = 6), and phosphatidylcholines (n = 5) (Figures 3I, J). Glycine was the most significantly associated metabolite (Pearson correlation coefficient 0.91, p value 0.05).

Analysis of cross-sections at follow-up: The metabolome changes in response to GLP-1 RAs and the implications for glycerophospholipid metabolism remodeling

Post-treatment samples (25 cases out of 39 samples in the DU group) were used to gain further insight into the metabolic changes associated with GLP-1RAs, while 8 cases out of 13 samples in the LIGA group) were further analyzed cross-sectionally to identify the GLP-1RAs-associated metabolites. Age, BMI, FBG, and HbA1c at baseline did not differ significantly between the groups (Figures S3A, B, E, F), as well as other key clinical characteristics, including blood pressure, liver function, kidney function, and blood lipids (Figure S3). Compared to baseline, participants in the DU and LIGA groups after 12 weeks of follow-up showed significantly lower levels of FBG, 2-hour postprandial blood glucose, body weight, and HbA1c (Figures 4A, B, S4A, C). The glucose metric analysis in both groups was calculated using CGMS data (n=20 in the DU group, n=10 in the LIGA group) during 4 weeks of treatment. The quality of the glucose control, as evaluated by the percentage of time in the range of 8.0 to 10.0mmol/L, did not differ statistically between the DU group and the LIGA group (Figure 4C). An increment in HOMA2%β after 12 weeks of treatment was observed in both groups (Figure 4D). Improvements in glycemic control, insulin resistance, and body weight were similar in two groups (Figures 4A, S4A–D).

Furthermore, in both the DU and LIGA groups, the well-separated metabolic profiles before and after treatments (4 weeks or 12 weeks of treatment) in the PLS-DA score plot indicate the significant effect of GLP-1RAs on metabolomics (Figures S5A–D). Differential metabolites were visualized in the volcano plot (Figures 4E, F). After 12 weeks, the DU and LIGA groups had 46 and 45 differentially regulated metabolites, respectively (log2FC >1.2, p value<0.05) (Figures 4G, H). In order to gain a deeper understanding of T2D metabolic pathways, all differential metabolites were analyzed using the KEGG pathway analysis. As indicated by KEGG pathway analysis, glycerophospholipid metabolism was profoundly affected in both groups (p value = 0.001) (Figures 4I, S5E, F), suggesting its important role in association with dulaglutide and liraglutide treatments. For dulaglutide treatment, other metabolic pathways, including the citrate cycle (TCA cycle), β-alanine metabolism, glycolysis, histidine metabolism, and pyruvate metabolism, were also enriched.

Integrating longitudinal analysis with case-control diabetes status to identify metabolic pathways associated with GLP-1RAs

To further examine metabolite dynamics and illuminate metabolic pathways, we traced the dynamic changes of metabolites during 12-week treatment in each group. All differentially expressed metabolites detected in the DU group and the LIGA group compared to the baseline were listed in Tables 1, 2, respectively. In the DU group, PC (16:1/22:6), PE (16:0/0:0), PS(O-20;0/14:0) were elevated before treatment, but showed a decrease pattern after 4 weeks and 12 weeks of treatment. On the contrary, lysoPC (P-16:0, P-18:0) and lysoPE (22:1) showed a greater decrease in T2D cases but increased after 12 weeks of treatments (an increase of 15%) (Figure 5A). Similar trends were observed in the LIGA group, such as PE (18:1/0:0), PS (16:0/22:1), PS (15:0/22:0) and PS (17:1/22:2) showed a greater decrease in T2D cases but increased after treatments (Figure 5B). Notably, like our findings from the cross-sectional analysis after treatment, most differentially regulated metabolites identified by longitudinal analysis also belong to glycerophospholipids, which emphasizes the association of remodeling glycerophospholipid metabolism and GLP-1RAs treatments. Our results suggested that there was a metabolic dysmetabolism of lipids present in the baseline status of the patients with newly onset T2D, which ameliorated partially during GLP-1RAs treatments. In the DU group, differential metabolites, such as the carboxylic acids and derivatives (e.g., Neuromedin N and L-Histidine), sphingolipids (e.g., TG (15:1/18:4) and Glc-GP (18:0/20:4) were also altered in response to 12 weeks of dulaglutide treatment (Figure S6A). Additionally, 4-hydroxybutyric acid (GHB) increased at baseline, but decreased at follow-up in the LIGA group (Figure S6B).

Compound-protein-pathway interactions were established to better understand metabolite pathways and their metabolic processes. Significant (p value < 0.05) pathways were chosen as potential targets. In particular, the insulin resistance pathway (p value < 0.05) and type 2 diabetes mellitus (p value < 0.05) were found to be enriched in both the DU and LIGA groups (Figures 5C, D). Multiple pathways involved in energy metabolism are expressed in the DU group, including apelin metabolism, phospholipase signaling pathway, Foxo signaling pathway, glucagon signaling pathway, and regulation of lipolysis in adipocytes. The pathways with p value < 0.05 in the LIGA group were associated with apelin metabolism and the sphingolipid signaling pathway. Interestingly, the DU group was significantly more enriched in signals related to aldosterone synthesis, thyroid hormone production, and cortisol production, which may suggest a possible correlation with regulation of endocrine hormones. In the LIGA group, the finding of adrenergic signaling in cardiomyocyte metabolism indicated the role of LIGA in cardiovascular disease.

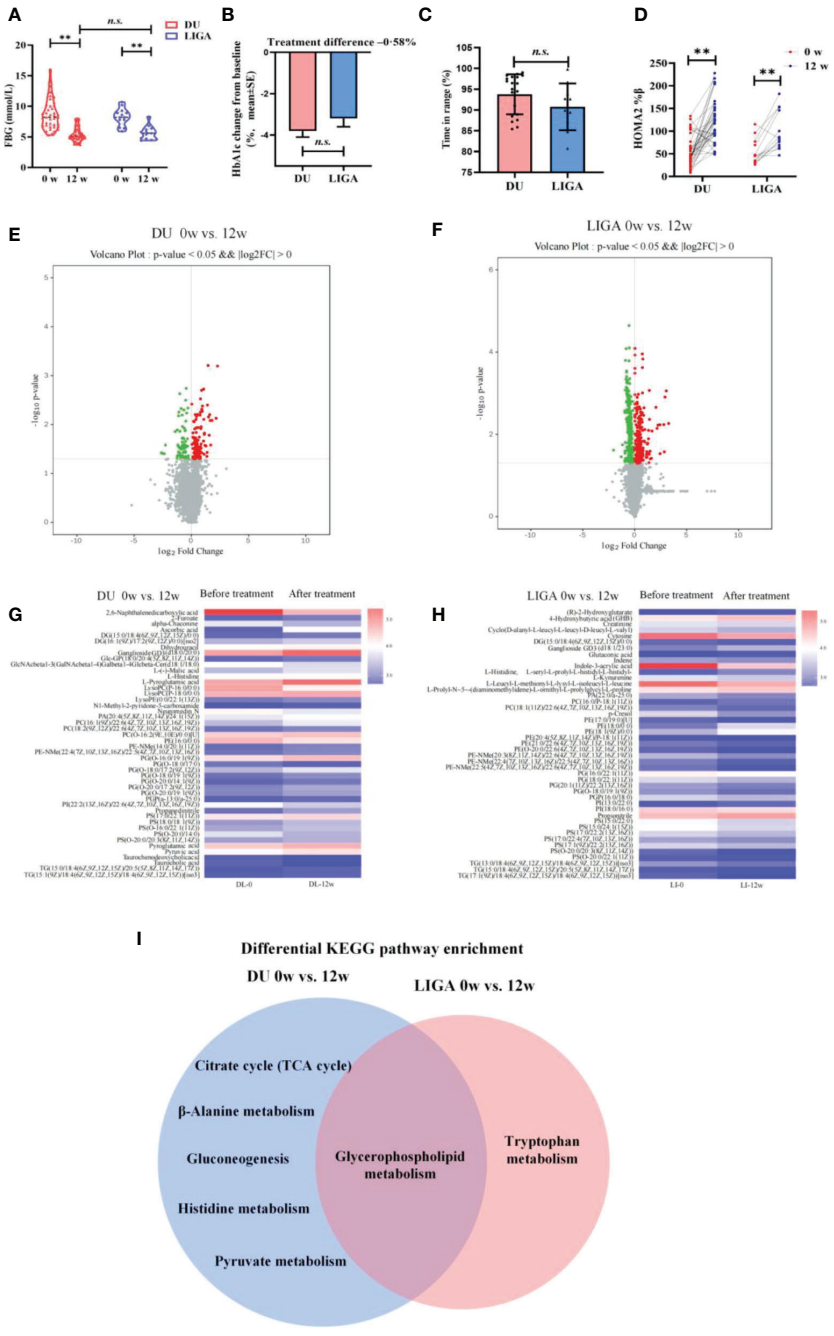


FIGURE 4
Cross-sectional analysis of metabolome changes in response to GLP-1RAs and implications for glycerophospholipid metabolism remodeling. **(A)** The levels of FBG at baseline and 12 weeks after treatment. **(B)** The decreasing levels of HbA1c after treatment with DU and LIGA at baseline and 12th weeks. **(C)** Comparison of the quality of the glucose control, as evaluated by the percentage of time in the range of 8.0 to 10.0mmol/L. **(D)** Change in HOMA2%β index after treatment with DU and LIGA at baseline and 12th week. **(E)** Volcano plot of -$\log_{10}$ p-value against $\log_2$-fold change of 46 metabolites in the DU group at baseline and 12th week. **(F)** Volcano plot of -$\log_{10}$ p-value against $\log_2$-fold change of 45 metabolites in LIGA group at baseline and 12th week. **(G, H)** Comparison of differential metabolites before and after treatments in the DU and LIGA groups. Fold changes are log transformed and indicated by color scale in matrix. **(I)** Venn diagram depicting KEGG pathway analysis of differential metabolites in the DU and LIGA groups. Differences between T2D and CTL were tested by independent sample t test (normally distributed variables). **p < 0.01. n.s. the difference is not statistically significant. BMI, body mass index; FBG, fasting blood glucose; HbA1c, glycated hemoglobin; DU, dulaglutide; LIGA, liraglutide; HOMA2%β, the Homeostasis Model Assessment (HOMA) estimates steady state beta cell function (%β).

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TABLE 1 The longitudinal trajectories of 22 significant metabolites in response to dulaglutide overlapped with those of T2D at baseline.

Name	Class
PG(O-20:0/14:1(9Z))	Glycerophospholipids
PE(16:0/0:0)	Glycerophospholipids
PG(O-18:0/17:2(9Z,12Z))	Glycerophospholipids
LysoPC(P-16:0/0:0)	Glycerophospholipids
PI(22:2(13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Glycerophospholipids
LysoPC(P-18:0/0:0)	Glycerophospholipids
LysoPE(0:0/22:1(13Z))	Glycerophospholipids
PC(16:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Glycerophospholipids
PS(O-20:0/14:0)	Glycerophospholipids
PG(O-16:0/19:1(9Z))	Glycerophospholipids
PS(O-16:0/22:1(11Z))	Glycerophospholipids
Neuromedin N	Carboxylic acids and derivatives
L-Histidine	Carboxylic acids and derivatives
Pyroglutamic acid	Carboxylic acids and derivatives
DG(16:1(9Z)/17:2(9Z,12Z)/0:0)	Fatty Acyls
TG(15:1(9Z)/18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))	Glycerolipids
Glc-GP(18:0/20:4(5Z,8Z,11Z,14Z))	Glycerolipids
alpha-Chaconine	Steroids and steroid derivatives
Ganglioside GD3 (d18:0/20:0)	Sphingolipids
L-(-)-Malic acid	Hydroxy acids and derivatives
2-Furoate	Furans
Dihydrouracil	Diazines

TABLE 2 The longitudinal trajectories of 14 significant metabolites in response to liraglutide overlapped with those of T2D at baseline.

Name	Class
PGP(16:0/18:0)	Glycerophospholipids
PC(18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	Glycerophospholipids
PE(17:0/19:0)	Glycerophospholipids
PS(15:0/22:0)	Glycerophospholipids
PS(17:1(9Z)/22:2(13Z,16Z))	Glycerophospholipids
PS(17:0/22:2(13Z,16Z))	Glycerophospholipids
PG(16:0/22:1(11Z))	Glycerophospholipids
PI(13:0/22:0)	Glycerophospholipids
PA(22:0/a-25:0)	Glycerophospholipids
Glutaconic acid	Carboxylic acids and derivatives
PE(18:1(9Z)/0:0)	Carboxylic acids and derivatives
4-Hydroxybutyric acid (GHB)	Fatty Acyls
Cytosine	Diazines
(R)-2-Hydroxyglutarate	Hydroxy acids and derivatives

Further, the topological parameter was used to investigate the core network integrating metabolites and proteins in order to identify significant proteins that correlate with the metabolites found in the study. In the core network, all nodes had a degree greater than 3. Table 3 shows that these proteins may be potential targets for dulaglutide or liraglutide. For example, GLP1R1 and GNAS proteins were related to insulin resistance. These potential targets provided us with some clues about the metabolic mechanism of GLP-1RAs in the treatment of T2D.

Discussion

In our study, we offered a comprehensive view of the metabolite changes in T2D. Then we determined the effects of pharmacologically stimulated GLP-1 receptor function on metabolic remodeling and compared the differences in improved metabolism during 12-week treatment by longitudinal analysis. This is the first study to implement metabolomics to comprehensively explore the dynamic metabolic responses of T2D patients treated with GLP-1RAs, and to distinguish the differences in the metabolic remodeling of two GLP-1RA drugs (i.e., dulaglutide and liraglutide).

Compared to metabolically healthy subjects with normal weight, 198 differentially regulated metabolites were identified in T2D cases, and the major classes including glycerophospholipids, carboxylic acids, and derivatives (i.e., BCAAs, glycine, and organic acids), were significantly regulated (Figures 3F, G). Increasing evidence from human and animal studies indicates that a number of metabolites could contribute to specific metabolic disturbances in the pathogenesis of metabolic syndrome (e.g., glucose tolerance, obesity, insulin resistance, and NAFLD) (19, 20). The level of BCAAs among adults increased before the onset of T2D, which could impair pancreatic beta cell function and affect insulin signaling (21, 22). An analysis of multi-metabolite signatures of T2D revealed altered metabolic fingerprints associated with the metabolism of amino acids, carbohydrates, and microbiota metabolism (23). Many of the T2D-associated metabolites (e.g., glycerophospholipids, sphingolipids, and amino acids), which were revealed in other studies, were also validated in our study (24, 25). Among the altered metabolites in the T2D cases, the major class was glycerophospholipids. In addition, a small group of glycerophospholipids was also identified for their association with the treatment of dulaglutide or liraglutide. Glycerophospholipid perturbance has been linked to the pathogenesis of diabetes in animal studies, as well as in prospective studies in humans (26). Our findings are in line with the known relationships between glycerophospholipid disturbance and the pathogenesis of diabetes. Recently, in a prospective study, eight glycerophospholipids, especially PCs, were associated with the *de novo* lipogenesis pathway and positively correlated with incident diabetes among Asians (27).

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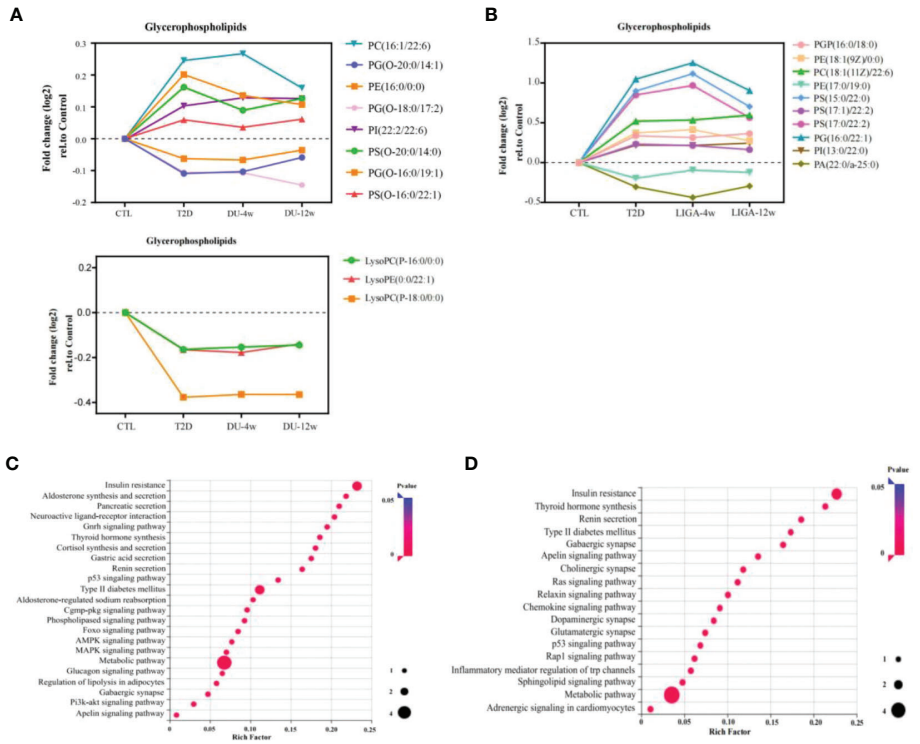


FIGURE 5
Integrating longitudinal analysis concurrent with diabetes case-control status to reveal GLP-1RAs-associated metabolic pathways and potential targets. **(A, B)** Longitudinal trajectories of significant metabolites involved in glycerophospholipid metabolism in response to DU and LIGA, respectively. **(C, D)** Regulated pathways associated with DU **(C)** and LIGA **(D)**. DU, dulaglutide; LIGA, liraglutide.

Compared to the patients with CTL, patients with recent-onset T2D had significantly higher levels of PC, whereas, surprisingly, the concentrations of lysoPC were lower in the T2D group. In a recent study, serum lysoPCs were associated with a reduced risk of type 2 diabetes (28). Previous studies found that lysoPC levels were lower in individuals with obesity, insulin resistance, and T2D (29–31). These findings suggest that the dynamic alterations of lysoPCs may potentially be associated with the pharmacological actions of GLP-1 receptor signaling by dulaglutide and liraglutide on glucose metabolism and lipid handling in T2D patients. Serum LysoPCs are primarily generated by secretory or lipoprotein-bound phospholipase A2 from lipoproteins or membrane-derived PCs. A second source of plasma lysoPC is endothelial lipase, which also creates HDL and oxidized LDL by secreting lecithin-cholesterol acyltransferase from the liver. On the other hand, lyso-PCs can also be reacylated into PC by lysoPC acyltransferase. Previous studies have reported that both lysoPCs and lysoPEs, grouped as LPs, were associated with a reduced risk of T2D (32). In adipocytes, lysoPC(C16:0) enhanced glucose uptake in an insulin-independent and protein kinase C-dependent manner (33). These findings provide evidence of the beneficial effects of lysoPC in metabolic diseases *in vivo* and *in vitro*. Furthermore, A significant change in amino acid metabolism occurred during the pathogenesis of T2D, in which some amino acids served as substrates for gluconeogenesis. It is worth noting that the metabolic pathways associated with T2D differed between studies due to the different inclusion criteria, heterogeneity regions, sample sizes, or analytical methods.

It is the first study to determine the trajectory changes of the metabolites in the cohorts after treatment with different GLP-1RAs. Both dulaglutide and liraglutide are widely advocated as means of improving glycemic control outcomes, but little is known about the metabolic remodeling in serum responsible for its beneficial effects. The metabolic profiles here revealed a common metabolic pathway, glycerophospholipid metabolism associated with dulaglutide or liraglutide treatment. At 12 weeks of follow-up, the levels of lysoPCs were partially attenuated by dulaglutide. LysoPC can also be transformed into lysophosphatidic acid, sphingosine-1-phosphate, and other extracellular signaling lipids by plasma lysophospholipase D/autotoxin (34). A previous study has reported that the serum levels of lysoPC and lysoPE, which were associated with higher risk of cardiovascular diseases, were decreased in T2D patients compared to controls (35). Circulating lysoPCs have been identified as markers of a metabolically benign nonalcoholic

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TABLE 3 The proteins of key protein-metab1ite network in the DU and LIGA groups.

DU group			LIGA group		
No.	Protein	Degree	No.	Protein	Degree
1	GLP1R	3.5759	1	GLP1R	3.5604
2	ARRB1	3.7672	2	GNAS	3.7517
3	GNAS	3.3513	3	LEG3	3.3358
4	GNGT1	3.0886	4	EGFR	3.0731
5	GCG	3.6718	5	TAP1	3.6563
6	GSR	3.9392	6	AMPK	3.9237
7	DCK	3.1178	7	HXK4	3.1023
8	FIBG	3.8304	8	GMPR1	3.8149
9	MAPK	3.3545	9	FKB1A	3.339
10	GSTT2	3.5978	10	AMY1A	3.5823
11	ABL1	3.4052	11	CCL5	3.3897
12	IMPA1	3.1767	12	Arl5b	3.1612
13	RAN	3.3574	13	BCAT2	3.3419
14	EGFR	3.5094	14	KTHY	3.4939
15	APAF	3.6077	15	AKT1	3.5922
16	GMPR2	3.5722	16	HINT1	3.5567
17	CHIT1	3.5137	17	HXK1	3.4982
18	ITPKA	3.9462	18	P07741	3.9307
19	ADK	3.0662	19	P06737	3.0507
20	DAPK1	3.0916	20	CBR1	3.0761

DU, dulaglutide; LIGA, liraglutide.

fatty liver, suggesting that lysoPCs may play an important role in the pathophysiology of nonalcoholic fatty liver disease and its progression (36). The patterns observed in these metabolites indicate alterations in glycerophospholipid metabolism by dulaglutide that could be involved in the mechanisms linking GLP-1RAs action to a beneficial role in the cardiovascular system and attenuation of nonalcoholic steatohepatitis (NASH). Even though GLP-1R agonists are beneficial for modifying risk factors such as body weight, blood pressure, and lipids, these changes over time do not fully explain their effects on the reduction in MACE. Currently, there is no clear understanding of how GLP-1R agonists attenuate NASH, and whether glycerophospholipids are indicative of the protective effect in liver should be further determined.

In Table 3, these proteins seem to be potential targets of dulaglutide and liraglutide. In terms of hypoglycemic effect and improvement in pancreatic function, dulaglutide on a weekly basis is not inferior to liraglutide daily. However, the metabolic pathways involved in the pathological mechanism of dulaglutide and liraglutide were distinct. The insulin resistance signaling pathway was enriched in both the DU and LIGA groups (Figures 5C, D). We have illustrated the possible molecular mechanisms involved in GLP-1 dependent insulin sensitivity, including the amelioration of oxidative stress, inflammatory responses, plasma lipid profile, and the induction of insulin signaling pathways (37–40). Unexpectedly, the neuroactive ligand-receptor interaction signaling pathway was enriched in the DU group, while several neurotransmitter related signals such as the GABAergic synapse, dopaminergic synapse, and glutamatergic synapse were enriched in LIGA group. *In vitro* and *in vivo* studies using GLP-1RAs have demonstrated the neurotrophic and neuroprotective effects of GLP-1 receptor stimulation (41–43). In addition, adrenergic signaling in cardiomyocyte signaling and p53 signaling pathway were significantly related in the LIGA group. Impressively, the roles of the β-adrenergic receptor-mediated signaling pathway and the p53-mediated signaling pathway in the apoptosis of cardiomyocytes have been reported as major factors in the pathogenesis of heart failure (44). Hence, the reduction in non-fatal myocardial infarction detected with liraglutide is unlikely to be explained solely by GLP-1 receptor-mediated actions; A better understanding of how GLP-1R activation reduces the risk of myocardial infarction and cardiovascular death is needed. GLP-1RAs may target these proteins, as shown in Table 3. GLP1R, GNAS, and GCG were related to insulin resistance, which were verified by the previous studies (45). Recently, Yulia A et al. have reviewed recent progress and discoveries of T2D pharmacogenetics, including GLP-1RAs, and found that *GLP1R*, *TCF7L2*, *CNR1*, *SORCS1* and *WFS1* are associated with therapeutic responses to GLP1-RAs. Several candidate gene studies have been conducted to assess the role of GLP1R gene variants in the efficiency of GLP-1RAs treatment (46–48). The findings indicate that the different mechanisms underlined in the effects of GLP-1RAs used for T2D treatment

are likely multifactorial. Although the proteins with high degrees of nodes may not represent the significantly altered proteins between the DU and LIGA groups, they might provide some insight into the underlying metabolism.

This study has three main strengths. First, since metabolic remodeling has been investigated in a well-characterized cohort with recent-onset T2D (49), our study was the first to compare two GLP-1RAs in metabolic remodeling in addition to the results of glycemic and extra-glycemic effectiveness. Second, we traced and profiled metabolic changes within individuals and performed a longitudinal analysis between two different long-acting GLP-1RAs. We found that glycerophospholipid remodeling acts as a metabolomic signature of dulaglutide and liraglutide. Third, the broader spectrum of glycerophospholipids in the cohort study allowed us to identify novel biomarkers and to uncover comprehensively the relationships of glycerophospholipids with GLP-1RAs and associated metabolic pathways, as a means of supporting the development of stratified and precision medicine.

There were several limitations to this study. First, the metabolic study had a relatively small sample size. Although the DU group was matched in age, sex, BMI, and key clinical indicators with the LIGA group, unmeasured factors such as dietary habits, medication, or supplements may also contribute. Second, the duration of 12 weeks of follow-up, although like that of phase III randomized controlled studies, was still relatively short. After 12 weeks of treatment, there was no clinically significant difference in body weight between the DU and LIGA groups. One potential explanation is that the duration of GLP-1RAs and the relatively small sample size could play a role, especially in the LIGA group. Finally, the DU group received the same guidance (low-calorie diet) from a dietitian as the LIGA group according to the clinical guideline of T2D. However, we did not collect data on diet composition and exercise record during the treatments.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Internal Review and Ethics Boards of Tongren Hospital, which is affiliated with Shanghai Jiao Tong University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Studies were designed by XRG, XW and SH. JD wrote the manuscript. And ZZ did the metabolomics study. Sample collection was performed by LX, XXG, WYL, WP, XJ, WL, NZ. Data was analyzed by JD and ZZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1097612/full#supplementary-material

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Adiponectin, IGFBP-1 and -2 are independent predictors in forecasting prediabetes and type 2 diabetes

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Objective: Adiponectin and insulin-like growth factor (IGF) binding proteins IGFBP-1 and IGFBP-2 are biomarkers of insulin sensitivity. IGFBP-1 reflects insulin sensitivity in the liver, adiponectin in adipose tissue and IGFBP-2 in both tissues. Here, we study the power of the biomarkers adiponectin, IGFBP-1, IGFBP-2, and also included IGF-I and IGF-II, in predicting prediabetes and type 2 diabetes (T2D) in men and women with normal oral glucose tolerance (NGT).

Design: Subjects with NGT (35-56 years) recruited during 1992-1998 were re-investigated 8-10 years later. In a nested case control study, subjects progressing to prediabetes (133 women, 164 men) or to T2D (55 women, 98 men) were compared with age and sex matched NGT controls (200 women and 277 men).

Methods: The evaluation included questionnaires, health status, anthropometry, biochemistry and oral glucose tolerance test.

Results: After adjustment, the lowest quartile of adiponectin, IGFBP-1 and IGFBP-2 associated independently with future abnormal glucose tolerance (AGT) in both genders in multivariate analyses. High IGFs predicted weakly AGT in women. In women, low IGFBP-2 was the strongest predictor for prediabetes (OR:7.5), and low adiponectin for T2D (OR:29.4). In men, low IGFBP-1 was the strongest predictor for both prediabetes (OR:13.4) and T2D (OR:14.9). When adiponectin, IGFBP-1 and IGFBP-2 were combined, the ROC-AUC reached 0.87 for women and 0.79 for men, higher than for BMI alone.

Conclusion: Differences were observed comparing adipocyte- and hepatocyte-derived biomarkers in forecasting AGT in NGT subjects. In women the strongest predictor for T2D was adiponectin and in men IGFBP-1, and for prediabetes IGFBP-2 in women and IGFBP-1 in men.

KEYWORDS

biomarkers, prospective study, abnormal glucose tolerance, adiponectin, IGFBP-1, IGFBP-2, gender

Introduction

Type 2 diabetes (T2D) is a multi-factorial disease represented by insulin resistance, impaired insulin secretion and hyperglycemia with obesity as the most common cause of insulin resistance. Although insulin production and sensitivity are the primary regulators of glucose homeostasis, other glucose-regulating factors are likely to play a role in the development of abnormal glucose tolerance (AGT). In this context, components of the insulin-like growth factor (IGF) system, which exert glucose metabolic effects, have been in focus (1–11). Recent studies showed that subjects with either low or high serum IGF-I levels are at increased risk of developing T2D (5, 7, 8). IGF-II has also been suggested to be involved in metabolic disorders and diabetes (4, 9). The IGF binding proteins, IGFBP-1 and -2, may influence glucose regulation and homeostasis by modulating IGF bioavailability (1, 2, 10, 11). Circulating IGFBP-1, mainly produced in the liver where insulin suppresses its production, reflects insulin production, hepatic and whole-body insulin sensitivity (2, 11–14). Low IGFBP-1 concentrations are predictive of future development of T2D (5, 15–18) and a stronger predictor of T2D than fasting plasma insulin and glucose (16, 17). IGFBP-2 is secreted by both liver and adipose tissues, and its secretion is regulated by leptin and nutrition (19–21). Serum IGFBP-2 is associated with insulin sensitivity with levels inversely correlated with plasma insulin and free IGF-I (18–20). Furthermore, low serum IGFBP-2 associates with increased fat mass, elevated triglyceride levels and increased risk of T2D (6, 18, 19, 21–23).

Adiponectin is an insulin-sensitizing peptide, secreted mainly by adipocytes (24). Serum levels of adiponectin are reduced in obese subjects with increased visceral fat (25). Adiponectin is primarily regulated by inflammatory cytokines and angiogenic factors. There is a close association between low adiponectin levels and future as well as present T2D (24–28).

In a recent study, a panel of six biomarkers including inflammatory markers, HDL cholesterol, IGFBP-2 and adiponectin improved prediction of future T2D when added to a clinical model including HbA1c, which was used instead of fasting glucose and insulin (29). However, according to baseline HbA1c levels the studied cohort included subjects with prediabetes. In the study by Schiffman et al., an insulin resistances score (IRScore) based on insulin and C-peptide improved diabetes risk assessment in older European subjects compared to established risk factors plus fasting glucose at baseline. The IRScore predicted T2D regardless of prediabetes (30).

The aim of our prospective study of middle-aged NGT healthy subjects, many with a family history of diabetes (FHD), was to investigate the predictive power of the liver and adipose tissue specific biomarkers of insulin sensitivity, IGFBP-1, IGFBP-2 and adiponectin, as well as of IGF-I and IGF-II, to forecast development of prediabetes and T2D. Moreover, we thought it was of interest to study men and women separately, since many of these biomarkers express gender differences.

Material and methods

Study population

This is a nested case control study based on a larger prospective cohort. The study population participated in an epidemiological survey, the Stockholm Diabetes Prevention Program. The design of the study has been described previously (31). Briefly, individuals aged 35-56 years without known diabetes were recruited for the baseline study performed in 1992-94 for men and in 1996-98 for women. Due to the study design, a selection was made so that the study cohort was enriched with participants with FHD (see Supplemental Flow Diagram). Thus, at baseline about 50% of the participants had a positive FHD, defined as known diabetes in at least one first-degree relative (father, mother, brother or sister) or at least two second-degree relatives (grand-parents, uncles or aunts). Eight to ten years later; in 2002-2004 for men and in 2004-2006 for women, a follow-up study was undertaken. All baseline participants were invited with the exception of those, who were diagnosed with T2D at baseline, had moved out of the Stockholm area or were deceased. In total 4,821 women and 3,128 men participated in the baseline study. Corresponding figures for the follow-up study were 3,329 (76% of invited) and 2,383 (87% of invited), respectively. Baseline and follow-up studies consisted of an extensive questionnaire covering lifestyle factors, a health examination including measurements of blood pressure, weight, height, hip and waist. Blood samples were taken after an overnight fast and 2-h after drinking 75 g

glucose (oral glucose tolerance test (OGTT)). Individuals, who progressed from normal glucose tolerance (NGT) at baseline to AGT at follow-up, were selected as cases. Thus, cases consisted of persons who developed prediabetes (IGT and IFG+IGT) (133 women, 164 men) or overt T2D (55 women, 98 men). A similar number of controls were randomly selected among subjects having NGT and negative FHD at both baseline and follow-up (200 women, 277 men), matched to cases by sex and age in five-year interval. Only subjects having data on all variables were included in the analyses.

The study, approved by the Ethics Committee at Karolinska University Hospital, was carried out in accordance with the Declaration of Helsinki. All participants gave informed consent.

Classification of glucose tolerance

Individuals were categorized after an OGTT according to 1999 WHO criteria (WHO 1999) as having NGT, impaired fasting glucose (IFG, between 6.1 and 7.0 mmol/L), impaired glucose tolerance (IGT, 2-h value between 7.8 and 11.1 mmol/L) or T2D.

Subjects having IGT or IFG plus IGT were defined as having prediabetes. Moreover, subjects classified as having T2D at follow-up are those diagnosed at the follow-up examination as well as those, who were diagnosed by a physician during the period between baseline and follow-up examinations. *The physicians in primary care diagnosed T2D when the fasting glucose levels were higher than 7 mmol/l at two or more occasions or symptoms accompanied by glucose levels higher than 11.1 mmol/l or HbA1c above 47 mmol/mol. All subjects categorized as having prediabetes in the present study were newly diagnosed by the OGTT at the follow up occasion.*

Classification of established diabetes risk factors and potential confounders

Body mass index (BMI, kg/m^2) was divided into three groups (<25.0, 25.0-29.9 and ≥30.0). Similarly, waist was categorized in three groups: <80, 80-87, and >87 cm for women, and <94, 94-101, and >101 cm for men. Physical activity during leisure time and smoking, based on the response alternatives were categorized into three groups (Table 1). Socioeconomic position, based on self-reported

TABLE 1 Characteristics at baseline in women and men according to glucose tolerance at follow-up.

	Controls mean (95% CI)	Future Prediabetes mean (95% CI)	Future T2D mean (95% CI)	p-value[a]
WOMEN, n	200	133	55	
Age (yrs)	49.0 (48.4-49.6)	49.1 (48.4-49.8)	49.3 (48.1-50.5)	0.899
BMI (kg/m^2)	24.4 (23.8-25.0)	27.6 (26.8-28.4)***	29.9 (28.3-31.6)***++	<0.001
Waist (cm)	78 (77-79)	86 (85-88)***	92 (89-96)***++	<0.001
Systolic BP (mm Hg)	119 (117-121)	128 (125-131)***	128 (124-131)**	<0.001
Diastolic BP (mm Hg)	74 (72-75)	80 (78-81)***	79 (77-81)**	<0.001
Glucose, fasting (mmol/l)	4.6 (4.5-4.6)	4.9 (4.9-5.0)***	5.2 (5.1-5.3)***++	<0.001
Glucose, 2h (mmol/l)	4.3 (4.2-4.4)	5.7 (5.5-5.9)***	5.8 (5.4-6.1)***	<0.001
Insulin, fasting (pmol/l)[b]	54 (51-57)	73 (68-79)***	91 (81-103)***++	<0.001
Insulin, 2h (pmol/l)[b]	173 (162-185)	289 (261-319)***	352 (297-419)***	<0.001
IGFBP-1, fasting (μg/l)[b]	43 (40-46)	29 (26-32)***	24 (21-28)***	<0.001
IGFBP-1, 2h (μg/l)[b]	22 (20-23)	15 (14-16)***	14 (12-15)***	<0.001
IGFBP-2 (μg/l)[b]	234 (221-248)	153 (141-165)***	144 (127-162)***	<0.001
Adiponectin (mg/l)[b]	13.9 (13.2-14.7)	10.0 (9.3-10.7)***	8.6 (7.8-9.5)***	<0.001
IGF-I (μ/l)[b]	175 (169-182)	186 (178-195)	168 (155-183)	0.038
IGF-II (μ/l)[b]	799 (778-820)	834 (807-862)	796 (755-839)	0.106
Physical activity, n (%)				
sedentary	19 (9.5)	23 (17.3)	16 (29.1)	

(Continued)

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TABLE 1 Continued

	Controls mean (95% CI)	Future Prediabetes mean (95% CI)	Future T2D mean (95% CI)	p-value[a]
moderate	109 (54.5)	85 (63.9)	28 (50.9)	
regular	72 (36.0)	25 (18.8)	11 (20.0)	<0.001
Smoking, n (%)				
never	82 (41.0)	57 (42.8)	16 (29.1)	
former	67 (33.5)	38 (28.6)	22 (40.0)	
current	51 (25.5)	38 (28.6)	17 (30.9)	0.384
Socioeconomic status, n (%)				
low	45 (22.5)	42 (31.6)	17 (30.9)	
middle	44 (22.0)	38 (28.6)	21 (38.2)	
high	106 (53.0)	50 (37.6)	14 (25.4)	
self-employed	5 (2.5)	3 (2.2)	3 (5.4)	0.006
Hypertension[c], n (%)				
no	167 (83,5)	89 (66.9)	38 (69.1)	
yes	33 (16.5)	44 (33.19	17 (30.9)	0.001
MEN, n	277	164	98	
Age (yrs)	47.6 (47.0-48.2)	47.6 (46.8-48.3)	47.8 (46.9-48.8)	0.932
BMI (kg/m^2)	25.2 (24.8-25.5)	27.5 (27.0-28.1)***	27.6 (26.9-28.4)***	<0.001
Waist (cm)	91 (90-92)	96 (94-97)***	96 (94-98)***	<0.001
Systolic BP (mm Hg)	123 (121-125)	131 (128-133)***	130 (127-133)***	<0.001
Diastolic BP (mm Hg)	79 (78-80)	83 (82-85)***	82 (80-84)**	<0.001
Glucose, fasting (mmol/l)	4.5 (4.5-4.6)	4.8 (4.6-4.8)***	5.0 (4.9-5.1)***++	<0.001
Glucose, 2h (mmol/l)	4.4 (4.2-4.5)	5.2 (5.1-5.4)***	5.6 (5.4-5.9)***+	<0.001
Insulin, fasting (pmol/l)[b]	99 (94-103)	121 (113-129)***	131 (121-143)***	<0.001
Insulin, 2h (pmol/l)[b]	247 (231-264)	411 (374-451)***	397 (346-455)***	<0.001
IGFBP-1, fasting (µg/l)[b]	29 (27-31)	14 (13-16)***	14 (13-16)***	<0.001
IGFBP-1, 2h (µg/l)[b]	15 (14-16)	6 (6-7)***	7 (6-8)***	<0.001
IGFBP-2 (µg/l)[b]	185 (176-195)	131 (121-142)***	130 (117-144)***	<0.001
Adiponectin (mg/l)[b]	8.6 (8.2-9.0)	7.5 (7.0-8.0)**	7.0 (6.4-7.6)***	<0.001
IGF-I (µ/l)[b]	185 (179-191)	187 (179-195)	185 (175-196)	0.919
IGF-II (µ/l)[b]	939 (918-961)	959 (929-989)	995 (956-1037)*	0.043
Physical activity, n (%)				
sedentary	21 (7.6)	15 (9.1)	14 (14.3)	
moderate	125 (45.1)	101 (61.6)	54 (55.1)	
regular	131 (47.3)	48 (29.3)	30 (30.6)	<0.001
Smoking, n (%)				
never	117 (42.2)	57 (34.8)	30 (30.6)	

(Continued)

TABLE 1 Continued

	Controls mean (95% CI)	Future Prediabetes mean (95% CI)	Future T2D mean (95% CI)	p-value[a]
former	104 (37.5)	64 (39.0)	29 (29.6)	
current	56 (20.2)	43 (26.2)	39 (39.8)	0003
Socioeconomic status, n (%)				
low	82 (29.6)	54 (32.9)	33 (33.7)	
middle	42 (15.2)	33 (20.1)	23 (23.5)	
high	133 (48.0)	71 (43.3)	37 (37.8)	
self-employed	20 (7.2)	6 (3.7)	5 (5.1)	0.240
Hypertension[c], n (%)				
no	209 (75.5)	93 (56.7)	53 (54.1)	
yes	68 (24.5)	71 (43.3)	45 (45.9)	<0.001

[a]Comparison between groups was for continuous variables performed by ANOVA, and if significant followed by Scheffé post-hoc test: *P<0.05, **P<0.01, ***P<0.001 vs control; +P<0.05, ++P<0.01, +++P<0.001 vs prediabetes, and for categorical variables by chi-square test. [b]geometric mean [c]hypertension; no=normal blood pressure and no hypertension treatment, yes=high blood pressure and/or hypertension treatment.
All subjects had normal glucose tolerance (NGT) at baseline. The Controls stayed NGT at follow up.

occupation, classified according to the standard system from Statistics Sweden, was categorized into four groups (unskilled/skilled manual workers, low-level non-manual employees, medium- and high-level non-manual employees and self-employed/farmers). Education comprised three categories; low (elementary school), middle (senior high school, technical and vocational school), and high (college, university). Blood pressure was dichotomized into normal (diastolic and systolic blood pressure below 90 and 140 mmHg, respectively, without hypertension treatment) and high (systolic blood pressure ≥140 and/or diastolic blood pressure ≥ 90 mmHg and/or on anti-hypertensive treatment).

Assays

All samples were assayed in duplicate. Serum IGFBP-1 was measured by an in-house radioimmunoassay (RIA) using a polyclonal antibody and human IGFBP-1 as standard (32). Intra- and inter-assay CV were 3% and 10%, respectively. Serum IGFBP-2 was determined by an in-house time-resolved immunofluorometric assay (TR-IFMA) based on commercial reagents as previously described, with intra- and inter-assay CVs averaging 5 and 12%, respectively (33). Serum adiponectin was determined by an in-house TR-IFMA based on commercial antibodies as previously described, with intra- and inter-assay CVs averaging <5 and <13%, respectively (34). This assay was later demonstrated to detect all major forms of adiponectin in human serum (high-molecular, medium molecular and low molecular weight forms) (34). Serum IGF-II was determined following acid ethanol extraction of serum, using a highly sensitive TR-IFMA as previously described, intra- and inter-assay CVs averaging <5 and <15%, respectively (35). IGF-I was measured in serum by RIA after acid-ethanol extraction and cryo-precipitation, using des-(1-3)-IGF-I as tracer to minimize interference by IGFBPs (36). Intra- and inter-assay CV were 4% and 11%, respectively.

Venous serum glucose was assayed using the glucose oxidase method (Yellow Springs Glucose Analyzer, Yellow Springs, OH, USA).

Immunoreactive insulin was assayed by in-house RIA, using a polyclonal antibody, human insulin as standard and charcoal addition to separate antibody-bound and free insulin. The intra-assay CV was 5.8-8.4% and the inter-assay CV was 11.5-16.9%. In this assay proinsulin has a 100% cross reactivity (37).

Data analysis

Results are presented separately for women and men. Clinical characteristics are presented as means and 95% confidence intervals (CI). Variables not normally distributed (i.e. adiponectin, IGFBP-1, IGFBP-2, IGF-I, IGF-II and insulin) were log-transformed prior to analyses. Differences between more than two groups were for continuous variables analyzed by one-way ANOVA and if significant, followed by Scheffé *post hoc* test and for categorical variables by chi-square test. Paired t-test was performed when comparing two occasions within group and unpaired t-test when comparing two groups. Odds ratios (ORs) together with 95% confidence intervals (CIs) were calculated by multiple logistic regression analyses to explore

associations between serum variables and an abnormal glucose regulation.

Potential confounders (BMI, waist, physical activity during leisure time, smoking, socioeconomic position, education and blood pressure) were tested separately in logistic regression models including the exposure variable and age. The change-in-estimate method was used, meaning that variables that contributed to at least a 10% change of the age-adjusted crude estimate in any of the outcome measures were included in the final multi-adjusted model. Waist and BMI, just like socioeconomic position and education, had similar influence on the crude estimates and accordingly BMI and socioeconomic position were chosen for the final model. Thus, two logistic regression models are given; model 1 adjusted for age and model 2 adjusted for age, BMI, physical activity, smoking, socioeconomic position and blood pressure.

In the regression model serum variables are categorized in quartiles according to the distribution within sex or used as continuous ^{2}log-transformed variables and thus reported per halving (adiponectin, IGFBP-1 and IGFBP-2), or per doubling (IGF-I and IGF-II). Co-linearity between biomarkers was evaluated by estimating variance inflation factor (VIF) which was found to not exceed 2.0.

Receiver operating characteristics (ROC) curve analyses were used to evaluate and compare the predictive power of the biomarkers. In PROC LOGISTIC (SAS statistical package) the ROCCONTRAST statement, implementing a nonparametric approach, was used to analyze differences between the respective areas under the curve (AUC). The analyses were performed using SAS statistical package version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA) and Statistica StatSoft version 10 (Tulsa, OK, USA). P-values <0.05 were considered statistically significant.

Results

Women

At baseline, all subjects had normal fasting glucose, OGTT and blood pressure. Significant differences were observed in all continuous parameters except for age and serum IGF-II levels in those who later developed prediabetes or T2D compared to the controls, who remained NGT (Table 1). FHD was reported at baseline in 71% of women, who later developed prediabetes, and in 80% of women, who developed T2D. BMI and waist circumference were at baseline progressively higher in those, who later developed prediabetes or T2D. Conversely, baseline levels of adiponectin, IGFBP-1 and IGFBP-2 were significantly lower in individuals, who later developed prediabetes or T2D compared to the controls.

Those who later developed T2D were at baseline more sedentary with less regular physical activity and they had lower socioeconomic status compared to the controls (Table 1). Hypertension was more common in those who later developed prediabetes or T2D.

Eight years later at follow up the number with known FHD had increased to 75% of those with prediabetes and to 84% of those with T2D.

After adjustment for known risk factors, significant ORs for future T2D were obtained for the lowest quartiles of adiponectin (OR:29.42), IGFBP-2 (OR:9.52) and IGFBP-1 (OR:5.41), and for the highest quartile of IGF-I (OR:3.79). IGF-II did not predict T2D. For the prediction of prediabetes, significant ORs were obtained for all five proteins, the strongest for IGFBP-2 (OR:7,48) and adiponectin (OR:6,06) (Table 2).

The OR for T2D associated with low adiponectin was not affected by the adjustment for confounding risk factors in contrast to that of IGFBP-1 and -2, which decreased after extended adjustment (Table 2 and Supplemental Table S1).

Men

The control men, who stayed NGT, demonstrated compared to the control women higher concentrations of insulin and IGF-II ($p<0.001$) and lower levels of fasting adiponectin, IGFBP-1 and IGFBP-2 ($p<0.001$) (Table 1). The glucose levels were not different.

At baseline all subjects had normal fasting glucose, OGTT and blood pressure. Significant differences were observed in all continuous parameters except for age and serum IGF-I levels in those NGT men, who later developed prediabetes or T2D compared to controls, who stayed NGT (Table 1). At baseline FHD was reported in 63% of men, who later developed prediabetes, and in 77% of the men, who developed T2D. BMI and waist measurements were higher in those who later developed prediabetes compared to controls, but in contrast to women, with no further increments in those who later developed T2D. Fasting and 2h OGTT plasma glucose levels differed at baseline between men, who later developed prediabetes or T2D (Table 1). As for women, levels of adiponectin, IGFBP-1 and IGFBP-2 were significantly lower at baseline in those who later developed prediabetes or T2D as compared to controls.

Among men, who later developed T2D, there were more sedentary activities and more current smokers (Table 1). Hypertension was more common in those, who later developed prediabetes or T2D.

At follow up, 10 years later, the number with known FHD had increased to 66% of those with prediabetes and to 82% of those with T2D.

After adjustment for known risk factors, significant ORs for future T2D were obtained for the lowest quartiles of IGFBP-1 (OR:14.89), IGFBP-2 (OR:7,63) and adiponectin (OR: 2.35). Neither IGF-I nor IGF-II predicted AGT. For the prediction of prediabetes, only IGFBP-1 (OR:13.44) and IGFBP-2 (OR:4.03)

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TABLE 2 Odds ratios (ORs) for decreasing baseline values of IGFBP-2, adiponectin and IGFBP-1 and increasing baseline values of IGF-I and IGF-II in subjects having normal glucose tolerance (NGT) at baseline in the association to development of prediabetes and type 2 diabetes at follow-up compared to remaining NGT.

WOMEN								MEN							
	NGT	Prediabetes			Type 2 diabetes				NGT	Prediabetes			Type 2 diabetes		
	n	n	OR	95% CI	n	OR	95% CI		n	n	OR	95% CI	n	OR	95% CI
IGFBP-2, µg/l								**IGFBP-2,** µg/l							
>268	79	14	1.00		5	1.00		>224	92	28	1.00		14	1.00	
193-268	52	34	2.70	1.25-5.84	8	0.92	0.22-3.87	161-224	95	27	0.96	0.50-1.83	17	1.37	0.59-3.17
141-192	47	32	2.15	0.97-4.79	16	2.81	0.77-10.26	110-160	58	48	2.54	1.34-4.82	29	4.39	1.89-10.23
<141	22	53	7.48	3.17-17.64	26	9.52	2.59-35.03	<110	32	61	4.03	1.97-8.25	38	7.63	3.13-18.59
continuous, ^{2}log	200	133	3.74	2.32-6.03	55	5.31	2.43-11.58	continuous, ^{2}log	277	164	2.46	1.68-3.60	98	3.17	1.99-5.07
Adiponectin, mg/l								**Adiponectin,** mg/l							
>15.62	74	18	1.00		5	1.00		>10.55	84	36	1.00		17	1.00	
11.60-15.62	62	28	1.48	0.69-3.16	5	1.19	0.25-5.69	7.95 - 10.55	70	41	1.17	0.64-2.14	20	1.33	0.60-2.91
8.56-11.59	43	42	2.63	1.24-5.57	14	5.98	1.42-25.16	6.04 – 7.94	74	36	0.96	0.52-1.75	27	1.56	0.73-3.32
<8.56	21	45	6.06	2.71-13.57	31	29.42	7.11-121.77	<6.04	49	51	1.70	0.93-3.12	34	2.35	1.11-4.99
continuous, ^{2}log	200	133	3.30	2.04-5.33	55	10.89	4.41-26.91	continuous, ^{2}log	277	164	1.46	1.02-2.10	98	1.92	1.23-2.98
IGFBP-1, µg/l								**IGFBP-1,** µg/l							
>49	73	20	1.00		5	1.00		>35	106	18	1.00		9	1.00	
35-49	63	28	1.49	0.71-3.10	8	1.60	0.42-6.04	23 - 35	88	23	1.62	0.79-3.31	20	3.00	1.22-7.35
24-34	42	39	2.83	1.35-5.96	18	4.84	1.36-17.19	14 - 22	57	47	4.16	2.10-8.21	29	6.33	2.56-15.62
<24	22	46	4.27	1.86-9.79	24	5.41	1.45-20.12	<14	26	76	13.44	6.33-28.54	40	14.89	5.61-39.48
continuous, ^{2}log	200	133	2.17	1.47-3.20	55	2.98	1.62-5.48	continuous, ^{2}log	277	164	3.23	2.39-4.35	98	3.19	2.20-4.60
IGF-I, µg/l								**IGF-I,** µg/l							
<150	52	28	1.00		18	1.00		<161	63	42	1.00		32	1.00	
150-182	57	29	0.94	0.44-2.01	12	0.62	0.21-1.81	161 - 185	82	36	0.60	0.32-1.10	17	0.32	0.15-0.68
183-214	53	32	1.42	0.67-2.98	12	1.14	0.38-3.38	186 - 218	62	42	1.10	0.60-2.04	22	0.71	0.33-1.53
>214	38	44	3.79	1.76-8.15	13	3.37	1.04-10.95	>218	70	44	0.81	0.43-1.53	27	0.70	0.33-1.47
continuous, ^{2}log	200	133	3.07	1.53-6.14	55	1.56	0.58-4.22	continuous, ^{2}log	277	164	1.09	0.61-1.95	98	1.08	0.53-2.20

(Continued)

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TABLE 2 Continued

WOMEN								MEN							
	NGT	Prediabetes			Type 2 diabetes				NGT	Prediabetes			Type 2 diabetes		
	n	n	OR	95% CI	n	OR	95% CI		n	n	OR	95% CI	n	OR	95% CI
IGF-II, µg/l								**IGF-II**, µg/l							
<711	58	25	1.00		15	1.00		<843	80	36	1.00		20	1.00	
711-803	53	32	1.70	0.81-3.56	12	0.93	0.33-2.63	843 - 958	77	38	1.07	0.58-1.98	21	1.06	0.49-2.30
804-929	46	38	2.24	1.06-4.72	15	1.66	0.58-4.68	959 - 1096	58	49	1.82	0.99-3.33	26	2.11	1.00-4.47
>929	43	38	2.39	1.13-5.03	13	0.90	0.30-2.68	>1096	62	41	1.06	0.57-1.96	31	1.80	0.86-3.76
continuous, ^{2}log	200	133	2.68	1.06-6.79	55	0.71	0.18-2.84	continuous, ^{2}log	277	164	1.08	0.51-2.26	98	2.49	0.99-6.31

Logistic regression models were adjusted for age, BMI (<25.0, 25.0-29.9,≥30.0), physical activity during leisure time (sedentary, moderate, regular exercise), smoking (never, former, current), socioeconomic status (low, middle, high, self-employed) and blood pressure (normal blood pressure and no hypertension treatment vs high blood pressure and/or hypertension treatment).

yielded significant ORs (Table 2). Extended adjustment for confounding risk factors did not significantly alter the ORs for T2D or prediabetes (Table 2 and Supplemental Table S1).

Combining biomarkers and comparisons to BMI

In both women and men, low serum levels of adiponectin, IGFBP-1 and IGFBP-2 showed a concentration dependent risk to develop T2D (Table 2). When included in the same regression model and following multivariate analysis and adjustment of established risk factors, only adiponectin and IGFBP-2 remained significantly and independently associated with future prediabetes and T2D in women, while only IGFBP-1 remained associated with future prediabetes and T2D in men (Table 3).

ROC curves for the three variables adiponectin, IGFBP-1 and IGFBP-2 were evaluated in comparison with the ROC curve for BMI. In women, the AUC-ROCs were; 0.81, 0.79, and 0.79 for adiponectin, IGFBP-1, and IGFBP-2, respectively, compared to 0.80 for BMI (Figure 1A). These AUCs did not differ significantly. In men, the corresponding AUC-ROCs were 0.64, 0.78, 0.71, respectively, and for BMI 0.69 with the value for IGFBP-1 being significantly higher compared to the other proteins ($p<0.001$ to $p=0.003$). When adiponectin, IGFBP-1, and IGFBP-2 were combined the ROC-AUC reached 0.87 for women and 0.79 for men, which was significantly higher than for BMI alone, $p=0.049$ for women and $p=0.002$ for men. Including BMI in the model did not markedly change the AUC ROCs (Figure 1B).

Thus, in women, the sensitivity was similar for BMI and the three biomarkers. However, when adiponectin, IGFBP-1 and IGFBP-2 were combined, they were together more sensitive than BMI. In contrast to women, in men IGFBP-1 showed a significantly higher sensitivity than adiponectin, IGFBP-2 or BMI. When combined the three biomarkers were as in women resulting in a higher sensitivity than BMI.

Discussion

In this study investigating the risk for healthy middle-aged NGT men and women, many with a positive FHD, to develop prediabetes or overt T2D over a period of 8-10 years, we focused on biomarkers of insulin sensitivity and insulin production, i.e. adiponectin, IGFBP-1, IGFBP-2, IGF-I and IGF-II; proteins being linked to AGT (5–10, 15–18, 21, 22, 25–29). Low plasma concentrations of adiponectin, IGFBP-1 and -2 associated with future AGT in both genders. Included in the same regression model following adjusted multivariate analyses low levels of adiponectin and IGFBP-2 remained independently associated with development of AGT in women, while low levels of the liver specific IGFBP-1 remained the only associated biomarker in men. Thus, the biomarkers showing insulin resistance were present at baseline in those NGT men and women, who 10-8 years later developed AGT. Finally, in ROC curve analyses the combination of adiponectin, IGFBP-1 and IGFBP-2 yielded AUCs higher than that of BMI in both men and women, and inclusion of BMI did not increase the AUCs any further, although BMI is an accepted surrogate for HOMA-IR (WHO Expert Consultation December 2008, ISBN 9789241501491). Thus, this longitudinal study performed in a well-described large cohort confirms that in normoglycemic women, low levels of adiponectin and IGFBP-2 are predictors of T2D (18, 21, 22, 26–28), whereas in normoglycemic men, low levels of IGFBP-1 predict future T2D (16).

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TABLE 3 Odds ratios (ORs) for decreasing values of IGFBP-2, adiponectin and IGFBP-1 measured at baseline and included in the same regression model in the association to development of prediabetes and type 2 diabetes at follow-up in women and men.

	NGT	Prediabetes				Type 2 diabetes			
	n	n	OR[c]	95% CI	p	n	OR[c]	95% CI	p
WOMEN									
Model 1[a]	200	133				55			
IGFBP-2, ^{2}log			3.19	1.96-5.19	<0.0001		3.03	1.50-6.13	0.0021
Adiponectin, ^{2}log			2.74	1.69-4.42	<0.0001		4.90	2.29-10.51	<0.0001
IGFBP-1, ^{2}log			1.47	0.99-2.16	0.0535		2.39	1.30-4.37	0.0499
Model 2[b]	200	133				55			
IGFBP-2, ^{2}log			2.70	1.59-4.57	0.0002		3.04	1.23-7.52	0.0158
Adiponectin, ^{2}log			2.40	1.45-3.97	0.0007		7.75	2.94-20.43	<0.0001
IGFBP-1, ^{2}log			1.32	0.86-2.02	0.2070		1.39	0.69-2.79	0.3629
MEN									
Model 1[a]	277	164				98			
IGFBP-2, ^{2}log			1.28	0.85-1.95	0.2392		1.36	0.83-2.25	0.2247
Adiponectin, ^{2}log			1.06	0.71-1.57	0.7768		1.43	0.89-2.29	0.1423
IGFBP-1, ^{2}log			3.21	2.33-4.43	<0.0001		2.86	1.97-4.15	<0.0001
Model 2[b]	277	164				98			
IGFBP-2, ^{2}log			1.21	0.77-1.89	0.4139		1.60	0.93-2.77	0.0924
Adiponectin, ^{2}log			0.98	0.64-1.48	0.9068		1.25	0.76-2.05	0.3901
IGFBP-1, ^{2}log			3.05	2.18-4.25	<0.0001		2.62	1.73-3.96	<0.0001

[a]Model 1: adjusted for age (36-40, 41-45, 46-50, 51-56 yrs)
[b]Model 2: adjusted for age, BMI (<25.0, 25.0-29.9, ≥30.0), physical activity during leisure time (sedentary, moderate, regular exercise), smoking (never, former, current), socioeconomic status (low, middle, high, self-employed) and blood pressure (normal blood pressure and no hypertension treatment vs high blood pressure and/or hypertension treatment).
[c]OR for the association between glucose tolerance and decreasing values of the variable.

The finding that low adiponectin, which reflects unhealthy adiposity (24, 38), was the strongest predictor (OR:29.42) for future T2D in overweight NGT women agrees with our previous finding of waist index as a stronger predictor than both IGFBP-1 and insulin in women (17). Our finding also agrees with other studies (24–27, 39–41) showing that increasing amount of visceral adiposity associates with insulin resistance and a great risk to develop AGT in women. Noteworthy, the role of adiponectin was recently supported by genetic studies (38). Visceral adipose tissue secretes relatively high amounts of inflammatory cytokines, which inhibit the adiponectin secretion and amplify the risk for AGT (15, 25–28). In a population-based study of gender differences, adiponectin correlated inversely with markers of inflammation (CRP and sedimentation rate) in women but not in men (39). This is in line with the report by Saltevo et al. showing that inflammatory markers are higher in women than in men with AGT (42).

In NGT women the OR for prediabetes was highest for low IGFBP-2 (OR:7.48), closely followed by low adiponectin (OR:6.06). These NGT women had at baseline lower BMI, waist circumference, fasting glucose and insulin than those who later developed T2D. Low IGFBP-2 levels suggest leptin resistance and excess nutrition (19, 20). IGFBP-2 has achieved increasing interest due to its metabolic involvement (6, 40, 43, 44). Preclinical studies show that IGFBP-2 inhibits adipogenesis (19) and protects against the development of obesity and insulin resistance (6, 40, 41, 43, 44). Several studies demonstrate that IGFBP-2 is inversely related with BMI and insulin (6, 18–21, 23, 40, 43). However, the mechanisms by which IGFBP-2 exert its beneficial metabolic actions remain to be fully clarified. One possible explanation is that IGFBP-2 acts through sequestration of IGFs. However, this mode of action appears less likely. Another, and in our view more likely explanation is that IGFBP-2 possesses IGF-independently beneficial metabolic effects (6, 19) as recently reviewed (43–45). Supportive of this, approximately 50% circulating IGFBP-2 is indeed not carrying an IGF molecule (41).

In contrast to our findings in women, the strongest predictor of both prediabetes and T2D in NGT men was a low fasting IGFBP-1. This suggests that hepatic and probably whole-body insulin resistance and peripheral hyperinsulinemia (13, 14, 16, 19), are the most important factors predicting development of AGT in men.

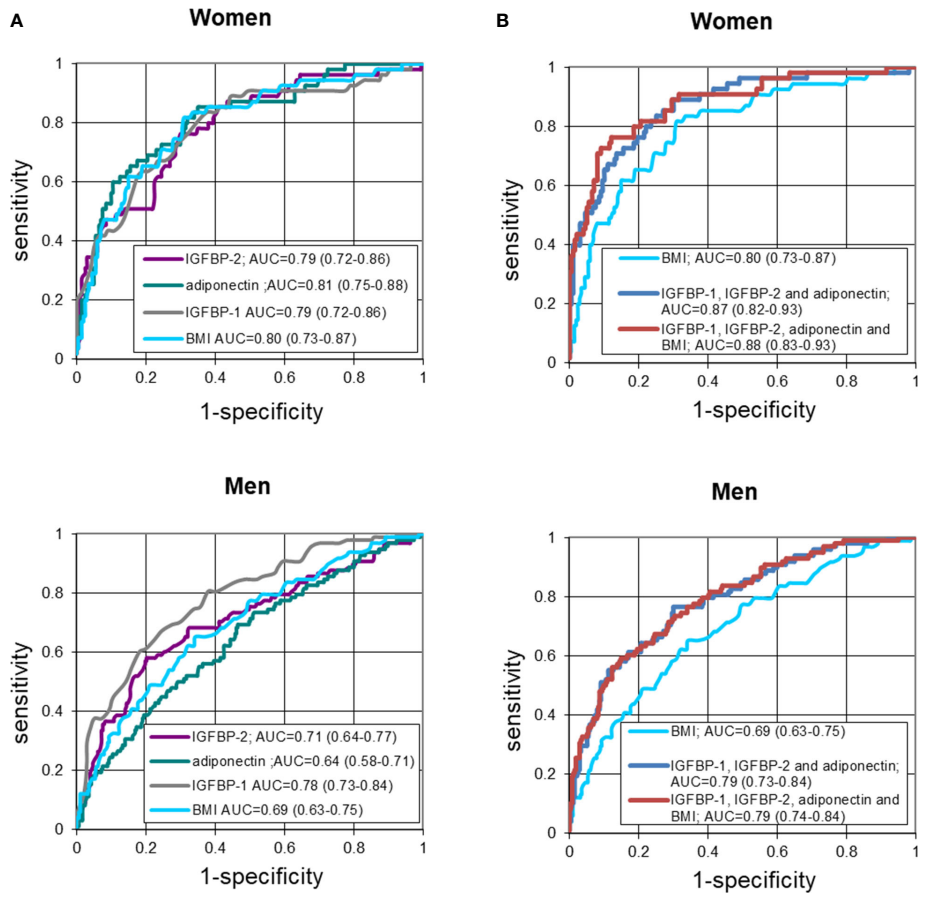


FIGURE 1
(A) Receiver-operating characteristic (ROC) curves of fasting IGFBP-2, adiponectin, IGFBP-1 and BMI in detecting T2D in women and men. Logistic regression models not adjusted for confounders. **(B)** Receiver-operating characteristic (ROC) curves of fasting IGFBP-2, adiponectin and IGFBP-1 combined, as compared to BMI alone or included, in detecting T2D in women and men. Logistic regression models not adjusted for confounders.

Fasting and 2h OGTT plasma glucose levels were the only variables that differed at baseline between the NGT men, who later developed prediabetes or T2D. It was not insulin, BMI or waist circumference as in women. These findings suggest that in NGT men with FHD, who later developed T2D, there was an early involvement of dysfunctional beta cells, being less able to further increase insulin secretion to compensate for increased glucose and hepatic insulin resistance indicated by low IGFBP-1 levels (14). This is most probably genetically determined. Interestingly, in the present NGT control group, fasting IGFBP-1 levels in men without FHD were higher than the fasting levels found in NGT control men with FHD (16). Thus, FHD was associated with lower fasting IGFBP-1 in NGT men, who stayed NGT. The fasting levels were further reduced in those, who later developed AGT. Genetic factors explain 35% of IGFBP-1 serum concentrations (46). Thus, the road to prediabetes and T2D appears to be different in several ways when comparing men and women.

IGFBP-1 functions both as an IGF-regulating protein, and as an IGF-independent protein with effects similar to IGFBP-2 (6, 43–45). Due to its RDG sequence, IGFBP-1 like IGFBP-2 can bind to an integrin receptor, which stimulates metabolic and anabolic pathways (6, 43–45). IGFBP-1 and IGFBP-2 have effects *via* inhibition of the IGF effect on adipose tissue, where they inhibit preadipocyte expansion and differentiation and lipid accumulation (11, 44, 47). IGFBP-2 has a direct effect *via* its heparin-binding domain-2 on adipogenesis, especially visceral. Mice overexpressing IGFBP-1 in endothelial cells were characterized by stimulated NO synthesis and protection against development of AGT *via* its RDG sequence (6, 45, 48). Protection against T2D has also been seen in transgene mice overexpressing IGFBP-2 and in humans overexpressing IGFBP-1 and adiponectin, respectively (40, 48–51). Thus, these three proteins could *via* cellular effects be protective against AGT and when decreased production this may be involved in the pathogenesis of AGT (6, 27, 28, 40, 43–45, 48–51).

After adjustment, the associations of high baseline levels of total IGF-I and IGF-II, respectively, with future AGT were weak and only present in women. IGF-II serum levels are elevated in

obesity, irrespective of concomitant presence of T2D, and decline after diet-induced weight loss (4). This association with obesity can explain the present finding. Previous studies in cohorts consisting of men and women have shown inconsistency in the predictive value of serum IGF-I for incident T2D (5, 7, 8, 18). However, when only females were studied, an elevated serum IGF-I predicted gestational diabetes and T2D (18, 22, 52). IGF can stimulate adiposity if IGFBP-1 and -2 are suppressed (47), a strong risk factor for AGT in women. High serum levels of *free* IGF-I levels have indeed been shown to predict AGT, which is in line with our findings of high total IGF-I (positively correlated with free IGF-I) and low IGFBP-1 (negatively correlated with free IGF-I) as predictors of prediabetes and T2D in women (18, 53).

The present findings support that hyperinsulinemia and insulin resistance exists in healthy NGT subjects with FHD long before the appearance of prediabetes and overt T2D (54). It also suggests that in NGT women with FHD the increasing amount of unhealthy adiposity drives the development to T2D concomitant with a declining beta-cell function (17).

Our study has limitations. First, all studied subjects were Caucasians. Furthermore, due to the study design, the cohort was enriched with subjects having FHD, our findings cannot be generalized to the general population. Additionally, we did not analyze lipids, HbA1c or controlled for HOMA-IR, which could have added further value to the study. However, we have previously shown that fasting IGFBP-1 was a stronger predictor than both fasting insulin and glucose (16, 17). We controlled for BMI, a known strong risk factor, which is closely associated with HOMA-IR. Moreover, the selected biomarkers have previously been shown to associate well with HOMA-IR (e.g. 5, 7, 9, 13, 15, 18, 23, 25).

The strengths of our study were that OGTT were performed in all subjects at baseline and at follow-up to confirm glucose tolerance. The findings were controlled for well-established risk factors for T2D. Finally, our studied biomarkers may have direct pathogenic effects on the development of AGT, more than BMI and HOMA-IR *per se*.

In conclusion, among biomarkers associated with insulin sensitivity and secretion, low serum adiponectin was the strongest predictor for T2D in women with FHD, whereas low serum IGFBP-1 was the strongest predictor in men. Neither IGF-I nor IGF-II showed any strong predictive value. The strongest predictor for prediabetes was in men low IGFBP-1 and in women low IGFBP-2. In both genders, the combination of adiponectin, IGFBP-1 and IGFBP-2 yielded a stronger prediction for future T2D than BMI. Thus, this study supports the concept that in subjects with FHD, a strong risk factor for AGT is in women adiposity, especially visceral, and in men hepatic insulin resistance.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee at Karolinska University Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

KB performed the measurements of IGFBP-1 and IGF-I and wrote the manuscript, KB, JF and C-GÖ designed the study, supervised experimental data collection, analyzed and interpreted data and edited the manuscript, AH did the statistical calculation, interpreted data and edited the manuscript, IA reviewed/edited the manuscript. JF and AF supervised and performed the measurements of IGF-II, adiponectin and IGFBP-2 and edited the manuscript. KB is the guarantor of this work and as such had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1092307/full#supplementary-material

SUPPLEMENTAL FLOW DIAGRAM

Study design Baseline and follow-up study of men and women in Stockholm Diabetes Prevention Program and selection for the present study. FHD, family history of diabetes; T2D, type 2 diabetes; NGT, normal glucose tolerance. *Only subjects in whom heredity for diabetes was certain at follow-up are included. **Included are only subjects having data on all variables. ***These subjects were randomly selected among subjects having NGT and negative FHD at both baseline and follow-up. ****Subjects having only impaired fasting glucose were not included in the present study.

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Stratified glucose-lowering response to vildagliptin and pioglitazone by obesity and hypertriglyceridemia in a randomized crossover trial

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Background: Understanding which group of patients with type 2 diabetes will have the most glucose lowering response to certain medications (which target different aspects of glucose metabolism) is the first step in precision medicine.

Aims: We hypothesized that people with type 2 diabetes who generally have high insulin resistance, such as people of Māori/Pacific ethnicity, and those with obesity and/or hypertriglyceridemia (OHTG), would have greater glucose-lowering by pioglitazone (an insulin sensitizer) versus vildagliptin (an insulin secretagogue).

Methods: A randomised, open-label, two-period crossover trial was conducted in New Zealand. Adults with type 2 diabetes, HbA1c>58mmol/mol (>7.5%), received 16 weeks of either pioglitazone (30mg) or vildagliptin (50mg) daily, then switched to the other medication over for another 16 weeks of treatment. Differences in HbA1c were tested for interaction with ethnicity or OHTG, controlling for baseline HbA1c using linear mixed models. Secondary

outcomes included weight, blood pressure, side-effects and diabetes treatment satisfaction.

Results: 346 participants were randomised (55% Māori/Pacific) between February 2019 to March 2020. HbA1c after pioglitazone was lower than after vildagliptin (mean difference -4.9mmol/mol [0.5%]; 95% CI -6.3, -3.5; p<0.0001). Primary intention-to-treat analysis showed no significant interaction effect by Māori/Pacific vs other ethnicity (1.5mmol/mol [0.1%], 95% CI -0.8, 3.7), and per-protocol analysis (-1.2mmol/mol [0.1%], 95% CI -4.1, 1.7). An interaction effect (-4.7mmol/mol [0.5%], 95% CI -8.1, -1.4) was found by OHTG status. Both treatments generated similar treatment satisfaction scores, although there was greater weight gain and greater improvement in lipids and liver enzymes after pioglitazone than vildagliptin.

Conclusions: Comparative glucose-lowering by pioglitazone and vildagliptin is not different between Māori/Pacific people compared with other New Zealand ethnic groups. Presence of OHTG predicts greater glucose lowering by pioglitazone than vildagliptin.

Clinical trial registration: www.anzctr.org.au, identifier (ACTRN12618001907235).

KEYWORDS

dipeptidyl peptidase inhibitor, Māori, Pacific, pioglitazone, precision medicine, stratified drug response, thiazolidinedione, obesity

Introduction

The epidemic of type 2 diabetes (T2D) is of significant concern due to increased morbidity, mortality, and health care costs. Current treatment guidelines recommend early use of sodium glucose transport protein 2 inhibitors (SGLT2i) and glucagon like peptide 1 receptor agonists (GLP1RA) for those with high cardiovascular risk, heart failure or renal disease (1). In the remainder, metformin, followed by a range of other medications are recommended for glucose lowering on a sequential, additive basis, influenced by cost, side effect profiles, and average treatment effects from clinical trials (1). Consideration of personal characteristics such as comorbidities, and circumstances such as food security, financial stability, alongside setting an appropriate target HbA1c for individuals is part of individualising treatment of T2D. Identifying subgroups or strata of patients with similar characteristics who respond better to one type of glucose-lowering therapy over another, and/or have altered risk of treatment-specific side effects, has been termed stratification of treatment response, which represents the first step towards precision medicine for T2D (2).

The glucose lowering response to non-insulin therapies that target discrete aspects of glucose metabolism is, extremely variable between individuals with T2D. Knowledge of which medications are likely to be most effective for individuals with T2D is important to minimise cost, side effects and periods of hyperglycaemia associated with initiating an ineffective medication. In the absence of knowledge about which subgroups of people with T2D respond better to a particular medication, relatively low glucose lowering response to certain treatments may be inappropriately attributed to non-adherence. There is emerging evidence for stratification of glucose lowering response to certain medications for T2D by baseline characteristics, such as ethnicity, obesity and triglyceride levels.

Ethnicity as a marker for differential medication responsiveness has been investigated as a simple tool for therapeutic stratification of T2D medications. Both SGLT2i and dipeptidyl peptidase 4 inhibitor (DPP4i) have been reported to be more effective in lowering HbA1c in people of Asian compared with European ethnicity (3). Metformin may be more effective in African Americans with younger onset T2D compared to Americans of European ethnicity (4). Alpha-glucosidase inhibitors appear to work better in South East Asians than Europeans (5).

It is unclear whether differences in treatment response by ethnicity are due to biological differences in underlying T2D aetiology, or whether ethnicity serves as a proxy for residual

confounders such as level of glycaemia, concomitant diabetes therapies, dietary factors, medication adherence or other health inequities. Biological differences in underlying T2D aetiology using routinely assessed clinical markers of insulin resistance, such as obesity and/or high triglycerides (OHTG), have been shown to predict less glucose-lowering to DPP4i (6), which amplifies glucose-stimulated beta cell insulin secretion through the endogenous incretin pathway. Those with obesity have been reported to have greater glucose-lowering with thiazolidinediones (6, 7), which act to improve insulin sensitivity and lipid metabolism by stimulating the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARG).

Māori are the indigenous people of Aotearoa, New Zealand and share common Polynesian ancestry with Pacific peoples. Obesity and elevated triglyceride levels are prevalent in people of Māori and Pacific ethnicities (8, 9). There are disparities in diabetes outcomes for Māori and Pacific peoples (10), hence research into biological and other factors underlying these disparities are needed. To date glucose lowering response to different medications in people of Māori or Pacific ethnicity with T2D has not been studied. In this T2D medication, Which One is Right Here (WORTH) trial, we tested the potential stratification of comparative glycaemic response to pioglitazone (thiazolidinedione) and vildagliptin (DPP4i), by Māori or Pacific ethnicity and by OHTG status.

Materials and methods

Participant eligibility

Patients with T2D for >1 year, who had been on stable doses of metformin and/or sulfonylurea for >3 months were eligible to participate if they were aged 18–80 years inclusive, had HbA1c > 58 mmol/mol [> 7.5%] and < 111 mmol/mol [<12.3%], had never been on DPP4i or thiazolidinedione, had no insulin use, had no active infection requiring antibiotics, had no contraindication to either trial medication, and were able to give written informed consent. These eligibility criteria were modified to enable those on stable doses of two other types of oral glucose-lowering medications to participate in this trial (acarbose or dapagliflozin) as dual therapy with either metformin or sulfonylurea. Recruitment targeted 40% of participants being of Māori and/or Pacific ethnicity.

Settings and location

The trial participants were recruited at nine sites across urban (Auckland and Waikato) and rural settings (Te Tai Tokerau (Northland) and Te Tairāwhiti (East Coast of the North Island) in NZ.

Trial design

We conducted an investigator-initiated, multi-centre, open-label, two-treatment (pioglitazone 30mg once daily [P], vildagliptin 50mg once daily [V]), two-period (16-weeks each), randomised, crossover trial. There was no washout period between medications because medication withdrawal could have caused detrimental rebound hyperglycaemia and this was deemed unnecessary due to neither of the two medications having a continuing glucose lowering effect beyond 4 weeks. The primary outcome of HbA1c measurement, which reflects glycaemia over the preceding 8-12 weeks, would therefore reflect results only from the last medication during the 16-week treatment period, without any carry over effect from the first medication. The strength of the PV/VP crossover design is that both interventions were evaluated for each participant, which allows comparison at the individual rather than group level alone (11). In addition, participants could express preferences by comparing their experiences of both interventions which is not possible in a parallel group design. The trial protocol was approved by the NZ Health and Disability Ethics Committee, and has been published (12). All participants gave written informed consent.

Outcome and baseline measures

The primary outcome measure was HbA1c after each 16-week treatment period. The primary analysis was to test stratification in HbA1c response by Māori or Pacific ethnicity and the pre-specific secondary analysis was to test stratification in HbA1c response by OHTG status. To minimise confounding by adherence or treatment change, protocol adherence was considered to be those who on assessment had >75% adherence to the trial medication based on pill counts, had not commenced any additional glucose-lowering therapies since the baseline eligibility visit, and had not stopped any baseline glucose-lowering medication. HbA1c data were considered invalid if they were obtained more than 7 days after completing the study medication phase. Secondary outcome measures included body weight, blood pressure (BP), frequency of side effects, and Diabetes Treatment Satisfaction Questionnaire (DTSQ) (13) total scores, change in scores, and patient preference for either medication at the end of each treatment period. Serious adverse events (AEs) were collected throughout the trial period.

Self-reported ethnicity was recorded at the baseline visit. Participants were asked to tick all of the following categories that applied: Māori, Pacific, NZ European, Other European, Indian, Other Asian, Other (asked to specify). Prioritised ethnicity classification as Māori or Pacific was defined if either of these ethnicities were ticked or specified in "Other". Diabetes diagnosis date, current and past diabetes medications, co-

morbidities and other medications were verified by medical records. Fasting blood tests were performed to assess HbA1c, glucose, lipids, renal, liver function at baseline and were repeated at the end of each treatment period. OHTG was defined by the presence of BMI ≥30kg/m^2 and/or high triglycerides ≥2.3mmol/L. Fasting C-peptide, diabetes autoantibodies and blood for genetic analysis were taken at baseline. All biochemical analyses were performed at local diagnostic laboratories with validated and accredited assays. The 3-screen islet cell autoantibody Enzyme-Linked Immunosorbent Assay (ELISA) kit (RSR limited, Cardiff, UK) was used for combined quantitative determination of glutamic acid decarboxylase (GAD), islet tyrosine phosphatase-2 (IA-2) and zinc transporter8 (ZnT8) autoantibodies in human serum. All samples testing above the assay concentration threshold of 20u/mL were tested again using separate ELISA assays for each autoantibody (Anti-GAD and Anti-IA2, Euroimmun AG, Lübeck, Germany; ZnT8, ElisaRSR, Cardiff, UK).

Randomisation

Eligible participants were randomised 1:1 to one of the two sequences (VP or PV) by a central trial pharmacist using a secure trial database. The randomised study medication was couriered to the participant's nominated address by the central trial pharmacy. Randomisation lists were prepared by the trial statistician, using permuted block randomisation with variable block sizes (2 or 4) and stratified by recruiting region and self-reported ethnicity (Māori and Pacific vs non-Māori/non-Pacific, including New Zealand European, other European, Indian, Asian, and other groups). This was an open-label trial, where both participants and research staff were aware of the treatment sequences [VP/PV] after randomisation.

Statistical analysis

We aimed to recruit a total of 300 participants, with a target sample size of 40% Māori and Pacific people. This sample size would provide 80% power at 5% significance level to detect a minimal effect size of 0.35 standard deviation (SD) between Māori and Pacific vs non-Māori/non-Pacific groups on the difference in HbA1c between two test medications, allowing for 10% loss to follow up. Full details were reported in the published trial protocol (12).

All randomised participants were included in the primary outcome analysis, following the intention to treat (ITT) principle. Missing HbA1c data after each treatment were imputed using the baseline value or any lab data collected post-baseline before the scheduled assessment window. Per protocol (PP), analysis was also performed on the primary outcome, including only those participants who provided complete outcome data with no major protocol violations. All outcomes were assessed using valid visit data collected within the scheduled assessment window. No imputation was considered on secondary outcomes. Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

Baseline demographic and clinical characteristics of all randomised participants were summarised overall and by ethnicity. Continuous variables were presented as mean and SD. Categorical variables were presented in frequencies and percentages. Whether the difference in achieved HbA1c for the two medications was different between Māori and Pacific vs non-Māori/non-Pacific participants was tested using a linear mixed model with both fixed and random effects. The fixed effects included the baseline outcome value, period, medication class, patient group and its interaction with the medication. The random effect included patients as the cluster. The model-adjusted mean difference between two medications was estimated for each patient group, and the interaction effect between medications and patient groups was estimated with a 95% confidence interval (CI). Similar regression analyses were conducted to test the medication response with other patient groups of interest, including OHTG status (high BMI ≥30kg/m^2 and/or high triglycerides ≥2.3mmol/L vs BMI <30kg/m^2 and triglycerides <2.3mmol/L). As a secondary analysis, the overall treatment effect was compared between the two medications without the interaction with patient group term and potential carryover effect was tested. Generalised linear regression was used on other secondary outcomes collected at the end of the trial, including patient medication preference and DTSQ change score. Statistical tests were two-sided at 5% significance level. The CONSORT 2010 statement extension for randomised crossover trials was followed (14).

Results

Participant flow

A total of 346 participants who completed eligibility screening between February 2019 and March 2020, were randomised to VP or PV sequence (173 each). Of these, valid HbA1c data were obtained in 261 participants after vildagliptin and 237 participants after pioglitazone, with 203 participants completing both assessments (Figure 1). The large proportion of invalid HbA1c data was due to pandemic related delays in obtaining timely blood tests within 7 days of completing the study medication phase. There were 16 participants who withdrew during pioglitazone treatment and 15 who withdrew during vildagliptin treatment.

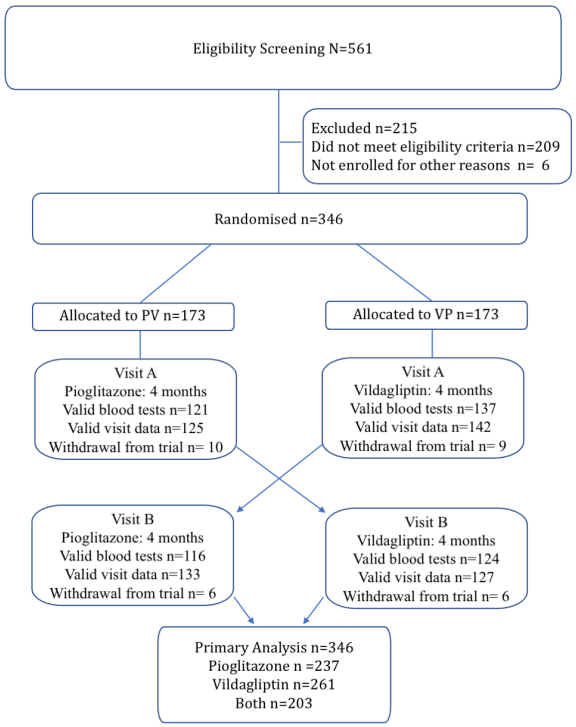


FIGURE 1
Flow diagram of the study participants included in this analysis.

Baseline data

The mean (SD) for age was 57.5 (10.9) years, HbA1c 75 (12) mmol/mol), [9% 1.1)], diabetes duration 9 (6) years; 41% of participants were women, 55% were Māori and/or Pacific (Table 1). Of all trial participants, 78 (23%) were Māori, 111 (32%) were Pacific people, 90 (26%) were NZ European, 67 (26%) were from other ethnic groups. There was no difference in characteristics by sequence PV vs VP. OHTG was present in 88% of Māori and Pacific participants. (Table 2). Nine participants tested positive in the triple autoantibody screening assay, verified as only GAD, (Supplementary Table 1). All had preserved fasting C-peptide (320-1983pmol/L), and were included in the analyses.

Outcomes

Overall, a greater mean decrease in HbA1c was observed after pioglitazone than after vildagliptin treatment (adjusted mean difference -4.9mmol/mol [-0.5%], 95%CI -6.3, -3.5; p < 0.0001) (Table 3). No carry-over effect was found (p=0.94). The primary ITT analysis showed no interaction effect in HbA1c response between treatments (pioglitazone vs vildagliptin) by ethnicity (Māori and Pacific -2.1mmol/mol [0.19%] vs non-Māori/non-Pacific -3.6 mmol/mol [0.33%]; interaction effect 1.5mmol/mol [0.14%], 95%CI -0.8, 3.7, p= 0.2). This was consistent with the PP analysis (Māori and Pacific -6.1mmol/mol [0.56%] vs non-Māori/non-Pacific -4.9mmol/mol [0.45%]; interaction effect -1.2mmol/mol [0.11%], 95%CI -4.1, 1.7, p=0.40); and the analysis of all valid data (Māori and Pacific -5.4mmol/mol [0.5%] vs non-Māori/non-Pacific -4.4mmol/mol [0.4%], interaction effect -1.1mmol/mol [0.1%], 95%CI -3.8, 1.7; p=0.4) (Table 4).

A treatment difference in HbA1c response was observed in participants with OHTG (-5.9mmol/mol [0.54%]) compared with those without OHTG (-1.2mmol/mol [0.11%]), with an estimated interaction effect of -4.7mmol/mol [0.43%], 95% CI -8.1, -1.4, p=0.005 (Table 4).

Mean weight after pioglitazone was higher than after vildagliptin treatment, with an adjusted mean difference of 1.6kg (95%CI [1.1, 2.0]; p<0.0001). There was no interaction in weight change response to pioglitazone versus vildagliptin by ethnicity or OHTG status (Table 4). BP and DTSQ did not differ by treatment group in the overall analysis, by ethnicity, or OHTG status (Tables 4, 5). There was no significant carryover effect detected for weight (p=0.66). There were 27 (7.8%) participants reporting new or worsening pedal edema after pioglitazone and 15 (4.3%) after vildagliptin (Chi-square p=0.06).

Hepatic enzymes, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) were lower after pioglitazone but remained unchanged after vildagliptin (difference p<0.0001). The difference in hepatic enzymes after pioglitazone vs vildagliptin, did not differ significantly by Māori and Pacific ethnicity or OHTG. Triglycerides reduced after pioglitazone compared with after vildagliptin (mean difference -0.3mmol/L, p<0.0001), with no interaction by ethnicity or OHTG grouping. HDL-C increased more after pioglitazone than after vildagliptin (+0.1mmol/L, p<0.0001).

At the final trial visit, 257 participants indicated their preferred medication: 98 (38%) preferred pioglitazone, 87 (34%) preferred vildagliptin, and 72 (28%) indicated either no preference (n=62) or neither (n=10). No differences in medication preference were found by ethnicity, or OHTG strata after adjusting for their treatment sequence (PV or VP), the change in HbA1c, weight and DTSQ score between two treatments.

There were 15 serious adverse events: 3 deaths (2 strokes and 1 myocardial infarction) and 12 hospitalisations. None were deemed to be due to the trial medication by an independent data safety monitoring committee.

Discussion

This multicentre, two-way, randomised crossover trial showed that the glucose-lowering response to pioglitazone

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TABLE 1 Baseline demographics and clinical characteristics.

	Overall	Māori and Pacific	Non Māori and non-Pacific
	n=346	n=189 (55%)	n=157 (45%)
Age (years)	57.5 (10.9)	56.2 (10.9)	59.1 (10.7)
Sex			
Female	141 (40.8%)	92 (48.7%)	49 (31.2%)
Male	205 (59.2%)	97 (51.3%)	108 (68.8%)
OHTG			
Yes	273 (78.9%)	166 (87.8%)	107 (68.2%)
No	72 (20.8%)	22 (11.7%)	50 (31.8%)
Missing	1 (0.3%)	1 (0.5%)	0 (0%)
Duration of diabetes (years)	9.0 (6.3)	8.4 (6.4)	9.6 (6.1)
Current smoker	49 (14.2%)	36 (19.0%)	13 (8.3%)
Baseline diabetes medication			
Metformin	339 (98.0%)	186 (98.4%)	153 (97.5%)
Sulfonylureas	216 (62.4%)	120 (63.5%)	96 (61.1%)
Other*	13 (3.8%)	7 (3.7%)	6 (3.8%)
BMI (kg/m^2)	35.5 (7.8)	38.0 (7.9)	32.5 (6.6)
Mean BP systolic (mmHg)	132.6 (15.1)	131.4 (15.6)	133.99 (14.4)
Mean BP diastolic (mmHg)	80.9 (8.5)	80.7 (8.8)	81.20 (8.3)
Fasting Glucose (mmol/L)	10.5 (3.0)	10.5 (3.1)	10.46 (2.9)
Fasting TG (mmol/L)	2.1 (1.5)	2.1 (1.7)	2.00 (1.3)
Creatinine (umol/L)	81.1 (18.5)	81.6 (20.1)	80.5 (16.3)
Fasting C-peptide (pmol/L)	1162 (494)	1201 (469)	1117 (520)
GAD antibodies	n=7/341 (2.1%)	n=4/185 (2.2%)	n=3/156 (1.9%)

Data are n (%) or mean (SD). OHTG refers to obese (BMI ≥ 30kg/m^2) and/or high triglycerides > 2.3mmol/L (TG). GAD, Glutamic acid decarboxylase.
*Other medication includes acarbose in 10 cases and dapagliflozin in 3 cases.

relative to vildagliptin was not different between people of Māori or Pacific ethnicity and other New Zealand ethnicities. However, there was a significant interaction in relative glucose-lowering by OHTG status, with 4.7mmol/mol greater reduction in HbA1c by pioglitazone relative to vildagliptin among those with OHTG. Those without OHTG had similar glucose lowering to each medication. Importantly, both treatments generated similar treatment satisfaction scores although there was greater weight gain and greater improvement in lipids and liver enzymes after pioglitazone than vildagliptin. No severe adverse events were directly related to these treatments.

While people of Māori or Pacific ethnicity had higher prevalence of OHTG, such ethnicity grouping did not predict altered glucose lowering response to pioglitazone versus vildagliptin. Within Māori and Pacific populations, there is genetic heterogeneity in risk and aetiology of T2D. A genetic variant (*rs373863828*, p.Arg457Gln) in the *CREBRF* gene, specifically found in approximately 25% people of Māori and Pacific ancestry, is associated with increased body mass index (15, 16) yet 40% lower odds of T2D (15, 16), due to enhanced insulin secretion capacity (17). Testing whether *CREBRF* gene variant status has utility in predicting stratified glucose lowering responses to T2D medications among people of Māori and Pacific ethnicity is currently underway (12). OHTG status has been reported to predict lower glucose response in studies with European participants (6).

In contrast to rosiglitazone, which is no longer used due to its associated increased risk of myocardial infarction and overall mortality (18), pioglitazone has demonstrable cardiovascular benefits (19, 20). This is thought to be partly due to its favourable impact on lipids (21, 22), which was also observed in this trial. In addition, our finding of improvement in liver

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TABLE 2 Descriptive data at baseline and after each treatment.

	Baseline (B)	Pioglitazone (P)	Vildagliptin (V)
	N, mean (SD)	N, mean (SD)	N, mean (SD)
HbA1c in mmol/mol [%]			
Overall cohort	346, 74·9 (11·5) [9.0]	237, 60·6 (12·1) [7.7]	261, 65·8 (13·4) [8.2]
Māori/Pacific	189, 76·0 (12·2) [9.1]	114, 61·6 (12·5) [7.8]	135, 67·4 (15·0) [8.3]
Non Māori/Pacific	157, 73·5 (10·6) [8.9]	123, 59·6 (11·6) [7.6]	126, 64·1 (11·3) [8.0]
OHTG	273, 75·5 (11·4) [9.1]	184, 60·7 (12·6) [7.7]	206, 67·0 (13·9) [8.3]
No OHTG	72, 72·6 (11·9) [8.8]	52, 60.2 (10.3) [7.7]	54, 61.4, (10.3) [7.8]
Female	141, 75·6 (11·9) [9.1]	97, 58.5 (12·1) [7.5]	109, 65.4 (13.2) [8.1]
Male	205, 74.3 (11.3) [8.9]	140, 62.0 (11.9) [7.8]	152, 66.2 (13.5) [8.2]
Weight in kilograms			
Overall cohort	346, 103.0 (24.8)	222, 105.5 (26.9)	235, 103.2 (26.1)
Māori/Pacific	189, 109.3 (25.4)	107, 113.7 (29.4)	116, 112.1 (28.5)
Non Māori/Pacific	157, 95.3 (21.8)	115, 97.8 (21.9)	119, 94.6 (20.1)
OHTG	273, 109.7 (23.2)	173, 113.1 (25.3)	181, 110.8 (24.6)
No OHTG	72, 77.6 (9.6)	49, 78.6 (10.6)	53, 77.6 (9.7)
Female	141, 99.5 (23,5)	92, 102.6 (26.2)	92, 99.5 (24.5)
Male	205, 105.3 (25.4)	130, 107.5 (27.4)	143, 105.7 (26.9)
Systolic blood pressure in mmHg			
Overall cohort	346, 132.6 (15.1)	220, 129.6 (15.3)	231, 130.9 (13.9)
Māori/Pacific	189, 131.4 (15.6)	107, 128.8 (16.8)	114, 129.6 (14.4)
Non Māori/Pacific	157, 134.0 (14.4)	113, 130.4 (13.8)	117, 132.1 (13.4)
OHTG	273, 133.0 (14.8)	172, 130.1 (14.4)	179, 130.6 (13.2)
No OHTG	72, 131.2 (16.1)	48, 128.0 (18.4)	51, 132.0 (16.3)
Female	141, 131.2 (15.3)	90, 128.9 (14.8)	89, 129.3 (13.1)
Male	205, 133.5 (14.9)	130, 130.1 (15.8)	142, 131.9 (14.4)
Diabetes Treatment Satisfaction Questionnaire (DTSQ) total score*			
Overall cohort	346, 28.6 (6.0)	256, 30.5 (5.9)	268, 30.0 (6.5)
Māori/Pacific	189, 29.4 (6.0)	128, 30.3 (6.0)	133, 30.1 (6.8)
Non Māori/Pacific	157, 27.6 (5.8)	128, 30.7 (5.8)	135, 30.0 (6.3)
OHTG	273, 28.2 (6.2)	198, 30.2 (6.2)	208, 29.8 (6.6)
No OHTG	72, 29.8 (5.0)	57, 31.4 (4.5)	59, 30.8 (6.2)
Female	141, 28.9 (6.4)	111, 31.1 (5.7)	110, 30.4 (6.9)
Male	205, 28.3 (5.6)	145, 30.0 (6.0)	158, 29.8 (6.3)

*Questions contained in the DTSQ are shown in Table 5.

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TABLE 3 The estimated medication effects on HbA1c and its interaction with ethnicity (primary outcome analysis).

HbA1c	Pioglitazone versus Vildagliptin (PV)		Difference in PV between Ethnicity	
	Mean estimate (95% CI)	p-value	Mean estimate (95% CI)	p-value
ITT analysis				
Māori/Pacific (mmol/mol)	-2.1 (-3.6,-0.6)	0.0070	1.5 (-0.8, 3.7)	0.2
non-Māori/Pacific (mmol/mol)	-3.6 (-5.2, -1.9)	< 0.0001		
Māori/Pacific (%)	-0.19 (-0.33, -0.05)	0.0070	0.14 (-0.07, 0.34)	0.2
non-Māori/Pacific (%)	-0.33 (-0.48, -0.17)	< 0.0001		
PP analysis				
Māori/Pacific (mmol/mol)	-6.1 (-8.2,-4.0)	<0.0001	-1.2 (-4.1, 1.7)	0.4
non-Māori/Pacific (mmol/mol)	-4.9 (-6.9, -2.9)	<0.0001		
Māori/Pacific (%)	-0.56 (-0.75, -0.37)	<0.0001	0.01 (0, 0.03)	0.4
non-Māori/Pacific (%)	-0.45 (-0.63, -0.27)	<0.0001		
HbA1c	**Pioglitazone**	**Vildagliptin**	**Pioglitazone versus Vildagliptin (PV)**	
	Mean estimate (95% CI)	**Mean estimate (95% CI)**	**Mean estimate (95% CI)**	**p-value**
Overall medication effect (mmol/mol)	61.2 (59.8, 62.5)	66.1 (64.7, 67.4)	-4.9 (-6.3, -3.5)	<0.0001
Overall medication effect (%)	7.8 (7.6, 7.9)	8.2 (8.1, 8.3)	-0.45 (-0.58, -0.32)	<0.0001

ITT, intention to treat; PP, per protocol; CI, confidence interval.

enzymes with pioglitazone treatment is consistent with its reported benefits in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. Whilst the weight gain with pioglitazone has been highlighted as a caution for its use, our data suggests the weight gain was mild and did not reduce patient satisfaction.

The lack of interaction of reduction in liver enzymes or lipids after pioglitazone with baseline OHTG is interesting. One explanation is that the glucose lowering action of pioglitazone is mediated by ligand binding to nuclear receptor PPARG which are most abundant in adipocytes and hence greater in people with obesity (23). In contrast, the liver enzyme or lipid lowering action of pioglitazone may be mediated by other pathways that are not directly correlated with baseline OHTG status such as PPAR alpha expression (24).

Comparative medication glucose response data from this crossover study design provides evidence-based understanding of how patients would respond if they were to switch from one medication to another. While this is something that happens frequently in diabetes care, there is very limited clinical data about how patients' glycaemic control changes when this happens or which medication they prefer. In addition, several consensus reports describe that when making treatment decisions for patients with type 2 diabetes it is important to consider not only the efficacy and safety of each medication, but also patient preference. This open-label cross-over study provides the added value of patient preference outcomes between pioglitazone and vildagliptin in the real-world setting. The protocol for a three-way crossover study including a DPP4 inhibitor, thiazolidinedione and a SGLT2 inhibitor has been described, which will provide further information on stratification of glucose response to three diabetes medications by routine clinical features ascertained at baseline (25).

A key limitation of this trial was the low follow up rate resulting in only 203 participants with valid HbA1c data after both treatments. Some of this related to difficulties encountered during the pandemic in obtaining a timely HbA1c result at the end of each study medication treatment phase and in conducting in person assessments. This is unlikely to have introduced any retention bias as each person contributed their own comparative data for both medications. Furthermore, the analysis was conducted with and without imputation on the primary analysis population, to test the robustness of the findings. Secondly, the open-label nature of the trial was selected for lower cost and complexity, which may have introduced observer and participant bias, but increased external validity. Thirdly, the crossover trial design might have led to the inappropriate estimation of the risk of weight gain with each medication, although the sensitivity analysis showed no significant carryover effect. The crossover design was selected as the best method to test differential glucose-lowering responses using HbA1c as the primary outcome measure between two diabetes medications in

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TABLE 4 The estimated medication effects on patient outcomes and its interaction with patient groups.

	Pioglitazone versus Vildagliptin (PV)		Difference in PV between Patient groups	
	Mean estimate (95% CI)	p-value	Mean estimate (95% CI)	p-value
HbA1c (mmol/mol)				
Māori/Pacific	-5.4 (-7.4, -3.5)	<0.0001	-1.1 (-3.8, 1.7)	0.4
non-Māori/Pacific	-4.4 (-6.3, -2.5)	<0.0001		
OHTG	-5.9 (-7.5, -4.4)	<0.0001	-4.7 (-8.1, -1.4)	0.005
non-OHTG	-1.2 (-4.1, 1.8)	0.4		
HbA1c (%)				
Māori/Pacific	-0.49 (-0.68, -0.32)	<0.0001	-0.10 (-0.35, 0.16)	0.4
non-Māori/Pacific	-0.40 (-0.58, -0.23)	<0.0001		
OHTG	-0.54 (-0.69, -0.40)	<0.0001	-0.43 (-0.74, -0.13)	0.005
non-OHTG	-0.11 (-0.38, 0.16)	0.4		
Weight (kg)				
Māori/Pacific	1.6 (0.9, 2.3)	<0.0001	0.1(-0.9, 1.0)	0.9
non-Māori/Pacific	1.5 (0.9, 2.2)	<0.0001		
OHTG	1.6 (1.1, 2.2)	<0.0001	0.2 (-0.9, 1.4)	0.7
non-OHTG	1.4 (0.4, 2.4)	0.007		
Systolic blood pressure (mmHg)				
Māori/Pacific	0.1 (-2.9, 3.2)	0.9	0.9 (-3.3, 5.1)	0.7
non-Māori/Pacific	-0.8 (-3.7, 2.1)	0.6		
OHTG	0.1 (-2.2, 2.5)	0.9	2.4 (-2.7, 7.4)	0.4
non-OHTG	-2.2 (-6.7, 2.3)	0.3		
Diabetes Treatment Satisfaction Questionnaire (DTSQ) total score*				
Māori/Pacific	0.2 (-1.0, 1.5)	0.7	-0.5 (-2.2, 1.2)	0.6
non-Māori/Pacific	0.7 (-0.5, 1.9)	0.2		
OHTG	0.4 (-0.6, 1.4)	0.4	-0.1 (-2.2, 2.0)	0.9
non-OHTG	0.5 (-1.3, 2.4)	0.6		

All valid patient data collected at baseline and after each medication treatment were used in the analysis; missing data were not imputed.
*Questions contained in the DTSQ are shown in Table 5.

different patient subgroups (11). A longer treatment duration of each period could have mitigated against the slow onset and offset of each medication in absence of wash-out period. However, the 16-week consecutive treatment period was considered the most efficient study design.

It is well recognised that overall glucose lowering effects of pioglitazone are typically greater than vildagliptin. The important finding of this study is that a superior glucose-lowering response to pioglitazone versus vildagliptin is observed mainly in those with OHTG. Whilst OHTG is more prevalent in those of Māori and Pacific ethnicity, the glucose-lowering response was not significantly different by such ethnicity group in a multi-ethnic New Zealand population. This has several clinical implications. Firstly, these findings suggest that ethnicity is not a sufficient proxy for stratified medicine for pioglitazone and vildagliptin in T2D. Rather, clinical markers of insulin resistance that are routinely available in clinical practice such as BMI and triglyceride levels are able to predict whether superior glucose lowering response to pioglitazone relative to vildagliptin is expected. The recent progress in better defining novel T2D subtypes based on underpinning disease mechanisms such as age at diabetes

TABLE 5 DTSQ scores at baseline and after each treatment.

	DTSQ scores as mean (SD)		
	Baseline (B)	Pioglitazone (P)	Vildagliptin (V)
Overall DTSQ score*	28.6 (6.0) N=345	30.6 (5.8) N=232	29.9 (6.6) N=245
1.How satisfied are you with your current treatment	4.7 (1.4) N=346	5.0 (1.4) N=232	4.9 (1.4) N=245
2. How often have you felt your blood sugars have been unacceptably high recently?	3.3 (2.0) N=346	1.3 (1.7) N=243	1.7 (1.9) N=256
3. How often have you felt your blood sugars have been unacceptably low recently?	1.1 (1.6) N=346	0.7 (1.2) N=243	0.6 (1.2) N=256
4. How convenient have you been finding your treatment recently?	4.9 (1.4) N=346	5.3 (1.2) N=243	5.1 (1.5) N=256
5. How flexible have you been finding your treatment recently?	4.6 (1.5) N=346	5.2 (1.3) N=243	5.1 (1.4) N=256
6. How satisfied are you with your understanding of your diabetes?	4.9 (1.3) N=346	5.1 (1.3) N=243	5.2 (1.1) N=256
7. Would you recommend this form of treatment to someone else with your kind of diabetes?	4.8 (1.7) N=345	4.9 (1.8) N=243	4.7 (1.9) N=256
8. How satisfied would you be to continue with your present form of treatment?	4.6 (1.6) N=346	5.0 (1.6) N=232	4.9 (1.7) N=245

*Derived from the sum of responses to question 1 and questions 4-8 each rated on a scale from 0 to 6, with a high score (maximum 36) representing high treatment satisfaction.

diagnosis, GAD antibodies, C-peptide and genetic factors need to be explored for their utility in predicting other stratified glucose lowering responses (26). However, the use of non-routine biomarkers are less easily adopted in clinical practice. Secondly, the participant satisfaction data suggests people are generally not troubled by the small weight increase from pioglitazone so this medication is best selected for those with OHTG to maximise risk vs benefits. Finally, T2D treatment guidelines could include consideration of OHTG to inform treatment selection between vildagliptin and pioglitazone. Including a more prominent role for pioglitazone over vildagliptin in the presence of OHTG would be reasonable since this medication produces much greater glucose lowering in this subgroup. This is a robust finding that has now been replicated in a non-European population. Pioglitazone treatment selection will need to be considered in the context of other common indications for this medication such as fatty liver or dyslipidaemia, and relatively uncommon contraindications such as heart failure, osteoporosis and macular oedema, as part of individualised care based on patient characteristics.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by NZ Health and Disability Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RM designed the trial. YJ was responsible for the statistical analysis of the data. TRM was responsible for the genetic analysis plan. ML designed the population-specific genetic variant panel. TM assisted with genetic analysis of the data. RM and PRS obtained funding. RB, RY, RT-C, KS, GD, KAM, RD, PC, AM, NN, FK, JH, BO-W, and RP were responsible for trial implementation and data acquisition. RB and RM wrote the first draft of this manuscript and RM handled the revisions. All authors reviewed the manuscript and approved the final version for submission.

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Conflict of interest

Authors RP and RM have received speaking honoraria from Lilly, Boehringer Ingelheim, Astra Zeneca, Sanofi, Novo Nordisk, also Novartis (RM) and Inova pharmaceuticals (RP). RP is a member of the Lilly, Novo Nordisk and Dexcom New Zealand advisory boards. Authors KAM, RD, PC were employed by Ventures/Pinnacle Incorporated, a primary health care organisation which has no relevant commercial or financial relationships.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1091421/full#supplementary-material

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Case report: Diabetic muscle infarction with diabetic ketoacidosis: A rare complication of diabetes

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Background: Diabetic muscle infarction (DMI), which is also referred to as diabetic myonecrosis, is a rare and long-term complication of poorly controlled diabetes mellitus, while we found that acute diabetes decompensation, such as diabetic ketoacidosis (DKA), could also stimulate the occurrence and development of DMI.

Case presentation: A 23-year-old woman with type 1 diabetes presented with a 10-day history of nausea, vomiting, pain, and swelling of her left leg. Her urine ketone test was positive. The 3-beta-hydroxybutyrate and leukocyte counts and creatine kinase levels were elevated. Magnetic resonance imaging of the left thigh revealed extensive deep tissue oedema and an increase in the T2 signal in the involved muscles. Once the diagnosis of DMI was made, she was managed with rest, celecoxib, clopidogrel and aggressive insulin therapy. Three months after treatment, the patient reported complete resolution of symptoms.

Conclusion: DMI is a rare DM complication with a high recurrence rate, commonly presenting with chronic complications, while our case report shows that acute diabetes decompensation, such as DKA, can stimulate the occurrence and development of DMI. Timely diagnosis and appropriate treatment could shorten the recovery time.

KEYWORDS

diabetic muscle infarction, diabetic ketoacidosis, pathogenesis, complication, treatment

Introduction

Diabetic muscle infarction (DMI), which is also referred to as diabetic myonecrosis, is a rare and long-term complication of poorly controlled diabetes mellitus. Angervall and Stener described it in 1965 for the first time and fewer than 200 cases have been reported in the literature (1). It is typically characterized by lower-limb pain that has an acute

onset. The diagnosis and treatment of DMI are often delayed as a result of its nonspecific clinical presentation (1). Diabetic ketoacidosis (DKA) is one of the most common serious acute complications of diabetes; it most often occurs in people with uncontrolled type 1 diabetes and is often accompanied by varying degrees of circulatory volume loss (2). Although most cases of DMI occurring in association with other chronic complications of diabetes have been reported (1), few cases of associated DKA have been reported thus far. We present a case of a 23-year-old woman presenting with vomiting, nausea, left leg pain and swelling who was diagnosed with DKA and DMI, and our review covers the risk factors, as well as the general prognosis and management, of the disorder. Diabetic patients with poorly controlled glucose and limb pain should be kept on high alert for DMI.

Case presentation

A 23-year-old woman with a 9-year history of type 1 diabetes was admitted with vomiting, nausea, pain and swelling of her left leg for 10 days. She was diagnosed with DKA and treated in the local hospital for 3 days without significant improvement; then, she was transferred to our hospital for further management. The patient denied prior episodes of leg pain or trauma without medication with statins, and no peripheral vascular disease was reported. She was treated with insulin to control her glucose level. Her pain worsened with exertion and improved partially with rest. She denied fevers, chills, and weakness of the left leg.

On physical examination, vital signs were notable for temperature of 36.4°C, blood pressure of 116/93 mm Hg, heart rate of 120 bpm, height of 152 cm, weight of 50 kg, and BMI of 21.6 kg/m^2. Other significant findings included swelling of the left leg, with mild warmth and without erythaema. On palpation, there was mild difficulty in the left leg. The pain restricted her muscle strength and motion of the left leg. The dorsalis pedis pulses were good bilaterally. She had no other musculoskeletal symptoms.

Her haemoglobin A1c was 13.7%, random glucose was 18.1 mmol/L, and 3-beta-hydroxybutyrate was 3.67 mmol/L. Her urine acetone bodies were 3+, and her urine protein was 2+. There was mild leucocytosis, and the white blood cell (WBC) count was 9.7× 10^9/L, with 69% neutrophils. She developed mild anaemia, and her haemoglobin (Hg) level was 99 g/L. Serum electrolytes and serum creatinine were within the normal ranges. Her erythrocyte sedimentation rate (ESR) was 95 mm/h, and her highly sensitive C-reactive protein level was 442.9 mg/L; both were elevated. Her creatine kinase (CK) was 1797 U/L on admission and decreased to 1356 U/L after 3 days. She also had a mildly reduced level of serum albumin (32 g/L). Her D-dimer level was normal. Her antinuclear antibodies and anticardiolipin antibodies were normal (Table 1). The fundoscopic examination shows haemorrhage, hard exudates and abnormal blood vessels and proliferative diabetic retinopathy (DR) was diagnosed (Figure 1).

Bilateral venous Doppler ultrasound revealed no abscess or deep vein thrombosis (DVT). Conventional coronal computerized tomography (CT) image and synthetic weighted images (T2WI) with fat suppression (FS) show severe soft tissue swelling in the left thigh. The CT images (Figure 2A) confirmed muscle swelling but no bone involvement of the femur. The corresponding areas of significant edema muscle with slightly increased signal intensity are also indicated on a coronal (Figure 2B) and axial T2WI (Figure 2C). The swelling muscle shows a slightly increased signal intensity on the T2WI map; A marked T2 hyperintensity occurred in the subcutaneous fascia of the left thigh on axial T2WI -FS (Figure 2C).

Intravascular volume correction and intensive insulin therapy were administered to treat the DKA of the patient. After the diagnosis of DMI was made, the treatment for the patient was rest, celecoxib, clopidogrel and insulin, and she improved after 10 days of treatment, with mild left leg swelling and pain. The patient was discharged with instructions for rest, celecoxib, clopidogrel and insulin. After taking celecoxib for one month and clopidogrel for three months, she recovered completely.

Discussion

Diabetic muscle infarction, which commonly presents with swelling and pain in the lower limb muscles, is a rare complication associated with poorly controlled diabetes mellitus (1). Neither the epidemiology nor pathophysiology of DMI is fully understood. A systematic review showed that the mean age at presentation for all DMI cases was 44.6 years old (1), demonstrating a female predominance in patients with DMI. For those with type 1 diabetes (T1DM), the mean age at presentation was 35.9 years old, while for those with type 2 diabetes (T2DM), the mean age at presentation was 52.2 years (1) old. For T1DM, the mean DM duration at the time of DMI diagnosis was 18.9 years, and for T2DM, it was 11.0 years (1). DMI most commonly affects the thigh without fever or trauma history. Previous reviews have identified thigh pain/swelling as the initial presentation in 83.7% and 80% (1) of cases. It was also common to experience calf pain/swelling as a presentation of DMI, and the thigh was the most commonly affected site (75–83.7%) (1, 3), while the calf was the second most commonly affected site (15–19.28%) (3). The patient was 23 years old and had T1DM for 9 years. According to Horton et al., 46.6% of patients had concurrent retinopathy, nephropathy, and neuropathy, and 65.8% had at least two complications, which suggests that DMI is often seen in patients with long-standing diabetes, indicating that DMI is frequently associated with severe microvascular complications (1). Our case showed positive urine protein and proliferative diabetic retinopathy, indicating that the patient suffered from chronic diabetic complications with poor glucose control.

The pathophysiology of DMI remains unclear. DMI can be caused by atherosclerosis, diabetic microangiopathy, vasculitis

TABLE 1 Laboratory findings.

Parameters	Value	Normal range
Complete blood count		
White blood cells (× 10^9/L)	9.7	3.5-9.5
RBC Red blood cells (× 10^12/L)	5.2	3.8-5.1
Haemoglobin (g/L)	99.0	115-150
Platelets (× 10^9/L)	297.0	125-350
Neutrophils (%)	69.0	40-75
Lymphocytes (%)	16.6	20-50
Monocytes (%)	12.9	3-10
Eosinophils (%)	1.1	0.4-8
Basophile (%)	0.4	0-1
Chemistry panel		
Glucose (mmol/L)	18.1	3.9-6.1
Haemoglobin A1c (%)	13.7	<6.5
3-beta -hydroxybutyrate (mmol/L)	3.67	0-0.28
Albumin (g/L)	32.0	40-55
Alkaline phosphatase (U/L)	128	30-128
Blood urea nitrogen (mmol/L)	3.6	2.8-7.6
Creatinine (mmol/L)	54.7	49-90
Uric acid (mmol/L)	325.0	155-357
Total cholesterol (mmol/L)	3.68	<5.18
Triacylglycerol (mmol/L)	1.45	<1.7
High sensitivity C-reactive protein (mg/L)	442.9	0-300
Creatine kinase (U/L)	1797	<145
Creatine kinase-MB (U/L)	70	0-25
Hypersensitivity troponin I (pg/mL)	1.7	0-26.2
Myoglobin (ng/mL)	248.5	<140.1
Lactate dehydrogenase (U/L)	306	110-245
Lactate (mmol/L)	4.92	0.5-2.5
Procalcitonin (ng/mL)	<0.05	<0.05
Erythrocyte sedimentation rate (mm/h)	95	0-20
Blood coagulation function		
Prothrombin time(S)	11.4	9.4-12.5
International normalized ratio	1.04	0.85-1.15
Thrombin time (S)	14.3	10.3-16.6
Fibrinogen-C (mg/dL)	757	238-498
D-dimer (ng/mL)	498	9.4-500

(Continued)

TABLE 1 Continued

Parameters	Value	Normal range
Anti-proliferating cell nuclear antigen	–	–
Antinuclear antibodies	–	–
Rheumatoid factors (kU/L)	<10.1	0.0-15.9
Anticardiolipin antibody-IgM (MPL/mL)	0.419	<12
Anticardiolipin antibody-IgG (GPL/mL)	3.955	<12
Anticardiolipin antibody-IgA (APL/mL)	1.544	<12
Insulin autoantibody	22.8	<20
Glutamic acid decarboxylase antibody	4.948	<30
Urine routines		
Glucose	++++	–
Ketones	+++	–
Protein	++	–
Leukocytes	+++	–

with thrombosis, or ischaemia-reperfusion injury (4). These conditions are associated with long-term poor glucose control with chronic complications. How acute complications such as DKA induce DMI has rarely been reported, and the pathophysiology remains unknown. A case of acute DMI subsequent to diabetic ketoacidosis was reported (5), which mentioned that the dehydration caused by DKA might have induced DMI, and no chronic diabetic complications were present in this patient. In addition to dehydration, some mechanisms might explain the occurrence of DMI in acute complications. Several studies have focused on myocardial infarction in patients with decompensated diabetes, such as those with DKA (6). As a result of hyperviscosity and increased coagulation, DKA precipitates a prothrombotic environment (7). In DMI, hypercoagulability and damage to vascular endothelial cells have been identified as changes in the

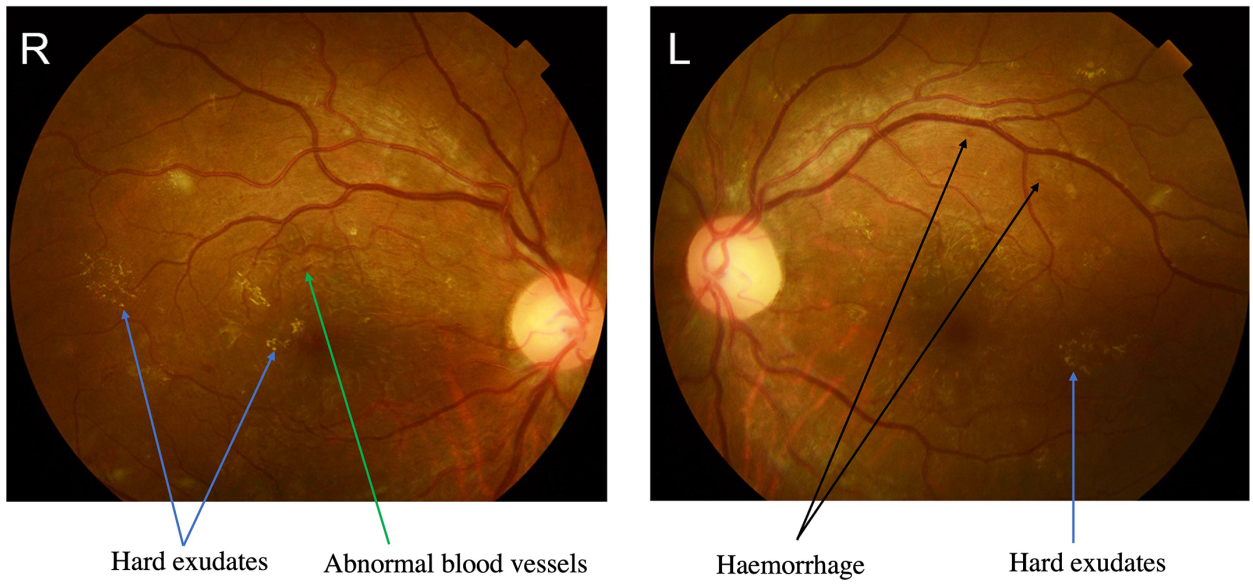


FIGURE 1
The fundus photographs showed severe proliferative diabetic retinopathy (DR). Proliferative DR showing abnormal blood vessels with green arrow, hard exudates with blue arrows and haemorrhage with black arrows.

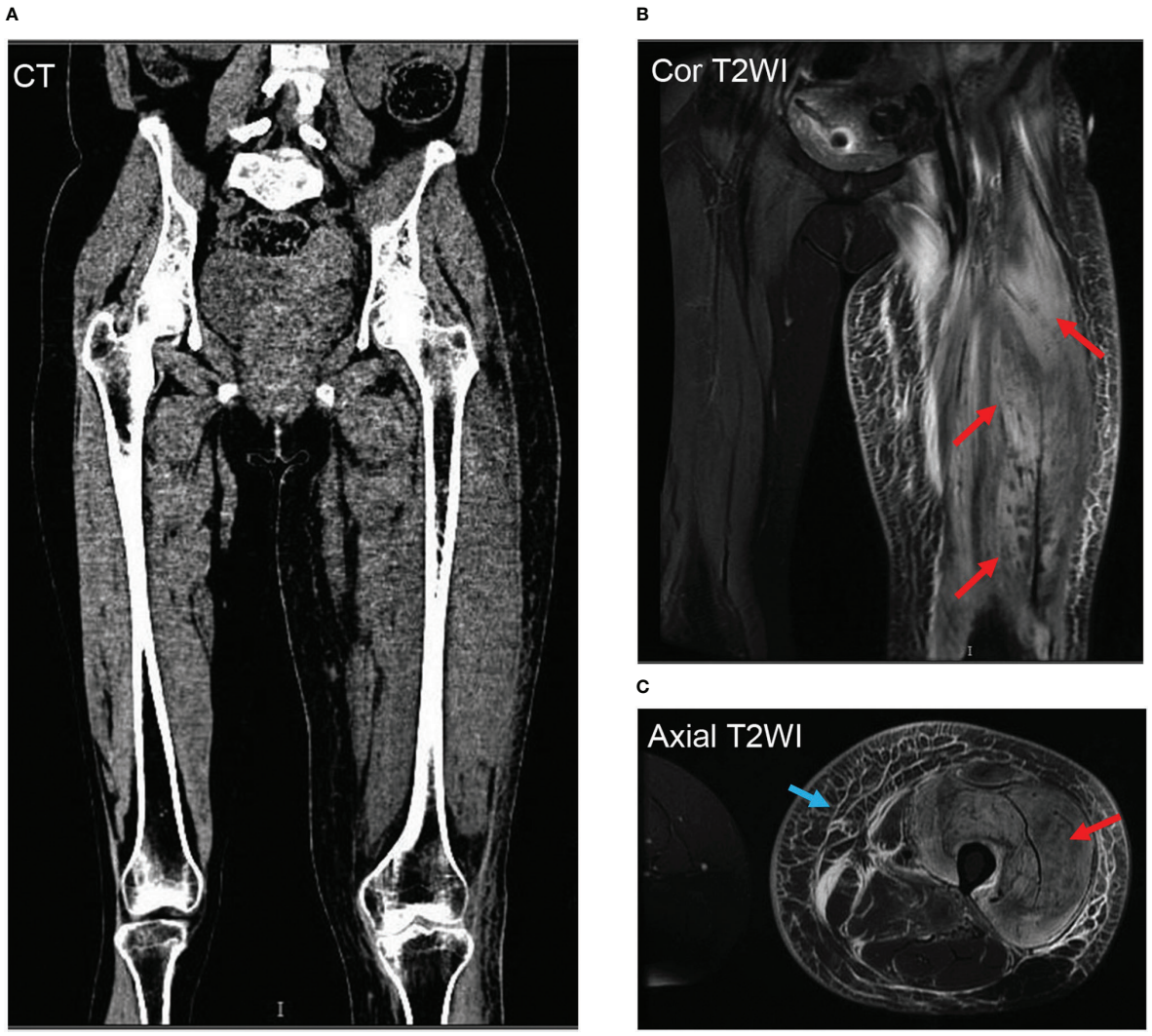


FIGURE 2
Representative thigh muscle images of conventional coronal computerized tomography (CT) image and synthetic weighted images (T2WI) with fat suppression (FS) show severe soft tissue swelling in the left thigh. The CT images **(A)** confirmed muscle swelling but no bone involvement of the femur. The corresponding areas of significant edema muscle with slightly increased signal intensity are also indicated on a coronal **(B)** and axial T2WI **(C)** with red arrows. The swelling muscle shows a slightly increased signal intensity on the T2WI map; A marked T2 hyperintensity occurred in the subcutaneous fascia of the left thigh on axial T2WI -FS with a blue arrow **(C)**.

coagulation-fibrinolysis system (8). Ketoacidosis can also cause coronary vasospasm due to endothelial dysfunction (9). In fact, even compensated diabetic patients with chronic hyperglycaemia have subclinical myocardial injury (10). Therefore, this type of injury might be detected in the other muscles of the body and is a reason why DMI always occurs in those patients with poor glucose control. In the setting of DKA/HHS, increased counterregulatory hormone levels result in an increase in myocardial oxygen, leading to a supply-demand mismatch, resulting in myocardial necrosis (11). The release of free fatty acids was also observed in acute diabetic decompensation (11, 12). By increasing the concentration of free fatty acids in the blood, fatty acids are incorporated into the lipid structure of the myocyte membrane, causing micelles to form, in turn causing the membrane to destabilize and rupture (13). This phenomenon of cellular toxicity may induce muscle infarction (14). These factors could all be involved in the occurrence and development of DMI in these patient, and acute factors could be a new pathogenesis for DMI (Table 2). It is important to exclude antiphospholipid syndrome, especially in patients with T1DM, due to its long-term implications (15), but the antiphospholipid test was negative in our patient.

In diabetic patients who present with lower extremity swelling and pain, DMI should be suspected. As a result of the nonspecific clinical findings of DMI, which are often mimicked by other conditions, such as DVT, pyomyositis, muscle abscesses, muscle

TABLE 2 Summary of possible pathogenesis of DMI.

Acute factors	Chronic factors
Hypercoagulability	Atherosclerosis
Endothelial dysfunction	Diabetic microangiopathy
Cellular toxicity	Vasculitis with thrombosis
	Ischaemia-reperfusion injury

neoplasms, statin-induced myopathy, peripheral vascular disease, osteonecrosis/necrotizing fasciitis, and haematomas, many cases could go undiagnosed or treated inappropriately (16). As a differential diagnosis for leg pain, rhabdomyolysis should be considered, which involves rapid breakdown of the skeletal muscles, allowing toxic cellular contents to leak into the circulation (17). Trauma, lipid lowering drugs, and infections are the most reported causes of rhabdomyolysis in adults, and our patient did not present these kinds of causes. Muscle pain, weakness, and dark tea-colored urine are classic clinical symptoms of rhabdomyolysis (17). In our case, the patient did not present left leg muscle weakness and the urine colour was normal. Acute kidney injury (AKI) is the most common systemic complication of rhabdomyolysis (17), and creatinine of our patient was normal. The pathogenesis of rhabdomyolysis is different from DMI. Rhabdomyolysis follows a common pathophysiological pathway, regardless of its cause. A muscle cell is affected either by direct damage to its membrane or by exhaustion of its energy reserves. Proteases and apoptosis pathways are activated by free ionized calcium entering the intracellular space. Ultimately, mitochondrial dysfunction results in cell death due to the production of reactive oxygen species (17).

No medication history of statins or peripheral vascular disease was reported by this patient. A lack of discriminating laboratory findings further compounds this diagnostic uncertainty. Laboratory investigations for DMI are relatively nonspecific (18). The white cell count (WCC) was within normal limits in 56.6% of cases, elevated in 42.5%, and decreased in 0.9%. In 67 of 126 cases, creatine kinase (CK) values were reported, and 68.4% were within normal limits. There is some evidence that erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) levels could aid in the diagnosis of DMI. In 60 of 126 cases, ESR values were elevated by 83.3%. Among the 30 reported cases, 27 (90%) had elevated CRP. An autoimmune workup was performed in only a few patients (1).

Diabetic limb pain can be associated with elevated white blood cell count, creatinine kinase, and inflammatory markers. The affected limb usually has an abnormal linear echotexture on ultrasonography (19). This review found that eighty-two of 83 (98.8%) cases had negative DVT ultrasonography findings (1). There are fewer specific CT findings in DMI than on MRI, but muscle enlargement with diminished attenuation, thickening of adjacent fascial planes and skin overlying the infarction, and increased subcutaneous attenuation could be observed (3). MRI has been shown to be an effective diagnostic tool in recent studies. In fat-suppressed T-2 weighted images, abnormal MRI findings have shown a sensitivity close to 100%. Diagnosis can be made with MRI due to its sensitivity and specificity (3). T2-weighted images usually show hyperintensity, whereas T1-weighted images usually show isointense to hypointense signals from the affected muscle, with associated subcutaneous, perifascial, and/or perimuscular oedema (3).

It is the gold standard of diagnosis to perform a muscle biopsy, but this procedure might not be necessary if classic history and MRI findings are present. Due to the increased time to symptom improvement and procedure-associated complications, muscle biopsy is not recommended for definitive diagnosis at this time (20). The pathology specimens for muscle biopsy usually show necrosis and oedema (1).

Intensive glycaemic control, rest, and NSAID treatment are the major recommendations for treatment. Shortening recovery time and improving pain might be possible with NSAIDs. The combination of rest plus NSAIDs resulted in a short recovery time. The antiplatelet therapy aspirin is commonly prescribed, but evidence regarding its efficacy is equivocal (1), and our patient was treated with clopidogrel. Physical therapy and surgery are not recommended (1) Physical therapy could prolong recovery time unless the lesion is fully healed. DMI patients with surgery had a mean time to symptom resolution of >80 days compared to patients with bed rest of approximately 40 days, and patients who underwent surgery had a higher recurrence rate (1). The risk of complications and recurrence should be decreased with adequate glycaemic control, rest, and NSAID use (1).

In summary, DMI is a rare DM complication with a high recurrence rate, commonly presenting with chronic complications, while our case report shows that acute diabetes decompensation, such as DKA, can stimulate the occurrence and development of DMI. Timely diagnosis and appropriate treatment could shorten the recovery time.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Zhongnan Hospital of Wuhan University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JT, ZD and Y-CX: conception and design of the work. QH, Y-WW and XL: data collection. LS, H-HD and J-ZS: Image analysis and interpretation. JT, LS manuscript writing. All authors were involved in revising manuscript critically. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effectiveness and safety of hydrogen inhalation as an adjunct treatment in Chinese type 2 diabetes patients: A retrospective, observational, double-arm, real-life clinical study

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Aim: To analyze the effectiveness and safety of hydrogen inhalation (HI) therapy as an adjunct treatment in Chinese type 2 diabetes mellitus (T2DM) patients in a real-life clinical setting.

Methods: This observational, non-interventional, retrospective, double-arm, 6-month clinical study included T2DM patients receiving conventional anti-diabetes medication with or without HI initiation from 2018 to 2021. Patients were assigned to the HI group or non-HI group (control group) after 1:1 propensity score matching (PSM). The mean change in glycated hemoglobin (HbA1c) after 6 months in different groups was evaluated primarily. The secondary outcome was composed of the mean change of fasting plasma glucose (FPG), weight, lipid profile, and homeostasis model assessment. Logistics regression was performed to evaluate the likelihood of reaching different HbA1c levels after 6-month treatment between the groups. Adverse event (AE) was also evaluated in patients of both groups.

Results: In total, 1088 patients were selected into the analysis. Compared to the control group, subjects in HI group maintained greater improvement in the level of HbA1c (-0.94% vs -0.46%), FPG (-22.7 mg/dL vs -11.7 mg/dL), total cholesterol (-12.9 mg/dL vs -4.4 mg/dL), HOMA-IR (-0.76 vs -0.17) and HOMA-β (8.2% vs 1.98%) with all $p< 0.001$ post the treatment. Logistics regression revealed that the likelihood of reaching HbA1c< 7%, ≥ 7% to< 8% and > 1% reduction at the follow-up period was higher in the HI group, while patients in the control group were more likely to attain HbA1c ≥ 9%. Patients in HI group was observed a lower incidence of several AEs including hypoglycemia (2.0% vs

6.8%), vomiting (2.6% vs 7.4%), constipation (1.7% vs 4.4%) and giddiness (3.3% vs 6.3%) with significance in comparison to the control group.

Conclusion: HI as an adjunct therapy ameliorates glycemic control, lipid metabolism, insulin resistance and AE incidence of T2DM patients after 6-month treatment, presenting a noteworthy inspiration to existing clinical diabetic treatment.

KEYWORDS

type 2 diabetes, observational study, hydrogen inhalation, glycemic control, real world study

1 Introduction

Diabetes is one of the most common chronic metabolic diseases with global morbidity of 9.3% in 2019 and expected 10.9% in 2045 (1), while Asian countries account for more than 60% of the diabetic population worldwide (2). China owns the largest number of diabetic patients in the world which is up to 109 million (3). T2DM contributes to more than 91% of all diabetes cases, which is estimated to cost more than 2 billion USD after a decade, while most of the costs are brought by complications which could be largely avoided by reaching and remaining glycemic target (4–6).

The current therapeutic strategy for T2DM mainly includes lifestyle amelioration and medication. If the patient fails to gain adequate glycemic control *via* lifestyle intervention composed of changes in diet and exercise (7), hypoglycemic medication initiation is recommended. As diabetes processes, Intensification treatment with multiple drugs will be utilized if required, which includes dual combination therapy, triple combination therapy or the addition of insulin (8).

However, the oral antidiabetic drug often results in insulin resistance or adverse event, leading to low efficacy (9). Optimal glycemic control is hard to obtain after insulin usage on account of the increasing risk of weight gain and hypoglycemia (10). Besides, the HbA1c target of< 7% is maintained by no more than half of the patients taking basal insulin in a meta-analysis (11). Although the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) recommended the glucagon-like peptide 1 receptor agonist as the first choice of injectable medication for individuals with T2DM, patients' compliance in the real-world study (RWS) is more than low, resulting in a non-negligible difference between RWS and randomized controlled trial (RCT) (5, 6, 12, 13). Despite antidiabetic medicine updating in the past few years, no more than half of Chinese diabetic patients have the capability to gain adequate glycemic control (14). Furthermore, comprehensive and effective therapy for patients with multiple metabolic disorders is especially demanded (15).

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the activity of the antioxidant defense system (16), which is widely concerned with multiple diseases including ischemia-reperfusion injury and inflammatory and neurological disease (17). Physiological ROS plays an important role in the maintenance and regulation of various physiological processes while redundant production of ROS would result in damage to proteins, DNA and lipids (18, 19). The development of metabolic disorders in diabetes are related to oxidative stress and inflammation (20). Thus, quenching or lowering ROS may be a potential antidiabetic means. Previous study suggested that antioxidant vitamins have a positive impact on glycemic control in T2DM patients (21). However, vitamin antioxidants application is associated with several adverse effects including mortality increase and heart failure (22, 23).

Hydrogen (H2) is a newly developed antioxidant which could diffuse across cytomembrane easily and selectively eliminate cytotoxic ROS without influencing ROS acting as parts of physiological function (24, 25). Furthermore, molecular hydrogen has been proved of a quantity of preventive and therapeutic effects in human and animal disease models, which include ischemia–reperfusion injuries (26, 27), neurodegeneration (28, 29), cardiovascular diseases (30, 31), metabolic syndrome (32), inflammation (33, 34) and cancer (35, 36). Recently, there are numerous reports about the prominent effect of molecular hydrogen in both T2DM patients and diabetic animal models (37–41). Previous RCT has already revealed the fact that the high-concentration hydrogen-rich water could attenuate the HbA1c and FPG levels of 60 patients with metabolic syndrome in 24-week treatment (42). Although RCT is the golden standard for evidence-based medicine, RWS displays clinical experience extensively and the diversity of patients' distribution, offering a better insight into the real-life medical situation.

There are plenty of molecular hydrogen ingestion methods, including oral ingestion by drinking hydrogen water, hydrogen-

saline injection, direct incorporation of molecular hydrogen by diffusion, maternal intake of hydrogen and hydrogen gas inhalation (43). With the progressive popularization of hydrogen medicine, the antidiabetic effect of molecular hydrogen is extensively acknowledged. While the utilization of medicinal gases as a therapeutic strategy is gaining ever-growing attention, hydrogen inhalation (HI) is widely accepted by the Chinese public, with a substantial amount of manufactory producing hydrogen generation machines (44, 45). An increasing number of Chinese diabetic patients are taking HI by applying the hydrogen-producing machine as a means of maintaining adequate glucose control. However, no research has focused on the effect of T2DM patients utilizing HI ever before to our knowledge.

Therefore, this retrospective study aims at revealing the effectiveness and safety of HI as an adjunct treatment in Chinese patients with type 2 diabetes in a real-world setting, adding more data to the evidence base of T2DM patients receiving HI treatment as explorational research.

2 Material and methods

2.1 Study design and patient population

This is a non-interventional, retrospective, multicenter, observational, double-arm study to analyze the real-world effectiveness and safety of HI as an adjunct treatment in Chinese patients with T2DM. The data were selected from electronic medical records in the multi-institutional health records database, which is composed of individual demographic characteristics, medical procedures, diagnoses (International Classification of Diseases, 10th Revision) and medicinal prescriptions from multiple hospitals and health examination centers in Tsingtao, China. All the eligible patients were anonymous by utilizing special study numbers.

Type 2 diabetes patients who kept conventional hypoglycemic treatment for at least 6 months with or without HI initiation between January 1, 2018 and December 31, 2021 were considered eligible for this study. Subjects were assigned to the HI group or non-HI group (control group) depending on previous treatment details. In the HI group, individuals without former molecular hydrogen treatment initiated and kept hydrogen therapy in the specific hydrogen therapy departments where the hydrogen gas was generated by the hydrogen-producing machine (HZS-2700A, Qingdao Haizhisheng Corp.,LTD, Tsingtao, China) which provided 100% hydrogen gas at the speed of 3000 ml/h by water electrolysis. The index date was defined as the time when HI was initiated or hypoglycemic medication was applied. The follow-up was defined as 6 ±; 1 months after the index date while 1 month before and after the index date was defined as the baseline.

Patients were included in the study in accordance with the following criteria: (1) Chinese adults (≥ 18 years of age) of both sexes. (2) diagnosed with T2DM. (3) at least one laboratory value in both periods of baseline and follow-up. (4) at least 25 hours of HI therapy per week or at least one hypoglycemic prescription at the baseline throughout the study. Meanwhile, the exclusion criteria are (1) type 1 or any other specific diabetes (e.g. gestational, secondary, steroid). (2) maintaining concurrent severe medical diseases or severe mental conditions. (3) pregnant or lactating. (4) participation in other studies simultaneously.

2.2 Data collection and assessments

Baseline characteristics included gender, age, HbA1c, diabetes duration, body mass index (BMI), lipid profile (triglyceride, total cholesterol, low-density lipoprotein and high-density lipoprotein), diabetes complications (i.e., diabetic retinopathy, neuropathy, nephropathy), cardiovascular diseases (i.e., angina pectoris, myocardial infarction, heart failure), antihyperglycemic treatment (i.e., biguanides, thiazolidinediones, insulin) and antihypertensive therapy (i.e., diuretics, beta-blockers, calcium channel blockers). HbA1c, FPG, lipid profile, homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β) and weight were assessed at the follow-up.

HOMA-IR and HOMA-β were determined as follows:

$$\text{HOMA} - \text{IR} = \text{FPG}\,(\text{mg/dL}) \times \text{FINS}\,(\mu\text{U/L})/405$$

$$\text{HOMA} - \beta = 360 \times \text{FINS}\,(\mu\text{U/L})/(\text{FPG}\,(\text{mg/dL}) - 63)\,(\%)$$

Safety was evaluated by recording hypoglycemia, adverse event (AE) and serious adverse event (SAE) during the study. All the AEs were extracted from the medical records documented by their attending physicians.

The change of HbA1c level from baseline to follow-up was evaluated primarily. The secondary endpoint included the change in FPG, weight, HOMA-IR, HOMA-β and lipid profile after 6 months. The proportion of patients with HbA1c level of< 7%, ≥ 7% to< 8%, ≥ 8% to< 9%, ≥ 9% and > 1% reduction after the 6-month follow-up period were also analyzed.

For subjects having more than one laboratory data in the baseline and follow-up period, the values closest to the index date and 6 months post index date were used.

2.3 Statistical analysis

To adjust the possible selective bias and to make the cohorts more comparable and homogeneous, the PSM was applied. Depending on the baseline parameters displayed in Table 1, a logistic regression model was established to estimate the propensity scores of different treatment groups.

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TABLE 1 Baseline characteristics of patients after propensity score matching in the study.

Variable	HI group (N = 544)	Control group (N = 544)	P-value
Gender (female)	278 (51.1%)	269 (49.4%)	0.182
Age, years	62.4 ± 8.9	63.1 ± 9.3	0.256
BMI, kg/m2	28.3 ± 3.5	28.8 ± 3.3	0.462
Diabetes duration, years	10.1 ± 3.0	9.9 ± 2.9	0.059
HbA1c, %	8.98 ± 0.85	9.03 ± 0.85	0.697
Lipid profile			
TG, mg/dL	160.0 ± 40.1	160.7 ± 41.0	0.783
TC, mg/dL	170.0 ± 37.2	169.1 ± 36.3	0.478
HDL, mg/dL	44.1 ± 7.8	44.3 ± 7.8	0.864
LDL, mg/dL	99.2 ± 15.0	97.9 ± 15.7	0.192
Diabetes complications			
Diabetic retinopathy	61 (11.2)	72 (13.2)	0.098
Diabetic neuropathy	108 (19.9)	116 (21.3)	0.752
Diabetic nephropathy	89 (16.4)	80 (14.7)	0.061
Cardiovascular diseases			
Hypertension	304 (55.9)	310 (57.0)	0.382
Hyperlipidemia	352 (64.7)	359 (66.0)	0.092
Coronary heart disease	112 (20.6)	118 (21.7)	0.216
Peripheral artery disease	12 (2.2)	10 (1.8)	0.546
Angina pectoris	127 (23.3)	125 (23.0)	0.896
Myocardial infarction	15 (2.8)	17 (3.1)	0.476
Heart failure	16 (2.9)	17 (3.1)	0.912
Dementia	2 (0.4)	3 (0.6)	0.745
Stroke	103 (18.9)	98 (18.0)	0.392
Cancer	56 (10.3)	51 (9.3)	0.298
Chronic obstructive pulmonary disease	34 (6.3)	36 (6.6)	0.465
Liver disease	9 (1.7)	8 (1.5)	0.341
Background anti-diabetes medications			
Biguanides	326 (59.9)	330 (60.7)	0.348
Sulphonylureas	168 (30.9)	162 (29.8)	0.548
Thiazolidinediones	82 (15.1)	83 (15.3)	0.786
α-glucosidase inhibitors	60 (11.0)	58 (10.7)	0.723
DPP-4 inhibitors	65 (11.9)	61 (11.2)	0.218
SGLT-2 inhibitors	38 (7.0)	39 (7.2)	0.812
GLP-1 receptor agonists	27 (5.0)	30 (5.5)	0.409
Insulin	351 (64.5)	356 (65.4)	0.293

(Continued)

TABLE 1 Continued

Variable	HI group (N = 544)	Control group (N = 544)	P-value
Glinides	11 (2.0)	9 (1.7)	0.582
Other	2 (0.4)	1 (0.2)	0.801
Blood pressure–lowering medications			
Diuretics	65 (11.9)	64 (11.8)	0.908
ACE inhibitors	54 (9.9)	57 (10.5)	0.168
ARBs	243 (44.7)	246 (45.2)	0.380
Beta-blockers	136 (25.0)	132 (24.3)	0.731
Calcium channel blockers	216 (39.7)	218 (40.1)	0.490
Other	4 (0.7)	3 (0.6)	0.812

All data are presented as mean ±; SD or as number (%).
HI, hydrogen inhalation; BMI, body mass index; HbA1c, glycated hemoglobin; TG, triglycerides; TC, Total Cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; DPP-4 dipeptidyl peptidase-4; SGLT-2, sodium-glucose co-transporter-2; GLP-1 glucagon-like peptide-1; ACE angiotensin-converting enzyme; ARB angiotensin-receptor blockers.

The PSM was performed with a greedy nearest neighbor 1:1 matching technique and a caliper of 0.05 on the propensity score scale.

Numerical data were expressed as the mean ± standard deviation (SD). Paired t-tests were applied to measure the differences in laboratory parameters from baseline to the end of the study within the same cohort, meanwhile, the independent t-tests were to analyze the changes in laboratory indicators between different groups. The means with 95% confidence interval (CI) were used to demonstrate the changes in laboratory parameters after 6-month treatment. Ordinal data were presented as frequency (percentage) and analyzed by the $\chi 2$ test. the logistic regression model was performed to examine the proportion of patients reaching HbA1c level< 7%, ≥ 7% to< 8%, ≥ 8% to< 9%, ≥ 9% and > 1% reduction after 6 months. odds ratio (OR) and 95% CI were calculated to compare the different cohorts.

To evaluate the robustness of the study, repeated analyses had been performed in subgroups based on several baseline values, including HbA1c (stratified by< 8% and ≥ 8%), BMI (stratified by< 28 kg/m2 and ≥ 28 kg/m2), usage of hypoglycemic drugs prior to the study (stratified by< 2 and ≥ 2), as previous studies showed that distinct BMI, glycemic level and former hypoglycemic treatment failures at the baseline may lead to different anti-diabetic treatment effect (46–49).

To display statistically significant differences in HbA1c change between different treatment groups with a power of 95%, each cohort demanded the sample size of 445 patients, assuming a minimum expected reduction in HbA1c of 0.1%, SD of 0.5% and 0.01 in two-sided significant level.

Statistical analyses were carried out with SAS 9.2 (SAS Institute, Cary, NY, USA). All statistical tests were two-tailed tests and considered the p-value<0.05 as statistically significant.

3 Result

3.1 patient flow and baseline characteristics

Depending on the inclusion and exclusion criteria, a total number of 1,603 patients were identified, composed of 708 subjects initiating HI therapy and 895 individuals treated with conventional hypoglycemic agents.544 pairs were selected for analysis after 1:1 propensity score matching (Figure 1). Patient demographic and clinical characteristics at baseline were comparable between the two cohorts, including gender, age, diabetes duration, HbA1c level, lipid profile, diabetes complications, cardiovascular diseases and concomitant hypoglycemic and antihypertensive medications (all p-value > 0.05) (Table 1).

3.2 Effectiveness evaluation

After 6-month treatment, both the levels of HbA1c and FPG decreased in HI group (-0.94%; 95% CI -1.04 to -0.85 and -22.7 mg/dL; 95% CI -27.0 to -18.5) and control group (-0.46%; 95% CI -0.56 to -0.35 and -11.7 mg/dL; 95% CI -16.5 to -7.0) with all p< 0.001. Meanwhile, greater reductions in HbA1c and FPG were observed in the HI group (p< 0.001 and p = 0.001 separately) (Figure 2).

HI therapy could lead to significant improvement in TC (-12.9 mg/dL; 95% CI -17.4 to -8.3), HDL (1.0 mg/dL; 95% CI 0.2 to 1.9) and LDL (-4.1 mg/dL; 95% CI -6.0 to -2.3). Meanwhile, it demonstrated reduction of LDL level (-3.4 mg/dL; 95% CI -5.3 to -1.6) with p< 0.001 in patients treated with conventional anti-diabetes medication. Compared to the control group, HI therapy only showed the favorable effect on TC (p=0.01) (Figure 3A).

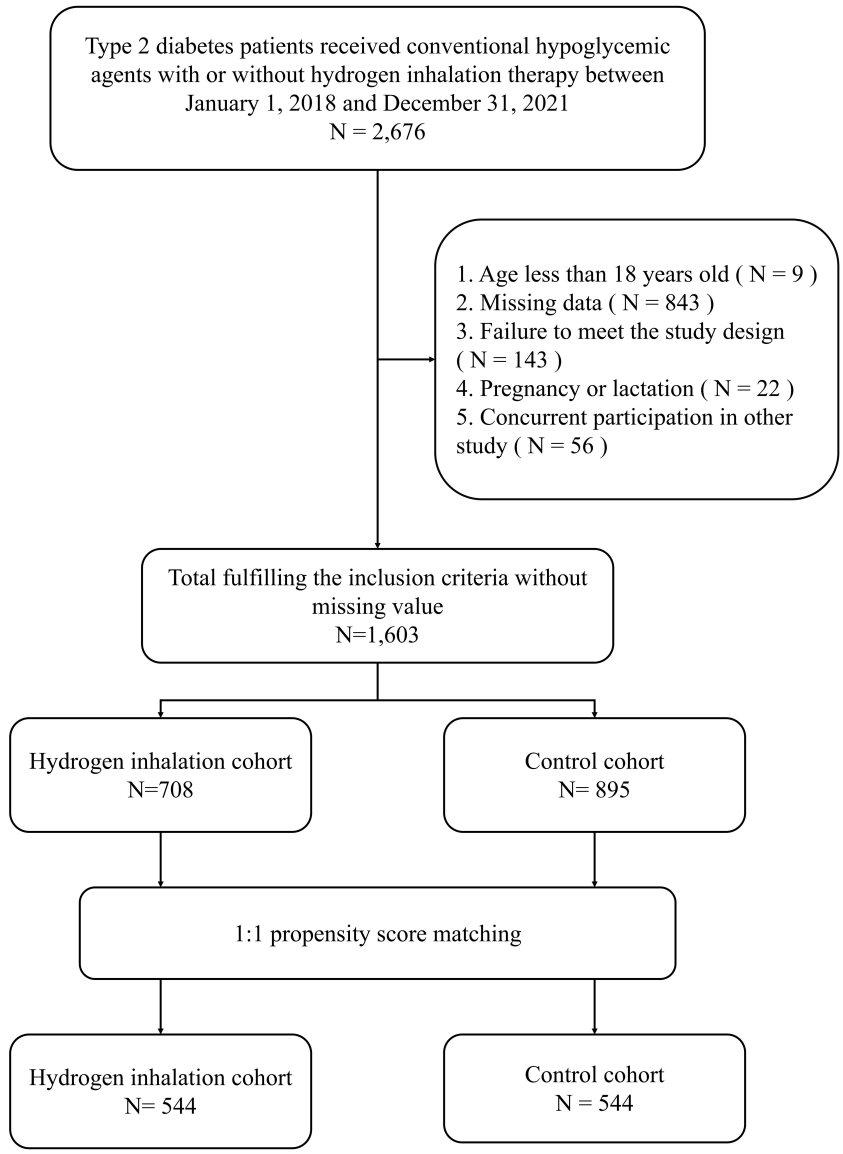


FIGURE 1
flowchart of patient selection.

There were significant improvements of both HOMA-IR and HOMA-β in HI group (-0.76; 95% CI -0.87 to -0.66 and 8.20%; 95% CI 7.28 to 9.11) and control group (-0.17; 95% CI -0.28 to -0.06 and 1.98%; 95% CI 1.09 to 2.87). fewer ameliorations were displayed in the control group of both indexes (all p< 0.001) (Figure 3B). Neither HI therapy nor conventional hypoglycemic agents could significantly influence body weight after 6 months (Figure 3C).

The likelihood of reaching HbA1c< 7%, ≥ 7% to< 8% and > 1% reduction at the follow-up period was higher in HI group with OR (95% CI) of 2.72 (1.50, 4.38), 2.02 (1.51, 2.69) and 4.35 (3.12, 5.48) separately, while subjects in control group were more likely to attain HbA1c ≥ 9% (all p< 0.001). The similar possibility of having HbA1c ≥ 8% to< 9% was presented in both cohorts (Figure 4).

3.3 Safety outcome

The gastrointestinal adverse event was the most common AE both in HI group (10.9%) and control group (15.9%). Compared to control group, the HI therapy leaded to lower incidence of several AEs including hypoglycemia (2.0% vs 6.8%, p< 0.001), vomiting (2.6% vs 7.4%, p< 0.001), constipation (1.7% vs 4.4%, p = 0.008) and giddiness (3.3% vs 6.3%, p = 0.023). There was no

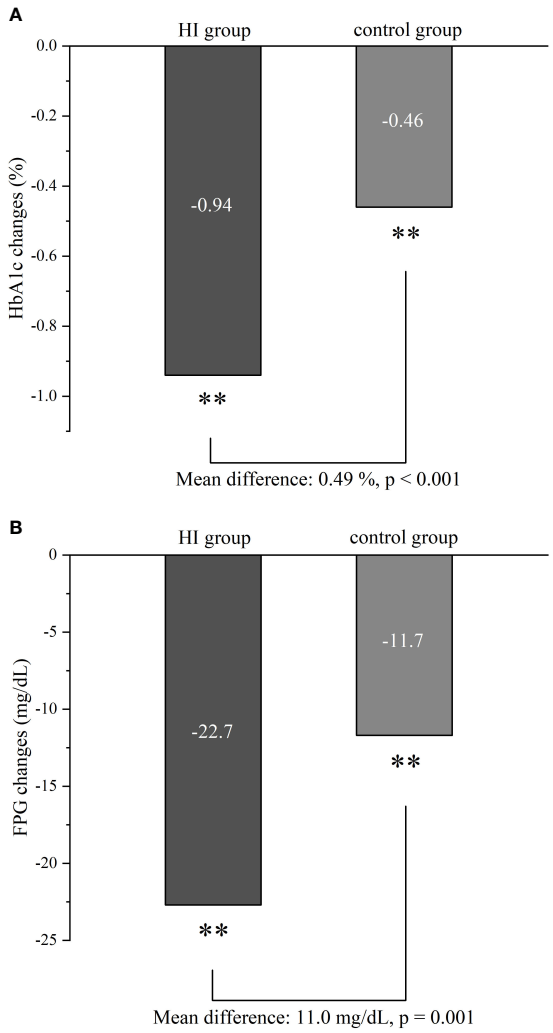


FIGURE 2
Comparison of glycemic control in HbA1c **(A)** and FPG **(B)** between HI group and control group from baseline to follow-up. **p < 0.001. Abbreviation: HI, hydrogen inhalation; HbA1c, glycated hemoglobin; FPG, fasting plasma glucose.

SAE recorded throughout the whole study. The frequency of adverse event episode incidence is summarized in Table 2.

4 Discussion

As no similar research has been carried out in this ever-growing field before, this observational, non-interventional, retrospective, double-arm, multicenter study focuses on revealing the effectiveness and safety of HI as an adjunct treatment in Chinese patients with type 2 diabetes in a real-world setting. Compared to conventional anti-diabetes medication, HI therapy is associated with greater amelioration in HbA1c, FPG, TC level and insulin resistance. Treatment with HI therapy versus control group leads to the higher likelihood of reaching HbA1c< 7%, ≥ 7% to< 8%, > 1% reduction and a lower possibility of achieving HbA1c ≥ 9% after 6 months.

Although hydrogen-water was often utilized in previous diabetic studies, low saturation of hydrogen would limit the body abortion (39). Otherwise, the comparison result which shows the effect of different hydrogen administration methods in normal rats also reveals that most serum biochemical factors alter more significantly in HI group than the hydrogen-water group, which is at least partially due to its longer period of hydrogen concentration maintenance (50, 51).

In comparison to patients treated with conventional hypoglycemic medication, significantly greater reductions of HbA1c and FPG are observed in subjects receiving HI therapy at the follow-up period. It is in accordance with the result of previous RCT revealing that hydrogen-rich water could ameliorate HbA1c and FPG level (42). However, a study applying hydrogen-rich water to 36 T2DM patients showed no significance in lowering the HbA1c concentration, which might be due to different usage of the molecular hydrogen and low sample size (41). Several previous animal researches also indicate that molecular hydrogen is associated with FPG-lowering effect (37, 38, 40). The glycemic improvement might be realized by activating phosphatidylinositol-3-OH kinase, protein kinase C and AMP-activated protein kinase (52).

As high blood glucose levels, insulin resistance and hyperinsulinemia often exist in T2DM patients, these factors would result in abnormal lipid metabolism (53, 54). Hydrogen gas shows a favorable effect in reducing TC level in the present study, showing the same trend as the study of hydrogen treatment before (37, 38, 40). HDL plays a beneficial role in transporting cholesterol reversely (55) which significantly increases in the HI group, but this effect is similar in patients without molecular hydrogen treatment. Previous hydrogen RCTs showed contradictory effects of HDL in metabolic syndrome (32, 42, 56). Although the significant reduction in LDL level presents in both the cohorts, no significant difference is determined between two groups. At the 6-month follow-up period, TG shows a decreasing trend without significance in both groups. It is consistent with previous molecular hydrogen study (52), while hydrogen-water was reported to restrain the increase of plasma TG in another diabetic mice model (57).

Hydrogen gas would result in greater recovery of insulin resistance and β-cell function as HOMA-β and HOMA-IR display a greater amelioration in the HI group. Multiple previous studies showed similar result (37–39, 41). Ectopic deposition of lipids in liver tissue is a non-negligible reason for insulin resistance (58). Hydrogen could suppress this process, which might be a reason for the insulin resistance improvement effect (40).

As ADA declares that the appropriate HbA1c goal for nonpregnant adults is reaching< 7% (53 mmol/mol) (59), Patients treated with HI achieve a 172% higher likelihood of reaching HbA1c< 7% at the follow-up period with an OR (95% CI) of 2.72 (1.50, 4.38) in comparison to the control group in this study.

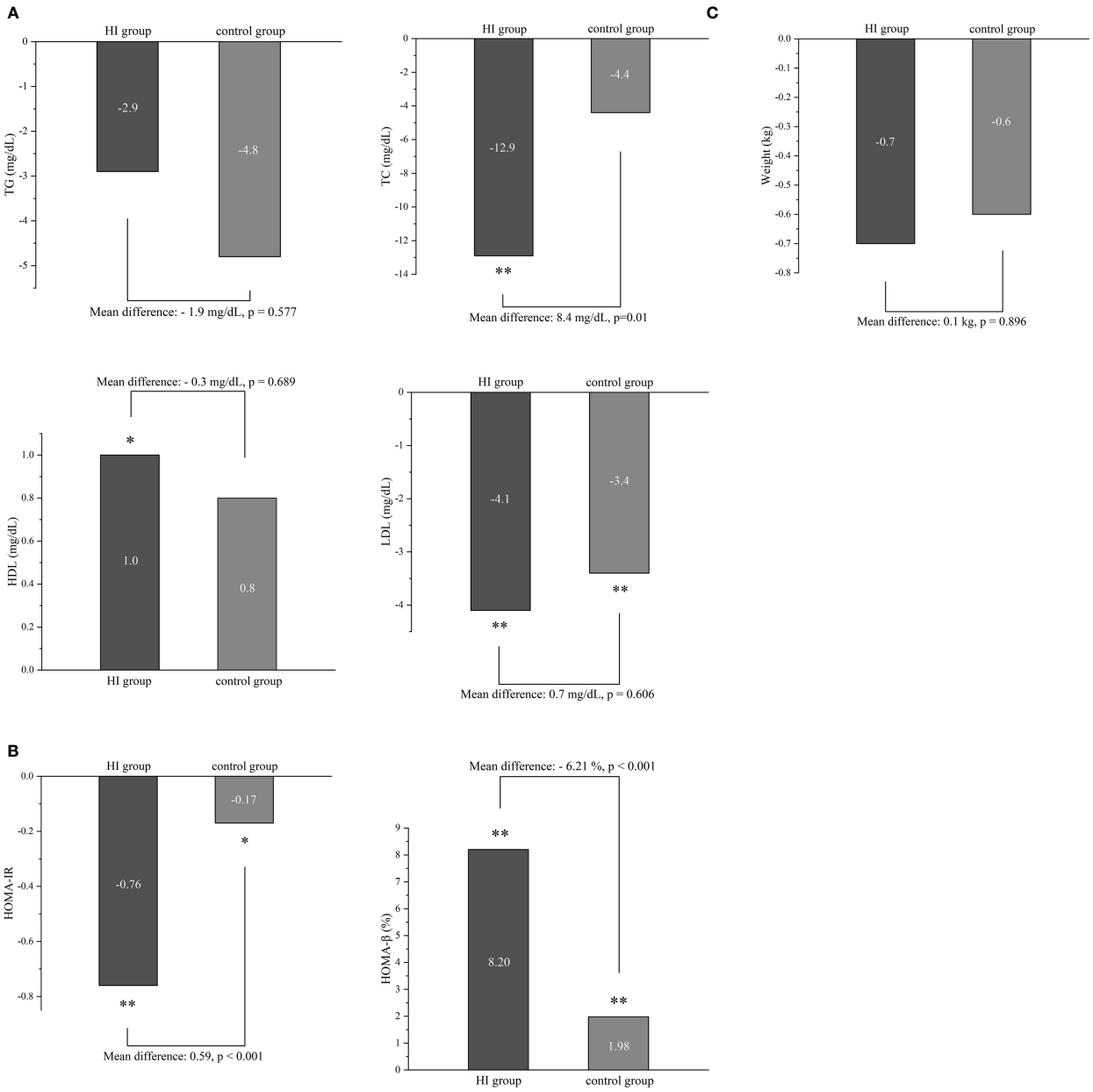


FIGURE 3
The difference in lipid profile **(A)**, insulin resistance **(B)** and body weight **(C)** between two groups during the study. *p < 0.05, **p < 0.001. Abbreviation: HI, hydrogen inhalation; TG, triglycerides; TC, Total Cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function.

The gastrointestinal adverse event is the most common AE in both cohorts while no severe adverse event is observed. Compared to the control group, HI therapy is associated with the lower incidence of several AEs including hypoglycemia, vomiting, constipation and giddiness, which indicates that molecular hydrogen treatment would result in fewer side effects and increase safety for human body. In previous studies, hydrogen treatment has been proved to be a safe therapy (60, 61).

The therapeutic mechanism of hydrogen is due to selectively address of OH·, which could damage cellular components and result in cellular necrosis and apoptosis with no targeted detoxification route (17, 24). Previous animal diabetic models proved that molecular hydrogen could improve diabetes and obesity by enhancing the expression of Hepatic fibroblast growth factor 21 (57). Recently, another study revealed that hydrogen may lower blood glucose and lipid *via* restraining TLR4/MyD88/NF-κB signaling (37).

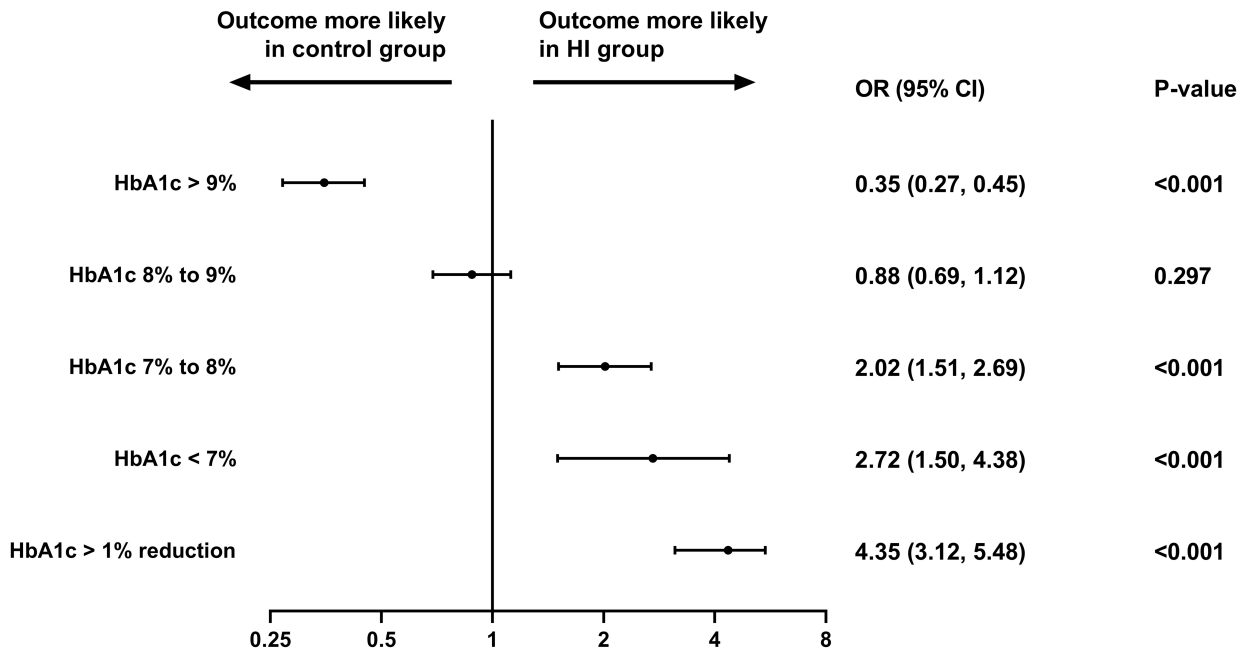


FIGURE 4
Different HbA1c level attainment at the follow-up period in the study. Abbreviation: HI, hydrogen inhalation; HbA1c, glycated hemoglobin; OR, odds ratio; CI, confidence interval.

TABLE 2 Adverse events recorded in the HI group and control group throughout the study.

Variable	HI group (N=544)	Control group (N=544)	P-value
Hypoglycemia	11 (2.0)	37 (6.8)	<0.001
Abdominal pain	7 (1.3)	15 (2.8)	0.085
Vomiting	14 (2.6)	40 (7.4)	<0.001
Bloating	4 (0.7)	7 (1.3)	0.363
Constipation	9 (1.7)	24 (4.4)	0.008
Urinary tract infection	21 (3.9)	27(5.0)	0.376
Palpitation	18 (3.3)	12 (2.2)	0.267
Giddiness	18 (3.3)	34 (6.3)	0.023

Data are displayed as number (%).
HI, hydrogen inhalation.

This study has several limitations: First of all, due to its retrospective nature, the evaluation of the therapeutic response of hydrogen treatment might be influenced by inevitable sample bias including but not limited to the variability of concurrent medication, deviation of patient selection and ambiguous metabolism within human body. Furthermore, the AEs are extracted from medical records, leading to the possibility of a few neglected incidents. Lastly, the present study only includes Chinese patients with T2DM, and it is necessary to verify the effects of hydrogen gas in populations from other countries. Despite these limitations, this observational study would provide valuable real-world data which have never been reported previously and present nonnegligible inspiration for existing clinical practice.

5 Conclusion

In summary, HI therapy as an adjunct treatment improves the glycemic control of Chinese T2DM patients in a real-world setting, ameliorating the lipid profile and insulin resistance, increasing the likelihood of reaching the HbA1c< 7% target after 6-month treatment with a lower incidence of AE. It reveals

an optional and effective strategy for the clinical treatment of T2DM.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Medical College of Qingdao University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZZ, HJ and TN contributed to the study concept and design. TN contributed to the data collection. ZL and RS performed the statistical analyses and interpreted the results. YL and YZ wrote the initial draft of the manuscript. TN reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Methylglyoxal products in pre-symptomatic type 1 diabetes

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Introduction: Progression to type 1 diabetes has emerged as a complex process with metabolic alterations proposed to be a significant driver of disease. Monitoring products of altered metabolism is a promising tool for determining the risk of type 1 diabetes progression and to supplement existing predictive biomarkers. Methylglyoxal (MG) is a reactive product produced from protein, lipid, and sugar metabolism, providing a more comprehensive measure of metabolic changes compared to hyperglycemia alone. MG forms covalent adducts on nucleic and amino acids, termed MG-advanced glycation end products (AGEs) that associate with type 1 diabetes.

Methods: We tested their ability to predict risk of disease and discriminate which individuals with autoimmunity will progress to type 1 diabetes. We measured serum MG-AGEs from 141 individuals without type 1 diabetes and 271 individuals with type 1 diabetes enrolled in the Fr1da cohort. Individuals with type 1 diabetes were at stages 1, 2, and 3.

Results: We examined the association of MG-AGEs with type 1 diabetes. MG-AGEs did not correlate with HbA1c or differ between stages 1, 2, and 3 type 1 diabetes. Yet, RNA MG-AGEs were significantly associated with the rate of progression to stage 3 type 1 diabetes, with lower serum levels increasing risk of progression.

Discussion: MG-AGEs were able to discriminate which individuals with autoantibodies would progress at a faster rate to stage 3 type 1 diabetes providing a potential new clinical biomarker for determining rate of disease progression and pointing to contributing metabolic pathways.

KEYWORDS

advanced glycation end products, methylglyoxal, biomarker, RNA adduct, type 1 diabetes

Introduction

Type 1 diabetes is a disease characterized by a myriad of causes including autoimmunity, beta-cell dysfunction, and metabolic dysfunction. Methylglyoxal (MG) is an abundant reactive electrophile produced from protein, fatty acid, and sugar metabolism, with levels ~4 μM/day in basal conditions (1). MG forms covalent adducts on DNA, RNA, and protein termed MG-advanced glycation end products (MG-AGEs). MG-AGEs are associated with type 1 diabetes and the risk of developing complications, but their ability to predict progression to clinical type 1 diabetes is not clear (2, 3). As MG is produced from a variety of metabolic pathways, MG-AGEs are proposed to provide a more comprehensive measure of metabolic changes associated with type 1 diabetes compared to hyperglycemia alone. Several previous studies show that children have elevated blood glucose levels long before the clinical manifestation of type 1 diabetes (4–6). We hypothesized that MG-AGEs may be associated with the clinical onset of type 1 diabetes and correlated with the rate of disease progression. We therefore investigated serum samples of children participating in the public health islet autoantibody screening study Fr1da (7).

Methods

N^2-carboxyethyl-guanosine (CEG) was quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) using isotopically labeled standards in the laboratories of S. Shuck and J. Termini through modification of a previously described method for N^2-carboxyethyl-deoxyguanosine CEdG measurement (8). A total of 20 μl of serum was used. Samples were blinded so that the investigators did not know the group assignment as defined in Table 1. The limit of detection of this assay was 0.01 ng/mL and the limit of quantitation was 0.05 ng/mL. Technical variation between independent sample workups was <2%. A representative chromatogram of CEG and CEdG can be found in ESM Figure 1).

Samples from children who participated in the Fr1da public health screening for islet autoantibodies were studied, including 271 with 2 or more islet autoantibodies (i.e. autoantibodies to insulin [IAA], glutamic acid decarboxylase [GADA], insulinoma antigen-2 [IA-2A], or zinc transporter 8 [ZnT8A]), and 141 who tested islet autoantibody negative (7, 9). All children with islet autoantibodies had participated in a staging visit in which an Oral Glucose Tolerance Test (OGTT) was performed resulting in 240 children diagnosed with stage 1, 22 with stage 2, and 9 with stage 3 type 1 diabetes (Table 1). Stages of type 1 diabetes were classified according to the consensus criteria of the JDRF, Endocrine Society and ADA of 2015, as previously described (4). Stage 1 type 1 diabetes was defined as positivity for multiple islet autoantibodies and normal glucose tolerance based on OGTT results. Stage 2 type 1 diabetes was defined as positivity for multiple islet autoantibodies accompanied by dysglycaemia based on OGTT results (fasting plasma glucose of 6.1– 6.9 mmol/l [110–125 mg/dl] or impaired 2 -h plasma glucose of 7.8–11.0 mmol/l [140–199 mg/dl], and/or plasma glucose ≥ 11.1 mmol/l [200 mg/dl] at 30, 60 or 90 minutes). Stage 3 type 1 diabetes was defined based on ADA criteria (10). The staging sample was used for measurement of CEG concentrations. Written informed consent was obtained from the children's parents or legal guardians. Children were prospectively followed for the development of stage 3 type 1 diabetes as previously described (7). CEG concentrations were compared between groups by Mann Whitney test. Correlations of CEG with HbA1c or age were calculated by Spearman test. The progression to clinical diabetes was assessed using the Kaplan–Meier time-to-event method. Children were censored when they developed stage 3 type 1 diabetes or reached the date of their final contact to ascertain diabetes status. Between-group comparisons in the Kaplan–Meier analyses were performed using the log rank test. For this analysis, CEG values were categorized into quartiles (Q). The survival analyses and plots were prepared using R version 4.0.2 and the package 'survival' v3.2-7.

TABLE 1 Characteristics of study population.

	IAb neg	Stage1 -T1D	Stage 2 -T1D	Stage 3 -T1D
n	141	240	22	9
Age (years), (median, IQR)	4.6 (3.3-5.7)	4.3 (3.3-5.6)	4.3 (3.2-5.2)	4.8 (4.2-8.2)
Sex m/f	68/73	131/109	16/6	5/4
Number of pos IAb				
2 3 4	/ / /	82 76 82	4 12 6	1 3 5
CEG (pmol/ml) (median, IQR)	0.039 (0.020, 0.169)	0.043 (0.020, 0.185)	0.045 (0.023, 0.167)	0.019 (0.000, 0.030)
Developed Stage 3 T1D during FU	0	55	14	/

*The study included 271 children with 2 or more islet autoantibodies (IAA, GADA, IA-2A, or ZnT8A) and 141 islet autoantibody-negative children.
IAb, islet autoantibody; T1D, type 1 diabetes; IQR, interquartile range; m, male; f, female; FU, follow-up.

Results

We first asked whether CEG levels were different between islet autoantibody-negative and islet autoantibody-positive children and whether CEG levels were related to the pre-symptomatic stage of type 1 diabetes. Median CEG levels did not differ between the 141 islet autoantibody-negative children (0.039 pmol/ml [interquartile range; 0.020, 0.169]) and the 271 islet autoantibody-positive children (0.042 pmol/ml [0.020, 0.181]; P = 0.94) or between islet autoantibody-negative children and children with stage 1 or stage 2 type 1 diabetes (Supplementary Figure 2A and Table 1). Children with stage 3 type 1 diabetes had slightly lower median CEG levels (0.019 pmol/ml [0.000, 0.030]; P = 0.05 compared to islet autoantibody-negative group), but the number of cases in this group was small (n = 9). CEG concentrations did not significantly correlate with HbA1c (r = -0.1204, P = 0.05) or age (r = 0.0345, P = 0.48) (ESM Supplementary Figures 2A, B) at the time of measurement. During prospective follow-up, 69 of 262 islet autoantibody-positive children with stage 1 or 2 type 1 diabetes progressed to stage 3 type 1 diabetes

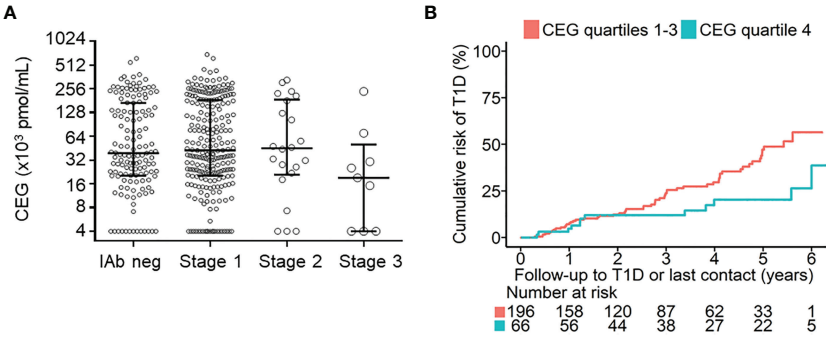


FIGURE 1
Relationship between CEG levels and islet autoantibody status, stage of pre-symptomatic type 1 diabetes (T1D), and risk of progression to clinical T1D (stage 3). **(A)** shows CEG levels for 141 islet autoantibody-negative children (IAb neg), 240 children with stage 1 T1D, 22 children with stage 2 T1D, and 9 children with stage 3 T1D. Median (IQR) values are shown for each group. **(B)** shows the cumulative risks of developing stage 3 T1D in children with stage 1 or 2 T1D for children with CEG levels in the highest quartile (Q4) of measurements (blue line) compared with children with lower CEG levels (Q1-Q3; red line) (P=0.001; log-rank test). Follow-up begins at initial staging by OGTTs. The number of children at risk is shown below each time point..

(Table 1). In these children, disease progression from stage 1 or stage 2 to stage 3 type 1 diabetes was significantly lower in children with CEG levels in the highest quartile (Q4) of measurements (20.3% [95% CI; 7.6 – 31.2] by 5 years follow-up vs. 47.1% [35.7 – 56.5] in children with CEG levels in Q1-Q3; $P = 0.001$) (Supplementary Figure 2B) or compared to children with CEG levels in Q1 (58.4% [95% CI; 36.1 - 72.6]; $P = 0.014$) (ESM Supplementary Figure 3). Similar results were obtained when children with stage 2 were excluded from the Kaplan-Meier analysis (Q4 16.1% [95% CI; 4.0 – 26.8] by 5 years follow-up vs. 43.1% [31.2 – 53.0] in children with CEG levels in Q1-Q3; P = 0.001 (ESM Supplementary Figure 4), and 55.4% [31.2 – 71.0] in children with CEG levels in Q1 (P = 0.011).

Discussion

Our results reveal the first association of MG-AGEs with progression to clinical type 1 diabetes (stage 3). Although we measured a panel of MG-AGEs, only the RNA adduct CEG was observed in serum (data not shown). The observation that MG-AGEs are negatively associated with progression to clinical type 1 diabetes may result from increased urinary excretion of these adducts resulting in lower serum levels or intracellular accumulation of adducts. Defining this relationship requires additional experimentation in human, mouse, and *in vitro* models, which we are currently investigating. Alternatively, counteractive mechanisms may affect the metabolic pathways generating MG-AGEs in an attempt to slow the latest stages in disease progression before clinical manifestation. Exploring these possibilities will help elucidate the extent to which CEG may be a driver of beta-cell destruction. One of the strengths of our study was the observation of a significant association of RNA MG-AGEs with the rate of type 1 diabetes progression as well as the large number of children with early disease stages investigated. RNA damage has not been significantly explored as a biomarker of disease but has emerging importance both as a marker and potential driver of disease. Furthermore, CEG may have utility in biomarker panels that measure metabolic changes that occur prior to clinical type 1 diabetes diagnosis. Limitations of this study include a lack of longitudinal samples and no matched serum and urine samples to investigate how excretion influences serum levels of MG-AGEs. The current results provide a framework for additional investigations of this adduct in metabolic disease models, including as predictors of diabetic complications.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SS and BR conceived the objective of this study. A-GZ is the principal investigator of the Fr1da study. A-GZ and PA were responsible for the study design. PA, CW, AW, SS, BR, and CH-C contributed substantially to data acquisition and analysis. A-GZ, PA, SS, and BR drafted the manuscript. SS takes responsibility for the integrity of the work as a whole. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1108910/full#supplementary-material

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Finerenone in type 2 diabetes and renal outcomes: A random-effects model meta-analysis

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Background: The nonsteroidal mineralocorticoid antagonist finerenone is a new addition to the list of agents (angiotensin converting enzyme inhibitors and sodium glucose cotransporter 2 inhibitors) conferring renal protection to patients with diabetic kidney disease. Two recent meta-analyses using the fixed effect model in patients with chronic kidney disease (both diabetic and nondiabetic populations) came to a conflicting conclusion on the effect of finerenone on eGFR decline. This meta-analysis was undertaken exclusively in the type 2 diabetes (T2D) population to explore the robustness and heterogeneity of the effect size by conducting a random effects model meta-analysis along with draft plots and prediction intervals.

Materials and methods: A database search was conducted using the Cochrane library, PubMed, and Embase to identify relevant citations. Analysis was conducted on the 14th of September 2022, using RevMan 5.4.1 and RStudio (2022.07.1, Build 554). The hazard ratio was used as the effect size for the renal composite, while the standardized mean difference (SMD) was used to estimate the effect size of eGFR decline and reduction in the urine albumin creatinine ratio (UACR). The Cochrane risk-of-bias was used to assess the quality of the studies. The primary outcome assessed was the renal composite defined as kidney failure, a sustained decrease of at least 40% in the eGFR from baseline, or death from renal causes.

Results: A pooled population of 13,943 patients from four citations was included for analysis. The Cochrane risk of bias was used to assess the quality of the studies. There was a significant 16% reduction in the renal composite (kidney failure, a sustained decrease of at least 40% in the eGFR from baseline, or death from renal causes) [HR: 0.84, 95% CI 0.77-0.92, ^{2}: 0, I^2: 0%). Finerenone was also associated with reduction in UACR (SMD: -0.49, 95% CI -0.53 to -0.46, τ^2: < 0.0001, I^2: 0%, prediction interval: -0.57 to -0.41) and prevention of decline in eGFR (SMD: -0.32, 95% CI -0.37 to -0.27, τ^2: < 0.0001, I^2: 0%, prediction interval: -0.43 to -0.21) without any evidence for significant heterogeneity. Except for an increase in hyperkalaemia (RR: 2.22, 95% CI 1.93-2.24), adverse events were observed with fineronone compared to placebo (RR: 1.00, 95% CI 0.98-1.01).

Conclusion: There are significant benefits in renal outcomes associated with finerenone treatment in T2D patients with established chronic kidney disease with a side effect profile comparable to placebo.

KEYWORDS

PROSPERO 2022 CRD42022360003, type 2 diabetes, finerenone, meta-analysis, renal outcomes, type 2 diabetes

1 Introduction

Diabetes mellitus has emerged as the leading cause of chronic kidney disease (CKD), accounting for almost 60% of cases of CKD worldwide. Nearly 30%-40% of patients with type 2 diabetes (T2D) develop CKD (1). Despite the effective implementation of reductions in the individual risk factors associated with diabetic CKD, the overall mortality and progression to end-stage renal disease (ESRD) are increasing (1). The major contributors to mortality from CKD are atherosclerotic cardiovascular disease and heart failure (2).

Metabolic control lies at the heart of preventing the development of CKD. A reduction in HbA1c by 0.9% from baseline accounted for a 24%-33% reduction in the development of diabetic nephropathy (3). In addition to metabolic control, researchers have focused on specific targets and interventions to alter the development and progression of CKD.

There are several mechanistic pathways responsible for the inflammatory and fibrotic insult to the renal architecture and vasculature (4). Traditionally, it was postulated that the activation of the renin-angiotensin system (RAS) by triggers such as insulin resistance resulted in ligand-dependent activation of the mineralocorticoid (MR) receptor by aldosterone, leading to salt retention and hypertension and consequent renal injury. This led to the strategy of RAS blockage, which was met with some success in reducing cardio-renal outcomes (5). However, with the discovery of "escape pathways" (incomplete RAS inactivation by RAS blockade), attempts were made to use dual blockade (ACEi and ARB) as well as direct renin blockade to obtain better cardio-renal benefits. Both these attempts proved to be abortive (6, 7). The recent understanding of nonligand-based activation of MR by Ras-related C3 botulinum toxin substrate 1 (Rac 1), hyperglycaemia, and excess sodium brought strategies blocking MR into focus (8). It seems plausible that additional nephropretection can be achieved with the concomitant use of MR antagonists in addition to ACEi/ARBs.

Nonsteroidal MR antagonists (MRA) were chosen since, being more MR selective, they are less likely to cause hyperkalaemia in contrast to steroidal MRA. This is obviously more suitable in the management of CKD (9). The dose finding ARTS (MinerAlocorticoid Receptor antagonist Tolerability Study) documented a significant impact of finerenone on albuminuria progression at doses of 10 mg-20 mg per day (10). With the adequate dose identified and its positive result on the cardio-renal components documented, finerenone was given FDA approval in 2021 for use in T2D patients with an aim to reduce cardio-renal adverse outcomes (11).

There were a couple of meta-analyses estimating the effectiveness of finerenone versus placebo on the renal composite as well as the individual components of urine albumin creatinine ratio (UACR) and estimated glomerular filtration rates (eGFR) (12, 13). Although both meta-analyses documented similar benefits regarding renal composite, there was discordance as far as benefits on eGFR reduction were concerned. Moreover, of the five included citations, one by Pitt el al did not include patients with T2D, while the other four did.

To explore the impact of finerenone on renal outcomes exclusively in the T2D population, this meta-analysis was undertaken. We intended to examine the data limited to the T2D population and look for any heterogeneity in the outcomes that could explain the divergent results in the two meta-analyses.

2 Materials and methods

This meta-analysis was conducted according to the recommendations of the PRISMA statement and registered with PROSPERO (CRD42022360003) (14).

2.1 Literature searches, search strategies and eligibility criteria

The web search was conducted using the PICO search pattern:
P: Type 2 diabetic patients with established chronic kidney disease
I: Finerenone
C: Control
O: (a). Renal composite (kidney failure, a sustained decrease of at least 40% in the eGFR from baseline, or death from renal causes), (b). Decrease in urine albumin creatinine ratio (UACR), (c). eGFR decline, (c). Adverse events associated with the active intervention (any adverse event, hyperkalemia, and acute renal injury).

2.2 Data extraction included assessment of the quality of the studies

Both authors conducted a web-based search for relevant citations dependent on the selected keywords. Additional filters included a cap on age above 18 years and clinical trials. No restrictions were placed based on language or date of publication. SG conducted the meta-analysis.

Having identified the four citations to be taken up for analysis, the data required for both analyses were entered into an Excel sheet. The chances of any error in entering the data were cross-checked by another author (BS). The quality of the selected citations was assessed using the Cochrane risk-of-bias algorithm, which included random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome data, incomplete outcome data, selective reporting, and other biases. All the selected citations were evaluated along with their supplementary data and scored individually by BS & SG. Any dispute was reassessed by SG and BS, and a final decision was made by consensus. Individual publication bias was analyzed using funnel plots. (Supplementary Figure S2)

After the initial process, a manual search was conducted jointly to identify the citations that met the inclusion criteria:

- Randomized controlled trials.
- Age limit: above 18 years, with type 2 diabetes mellitus and documented CKD defined as a UACR more than 200 mg/g or a decline in eGFR (<60 ml/min) for at least three months.
- Inclusion of placebo as the control arm.
- A minimum of 12 weeks of follow-up.
- Reporting of prespecified renal events (renal composite, UACR and eGFR).

- Reporting of adverse events related to the active intervention.

Exclusion criteria:

- Non-randomised trials, abstracts, review articles, and case reports were excluded from analysis.
- Age of recruitment below 18 years.
- A follow-up duration less than 3 months.
- Inclusion of active intervention in the comparator arm capable of influencing the renal outcomes.
- Any other type of diabetes (Type 1 diabetes, post-pancreatitis secondary diabetes).
- A clear documentation of exclusion of all acute renal events.

2.3 Patient approval and clearance from the ethical committee

In this systematic review and meta-analysis, there was no direct handling of patients. In addition, effect size estimates that were already published and in open web-based domains were used to conduct the meta-analysis. As a result, there was no requirement for patient or ethical committee consent.

2.4 Statistical analysis

The hazard ratio (HR) and standardized mean difference (SMD) risk were used as the preferred parameters of interest because of the differing patterns in reporting outcomes of interest in the included citations. The renal composite effect size was reported in two studies in the form of HR, and hence, the pooled mean effect size was analysed using HR. However, the other outcomes of interest (UACR and eGFR) were reported as raw mean values, and hence, an SMD was used to estimate the pooled effect size. Risk ratio was used as the effect size estimate for assessment of the adverse events since raw events were reported. In addition to the effect size, hypothesis testing was performed and reported in the form of a 95% confidence interval (95% CI) and p value. Since the p value does not represent the effect size distribution spectrum, a prediction interval was also planned to be assessed along with representation of the p value function distribution in the form of a drapery plot. The summary of results was reported as a forest plot, which also included the weightage of the individual studies. The analysis was conducted using the RevMan 5.4.1 and R studio (2022.07.1, Build 554) platforms. Heterogeneity was assessed using the prediction interval, Q statistic and Higgin's I^2 test. Heterogeneity was defined as a Q statistic value exceeding the degrees of freedom with a significance cut off p=0.1.

In view of the diverse population represented in the citations as well as selection of the citations from a universe of available data, a random effects model was used to estimate the effect size. This strategy also facilitated the process of assessing heterogeneity in contrast to the fixed effect model. A sensitivity and subgroup analysis were planned if significant heterogeneity related to the pooled effect size was encountered.

Prominent adverse events associated with finerenone compared to placebo were analysed using the risk ratio as the effect size. Since, UACR and assessment of eGFR are routinely performed in clinical practice, we included them as part of subgroup analysis. Both UACR as well as eGFR decline were part of the renal composite assessed as the primary outcome of interest.

3 Results

3.1 Identification of studies for analysis

The randomized prospective studies were identified through a thorough database search (Cochrane Library, PubMed, and Embase). The search was divided into three categories: (a) related to the intervention in question "Finerenone", "Nonsteroidal mineralocorticoid antagonist", (b) related to the primary disease in question "Type 2 Diabetes mellitus", "T2DM", "Chronic kidney disease", "CKD", "Diabetic kidney disease", "DKD", and (c) related to the outcomes assessed, which included the terms "Diabetic kidney disease", "Urine albumin creatinine ratio", "UACR", "eGFR decline", and "Renal composite"). Furthermore, the primary search filters included human data and clinical trials, although no search restrictions on time or language were used. While performing the Cochrane library search, the outcome keywords [(a), (b), and (c)] were clubbed using Boolean OR. The search results were then combined using Boolean AND to yield the first set of citations. The initial search was followed up by a detailed manual search. The main purpose of the manual search was to screen the initially filtered citations for duplicate publications, remove review articles, as well as identify those citations not conforming to the predetermined inclusion and exclusion criteria (Figure 1). The full search strategy is available in Supplementary Appendix 1.

Any citation that compared finerenone versus placebo was included for analysis.

3.2 Baseline characteristics of the studies

The preliminary web search resulted in the identification of 2130 citations, among which many duplicate (n=2027) studies were identified and removed. The screening process and search for relevant citations using the inclusion criteria resulted in the selection of four citations for the final analysis (15–18). A study by Pitt et al. (2013) was excluded because it was not conducted on patients with diagnosed T2D (10). The study by Bakris et al. (2015) was conducted on T2D patients with significant albuminuria optimized on an angiotensin-converting enzyme inhibitor or an angiotensin receptor blocker and primarily evaluated for reduction in UACR (15). The FIDELIO-DKD trial (Bakris et al., 2020) was conducted on patients with T2D with established DKD and primarily evaluated the impact of finerenone on the renal composite (16). The study by Katayama et al. evaluated the impact of finerenone on reduction of UACR at 90 days on T2D patients with diabetic nephropathy (17). The FIRAGO-DKD study (Pitt et al., 2021) expanded the scope of the inclusion criteria of the FIDELIO-DKD

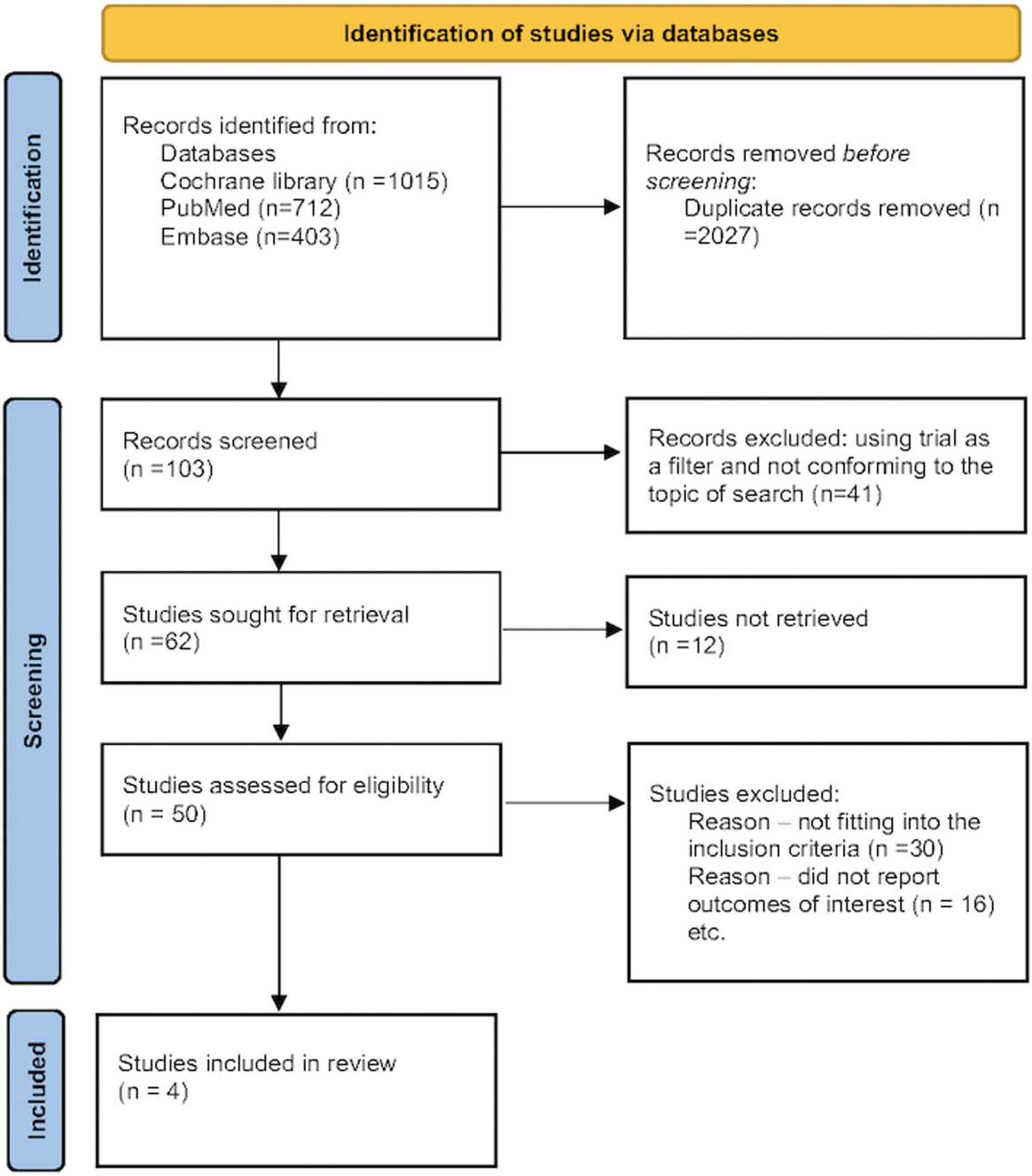


FIGURE 1
The study selection process.

study by recruiting T2D patients with moderate albuminuria and stage 2-4 CKD (18). The primary aim of this study was to evaluate the impact of finerenone on the renal composite (kidney failure, a sustained decrease of at least 40% in the eGFR from baseline, or death from renal causes) as well as reduction in UACR and retardation of decline in eGFR. The meta-analysis was conducted on a pooled patient population of 13,943 from 4 citations, divided into 7330 individuals on finerenone and 6613 patients on placebo. The duration of follow-up ranged from 3 to 40.8 months. The baseline characteristics of the citations included in the analysis are summarized in Table 1. Patients receiving a dose of finerenone ≥ 10 mg/day were included in the analysis.

3.3 Outcome measures: Renal composite

Only two of the four citations reported renal composite conforming to a universal definition. The pooled estimate of the mean effect size was significant, as indicated by a 16% reduction in the renal composite (HR:

TABLE 1 Baseline characteristics of the citations included for analysis.

Study	Year	Male/Female (Finerenone arm)	Mean Age (years)	Finerenone (n)	Finerenone ≥10 mg/day (n)	Baseline UACR/eGFR *	Placebo (n)	Follow up (months)
Bakris et al. (15)	2015	570/157	64.7 ± 9.26	727	342	202.7/66.0	94	3
Bakris et al. (16)	2020	1953/880	65.6 ± 9.1	2833	2833	302/67.6 ± 21.7	2841	31.2 (median)
Katayama et al. (17)	2017	67/17	64.0 ± 8.26	84	36	833/44.4 ± 12.5	12	3
Pitt et al. (18)	2021	2528/1158	64.1 ± 9.8	3686	3686	127.67/61.48	3666	40.8 (median)

0.86, 95% CI 0.77-0.92). A Q statistic of 0.38 with a df of 1 indicated the absence of significant heterogeneity in the true effect size (Figure 2). The prediction interval could not be assessed for the renal composite in view of the requirement of at least three studies.

3.4 Outcome measures: UACR

There was an impressive reduction in UACR from baseline in the finerenone arm compared to placebo (SMD: -0.49, 95% CI -0.53 to -0.43). A predictive interval of the effect size (-0.57 to -0.41) ruled out significant uncertainty in the true effect size (Figure 2).

3.5 Outcome measures: eGFR decline

There was an impressive retardation in eGFR decline from baseline in the finerenone arm compared to placebo (SMD: -032, 95% CI -0.37 to -0.27). A predictive interval of the effect size (-0.43 to -0.21) complemented the significant impact on the mean effect size (Figure 2).

3.6 Adverse events

Adverse events with finerenone were comparable to placebo (RR: 1.00, 95% CI 0.98-1.01), as was the risk of acute kidney injury or an

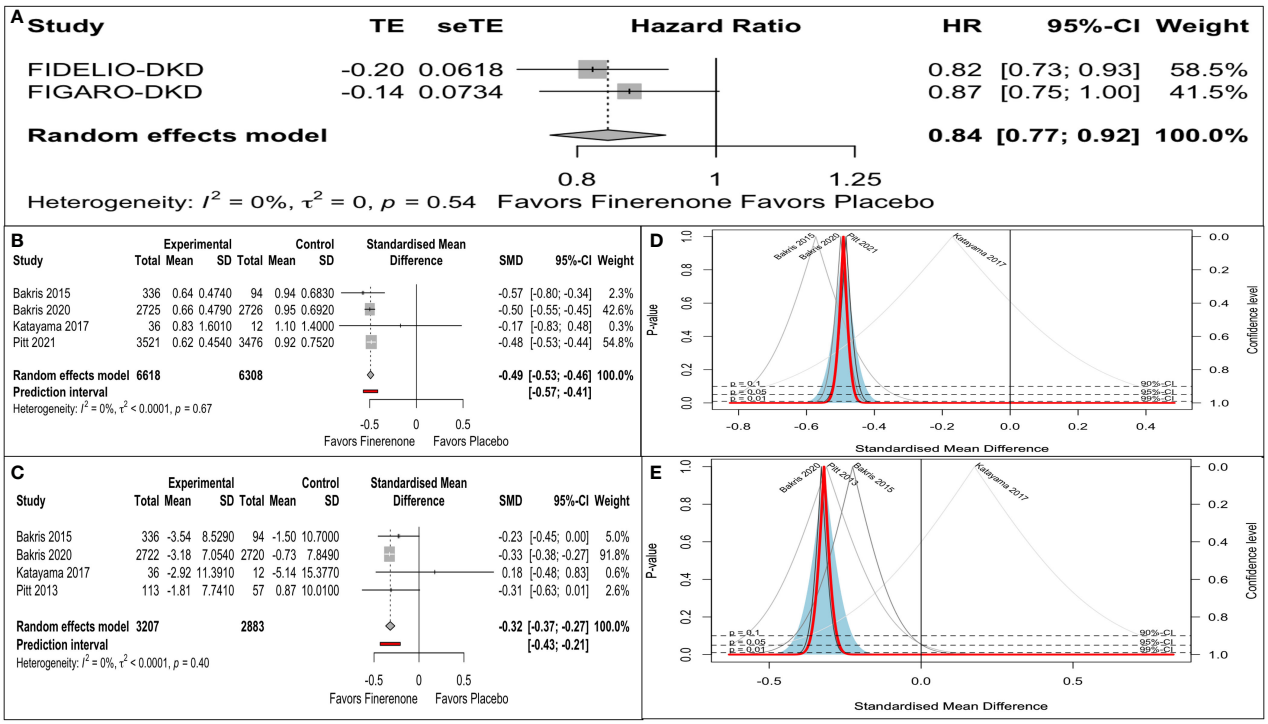


FIGURE 2
Finerenone versus placebo on: **(A)** Renal composite (Forest plot): hazard ratio (HR). **(B)** Reduction in UACR (Forest plot): Standardized mean difference (SMD). **(C)** eGFR decline (Forest plot): Standardized mean difference (SMD). **(D)** eGFR decline (Drapery plot). **(E)** Reduction in UACR (Drapery plot).

eGFR decline of ≥40% (RR: 0.98, 95% CI 0.78-1.11). However, the risk ratio of hyperkalaemia was significantly higher with finerenone (RR: 2.22, 95% CI 1.93-2.24) (Figure 3).

4 Discussion

Diabetic nephropathy is one of the leading causes of mortality and morbidity in patients with T2D. Targeting albuminuria and retarding the decline in eGFR have become the therapeutic strategy of choice in preventing cardiorenal outcomes. RAS inhibition remained the backbone of such a strategy. However, less than 50% of the target population in the RAS blockade arm of clinical trials achieved their primary endpoint in most of these trials, creating scope for newer strategies to tackle this unmet need (5). Recently, SGLT-2is and GLP1-RAs have been found to have a positive impact on cardio-renal outcomes (19). However, the search for taming the deleterious effects of aldosterone on target organs through activation of MR was not abandoned. Aldosterone is elevated approximately 4-fold in patients with CKD-Stage 4 or less, leading to inflammation and fibrosis in the tissues of target organs (20). Because MR is expressed in both the kidneys and the heart, MR receptor antagonism is considered as a prominent strategy to alter adverse cardio-renal outcomes (21). Nonsteroidal MRA are more selective in nature and less prone to gynaecomastia and hyperkalaemia. Finerenone is a prototype of an MR antagonist.

4.1 Literature review

The phase II program ARTS (MinerAlocorticoid Receptor antagonist Tolerability Study) was conducted in CKD patients with chronic heart failure and reduced ejection fraction (HFrEF), with spirololactone as a comparator (12). There was a comparable reduction in pro-brain natriuretic peptide (NT-Pro BNP) as well as UACR, with less gynaecomastia and hyperkalaemia. The ARTS-DN (MinerAlocorticoid Receptor antagonist Tolerability Study in Diabetic Nephropathy) comparing finerenone versus placebo documented a dose-dependent reduction in albuminuria at 90 days without a significant impact on eGFR decline (15). The FIDELIO-CDK study documented impressive benefits with the use of finerenone in reducing composite renal outcomes (kidney failure, a sustained decrease of at least 40% in the eGFR from baseline, or death from renal causes) in patients with T2D and established CKD (16). The FIRAGO-DKD study was the first to show a positive cardiovascular result using a 4-point MACE (composite of death from cardiovascular causes, nonfatal myocardial infarction, nonfatal stroke, or hospitalization for heart failure) in T2D patients with established CKD (18). A meta-analysis of five RCTs by Zhang et al. using the fixed effects

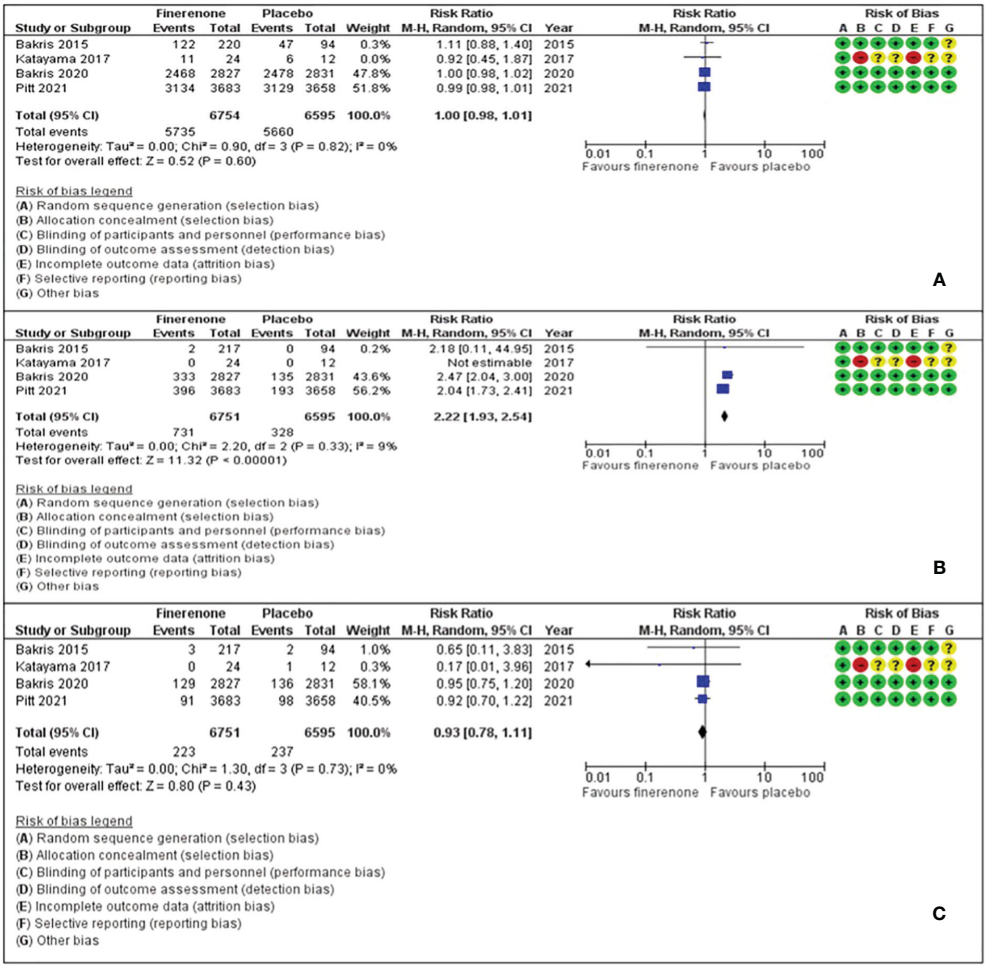


FIGURE 3
Effect of finerenone versus placebo on: **(A)** Any adverse events. **(B)** Hyperkalaemia. **(C)** Acute renal injury or eGFR decline of ≥40%.

model demonstrated a significant improvement in UACR reduction as well as retardation of eGFR decline with finerenone compared to placebo (12). However, another meta-analysis by Fu et al. with four studies using the fixed effects model demonstrated a positive impact of finerenone on UACR reduction but not on the retardation of eGFR decline (13).

4.2 Findings from our study

Our meta-analysis was conducted exclusively on T2D patients with established CKD. This is probably the first meta-analysis to explore the impact of finerenone on renal outcomes in T2D patients. In addition, we used the random effect model to conduct the meta-analysis, especially since there were significant differences in the baseline characteristics of the patients recruited in the individual studies, and to assess any heterogeneity and uncertainty in the true effect size. We documented a significant 16% reduction in the renal composite (kidney failure, a sustained decrease of at least 40% in the eGFR from baseline, or death from renal causes) [HR: 0.84, 95% CI 0.77-0.92, τ^2: 0, I^2: 0%) in the T2D patients with CKD receiving finerenone compared to the placebo arm. There was also an impressive reduction in UACR (SMD: -0.49, 95% CI -0.53 to -0.46, τ^2: < 0.0001, I^2: 0%, prediction interval: -0.57 to -0.41) and retardation of decline in eGFR (SMD: -0.32, 95% CI -0.37 to -0.27, τ^2: < 0.0001, I^2: 0%, prediction interval: -0.43 to -0.21) in the finerenone arm without any evidence for significant heterogeneity. Our meta-analysis endorses the robustness of the renal benefits in the T2D cohort as suggested in the RCTs, without any heterogeneity or uncertainty in the true effect size as assessed with the prediction interval. In addition, this study illustrated that fineronone usage was not associated with any adverse outcome or worsening of renal disease or acute kidney injury. However, as expected, since fineronone is after all an MRA, there is a significant increase in the rates of hyperkalaemia.

4.3 Limitations and strengths

Our meta-analysis has a few limitations. First, we analysed the reported mean data and did not have access to individual patient data. As a result, the bias ascertainment could have been impacted, since we were assessing data from published materials. Second, there are a couple of studies of very short duration in which the dramatic nature of the impact on outcomes could have skewed the overall result. Third, although renal composite was reported in a couple of trials on a time-dependent basis (HR), the reporting of UACR and eGFR decline were not time-dependent in nature.

The main strength of this meta-analysis was that it was conducted using the random effects model, taking into consideration the heterogeneity associated with the type of included citations and baseline characteristics of the patients. Another advantage was the use of the prediction interval, which helped us to assess any uncertainty in the true effect size. Finally, we segregated the T2D patients with CKD, unlike the pooled CKD analysis performed in previous meta-analyses, resulting in more specific reporting of the results.

5 Conclusion

The nonsteroidal MRA finerenone, by way of its unique mechanism of action, is a novel strategy in the fight against the progression of diabetic CKD. This meta-analysis convincingly demonstrates that finerenone not only produces an impressive reduction in the renal composite but also reduces UACR and retards the decline in eGFR in patients with T2D who have established CKD. Reassuringly, fineronone is well tolerated, except for an increased risk of hyperkalaemia, which must be monitored during fineronone therapy.

Ethics approval and consent to participate

We analyzed published data; hence, ethical approval was not needed. In addition, we did not deal with patients, and hence consent to participate was not applicable.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

SG and BS conceptualized the study. The meta-analysis was conducted by SG. BS cross validated the data and prepared the manuscript. SG and BS conducted an expanded literature review to contextualize the results from the meta-analysis. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1114894/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Risk of bias assessment using the Cochrane risk-of-bias algorithm.

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Altered dynamic functional architecture in type 2 diabetes mellitus

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Introduction: Type 2 diabetes mellitus (T2DM) can accelerate cognitive decline and even dementia so that the underlying mechanism deserves further exploration. In the resting state, brain function is still changing dynamically. At present, it is still unknown whether the dynamic functional connectivity (dFC) between various brain regions is in a stable state. It is necessary to interpret brain changes from a new perspective, that is, the stability of brain architecture.

Methods: In this study, we used a fixed dynamic time scale to explore the stability of dynamic functional architecture in T2DM, then the dynamic effective connectivity (dEC) was used to further explain how information flows through dynamically fluctuating brain architecture in T2DM.

Result: Two brain regions with decreased stability were found including the right supra-marginal gyrus (SMG) and the right median cingulate gyrus (MCG) in T2DM subjects. The dEC variation has increased between the left inferior frontal gyrus (IFG) and the right MCG. The direction of causal flow is from the right MCG to the left IFG.

Conclusion: The combination of stability and dEC can not only show the stability of dynamic functional architecture in brain but also reflect the fluidity of brain information, which is an innovative and interesting attempt in the field of neuroimaging. The changes of dynamic architecture in T2DM patients may present an innovative perspective and explanation for their cognitive decline.

KEYWORDS

type 2 diabetes mellitus, cognitive decline, resting state, functional architecture, dynamic functional stability, dynamic effective connectivity

1 Introduction

The incidence of type 2 diabetes mellitus (T2DM) is sharply increasing worldwide (1, 2). T2DM has crucial effects on cognition (3), and evidence has shown that T2DM is a generally acknowledged risk factor for Alzheimer's disease (AD) (4, 5). Some researchers have classified AD as type 3 diabetes (6), as there are some shared pathological mechanisms between T2DM and AD, including insulin resistance, mitochondrial dysfunction, and advanced glycation end products. Studies have shown that T2DM subjects have cognitive decline in the subclinical stage, such as executive function and memory and attention decline (7, 8). The early stage of cognitive decline in T2DM is a gradual and imperceptible process, and it is not always noticeable. Early assessment of cognitive function in T2DM patients is of great clinical significance for early prevention and intervention.

Recently, neuroimaging biomarkers have commonly been used for the early detection of numerous neurological and psychiatric diseases (9, 10). Resting-state functional magnetic resonance imaging (rs-fMRI) can sensitively capture the subtle functional alteration in the brain by collecting the blood oxygenation level-dependent (BOLD) signal in a non-invasive way (11). At this point, existing studies have shown that T2DM patients have abnormal regional and global functional changes, which is closely relevant to the decline of cognitive function in multiple aspects. A recent study revealed that reduced regional gray matter volume and increased functional connectivity (FC) may reveal the neurobiological mechanism underlying cognitive impairment in early-onset T2DM patients (12). Another study showed that abnormal volumes of hippocampal subregions in T2DM patients were associated with memory function, suggesting that reduced volumes of specific hippocampal subregions may be an underlying mechanism of memory dysfunction (13). At the same time, it has previously been shown that brain networks are time-variant during MRI scan (14). The brain is a temporally dynamic system. Even during a short scanning session, cognitive states and FC are continuously evolving (15). The most common approach is dynamic functional connectivity (dFC), which focuses on the distribution patterns and dynamics of signal fluctuations in neuronal activity (16–19). These reported developments in the field of dynamic network neuroscience have enabled quantifying time-variant changes in neural network connectivity, state transition, and functional organization and underscored the needs of revealing brain dynamic features. However, it is not known whether the dynamic pattern is in a steady state, which may be related to changes in cognition (20).

An important feature of consciousness is stability, characterized by the consistency of distributed neural activity and connectivity patterns over time (21). Functional stability can assist in the maintenance of brain function normally in dynamic changes and manifest from a dynamic viewpoint how functional organizations adapt to accomplish complicated natural tasks (22). Yan first clarified the stability of dFC in the human brain, that is, the stability distribution of functional architecture (20). Yan found that functional stability is unevenly distributed in the cerebral cortex and regions with strong stability were mainly distributed in the default mode network. Another study showed that amyotrophic lateral sclerosis patients had abnormal stability of brain dynamic functional architecture, which was relevant to the severity of the disease (23). We speculated that stability of dynamic functional architecture, which captured more sensitive and time-varying information, could be an objective and meaningful method to reveal underlying brain changes and evaluate cognitive function from a new perspective. It is innovative and interesting to investigate stability from a dynamic perspective to reveal the mechanisms of the dynamic functional architecture changes in T2DM.

During cognitive processing, specific messages are transmitted throughout the brain (21). Several complicated cognitive functions depend on constantly coordinating multiple aspects of information in the brain (20). There are continuous endogenous fluctuations in human brain regions that represent underlying brain functional architecture (24). It is essential that the process of transmission is fluid and directional. The related issues are that how information travels and flows through dynamically fluctuating brain functional architecture and whether information transmission is affected by changes in dynamic stability. The dFC represents the temporal consistency between endogenous dynamic fluctuations, but it cannot be used to show direct interactions among brain regions (24). Dynamic effective connectivity (dEC) reflects well the flow of information, emphasizing the directivity of information dissemination in functional dynamic networks. Granger causality analysis (GCA) is commonly used to measure dEC using multiple linear regression, which examines the prediction effect of current time-series values using all the information at some time series in the past (25). In recent years, GCA has been used to elucidate the causal relationship between brain regions (24, 26). However, there is no study combining dEC and stability to measure the dynamic functional architecture and directional connectivity changes in T2DM. In this study, we explored the stability of dynamic functional architecture at a fixed dynamic time scale to elucidate how stability was modified in T2DM. Then, dEC was used to effectively explore information flow in intricate dynamic brain networks, which was an interesting and innovative experiment. Finally, the relationships among the imaging indicators, clinical data, and neurocognitive measurements were further explored. This study was designed to investigate the underlying neuroimaging mechanism of cognitive decline in T2DM patients.

2 Materials and methods

2.1 Subjects

Fifty T2DM subjects and 58 healthy controls (HCs) were recruited. Details of the subjects are shown in Table 1. All subjects met the following requirements: 1) years of education >6; 2) Han Chinese; 3) right-handed. Subjects meeting the following criteria were excluded: 1) brain organic disease; 2) psychiatric or neurological disease; 3) claustrophobia. The exclusion criteria were consistent with our previous study (8). The T2DM criteria were set based on the American Diabetes Association, which contain the following: 1) A1C greater than or equal to 6.5%; 2) fasting plasma sugar (FPG) greater than or equal to 126 mg/dl; 3) after oral glucose tolerance test, blood sugar level greater than or equal to 200 mg/dl; 4) blood glucose level

TABLE 1 Demographic data and clinical biochemical indicators of all subjects.

	T2DM (n = 50)	HC (n = 58)	Statistics	P value
Age (years, x ± s)	50.60 ± 9.33	48.24 ± 7.43	t = 1.461	0.147
Sex (M/F)	29 (58)	30 (51.7)	χ^2 = 0.427	0.514
Education (years, x ± s)	10.5 (9,12)	10 (8.5, 12)	z = -0.006	0.995
HbA1c (%)	9.12 ± 2.09	N/A	N/A	N/A
FINS (μIU/mL)	7.12 (4.6875, 10.2675)	N/A	N/A	N/A
FPG (mmol/L)	7.825 (6.58, 8.915)	N/A	N/A	N/A
HOMO-IR	2.3569 (1.5149, 3.7948)	N/A	N/A	N/A
TG (mmol/L)	1.63 (0.975, 2.33)	N/A	N/A	N/A
TC (mmol/L)	4.57 ± 0.99	N/A	N/A	N/A
LDL (mmol/L)	3.045 (2.5875, 3.5525)	N/A	N/A	N/A

HC, healthy control; T2DM, type two diabetes mellitus; HbA1c, hemoglobin A1c; FINS, fasting insulin; FPG, fasting plasma glucose; HOMA-IR, homeostatic model assessment of insulin resistance; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; N/A, not applicable.

greater than or equal to 200 mg/dl. Each way usually needs to be repeated on a second day to diagnose T2DM. These examinations were performed by endocrinologists. All T2DM subjects were on insulin therapy *via* insulin pump or insulin injections. Age, sex, and education levels of HCs were matched with T2DM subjects.

2.2 Clinical and cognitive assessments

All subjects' clinical information containing age, sex, and education level was recorded. Biochemical information included FPG, hemoglobin A1c (HbA1c), fasting insulin (FINS), homeostatic model assessment of insulin resistance (HOMA-IR), total cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL) cholesterol levels. Comprehensive cognitive assessments were conducted, which include Montreal Cognitive Assessment (MoCA), Auditory Verbal Learning Test (AVLT, including immediate recall, short-term delayed recall, long-term delayed recall, and recognition), Trail Making Test (TMT, including parts A and B), Grooved Pegboard Test (GPT), Symbol Digit Test (SDT), the Clock Drawing Test (CDT), and the Digit Span Test (DST, including forward and backward).

2.3 Data acquisition

Rs-fMRI data were collected on a 3T MRI scanner (GE, SIGNA, USA). Conventional sequences were conducted to screen brain lesions, including T1-weighted and fluid-attenuated inversion recovery images. None of the participants were excluded at this stage. Then, the experimental sequences which contain BOLD and 3D-T1 were conducted for data processing. The scanning parameters of BOLD were as follows: repetition time (TR) = 2,000 ms, echo time (TE) = 30 ms, slices = 36, thickness = 3 mm, field of view (FOV) = 220 mm × 220 mm, acquisition matrix = 64 × 64, and flip angle = 90°. The scanning parameters of 3D-T1 were as follows: TR = 2,000 ms, TE = 2.6 ms, slices = 256, thickness = 1 mm, FOV = 250 mm × 250 mm, acquisition matrix = 256 × 256, flip angle = 12°.

2.4 Data preprocessing

Imaging data were processed by using DPABI and SPM on the basis of the MATLAB toolbox (27). The steps of preprocessing were as follows: 1) conversion of the image data formats: all the subjects' image data in DICOM format were converted to NIFTI format; 2) the functional images of the initial 10 time points were removed to stabilize the signal; 3) slice timing: reduce the difference caused by different image acquisition times; 4) head motion: subjects with head movement more than 2 mm or rotation more than 2° were excluded, and Friston 24 head movement parameters were obtained; 5) covariate of regression: interference covariates including linear trend, Friston 24 head motion parameter, white matter signal, and cerebrospinal fluid signal were regressed from the BOLD signal; 6) functional images were normalized to MNI space using DARTEL; 7) spatial smoothing (6-mm FWHM kernel); 8) band-pass filter (0.01–0.1Hz).

2.5 Computation of stability of dynamic functional architecture

The stability of dynamic functional architecture meant the consistency of dFC of a voxel with remaining voxels in the whole brain over time. For a given voxel j, the Pearson's correlation coefficients between the BOLD signal of voxel j and that of other voxels within the gray matter were calculated. Then, the dFC maps across time windows were generated for voxel j. The stability was quantified with the Kendall's coefficient of concordance (KCC) of these dFC maps, regarding the time windows as raters.

The specific calculation was as follows:

$$W = \frac{12S}{K^2(N^3 - N)}$$

$$S = \sum_{n=1}^{N} R_n^2 - \frac{1}{N}\left(\sum_{n=1}^{N} R_n\right)^2$$

W stands for Kendall's consistency coefficient, which ranges from 0 to 1. A higher W indicates that the results are more consistent. In other words, the dynamic functional stability of the brain is more stable in the field of neuroimaging. K is the number of sliding windows (raters), and N is the number of dFC between the given voxel and all atlases (respondents). R_n is the sum of the ranks of the nth dFC.

In this study, Craddock's atlas was used to divide the brain into 200 subregions (28). The calculation of the voxel-to-atlas dFC would generate a series of dFC vectors. The KCC of dFC vectors of voxel j was calculated with time windows as raters. The sliding-window approach was used to conduct dFC analysis (14), and the window length was 64 s and the sliding step was 4 s (29) (Figure 1). Different window lengths and sliding steps were set to 60 s/2 s and 96 s/8 s, respectively, to verify whether the result was affected by the window lengths and sliding steps.

2.6 Computation of dynamic effective connectivity

Brain regions with changed stability in T2DM were identified through the above steps. The regions which were obtained by group comparison of stability were extracted as region of interest (ROI) for further analysis. The dynamic BC toolbox was used to perform the GCA and calculate the dEC (30). The time series of each ROI was defined as x, and the time series of voxels in the whole brain was defined as y. The Granger causality effect between each ROI and each voxel in the whole brain was studied using bivariate coefficient GCA. Similarly, the sliding window method was used to estimate the dynamic GCA. For each subject, the average time course of GCA coefficients for each ROI was extracted and connected into a W × R matrix (where W represents the number of windows and R represents the number of ROIs). The dEC variability of each ROI was calculated by variance over the average time course of the GCA coefficients. The GCA model, based on the time series of the BOLD signal, describes the causal effect of the ROI on the rest of the voxels in the brain.

3 Statistical analysis

3.1 Demographic and clinical characteristics analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences version 22.0 (SPSS, Chicago, IL, USA). The statistical analysis of demographic and clinical characteristics was consistent with our previous studies (7, 8).

3.2 Intergroup comparison of dynamic functional stability and stability-based effective connectivity

The two-sample t-test in DPABI toolbox statistical software was used to compare the stability of the two groups. The gray matter mask

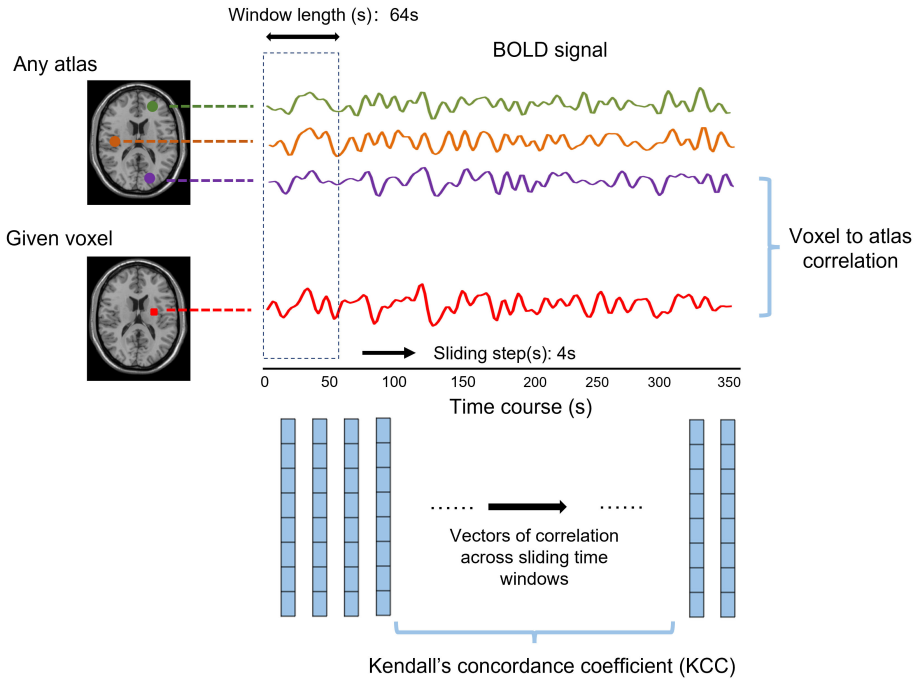


FIGURE 1
The abridged general view shows the calculation method of dynamic functional stability using the voxel-to-atlas way. The Pearson correlation between a given voxel and any atlas, namely, dynamic functional connectivity (dFC), was calculated for each window. The Kendall's concordance coefficient was computed based on cross-window dFC to state the functional stability.

automatically generated by preprocessing was used as the registration template, and sex, age, education level, and average head movement parameters were used as covariables to perform Gaussian random field (GRF) correction. Voxel level P< 0.001 and cluster level P< 0.05 were set. Subsequently, the same statistical method was used to conduct a statistical analysis on the dEC maps of the two groups.

3.3 The relationship between clinical biochemical indicators and neurocognitive scores

Partial correlation analysis was applied to analyze the correlation between various clinical biochemical indicators and scores of different neurocognitive function scales in the T2DM group after controlling for sex, age, and education level. P< 0.05 was considered statistically significant.

3.4 Correlation analysis

The Spearman correlation was conducted between the mean stability/dEC values of significant regions and cognitive function scores in T2DM subjects.

4 Results

4.1 Demographic, clinical, and cognitive characteristics

T2DM subjects and HCs were well matched across age, sex, and education level. For general cognitive status, the T2DM group performed significantly worse on AVLT (immediate), MoCA, CDT, and GPT(R&L) (P< 0.05) (Table 2).

4.2 Intergroup differences in stability in T2DM

Compared with HCs, T2DM subjects showed decreased stability in the right supra-marginal gyrus (SMG) and the right medial cingulate gyrus (MCG) with window length of 64 s and a step size of 4 s (Figure 2 and Table 3). The distribution comparison of stability was shown in the violin and box plots (Figure 3A). To further verify the validity and reliability of the results, validation tests were also processed by using a window length of 60 s and a step size of 2 s and a window length of 96 s and step size of 8 s, respectively (Supplementary Figures 1, 2). The location of the peak MNI point and the cluster were consistent, but the size of the cluster was slightly altered (Supplementary Table 1).

4.3 Intergroup differences in dynamic effective connectivity in T2DM

Compared with HCs, T2DM subjects showed that the dEC variability increased significantly between the left inferior frontal gyrus (IFG) and the right MCG. The direction of causal flow is from the right MCG to the left IFG (Figure 4 and Table 3). The distribution comparison of dEC is shown in the violin and box plots (Figure 3B).

4.4 Correlation analysis results

Partial correlation analysis found that AVLT immediate memory scores were negatively correlated with HbA1c (r = -0.309, P = 0.035) and LDL (r = -0.399, P = 0.004). However, there were no correlations between altered stability/dEC and scores of the neuropsychological tests.

TABLE 2 Neuropsychological result of two groups.

	T2DM (n = 50)	HC (n = 58)	Statistics	*P* value
AVLT (immediate)	20.16 ± 5.10	23.5 (18, 27)	*z* = -2.084	0.037*
AVLT (5 min)	7.56 ± 2.44	8 (7, 10)	*z* = -0.667	0.505
AVLT (20 min)	7.68 ± 2.77	8 (7, 9.5)	*z* = -0.289	0.772
AVLT (recognition)	11 (9, 12)	11 (10, 12)	*z* = -0.470	0.638
TMT-A (s)	53.5 (43.75, 75.25)	53 (41.5, 60)	*z* = -1.159	0.247
TMT-B (s)	45 (33.855, 61)	40 (34.95, 54.05)	*z* = -1.418	0.156
DST (forward)	8 (7,9)	8 (8, 9)	*z* = -1.753	0.080
DST (inverse)	4 (3,5)	4 (3, 4.25)	*z* = -1.328	0.184
CDT score	3 (2,3)	3 (3, 3)	*z* = -2.448	0.014*
MoCA score	26 (23,27.25)	27 (26, 29)	*z* = -3.039	0.002*
GPT (R) (s)	84 (73.125,99.6175)	72 (66.45, 81.85)	*z* = -3.853	<0.000*
GPT (L) (s)	90.35 (78.75,108.5)	80 (75, 89.25)	*z* = -3.381	0.001*

AVLT, Auditory Verbal Learning Test; TMT, Trail Making Test; DST, Digit Span Test; CDT, Clock Drawing Test; MoCA, Montreal Cognitive Assessment; GPT, Grooved Pegboard Test. *P < 0.05.

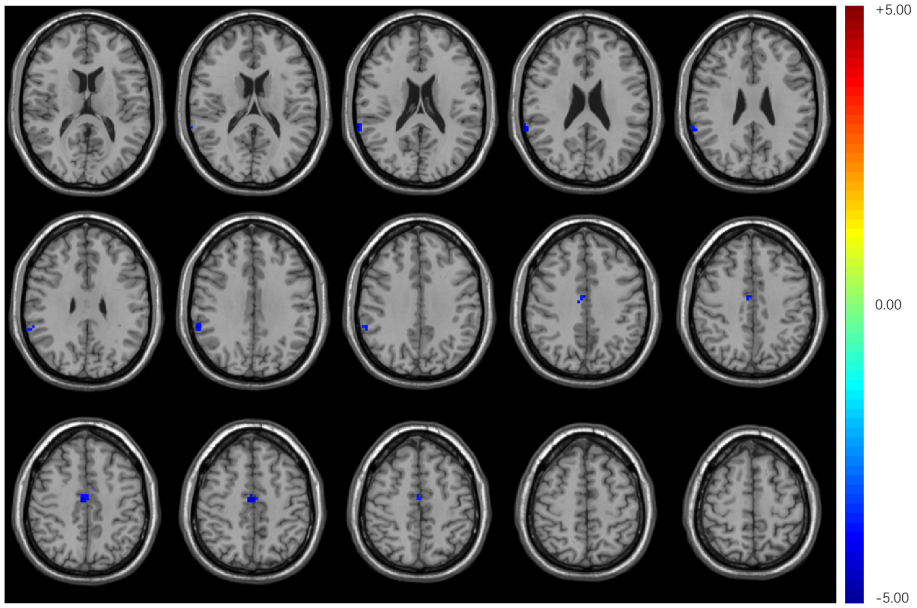


FIGURE 2
Compared with HCs, T2DM subjects exhibited decreased stability in the right supra-marginal gyrus and right medial cingulate gyrus with a window length of 64 s and a step size of 4 s.

TABLE 3 Brain regions with altered stability and EC in T2DM subjects.

Indicator	Cluster	Brain regions	MNI coordinates			Voxels	t-value
			x	y	z		
Stability	1	Right supra-marginal gyrus	69	-42	21	28	-4.5316
	2	Right median cingulum gyrus	3	-12	48	26	-4.746
EC	1	Left inferior frontal gyrus	-48	21	18	23	4.4585

MNI, Montreal Neurological Institute; X, Y, and Z, coordinates of primary peak locations in MNI space.

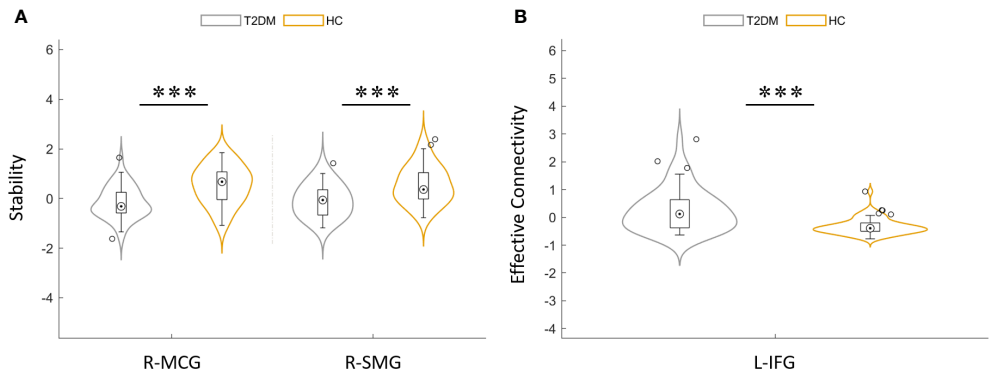


FIGURE 3
(A) Distribution comparison of dynamic functional stability between T2DM group and HCs. **(B)** Distribution comparison of dynamic effective connectivity between the T2DM group and HCs. MCG, median cingulate gyrus; SMG, supra-marginal gyrus; IFG, inferior frontal gyrus; L, left; R, right. ***P< 0.001.

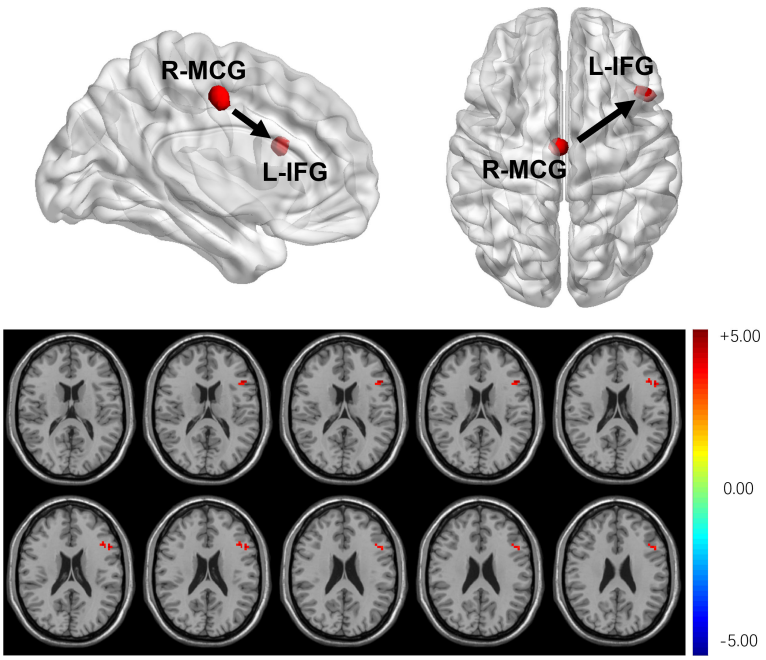


FIGURE 4
T2DM subjects showed that dEC has increased significantly between the left IFG and the right MCG. The direction of causal flow is from the right MCG to the left IFG.

5 Discussion

In the resting state, brain function is still changing dynamically (18). In this study, we explored the dynamic stability and dEC variability of the brain in T2DM. We found that there were two brain regions with decreased stability which contain right the SMG and the right MCG in T2DM subjects. DEC variation increased significantly between the left IFG and the right MCG in the T2DM group, which is further evidence of decreased stability in the brain of T2DM.

5.1 Altered functional architecture in T2DM

Stability, which reflects dFC without changing frequently in a continuous state, may provide an effective ability to coordinate information over time and facilitate rapid responses (21). The functional architecture of each region adjusts and changes dynamically and automatically as requested by physical need (31, 32). It is largely unclear whether the functional architecture of the brain in T2DM is stable over time. This study has well proved this point.

In this study, we found that there were two brain regions with decreased stability which contain the right SMG and the right MCG in T2DM subjects. SMG belongs to Brodmann area 40 which is involved in the motor attention and language processes, and lesions in it may lead to receptive aphasia (33). It is also reported that the right SMG was associated with poor melodic perception (34). In addition, the right SMG is a key region controlling spatial attention and working memory (35). The cingulate bundle is the main intermediate bundle, and it is one of the main white matter (WM) structures to transmit information (36). Moreover, the right MCG is contained in the frontoparietal network (FPN), which is involved in top-down attention and control task execution (37). It was found that WM fibers in cingulate gyrus were damaged in AD (38). The amplitude of low-frequency fluctuation (ALFF) is considered to be an effective method for detecting the intensity of spontaneously fluctuating regions and reflecting spontaneous brain activity in the brain. A study suggested that as cognitive function declines, the ALFF value of MCG decreased, which means the weakening in the neural activity of MCG. This finding represents decreased spontaneous neural activity or downregulation of excitability in MCG (36). Regions with high stability receive neural integration across modes and time so that they may conduct more function. Instead, the dynamic stability of these two brain regions decreased, which may represent impairment of cognitive functions (motor attention, language, executive function, etc.).

DEC variation increased significantly between the left IFG and the right MCG in the T2DM group, which is further evidence of decreased stability in the brain of T2DM. The frontal lobe is an important part of brain development and is responsible for higher-order cognitive control. Several studies (39–45) have shown that IFG, as the core region of the FPN, was associated with motor control, language processing, attention, and execution, which partially overlaps with the MCG's functions. A study reported on decreased activation in the left IFG under low working memory load conditions in T2DM patients (46). In addition, patients with AD and MCI have reduced activation of IFG in several memory tasks compared with HCs (47). Eliasova found that regulation of IFG excitability by rTMS may result in improved attentional task performance in patients with early AD (48). It is therefore reasonable to speculate that both the

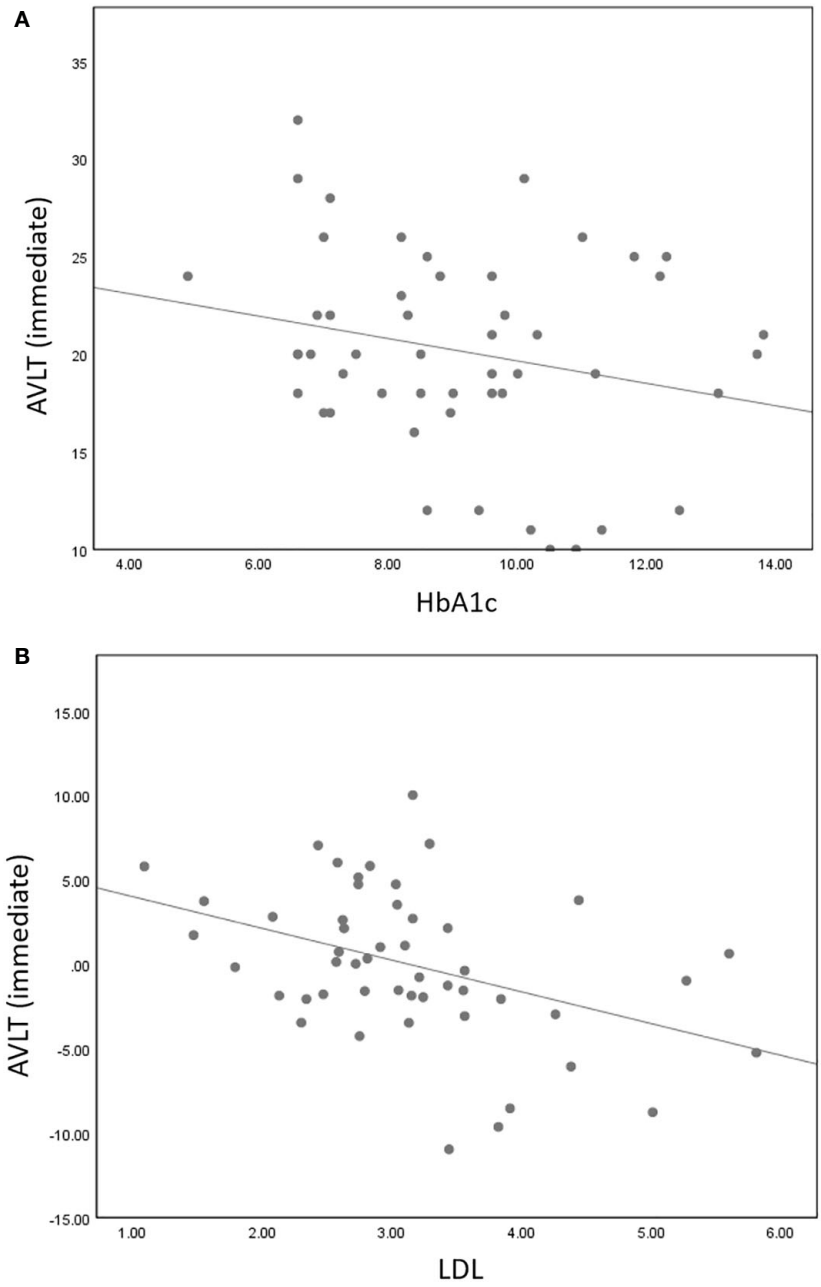


FIGURE 5
AVLT immediate memory scores were negatively correlated with HbA1c (r = -0.309, P = 0.035) **(A)** and LDL (r = -0.399, P = 0.004) **(B)**.

MCG and IFG may be involved in the control of certain cognitive brain functions, such as language processing, attention, and working memory.

At the same time, to verify this point, this study included a variety of cognitive function-related scales to explore the changes of cognitive function in T2DM patients. The results of our study showed that the overall cognitive function of T2DM patients decreased to varying degrees, which is consistent with previous research (7). Compared with the HCs, the scores of MoCA, AVLT immediate recall, CDT, and GPT (R and L) scales decreased significantly. This further confirms that T2DM does lead to accelerated cognitive decline. According to previous studies, altered brain regions in T2DM have been linked to visual motor attention, language processing, and memory function (49–51). Accordingly, we also found evidence in the neuroimaging measurements. MoCA is a comprehensive test scale (52); its memory test designs more words and tasks, which are more demanding and more sensitive to detect MCI (53). It assesses executive function, language, and visual-spatial processing. Verbal memory decline is the main sign of brain aging and an important character of AD. AVLT which includes immediate recall, 5-min recall, 20-min recall, and recognition mainly tests verbal memory with high sensitivity (54). CDT detects executive function as well as visuospatial function (55). GPT asks subjects to place each specially shaped peg in a

slot that fits it, which measures the performance speed of fine motor tasks (56). The decrease in these scores indicated an overall cognitive decline in T2DM patients.

We also found a close association between clinical indicators and cognitive tests in the correlation analysis (Figure 5). As the LDL and HbA1c levels increased, AVLT scores decreased. A higher level of LDL was associated with worse memory function [spice] (57). Research shows that increased LDL level is an independent risk factor for MCI (58). Many studies have found a link between plasma lipids and AD (59–61). In the case of hyperlipidemia, an elevated plasma cholesterol level results in the formation of free radicals that can damage the blood–brain barrier, leading to an elevated cholesterol level in the brain (61). Cholesterol is crucial in the formation of amyloid in the brain, leading to the formation of more beta-amyloid plaques. Eventually, it leads to neurodegeneration (61). Similarly, several studies have shown that HbA1c is associated with reduced cognitive function in diabetics and suggests neurological damage (62–64). HbA1c represents the percentage of glycosylated hemoglobin and is considered the gold standard for controlling the efficacy of treatments for diabetes (64). There is strong evidence that HbA1c is negatively associated with cognitive function and structural integrity of the brain in diabetic patients without dementia (65, 66). This study suggested that in T2DM, decreased LDL and HbA1c levels may represent reduced cognitive function, especially memory function.

5.2 Methodological validity

Most of the previous studies used the AAL atlas of 116 regions to perform the atlas-to-atlas method. Since some clusters span multiple functional regions but are not visible using the AAL atlas, the atlas-to-atlas method is not detailed enough and may miss some details. The calculation of functional stability can be conducted by two ways containing "voxel to voxel" and "voxel to atlas." The names of the two methods can manifest the accuracy level of measurement and the derivation process. In either method, the former "voxel" suggests that resolution of measurement and stability is measured voxel by voxel. The latter "voxel" suggests resolution of features, and it is at the voxel level. That is, for given voxel j, its dFC is computed with all other voxel across sliding windows and then stability is calculated. In the meantime, "atlas" suggests that the dFC is computed between voxel j and atlas. The "voxel to voxel" method is computationally accurate, but it is a huge amount of work and is time-consuming. We want to not only simplify the heavy workload but also ensure accurate measurement. The computation and results of the "voxel-to-atlas" method are very familiar to "voxel-to-voxel" so that it is the better way to perform the computation at a very fast rate. Therefore, the "voxel to atlas" method was used to calculate the stability. In Yan's study, it is shown that the "voxel to atlas" method has high speed and precision (20). After comparing the two methods, the results showed that "voxel to atlas," which is reasonable and feasible, could capture the main changes in the signal and the key information would not be lost. In this study, the "voxel to atlas" method was conducted to represent functional stability and the atlas with 200 regions was chosen that may make up for the shortcomings of past research. In addition, different window lengths and sliding steps were conducted to eliminate confounding effect.

6 Conclusion

In our study, changed dynamic functional architecture and directional connectivity in T2DM were explored and measured combining stability and dEC, which is an innovative attempt. Compared with HCs, T2DM subjects showed decreased stability in the right SMG and right MCG and dEC variation has increased significantly between left IFG and right MCG. The direction of causal flow is from the right MCG to the left IFG. These results provide additional evidence for cognitive decline in T2DM.

7 Limitation and future expectations

There was no correlation between neuroimaging biomarkers and cognitive function scores. We speculate that the sample size is relatively small, and the correlation may not be linear. In the future, we will continue to increase the sample size and reveal the relationship between neuroimaging indicators (dynamic stability and dEC) and cognitive function scores by multiple statistical methods. In addition, because the onset of T2DM is hidden and the specific time of onset is not precise enough, the course of the disease was not included in the study. In future studies, a very accurate disease course will be concluded.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This protocol was approved by the Ethics Committee of Guangzhou University of Chinese Medicine (Approval number: K2020115). Each subject provided written informed consent according to the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YFL designed the whole experiment and completed the manuscript. ML was involved in data processing. KZ and YW participated in the analysis design. XT and CQ recruited the study subjects. YR, ZS, LG, and ZC contributed to the statistical analysis. YL and SQ revised the manuscript. SQ is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the final version of the manuscript and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1117735/full#supplementary-material

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Nanomaterials for diabetic wound healing: Visualization and bibliometric analysis from 2011 to 2021

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Background: Nanomaterials have recently been shown to have a considerable advantage in promoting wound healing in diabetic patients or animal models. However, no bibliometric analysis has been conducted to evaluate global scientific production. Herein, this study aimed to summarize the current characteristics, explore research trends, and clarify the direction of nanomaterials and diabetic wound healing in the future.

Methods: Relevant publications from 2011 to 2021 were collected from the Web of Science Core Collection on October 3, 2022. VOSviewer, CiteSpace, bibliometrix-R package, Origin 2021, and Microsoft Excel 2019 were used for bibliometric and visualization analyses.

Results: We identified 409 publications relating to nanomaterials and diabetic wound healing. The number of annual productions remarkably increased from 2011 to 2021, with China and Shanghai Jiao Tong University being the most productive. The most prolific authors were Hasan Anwarul. The leading journal was the International Journal of Biological Macromolecules, with 22 publications. The most popular keywords were "nanoparticles," "delivery," "*in vitro*," "electrospinning," "angiogenesis," and "antibacterial." Keyword burst analysis showed "cerium oxide," "matrix metalloproteinase 9," "composite nanofiber," "hif 1 alpha," and "oxide nanoparticle" were emerging research hotspots.

Conclusion: We found there has been a great progress in the application of nanomaterials in diabetic wound healing from 2011 to 2021. Although many researchers and institutions from different countries or regions contributed contributed to publications, it will be helpful or the development of this field if the degree of international cooperation can be enhanced. In the future, nanomaterials with powerful antioxidant and antibacterial qualities and promoting angiogenesis are the research hotspots.

KEYWORDS

diabetic non-healing wound, knowledge map, research trends, web of science, bibliometric

1 Introduction

Diabetes mellitus (DM) is the most prevalent chronic metabolic disease characterized by hyperglycemia due to insulin deficiency or insulin resistance (1). The 10th edition IDF Diabetes Atlas estimated that approximately 537 million individuals live with diabetes worldwide (2). China has the highest number of patients with diabetes, which may reach over 174 million by 2045 (3). Besides sustained hyperglycemia, various complications occur, including diabetic ulcers, diabetic cardiomyopathy, and diabetic nephropathy. Diabetic foot ulcers (DFU) or diabetic non-healing wounds are catastrophic complications, which put a great social and economic burden on patients and the healthcare system (4). DFU affects 19%–34% of patients with DM, leading to 15%–25% of amputations and increasing the five-year death rate to 80% (5–7). A seven-year single-center retrospective review reported that the average total cost per patient was ¥21,826.91 ($3089.14) (8).

Normal wound healing consists of four successive and overlapping stages: hemostasis, inflammation, proliferation, and remodeling, which involve many types of cells and cytokines (9). However, diabetic wound healing usually does not progress due to the complex microenvironment with hyperglycemia, hypoxia, poor angiogenesis, and bacterial infection (10). Conventional treatment, including debridement, dressing, wound off-loading, infection control, vascular assessment, and glycemic control were ineffective, with 14%–20% of patients eventually undergoing low limb amputations (11, 12). Development of new treatments, such as nanotechnology, are urgently needed to improve patients' quality of life. Recently, many studies have been performed to verify that nanomaterials with different biological characteristics can get encouraging results in diabetic wound healing (13–15). Some nanomaterial-based wound dressings, such as hydrogels and nanofibers, provide a wet atmosphere by imitating the extracellular matrix for wound healing and enable the cell migration and proliferation by loading some drugs, protein and cytokines; Some nanomaterials, such as bioglass nanoparticles and metal nanoparticles, are of great angiogenic and antibacterial activities to improve the antibacterial effect and reduce the abuse of antibiotics by acting as the smart delivery system (16–18). Currently, several reviews have discussed the advances in the link between nanomaterials and diabetic wound healing, but comprehensive evaluation of the research status in this field is challenging.

Bibliometrics is an interdisciplinary subject that can be applied to conduct quantitative and qualitative analyses of publications by using mathematical and statistical methods (19). It includes the contributions and influence of different authors, countries/regions, institutions, journals, and trends (20). To the best of our knowledge, no bibliometric analysis has summarized current research hotspots. This study aimed to examine publication trends in nanomaterials and diabetic wound healing from 2011 to 2021 and provide future research direction for new researchers *via* the Web of Science Core Collection (WoSCC).

2 Materials and methods

2.1 Data collection and retrieval strategies

We collected publication data and downloaded them as plain text from WoSCC on October 3, 2022. Data included the article title, authors, journal title, publication year, institutions, keywords, citation frequency, and other basic information. Figure 1 shows data acquisition and retrieval strategies.

2.2 Inclusion and exclusion criteria

The accuracy of data must be ensured for analysis. Two researchers independently retrieved literature, and disagreements were resolved by discussing with outside parties, if necessary.

2.2.1 Inclusion criteria

(1) The research topic should be the application of nanomaterials in diabetic ulcers or diabetic wounds. (2) The document type is an article or a review article. (3) The document language is limited to English. (4) The study was published from January 1, 2011, to December 31, 2021.

2.2.2 Exclusion criteria

(1) Irrelevant documents, (2) other chronic wounds, such as burns, lower extremity venous ulcers, pressure ulcers, and diabetic corneal wounds, (3) retraction, (4) lack of basic information required for bibliometric analysis.

2.3 Data analysis and visualization

CiteSpace is Java-based bibliometric software, a widely used tool for the visual exploration of scientific literature, and helps discover research hotspots and future trends (21). We used CiteSpace (6.1.4) to perform dual-map overlay of journals, produce clustering analysis of keywords and co-cited references by using the log-likelihood ratio (LLR) algorithm, create a timeline view of keywords and co-cited references, and conduct keywords and co-cited references with the strongest burst.

In 2009, Van Eck and Waltman of The Centre for Science and Technology Studies at Leiden University in the Netherlands developed VOSviewer, a Java-based free software (22). VOSviewer (1.6.18) was used to visualize the co-citation network of countries, institutions, and authors. In the visual map, the node size indicated numbers or frequency, and the line thickness represented the strength of the link.

The bibliometrix R package (https://www.bibliometrix.org) was used to generate the distribution map of global publications. Microsoft Excel 2019 and Origin 2021 were used to display annual publications produced, international links of countries, and changes in yearly volume in China, India, and the USA.

3 Results

3.1 General information

We included 409 documents meeting the inclusion and exclusion criteria from the Web of Science database in our bibliometric analysis. Figure 2A shows the annual production of publications. Only a few

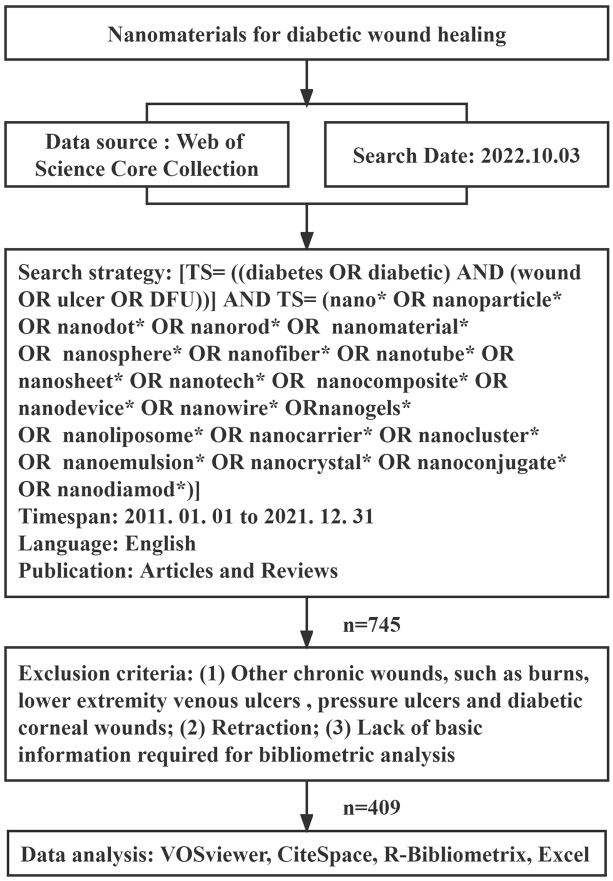


FIGURE 1
Flowchart for including and excluding publications.

articles were published in this field. Despite only five papers published in 2011, the number increased to 121 in 2021, with an increase of 2330%. Two phases were observed in the growth of literature published in the last decade: slow growth from 2011 to 2015, followed by rapid growth from 2016 to 2021. Carrot2 is an application for text clustering algorithms and organizing search results into topics. We divided 409 documents into 57 theme clusters for the Carrot2 analysis. We found that the top topics were "controlled release," "bacterial wound," "promoting angiogenesis," "nanoparticles promote," and "diabetic wound therapy" (Figure 2B).

3.2 Contributions of countries/regions

Fifty-one countries or regions contributed to publications in nanomaterials and diabetic wound healing. The distribution of published documents was shown on a world map, with the blue intensity representing the total number of publications (Figure 3). Figure 4A and Table 1 show that China published the most number of papers (n = 167, 40.83%), followed by India (n = 48, 11.74%), the USA (n = 32, 7.82%), Iran (n = 21, 5.13%), South Korea (n = 21, 5.13%), Egypt (n = 14, 3.42%), and Malaysia (n = 14, 4.42%). Other countries

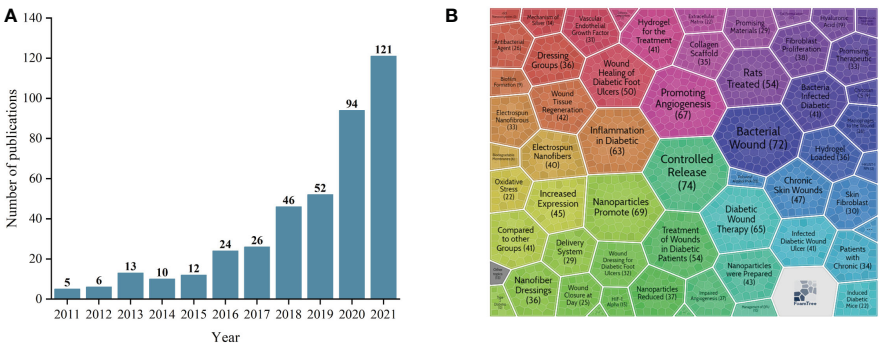


FIGURE 2
Annual number of publications **(A)** and theme clusters for the nanomaterials in diabetic non-healing wound by Carrot2 **(B)**.

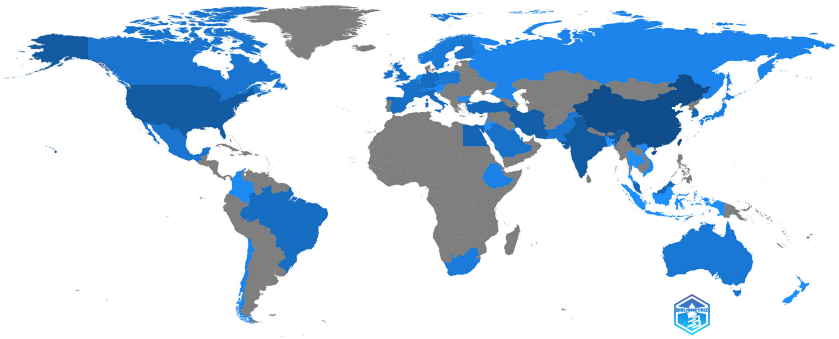


FIGURE 3
Map of global publications.

or regions published no more than five papers. In 2018, China's annual publication volume was significantly higher than that of India and the USA (Figure 4B). Among the top 10 countries, China also had the highest number of single-country papers, whereas the ratio of multiple-country papers ranked behind the USA and Brazil (Table 1). Moreover, the number of publications in China was significantly higher than that in other countries.

Figures 4C, D shows countries or regions with co-occurrence network. The size of the nodes represented the number of papers, and the line thickness showed cooperation strength. Of 51 countries, China and the USA had the strongest links. Accordingly, China was the most influential in the field based on publications and centrality.

3.3 Contributions of institutions

In the field of nanomaterials and diabetic wound healing, 699 institutions published 409 articles. Table 2 shows the top ten contributing institutions. Shanghai Jiao Tong University published the most papers (n = 17), followed by the Chinese Academy of Sciences (n = 16), Shanghai Normal University (n = 10), Sichuan University (n = 10), and Tehran University of Medical Sciences (n = 10). Other institutions published fewer than ten articles. Of the top ten institutions, eight were from China, and the remaining were from Iran and Qatar.

We used VOSviewer to demonstrate the co-authorship network of institutions (Figure 5). The minimum number of articles published by the institution was set to 5, and 13 clusters consisting of 27 institutions met the thresholds. Some of the 27 institutions were connected with others, with the largest set of connected clusters consisting of 11 institutions, of which Shanghai Jiao Tong University, Chinese Academy of Science, and Shanghai Normal University were the core. Research institutions in China were the leading organizations in nanomaterials and diabetic wound healing.

3.4 Authors

We retrieved 409 articles with 2508 authors, with an average of 6 authors per article. Table 3 lists 13 of the most effective authors. Xu He and Hasan Anwarul were the most prolific scholars, with 8 papers each, followed by Ke Qinfei (n = 7). In terms of total citations, Hasan Anwarul ranked first with 569 citations, followed by Jayakumar (n = 402) and Augustine Robin (n = 401). Although Xu He and Hasan Anwarul had the same number of publications, the average citations per paper of Hasan Anwarul (71.13 times) were significantly higher than those of Xu He (40.88 times).

H-index, g-index, and m-index were used to evaluate the academic level and influence of researchers, indicating that Hasan Anwarul made great achievements in nanomaterials and diabetic wound healing-related studies. VOSviewer was used to perform a visual analysis of the co-author (Figure 6). The minimum number of articles published by the author was set to 4. The largest clusters of connected authors consisted of six authors, and four of them were in the top 13.

3.5 Journals

These articles on nanomaterials and diabetic wound healing were published in 161 journals. Table 4 lists the top 15 journals with the most articles published, accounting for 38.14% (156/409). The International Journal of Biological Macromolecules, with 250 articles published, was the most productive journal, followed by Materials Science & Engineering C-Materials for Biological Applications currently known as Biomaterials Advances (n = 16, IF = 8.457), Acta Biomaterialia (n = 14, IF = 10.634). Based on the co-citation count, the International Journal of Biological Macromolecules (1217 citations), Acta Biomaterialia (871 citations), Biomaterials (581 citations), and Materials Science & Engineering C-Materials for Biological Applications (560 citations) were the top four journals. The others had <500 citations. Besides, 9 of 15 were found in the first quartile (Q1), and four had an IF impact of >10.

Citespace was used to show the topic distribution of relationships between journals by using the dual-map overlay. Figure 7 shows that the citing and cited journals are found on the left and right, respectively, and the colored line path represents the citation relationship, indicating the citation trajectory and knowledge flow. The map showed that molecular/biology/immunology journals generally cited articles published in molecular/biology/genetics and chemistry/materials/physics journals. The two pink routes showed that articles published in physics/materials/chemistry journals often cited molecular/biology/genetics journals and chemistry/materials/physics journals, indicating that articles on nanomaterials for diabetic

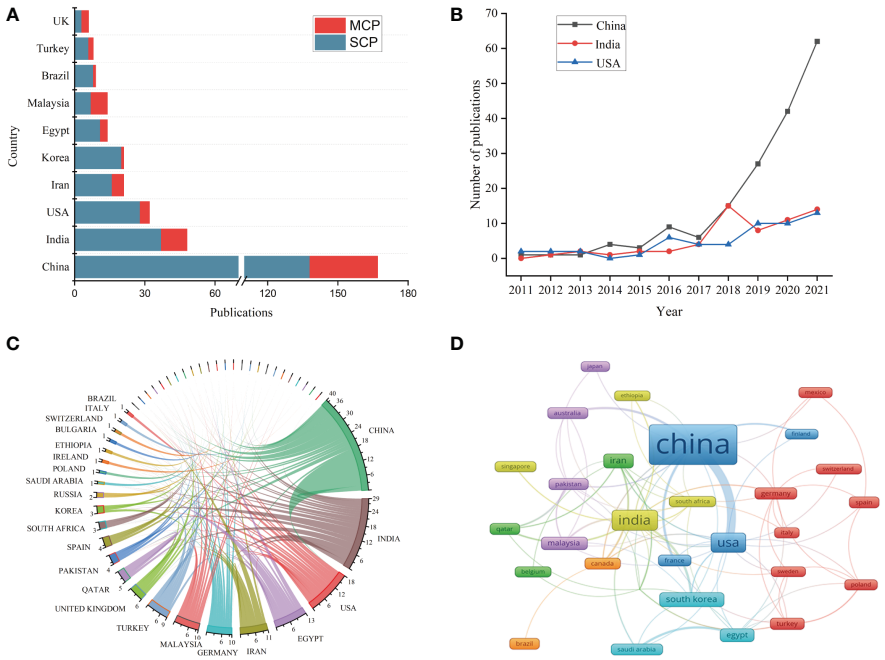


FIGURE 4
The distribution of single or multiple country papers in top 10 countries **(A)**; annual number of publications in China, India and USA **(B)**; The international cooperation networks between countries/regions **(C)**; VOSviewer network map of countries/regions **(D)**.

wound healing mainly focused on journals in molecular, biology, immunology, and physics, materials, and chemistry.

3.6 Keywords

The research hotspot in a certain knowledge field can be identified through keyword analysis. Subsequently, 1918 keywords were extracted from 409 selected articles, approximately 4.6 keywords per article. Of these, 172 keywords appeared over 5 times. After removing basic search terms, such as "wounds" and "diabetes," bibliometrics was used to create a word cloud map for keywords plus and author keywords (Figures 8A, B). "Electrospinning," "angiogenesis," "antibacterial," "nanoparticles," "drug-delivery," and "wound dressing" were common research hotspots in nanomaterials and diabetic ulcer healing.

In terms of keyword co-occurrence, CiteSpace software was used to perform keyword clusters by using the LLR algorithm. We selected the first 14 cluster labels (#0–#13) for analysis. The 14 clusters focused on 3 aspects: (1) diabetic wound healing process, including clusters #0, #1, #3, #4, #9, and #12; (2) nano-sized nanomaterials, including clusters #2, #7and #13; (3) mechanism of nanomaterials promoting diabetic wound healing, including clusters #5, #6, #8, #10 and #11.

The clustering time map can further show the appearance of each cluster. The end and time trends can reflect the importance, degree, and distribution period of a cluster. We found that cluster #9 stopped evolving since 2019. Although cluster #8 appeared the latest, it continued to evolve recently. In addition, clusters #5, #6, #10, and

TABLE 1 Total number of publications, single country publications, and multiple country publications of top 20 Countries by corresponding authors.

Country	Articles	Percentage of 409 articles	SCP	MCP	MCP Ratio
China	167	40.83%	138	29	0.174
India	48	11.74%	37	11	0.229
USA	32	7.82%	28	4	0.125
Iran	21	5.13%	16	5	0.238
Korea	21	5.13%	20	1	0.048
Egypt	14	3.42%	11	3	0.214
Malaysia	14	3.42%	7	7	0.5
Brazil	9	2.2%	8	1	0.111
Turkey	8	1.96%	6	2	0.25
United Kingdom	6	1.45%	3	3	0.5

TABLE 2 The top 10 institutions in the field of nanomaterials and diabetic wound healing.

Institution	Articles	Total citations	Country
Shanghai Jiao Tong Univ	17	551	China
Chinese Acad Sci	16	640	China
Shanghai Normal Univ	10	388	China
Sichuan Univ	10	261	China
Univ Tehran Med Sci	10	466	Iran
Jinzhou Med Univ	9	166	China
Qatar Univ	8	569	Qatar
Zhejiang Univ	8	443	China
Nankai Univ	7	218	China
Chang Gung Univ	7	296	China

#11 maintained a certain heat recently, indicating that the mechanism of nanomaterials in treating diabetic wounds is the current research trend. Moreover, keyword bursts were used to show research trends in the field. Figure 9 shows the top 30 most cited keywords. The keyword with the highest burst intensity was "silver" (2.8357), followed by "collagen" (2.7329) and "nanocomposite" (2.6773). The keyword with the longest duration was "regeneration", followed by "fibrous scaffold", "*Staphylococcus aureus*", "matrix metalloproteinase","curcumin", "collagen", and "proliferation."

3.7 Citation and co-cited reference

Citations are the standard way for authors to indicate the source, ideas, and findings of their research methods. The citation frequency of an article can measure its value to a certain extent. Table 5 shows the top 10 globally cited articles (23–32). "Engineering Bioactive Self-Healing Antibacterial Exosomes Hydrogel for Promoting Chronic Diabetic Wound Healing and Complete Skin Regeneration" published in Theranostics in 2019 had the largest number of citations (289 times).

Reference co-citations can be used to measure the degree of mutual influence among studies, among which the highly cited studies form the disciplinary basis of this field. Table 6 lists the top 10 co-cited references (26, 33–41). The most co-cited reference was "Wound healing and its impairment in the diabetic foot" by Falanga et al. in 200530. All references were clustered into the major 16 clusters, including "#0 diabetic foot ulcer", "#1 EGF," "#2 diabetic wound healing," and "#3 silver nanoparticle," and so on (Figure 10A). Figure 10B shows that "#11 nanofibers" and "#13 antibacterial properties" continuously evolved.

Figure 10C shows the top 18 references with the strongest citation bursts. "Fibrin-based scaffold incorporating VEGF- and bFGF-loaded nanoparticles stimulate wound healing in diabetic mice" by Losi et al. in 2013, and "Quantitative and reproducible murine model of excisional wound healing" by Galiano et al. in 2004 had the strongest burst (3.42) (26, 42). Additionally, the article "Effect of wettability and surface functional groups on protein adsorption and cell adhesion using well-defined mixed self-assembled monolayers" by Arima et al. had the longest burst period from 2011 to 2017 (43).

4 Discussion

Non-healing diabetic wounds, resulting in high mortality rates and disability in diabetic patients, have become a global public health

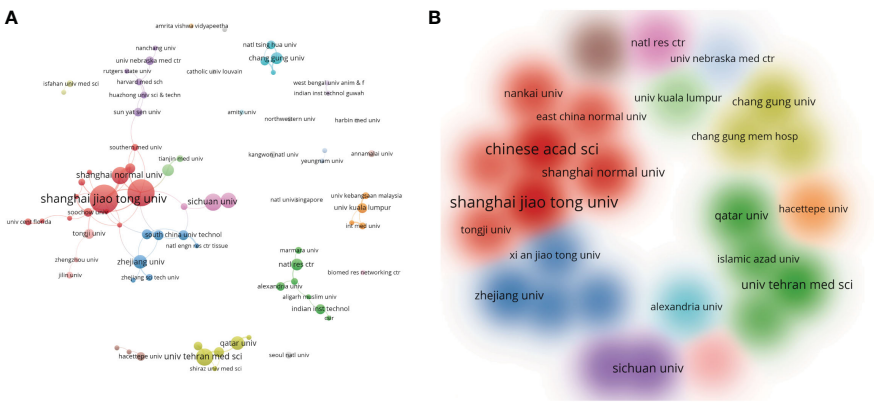


FIGURE 5
VOSviewer network map of institutions **(A)**; Density distribution map of institutions **(B)**. Minimum number of articles published by the institution was set to 5.

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TABLE 3 The top 13 authors in the field of nanomaterials and diabetic wound healing.

Rank	Author	Papers	Total citations	Average citations per paper	H-index	G-index	Ms-index
1	Hasan Anwarul	8	569	71.13	7	8	1.4
2	Xu He	8	327	40.88	7	8	1.167
3	Ke Qinfei	7	309	44.14	7	8	1
4	Chang Shang-hung	6	208	34.67	6	6	0.667
5	Hung Kuo-chun	6	208	34.67	6	6	0.667
6	Juang Jyuhn-huang	6	208	34.67	6	6	0.667
7	Liu Shih-jung	6	208	34.67	7	6	0.667
8	Yi Zhengfang	5	272	54.40	5	5	0.883
9	Augustine Robin	5	401	80.20	5	5	1
10	Chen zhenhua	5	108	21.60	4	5	1.333
11	Mei Xifan	5	108	21.60	4	5	1.333
12	Jayakumar R	5	402	80.40	5	5	0.455
13	Zhang Xinge	5	265	53.00	4	4	0.444

problem. Over the past years, many studies on the applications of nanomaterials in diabetic wound healing have been conducted to confirm that nanomaterials are effective pathways in treatment. Bibliometrics help researchers make comprehensive cognition in the field. For the first time, different bibliometrics software were combined to sort out the current characteristics and predict the development trends of nanomaterials and diabetic wound healing.

4.1 General information

The number of publications reflect the scientific activities in a field. Based on our study, 2508 authors from 699 institutions in 51 countries or regions have contributed 409 publications to the field of nanomaterials and diabetic wound healing from 2011 to 2021. Moreover, the global volume markedly increased. The number of annual output is almost twice as high as that in the previous year, indicating a promising prospect in the research regarding the application of nanomaterials in diabetic wound healing.

China is the most productive country, with 167 articles in terms of country publication volume. Compared with China, publications from other countries significantly decreased. China and the USA have the closest collaboration, indicating that China is the core country for international research on nanomaterials and diabetic wound healing. In addition, India has the highest MCP ratio in the top three productive countries. Moreover, India plays an important role in international cooperation. As the two most populous countries in the world, China and India have the highest number of diabetic patients. Thus, scholars are driven to explore the application of nanomaterials in diabetic non-healing wounds.

Furthermore, 699 institutions contributed articles to the application of nanomaterials in diabetic wound healing. The most prolific institution was Shanghai Jiao Tong University from China which published nine papers in 2021. Figure 6 shows that institutions in close geographical proximity usually collaborated actively. For example, no cooperation was found between Shanghai Jiao Tong University and Univ Tehran Med Sci, although they were ranked at the top. Regarding the author's contribution, Chinese scholars still played the lead role in publishing

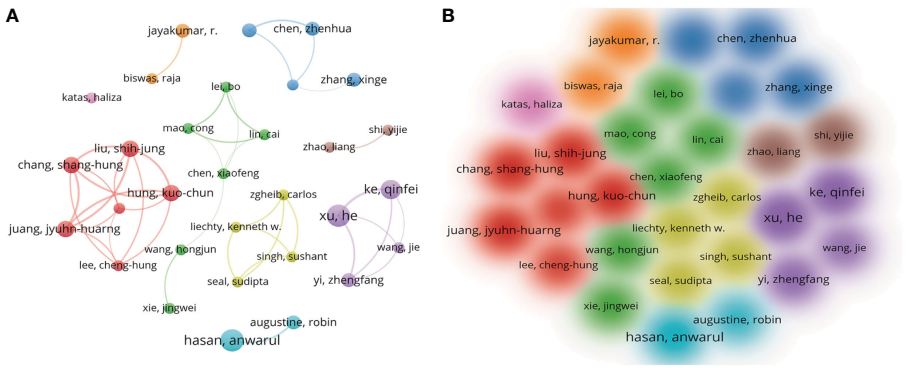


FIGURE 6
VOSviewer network map of authors **(A)**; Density distribution map of authors **(B)**.

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TABLE 4 The top 15journals in the field of nanomaterials and diabetic wound healing.

Journal title	Publications	Total citations	Average citation per paper	Impact factor(2021)	JCR	Country
Int J Biol Macromol	22	1217	55.31	8.0252	Q1	Netherlands
Mat Sci Eng C-Mater	16	560	35	8.4572	Q1	Netherlands
Acta Biomater	14	856	61.14	10.6335	Q1	England
J Mater Chem B	13	371	28.54	7.5711	Q1	England
Acs Appl Mater Inter	12	747	62.25	10.3831	Q1	USA
Biomater Sci-Uk	9	246	27.33	7.5896	Q1	England
J Drug Deliv Sci Tec	9	90	10	5.0619	Q2	France
Acs Biomater Sci Eng	8	219	27.375	5.395	Q2	USA
Chem Eng J	8	156	19.5	16.7438	Q1	Switzerland
Int J Nanomed	8	159	19.875	7.033	Q1/Q2	New Zealand
Rsc Adv	8	166	20.75	4.036	Q2	England
Sci Rep-Uk	8	261	32.625	4.996	Q2	England
Biomaterials	7	581	83	15.304	Q1	Netherlands
Colloid Surface B	7	244	34.85	5.999	Q1/Q2	Netherlands
Int J Pharmaceut	7	314	44.85	6.51	Q1	Netherlands

articles in the field. Table 3 shows that the most prolific authors were Hasan Anwarul. The latest article by Hasan Anwarul indicated that EGF-loaded PHBV-Gel MA hybrid patches could accelerate wound healing in diabetic mice by promoting the migration and proliferation of multiple cell types and angiogenesis (44). Similar to the status of the institutions' links, many cooperating groups exist among several scholars. To develop this field, institutions and researchers should focus on similar research topics to establish closer cooperation.

4.2 Research trends and hotspots

Keyword evolution and cited references map the dynamic changes in research hotspots. Therefore, we conducted multiple visualization knowledge mapping, including cluster analysis, timeline view, and citation bursts of the references and keywords. Analysis of keywords and co-cited references showed that nanomaterials, such as oxide nanoparticles with antibacterial and antioxidant qualities and promoting angiogenesis, are encouraging trends.

Nanotechnology is an emerging field in treating diabetic complications. Nano-sized materials, such as nanoparticles, liposomes, nanofibers, and hydrogel, have an advantage in delivering drugs, providing sustained release, prolonging action time, and reducing side effects (45, 46). Nanomaterials are ideal carriers for enhancing drug delivery efficiency and cell regeneration. Researchers showed great interest in these topics from 2011 to 2015. Figure 9 shows that "fibrous scaffold," "nanocrystalline silver," and "nanoparticle" appeared in the earliest burst keywords, as well as "migration" and "regeneration," which are important terms in progress in wound healing. Table 6 lists the top ten co-cited references. Most were reviews and aimed to outline the physiological and pathological processes of wound healing and summarize the negative factors that delay wound healing. Two original studies in the top ten co-cited references developed suitable carriers to deliver drugs or cytokines, such as EGF, VEGF, and bFGF (26, 36).

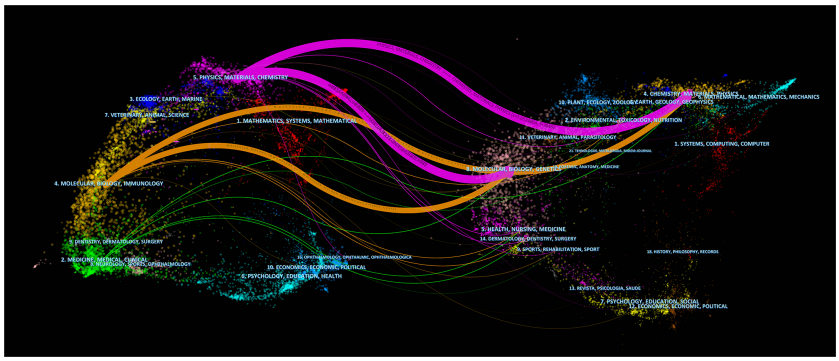


FIGURE 7
The dual-map overlay of journals contributed to publications regarding nanomaterials for diabetic wound healing from 2011 to 2021.

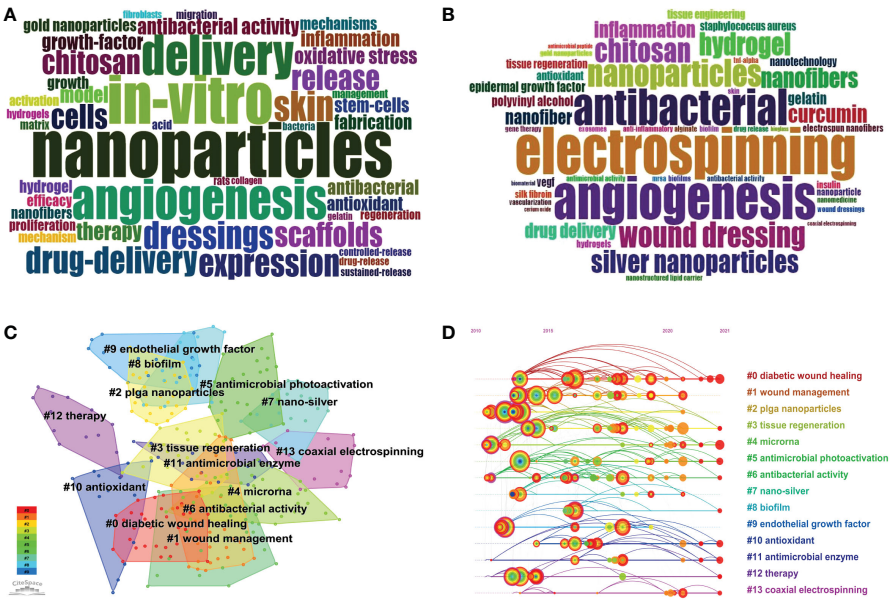


FIGURE 8
Word cloud of top 50 plus keywords **(A)**; Word cloud of top 50 author's keywords **(B)**; Map of keyword clusters **(C)**; Timeline view of keyword co-occurrence **(D)**.

Keywords	Year	Strength	Begin	End	2011 - 2021
fibrous scaffold	2011	2.2132	2012	2014	
staphylococcus aureus	2011	1.6158	2013	2015	
nanocrystalline silver	2011	1.3243	2013	2013	
silver dressing	2011	1.3243	2013	2013	
migration	2011	2.2944	2013	2014	
matrix metalloproteinase	2011	2.3182	2013	2015	
nanoparticle	2011	1.8571	2014	2015	
regeneration	2011	2.4141	2014	2017	
vegf	2011	1.309	2015	2015	
nanocomposite	2011	2.6773	2016	2017	
apoptosis	2011	1.2553	2016	2016	
chitosan	2011	1.652	2016	2017	
microsphere	2011	1.2553	2016	2016	
cell adhesion	2011	1.2553	2016	2016	
curcumin	2011	1.3831	2016	2018	
bacterial cellulose	2011	1.2614	2017	2017	
collagen	2011	2.7329	2017	2019	
microbial cellulose	2011	1.2614	2017	2017	
silver	2011	2.8357	2018	2019	
antimicrobial activity	2011	1.6169	2018	2019	
zinc oxide nanoparticle	2011	1.6693	2018	2018	
differentiation	2011	1.6693	2018	2018	
proliferation	2011	2.5045	2019	2021	
gold nanoparticle	2011	1.7441	2019	2019	
polysaccharide	2011	1.6266	2019	2019	
matrix metalloproteinase 9	2011	1.3521	2020	2021	
cerium oxide	2011	1.3521	2020	2021	
composite nanofiber	2011	1.3521	2020	2021	
hif 1 alpha	2011	1.3521	2020	2021	
oxide nanoparticle	2011	1.3521	2020	2021	

FIGURE 9
Top 30 keywords with the strongest citation bursts.

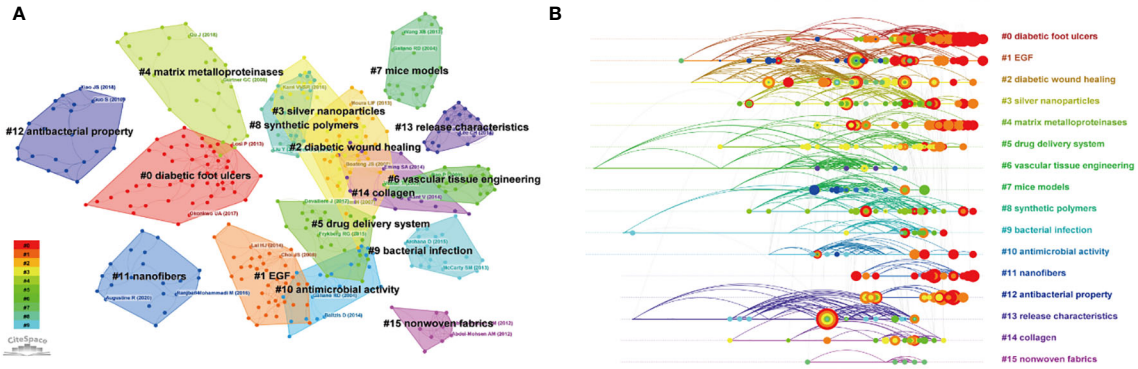
TABLE 5 Top 10 global cited articles in nanomaterials and diabetic wound healing.

Title	Journal title	First authors	Year	Total Citations
Engineering Bioactive Self-Healing Antibacterial Exosomes Hydrogel for Promoting Chronic Diabetic Wound Healing and Complete Skin Regeneration (23)	Theranostics	WANG CG	2019	289
Promotion of skin regeneration in diabetic rats by electrospun core-sheath fibers loaded with basic fibroblast growth factor (24)	Biomaterials	YANG Y	2011	256
Skin-inspired antibacterial conductive hydrogels for epidermal sensors and diabetic foot wound dressings (25)	Advanced Functional Materials	ZHAO Y	2019	219
Fibrin-based scaffold incorporating VEGF- and bFGF-loaded nanoparticles stimulates wound healing in diabetic mice (26)	Acta Biomaterialia	LOSI P	2013	217
Tailored design of electrospun composite nanofibers with staged release of multiple angiogenic growth factors for chronic wound healing (27)	Acta Biomaterialia	LAI HJ	2014	201
Novel electrospun chitosan/polyvinyl alcohol/zinc oxide nanofibrous mats with antibacterial and antioxidant properties for diabetic wound healing (28)	International Journal of Biological Macromolecules	AHMED R	2018	192
A cooperative copper metal-organic framework-hydrogel system improves wound healing in diabetes (29)	Advanced Functional Materials	XIAO JS	2017	175
Efficient angiogenesis-based diabetic wound healing/skin reconstruction through bioactive antibacterial adhesive ultraviolet shielding nanodressing with exosome release (30)	ACS Nano	WANG M	2019	169
Combined effect of PLGA and curcumin on wound healing activity (31)	Journal of Controlled Release	CHEREDDY KK	2013	167
Copper metal-organic framework nanoparticles stabilized with folic acid improve wound healing in diabetes (32)	ACS Nano	XIAO JS	2018	163

In addition, nanomaterials with antibacterial, anti-inflammatory, and antioxidant properties are another main reason for their application in improving diabetic wound healing. Figures 8C, D, 10A, B show that antibacterial or antimicrobial is the most important cluster that continuously evolves in diabetic wound healing. Besides, the ongoing burst keywords included "oxide nanoparticle," "hif 1 alpha," and "cerium oxide" which reflected the latest research trends. HIFP-1α is an important factor in diabetic wound healing, and the degradation of polyubiquitinated HIF-1α diminishes wound healing efficacy. Therefore, it is an effective treatment to stabilize the HIF-1α protein. Yang et al. designed an intelligent NIR-triggered NO nanogenerator to increase HIF-1α expression to enhance angiogenesis (47). Leung et al. also Introduced a small-molecule HIF-1α stabilizer to accelerate diabetic wound healing by enhancing angiogenesis (48). Metals and

TABLE 6 Top 10 co-cited references in nanomaterials and diabetic wound healing.

Title	Journal title	First authors	Year	Total Citations
Wound healing and its impairment in the diabetic foot (33)	Lancet	FALANGA V	2005	65
Cellular and molecular basis of wound healing in diabetes (34)	Journal of Clinical Investigation	BREM H	2007	47
Factors affecting wound healing (35)	Journal of Dental Research	GUO S	2010	38
In vivo wound healing of diabetic ulcers using electrospun nanofibers immobilized with human epidermal growth factor (EGF) (36)	Biomaterials	CHOI JS	2008	32
Recent advances on the development of wound dressings for diabetic foot ulcer treatment–a review (37)	Acta Biomaterialia	MOURA LIF	2013	29
Wound repair and regeneration (38)	Nature	GURTNER GC	2008	28
Wound healing dressings and drug delivery systems: a review (39)	Journal of Pharmaceutical Sciences	BOATENG JS	2008	26
Fibrin-based scaffold incorporating VEGF- and bFGF-loaded nanoparticles stimulates wound healing in diabetic mice (26)	Acta Biomaterialia	LOSI P	2013	26
Diabetes and wound angiogenesis (40)	International Journal of Molecular Sciences	OKONKWO UA	2017	24
Cutaneous wound healing (41)	New England Journal of Medicine	SINGER AJ	1999	24



C

References	Year	Strength	Begin	End	2011 - 2021
Blakytny R, 2006, DIABETIC MED, V23, P594, DOI 10.1111/j.1464-5491.2006.01773.x	2006	3.21	2011	2016	
Arima Y, 2007, BIOMATERIALS, V28, P3074, DOI 10.1016/j.biomaterials.2007.03.013	2007	2.63	2011	2017	
Lobmann R, 2002, DIABETOLOGIA, V45, P1011, DOI 10.1007/s00125-002-0868-8	2002	2.55	2012	2013	
Galiano RD, 2004, WOUND REPAIR REGEN, V12, P485, DOI 10.1111/j.1067-1927.2004.12404.x	2004	3.42	2013	2018	
Michaels J, 2007, WOUND REPAIR REGEN, V15, P665, DOI 10.1111/j.1524-475X.2007.00273.x	2007	2.86	2013	2017	
OLoughlin A, 2013, DIABETES, V62, P2588, DOI 10.2337/db12-1822	2013	2.57	2014	2015	
Martin P, 1997, SCIENCE, V276, P75, DOI 10.1126/science.276.5309.75	1997	3.36	2016	2018	
Moura LIF, 2014, ACTA BIOMATER, V10, P843, DOI 10.1016/j.actbio.2013.09.040	2014	2.83	2016	2018	
Yang Y, 2011, BIOMATERIALS, V32, P4243, DOI 10.1016/j.biomaterials.2011.02.042	2011	2.83	2016	2018	
Cavanagh PR, 2005, LANCET, V366, P1725, DOI 10.1016/S0140-6736(05)67699-4	2005	2.83	2016	2018	
Brem H, 2007, J CLIN INVEST, V117, P1219, DOI 10.1172/JCI32169	2007	2.66	2016	2019	
Losi P, 2013, ACTA BIOMATER, V9, P7814, DOI 10.1016/j.actbio.2013.04.019	2013	3.42	2017	2018	
Abrigo M, 2014, MACROMOL BIOSCI, V14, P772, DOI 10.1002/mabi.201300561	2014	2.9	2017	2019	
Boateng J, 2015, J PHARM SCI-US, V104, P3653, DOI 10.1002/jps.24610	2015	2.49	2017	2019	
Fonder MA, 2008, J AM ACAD DERMATOL, V58, P185, DOI 10.1016/j.jaad.2007.08.048	2008	2.93	2018	2019	
Falanga V, 2005, LANCET, V366, P1736, DOI 10.1016/S0140-6736(05)67700-8	2005	2.5	2018	2019	
Xiao JS, 2018, ACS NANO, V12, P1023, DOI 10.1021/acsnano.7b01850	2018	3.3	2019	2021	
Xu H, 2015, NANOSCALE, V7, P18446, DOI 10.1039/c5nr04802h	2015	2.7	2019	2021	

FIGURE 10
Map of cited references clusters **(A)**; Timeline view of cited references co-occurrence **(B)**; Top 18 references with the strongest citation bursts **(C)**.

their ions were beneficial to wound care. With the development of nanotechnology, metal oxide nanoparticles have attracted the interest of researchers. Cerium oxide nanoparticles were widely used in diabetic non-healing wounds because of their antioxidant properties. Augustine et al. designed a type of cerium oxide nanoparticle-loaded gelatin methacryloyl hydrogel to accelerate wound healing in diabetic mice (49). Dewberry et al. and Stager et al. also used cerium oxide nanoparticles as raw material in preparing a nanocarrier to promote diabetic wound healing (50, 51). Some metal oxide nanoparticles were utilized because of their antibacterial properties. Wang et al. developed a nanocomposite scaffold composed of iron oxide nanoparticles and showed antioxidant and substantial antibacterial properties in an *in vitro* study (52). Nanocomposites, including zinc oxide and magnesium oxide, seem to promote wound healing through their antibacterial activity (53, 54). Thus, developing materials with powerful antioxidant and antibacterial activities is challenging for researchers.

5 Conclusion

To the best of our knowledge, this is the first bibliometric analysis that investigated the application of nanomaterials in diabetic non-healing wounds. A comprehensive survey and analysis of countries, authors, keywords, journals, and literature was conducted. Bibliometric networks through authors, institutions, co-citation, and co-occurrence analyses were constructed using various scientific tools to reveal the bibliometric characteristics of the field. Moreover, the degree of international cooperation should be enhanced to develop this field. Nanomaterials with antibacterial and antioxidant qualities and promoting angiogenesis are the current research topics.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

Conception and design: ZW, JZ. Data collection and interpretation: JZ, TC, HL. Data analyses: YZ, XN. Manuscript draft and critical

review: all authors. Final approval of the study content and manuscript and accountability for data integrity: all authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The impact of oxidative stress-induced mitochondrial dysfunction on diabetic microvascular complications

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Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycaemia, with absolute insulin deficiency or insulin resistance as the main cause, and causes damage to various target organs including the heart, kidney and neurovascular. In terms of the pathological and physiological mechanisms of DM, oxidative stress is one of the main mechanisms leading to DM and is an important link between DM and its complications. Oxidative stress is a pathological phenomenon resulting from an imbalance between the production of free radicals and the scavenging of antioxidant systems. The main site of reactive oxygen species (ROS) production is the mitochondria, which are also the main organelles damaged. In a chronic high glucose environment, impaired electron transport chain within the mitochondria leads to the production of ROS, prompts increased proton leakage and altered mitochondrial membrane potential (MMP), which in turn releases cytochrome c (cyt-c), leading to apoptosis. This subsequently leads to a vicious cycle of impaired clearance by the body's antioxidant system, impaired transcription and protein synthesis of mitochondrial DNA (mtDNA), which is responsible for encoding mitochondrial proteins, and impaired DNA repair systems, contributing to mitochondrial dysfunction. This paper reviews the dysfunction of mitochondria in the environment of high glucose induced oxidative stress in the DM model, and looks forward to providing a new treatment plan for oxidative stress based on mitochondrial dysfunction.

KEYWORDS

diabetes mellitus, diabetic microvascular complications, mitochondria, oxidative stress, oxidative phosphorylation, mitochondrial DNA

1 Introduction

DM is characterized by chronic and persistent hyperglycemia and causes macrovascular and microvascular complications, which is a major cause of end-stage renal disease, blindness, and amputation today (1–4). As of 2014, there were approximately 387 million people with DM worldwide (8.3% of the world's population) and the number of people with DM is expected to increase to 640 million by 2040 (5, 6). The cost of diabetic microvascular complications is a major component of overall treatment costs and places a heavy burden on society and families (7).

The development and progression of DM has complex pathophysiological mechanisms, with inflammation, autophagy dysregulation, oxidative stress, and hemodynamic dysregulation all involved in the progression of the disease (8–11). In this regard, oxidative stress is an important part of disease development, and chronic hyperglycaemia promotes an imbalance between the production of free radicals and the scavenging capacity of the body's antioxidant system. Mitochondria play an important role in the process of oxidative stress in cells. Mitochondria are the main site of cellular respiration in the body and the central organelle for the production of adenosine triphosphate (ATP) (12). Continuous high glucose stimulation leads to impaired mitochondrial electron transport and promotes the production of ROS, which in turn causes damage to the mitochondria themselves and mitochondrial DNA (mtDNA), leading to impaired ATP synthesis, apoptosis, and activation of downstream inflammatory and fibrotic signaling pathways, contributing to disease progression (13–15). Scientists are now proposing that improving the antioxidant capacity of cells may be an important strategy for treating DM and complications, with some experiments in animals and humans and some results, but the evidence-based clinical support for anti-oxidative stress therapies is still insufficient (16–18). Based on this, the aim of this review is to explore together the important role of mitochondria in the process of oxidative stress in DM through abnormal mitochondrial oxidative phosphorylation (OXPHOS) and mtDNA damage, and to outline the efforts made by present-day scientists towards repairing mitochondrial function in the hope of providing new ideas for future scientific experiments.

2 Oxidative stress and ROS

The concept of oxidative stress was first introduced by the German scientist Helmut Sies and refered to the imbalance of oxidants and antioxidants that can cause damage to the organism (19). The concept of "oxidative stress" was later extended to refer to a pathological phenomenon resulting from an imbalance between the production of free radicals and the scavenging function of the antioxidant system, and is closely linked to the development of diseases such as chronic obstructive pulmonary disease, Alzheimer's disease, cancer, DM, hypertension and age-related diseases (20–26).

Structurally, free radicals are highly reactive substances containing at least one unpaired electron and are active derivatives of ROS and reactive nitrogen species (RNS), which are closely associated with the initiation of oxidative stress (27, 28). Both endogenous and exogenous stimulation lead to pathological increases in ROS and promote oxidative stress. Endogenous factors such as metabolic factors, mitochondrial damage, immune system dysregulation, and inflammatory products induce the production of ROS (29–32). Exogenous ionizing radiation, xenobiotics, ultraviolet light, alcohol abuse and smoking contribute to the onset and progression of aging and metabolic disease by promoting ROS production in the body (33–38) (Figure 1).

The intracellular ROS is mainly composed of $O_2^{\cdot-}$ [reduced from O_2 by electrons through the electron transfer chain (ETC)] and its derivatives. The three main reactive substances of ROS are superoxide anion ($O_2^{\cdot-}$), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2), with •OH being the most reactive (39, 40). Superoxide dismutase (SOD), the first line of defense of the cellular antioxidant defense

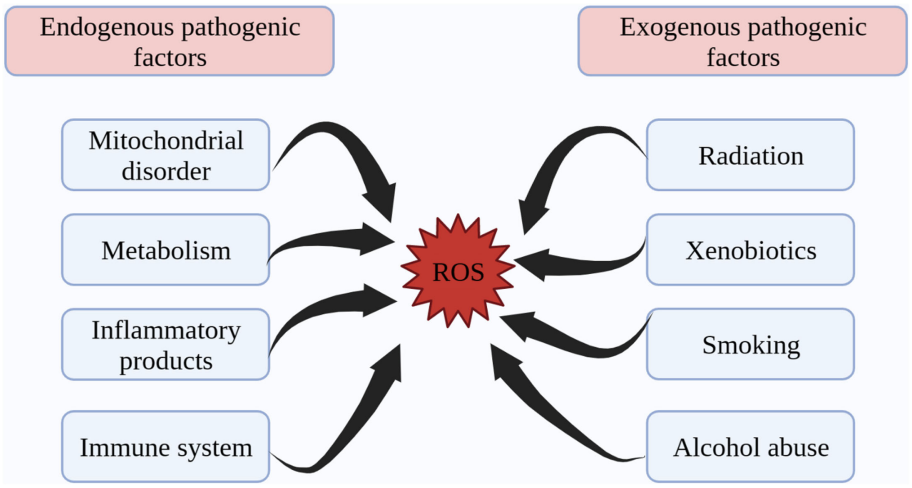


FIGURE 1
Endogenous and exogenous factors in ROS production. ROS generation is divided into two aspects: endogenous pathogenic factors and exogenous pathogenic factors. Endogenous pathogenic factors include mitochondrial disorder, metabolism, inflammatory products, immune system, etc. Exogenous pathogenic factors include radiation, xenobiotics, smoking, alcohol abuse, etc. ROS, reactive oxygen species.

system, promotes the production of the superoxide $O_2^{\cdot-}$ intermediating H_2O_2: $2O_2^{\cdot-}+2H^+ \rightarrow H_2O_2+O_2$ (41). Subsequently reduced by catalase (CAT) to non-toxic H_2O: $2H_2O_2 \rightarrow 2H_2O+O_2$; or catalyzed by glutathione peroxidase (GSH-Px) to produce H_2O: $2GSH+H_2O_2 \rightarrow 2H_2O+GSSG$, all of which complete the antioxidant scavenging process and constitute the antioxidant enzymatic response system of the organism, maintaining the intracellular redox balance (41, 42). Fe^{2+} and Cu^+ are capable of redox reactions with unpaired electrons, destroying the structure and function of proteins, nucleic acids and lipids and cross-linking with these macromolecules to produce toxic substances (19). Activation of mitochondrial permeability transition pore (MPTP) promotes the release of ROS (43). Also, the vicious cycle of oxidative stress is facilitated by an increase in toxic substances such as malondialdehyde (MDA), and 4-hydroxy-2-nonenal (4-HNE), which are lipid peroxidation products (44, 45). Likewise, advanced glycosylation end products (AGEs) further promote ROS production, induce overexpression of endothelial angiopoietin (Ang)-2, promote cellular sensitivity to pro-inflammatory factors such as vascular cell-adhesion molecule (VCAM)-1, and contribute to the progression of diabetes-related vascular disease (46). AGEs are non-enzymatic glycosylated forms of free amino acids that result from the interaction of glucose with lipids or proteins, bind to receptors to promote inflammation and oxidative stress, and are important in contributing to glomerulosclerosis and mesangial hypertrophy in DKD (47–49). On the other hand, ROS acts as an agonist of NF-κB signaling pathway to initiate the activation of downstream inflammatory signaling pathways and promotes the release of inflammatory factors such as IL-1β, TNF-α, intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) (50–54).

We know that mitochondria are the main site of ROS production and a target organelle for oxidative stress (55). The ETC is the central component of the mitochondria for functional operation, and the sequential transfer of electrons through the ETC to complex IV creates an electrochemical proton gradient that drives the F_1F_0 ATP synthase to produce ATP for OXPHOS (56). In addition, mitochondrial ROS production is increased and morphology is altered in high blood glucose environment of DM. Further, prolonged high glucose stimulation results in more electron donors such as NADH and $FADH_2$ being produced in the tricarboxylic acid (TCA) cycle, and too many electron donors entering the ETC, leading to a maximum mitochondrial voltage gradient (57). Uncoupling proteins (UCPs) reduce ROS production by dissipating the proton motive force and increasing the rate of electron transfer in the ETC (58). At the same time, molecular oxygen reacts prematurely to produce superoxide $O_2^{\cdot-}$, which exceeds the antioxidant scavenging capacity and leads to oxidative stress, causing impairment of cellular respiratory function, promoting apoptosis and ultimately leading to dysfunction of diseased tissues and systems (59, 60) (Figure 2). It is reported that NADH oxidoreductase (complex I) and cytochrome bc_1 oxidoreductase (complex III) are the main sites for the production of ETC superoxide (61). In addition to this, much of the literature has further linked mitochondrial OXPHOS function and mtDNA damage closely to oxidative stress (55, 62). High glucose-induced oxidative

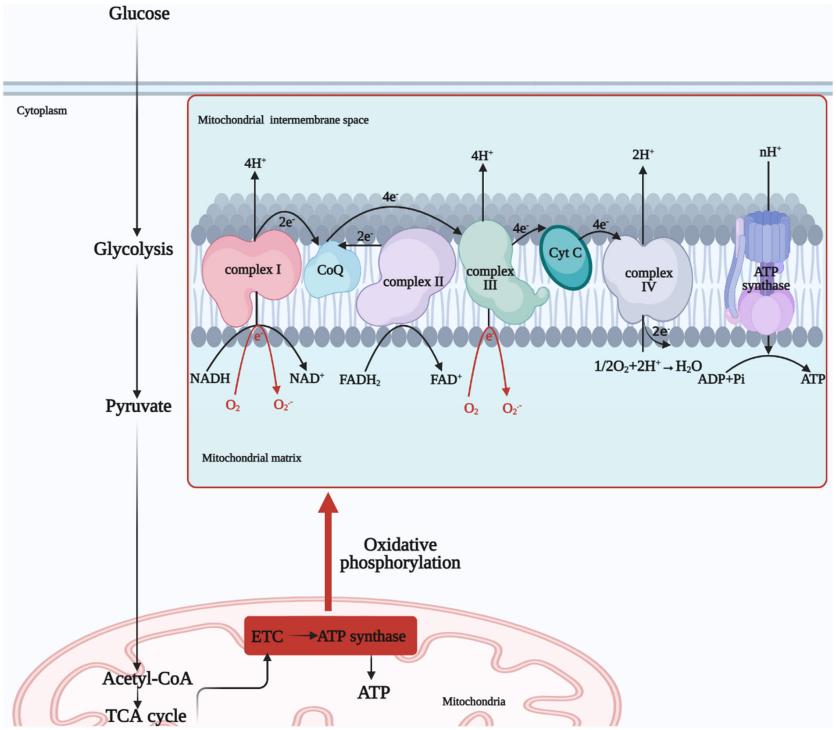


FIGURE 2
Mitochondrial OXPHOS process. Glucose undergoes glycolysis to produce pyruvate, which is oxidized to acetyl-coenzyme A or carboxylated to produce oxaloacetate, which enters the mitochondrial matrix for the TCA cycle. And finally NADH and $FADH_2$, electron donors, undergo electron transfer by the ETC to produce H_2O from O_2 and ATP by the action of ATP synthase, completing OXPHOS process. NADH, nicotinamide adenine dinucleotide; $FADH_2$, flavin adenine dinucleotide; ETC, electron transfer chain; TCA, tricarboxylic acid; ATP, adenosine triphosphate, ADP, adenosine diphosphate; OXPHOS, oxidative phosphorylation; Cyt C, cytochrome c; $O_2^{\cdot-}$, superoxide anion.

stress and mitochondrial dysfunction interact to promote the progression of DM and its complications (63). One article focused on the relationship between diabetic renal tubular injury and mitochondrial dysfunction, contributing to increased ROS production and metabolic abnormalities such as abnormal mitochondrial autophagy, and the article suggested that mitochondrial dysfunction may contribute to early diabetic tubulopathy (64). In addition to mitochondria, cytoplasmic NADPH oxidase (Nox) is the main source of cytoplasmic ROS (55). Further, cytoplasmic ROS could increase mitochondrial ROS production by continuously damaging mitochondria (6). Overall, mitochondria remain the main source of endogenous ROS (43). Therefore, we will next focus on the relationship between mitochondria and oxidative stress in the next sub-section.

It is important to note that the physiological level of ROS plays an important role in signaling, defense against infection and maintenance of redox homeostasis, only excessive ROS production leads to adverse effects of oxidative stress (65–67).

3 Physiological functions of mitochondria and oxidative damage

3.1 OXPHOS

In mitochondria, OXPHOS reactions use over 95% of O_2 to produce ATP, and a small amount of ROS are produced daily as OXPHOS by-products (68). However, when the production of ROS exceeds the scavenging capacity of the antioxidant defense system, it can lead to oxidative stress.

Glucose is one of the main sources of energy for the body and is glycolysed in the cytoplasm to produce pyruvate, which is oxidized to acetyl-coenzyme A or carboxylated to produce oxaloacetate, which enters the mitochondrial matrix for the TCA cycle (69). Subsequent production of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) provides the respiratory substrate for the ensuing OXPHOS process, which drives ATP production (70).

Mitochondria have a bilayer membrane structure, with the electron transport chain localized to the inner mitochondrial membrane (IMM), which is inlaid with four protein complexes, namely NADH oxidoreductase (complex I) (the largest subunits enzyme complex in the ETC), succinate dehydrogenase (complex II), cytochrome bc_1 oxidoreductase (complex III) and cytochrome c oxidase (complex IV) (71). There are also two free-moving electron transport carriers on the ETC, the lipid-soluble Q and the water-soluble cyt-c, and these six components together form the ETC supercomplex (72).

Two electrons from the TCA metabolite NADH in complex I are passed to Q and reduced to QH_2 (73). At the same time, the Fe-S cluster conformational change induces proton translocation and pumps four protons into the mitochondria intermembrane space (IMS) (74). Complex II is also an important carrier for the transfer of electrons, with $FADH_2$ transferring electrons to Q *via* the Fe-S cluster. Protons are required for the reduction of Q, so there is no net proton increase in the IMS (75). Complex III transfers electrons of QH_2 to cyt-c, cyt-c that gets electrons will be reduced, and the completion of the Q-cycle requires the pumping of four protons into the IMS (75). The reduced cytochrome carries electrons into complex IV, where they are eventually passed to the binuclear center of complex IV to complete the reduction of O_2, also known as one molecule of O_2 to produce two molecules of H_2O (76). Eight protons in the matrix are consumed in this process, four of which are pumped into the IMS (77). At this point, the high concentration of protons in the IMS constitutes the electrochemical gradient responsible for the energy storage of the mitochondria (78). F_1F_0 ATP synthase, also known as complex V, transfers protons from the IMS to the matrix and controls the threshold of the MMP, at which point ATP synthase undergoes structural changes that promote ADP phosphorylation to produce ATP (79, 80). In addition, UCPs lower the membrane potential by transferring protons from the IMS to the matrix and uncouple from the ATP synthesis process, forming a switch for ATP synthesis with ATP synthase (81) (Figure 2).

3.2 Structure and function of mtDNA

mtDNA is a double-stranded circular structure (consisting of light and heavy strands), localizes in the mitochondrial matrix, closing to IMM (82). The human mtDNA is 16,569 bp in length and consists of 37 genes, 22 tRNAs, 2 rRNAs and a non-coding region displacement-loop (D-loop) (83, 84). However, the non-coding region controls the transcription and translation of mitochondrial proteins, but the high sequence mutagenicity in this region makes the mtDNA mutation rate approximately 10-20 times higher than nuclear DNA, with relevance to diseases such as aging and cancer (85–87). Moreover, due to the tight arrangement of genes in the ring structure of mtDNA, some genes overlap, and lack of histone protection, it is vulnerable to ROS generated by oxidative stress process, resulting in persistent damage to mtDNA (88, 89).

Mitochondria have a different DNA genetic system from nuclear DNA. mtDNA is responsible for encoding some of the mitochondrial proteins (such as the protein complexes that make up the ETC) and involved in mitochondrial biogenesis and signaling, with semi-autonomous genetic characteristics (82, 90, 91). Therefore, mtDNA is important for OXPHOS. Mutations in mtDNA and epigenetic changes can lead to blocked electron transport in the ETC, reducing ATP synthesis and promoting apoptosis.

Further, transcription and packaging factor (TFAM), and transcription elongation factor (TEFM), essential cofactors for mtDNA replication and transcription, play an important role in the assembly and distribution of mtDNA-protein complexes and are thought to alleviate insulin resistance-induced oxidative stress (90, 92–94).

In addition, the organism equips mtDNA with DNA repair systems, and base excision repair (BER) is considered to be the main repair mechanism (95). BER maintains the normal structure of mtDNA by eliminating base mismatches caused by methylation, oxidation and alkylation, and by cleaving, gap-filling and connecting the structure of mtDNA (96). In addition, mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) are also important repair pathways of mtDNA (97). It has been documented that base mismatches caused by elevated ROS can be recognized and excised by the BER pathway to maintain the functional and structural integrity of mtDNA (98–100). Similarly, *in*

vitro and *in vivo* studies of the MMR pathway in high glucose environments have shown that high glucose environments induce damage to mtDNA and also impair the repair of the MMR pathway (101) (Figure 3).

4 The relationship between oxidative stress-induced mitochondrial damage and diabetic microvascular complications

Oxidative stress, an important trigger for the development of DM and its complications, is characterized by excessive ROS production and intracellular oxidative damage. Oxidative stress can lead to alterations in mitochondrial morphology and function, inducing structural changes and functional abnormalities in macromolecules such as proteins, lipids and nucleic acids, ultimately leading to apoptosis and accelerating the progression of diabetic microvascular complications such as diabetic retinopathy (22, 102–105). In the following we will focus on mitochondria as an entry point to explore the impact of oxidative stress-induced mitochondrial dysfunction on diabetic microvascular complications.

4.1 Impaired OXPHOS and recovery in diabetic microvascular complications

OXPHOS occurs in mitochondria and is the main process involved in supplying energy for cellular respiration and ATP synthesis (106). ETC is the central element of the OXPHOS process, with the sequence of complexes (I-IV) working to achieve electron and proton transfer, creating MMP to store energy for the next work of ATP synthase (75). However, prolonged hyperglycaemic stimulation leads to abnormal electron transfer, resulting in increased production of ROS such as superoxide, inducing the onset of oxidative stress (107). Therefore, excessive ROS is one of the main factors leading to impaired OXPHOS function.

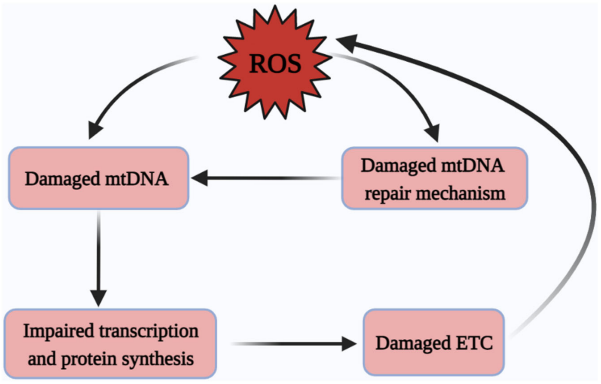


FIGURE 3
Excessive ROS damages mtDNA. ROS drives damage to mtDNA and the mtDNA repair systems, yet the damage of mtDNA repair systems is also an important contributor to mtDNA damage, followed by damage to mitochondrial protein-related transcription and synthesis pathways, ultimately leading to a vicious cycle of ETC abnormalities and subsequent the excessive generation of ROS. ROS, reactive oxygen species; mtDNA, mitochondrial DNA; ETC, electron transfer chain.

4.1.1 Diabetes kidney disease (DKD)

In high glucose induced podocytes, the superoxide levels were found to be increased while MMP expression and mitochondrial number were found to be decreased. However, overexpression of SIRT6 was shown to reverse this phenomenon (108). Dioscin effectively reduced blood glucose and markers of renal impairment in diabetic rats, reversed mitochondrial respiratory chain disorders, increased the activity of SOD and CAT antioxidant enzymes and reduced the level of ROS (109). Jujuboside A modulated mitochondrial respiratory chain complex protein expression in T2DM rats, improved respiratory chain function, reduced ROS levels, increased SOD, CAT and GPX expression, and downregulated apoptotic protein expression (110). In a study evaluating Abroma augusta L. (Malvaceae) leaf extract on T2DM-related nephropathy and cardiomyopathy in experimental rats, it was observed that redox homeostasis was disrupted, intracellular NAD and ATP levels were reduced and mitochondria-dependent apoptotic pathways were activated in T2DM state (111). Studies have shown that soluble klotho protein (referred to as rKL, known as an inhibitor of aging) reduced albuminuria, restored mitochondrial function and reduced ROS production in db/db mice. Moreover, in high glucose-induced mouse proximal tubular cells, rKL treatment alleviated OXPHOS impairment and induced mitochondrial repair *via* the PGC-1α-AMPK pathway (112). To investigate the effects of a high-fat diet on oxidative stress in wild-type and RAGE (receptors for AGEs) deficient mice, it was shown that RAGE can regulate mitochondrial respiratory chain function and oxidative stress in flounder muscle (113). Metformin, a classical hypoglycemic agent, promoted normalization of energy status and biochemical alterations, elevated ATP and lowered AMP, inhibited TNF-α and IL-6 pro-inflammatory gene expression, and exerted protective function of kidney in DKD rats (114). C3a induced mitochondrial fragmentation in podocytes, promoted mitochondrial depolarization, decreased SOD expression and increased ROS production, contributing to abnormal cellular energy metabolism, but this phenomenon could be inhibited by SS-31 (115). Knockdown of heat shock protein 60 (HSP60) in high-glucose-induced canine renal tubular cells showed that HSP60 regulated protein aggregation and ATP production in renal tubular cells (116). In high glucose and angiotensin II (ANG II)-induced HK-2 cells, increased p66Shc (promoter of apoptosis) and p-p66Shc were accompanied by increased ROS. The researchers made in-depth research and concluded that p66Shc mediated high glucose and ANG II-induced mitochondrial dysfunction *via* protein kinase C (PKC)-B and peptidyl-prolyl isomerase (Pin1) pathways, decreased MMP, promoted cyt-c leakage and increased the apoptotic protein caspase-9 (117). In another study, one of the mechanisms by which p66Shc promoted DKD was that p66Shc promoted disruption of mitochondrial dynamics, enhanced Mfn1-Bak interactions leading to loss of mitochondrial voltage potential, cyt-c release, excessive ROS production and apoptosis (118). In contrast, coagulation protease activated protein C and normalized MMP through epigenetic inhibition of p66Shc (119); also Obacunone exhibited nephroprotective effects that inhibited oxidative stress and mitochondrial dysfunction (120). Purple Rice Husk improved

mitochondrial function through the PGC-1α/SIRT3/SOD2 signaling pathway and reduced oxidative damage in renal tissue (121). Knockdown of the mitochondrial uncoupling protein UCP-2 increased uncoupling through adenine nucleotide transport proteins and reduced oxidative stress in the diabetic kidney in rat models (122). In STZ-induced diabetic mice, phillyrin reduced blood glucose and serum creatinine levels, increased Bcl-2/Bax ratio, reduced cyt-c leakage into the cytoplasm and inhibited apoptosis through the PI3K/Akt/GSK-3β signaling pathway (123). Genistein protected podocytes integrity, increased MMP, improved mitochondrial function and inflammatory status in rats with diabetic nephropathy by inhibiting the MAPK/NF-κB pathway (124). Telmisartan increased the MMP of glomerular endothelial cells induced by high glucose, and reduced the levels of 8-hydroxy-2-deoxyguanosine (8-OHdG) and MDA to alleviate oxidative stress (125). The complex I inhibitor rotenone (ROT) reduced ROS production and increased MMP and PGC-1α-controlled mitochondrial biogenesis in STZ and different inflammatory factor-induced mouse pancreatic β-cell line Min6 cells, suggesting that inhibition of complex I might be an effective strategy to protect β-cells in T1DM (126). Another study had shown that ROT could also correct over-activated biological processes, increasing the ratio of reduced glutathione (GSH) and nicotinamide adenine dinucleotide phosphate (NADPH) to its oxidized form, leading to redox balance (127). Resveratrol alleviated proteinuria, reduced ROS and MDA levels, restored SIRT1 and PGC-1α expression in kidney tissue. Resveratrol inhibited mitochondrial oxidative stress *via* SIRT1/PGC-1α, improved podocytes respiratory chain complex I and III activity, increased MMP and inhibited cyt-c release from mitochondria to the cytoplasm (128). In palmitic acid (PA) and oleic acid induced podocytes, PA was found to induce mitochondrial superoxide and H_2O_2 production (129). To investigate the role of SIRT3 deficiency on mitochondrial damage, researchers fed SIRT3-deficient mice a high-fat diet, resulting in mitochondrial dysfunction (involving abnormalities in OXPHOS, MMP and energy metabolism) and ultrastructural changes (130). In addition, a number of studies had also shown a close pathological link between impaired OXPHOS process and DKD (131–134).

The selective SIRT1 agonist BF175 was shown to prevent high glucose-induced mitochondrial damage and reduce superoxide production (135). Salvianolate effectively inhibited the generation of superoxide derived from NOX4 (mainly located in IMM) and reduced podocyte apoptosis (136). In the STZ-induced DKD rat model, the researchers observed a significant increase in blood creatinine and urine protein, as well as in ROS and MDA levels in the model group compared to the control group (137). In H_2O_2-induced HBZY-1 cells, Nepeta angustifolia inhibited H_2O_2 by increasing MMP, reducing ROS and MDA levels while inhibiting apoptosis (138). It had been shown that in addition to elevated biochemical parameters associated with kidney damage, STZ-induced diabetic rats also increased ROS production, reduced antioxidant defenses *in vivo*, and ultimately initiated mitochondria-dependent apoptosis (139). Increased ROS formation, elevated lipid peroxidation products and oxidative DNA damage, and mitochondrial apoptosis were observed in kidney tissue in STZ-induced diabetic mice, but dietary eicosapentaenoic acid inhibited this phenomenon by modulating hypoxia-inducible factor (HIF)-1α (140). Another study showed that Erythropoietin alleviated mitochondrial dysfunction, inhibited mitochondrial fragmentation and ROS production, and promoted autophagic flux *in vitro* (141). CAT deficiency increased ROS production and fibronectin expression in DKD mice and murine mesangial cells, demonstrating that endogenous catalase played an important role in the maintenance of mitochondrial function and protected the kidney from oxidative stress (142). Ferulic acid inhibited ROS production and apoptosis and induced autophagy in STZ-induced diabetic rats (143). In addition, other researchers found that Nox4 knockdown reduced NADPH oxidase activity, accompanied by reduction in high-glucose-induced superoxide, yet mitochondrial Nox4 expression was increased in the renal cortex of diabetic rats, demonstrating the role for Nox4 in the regulation of mitochondrial function (144). Adropin improved lipid metabolism and renal function in diabetic mice, regulated blood glucose and lipids, inhibited ROS production, improved lipid deposition and down-regulated lipoprotein expression (145). G Protein-Coupled Bile Acid Receptor TGR5 improved indicators of renal injury in db/db mice, upregulated regulators of mitochondrial biogenesis, reduced lipid accumulation and H_2O_2 production and increased SOD2 activity; similarly, similar results were observed in high glucose-induced podocytes (146).

4.1.2 Diabetic retinopathy (DR)

In a high glucose-induced retinal ganglion cells (RGC) model, Hesperidin (Citrus Flavonone) restored mitochondrial function, prevented loss of MMP and cyt-c release into the cytoplasm, prevented ROS production, increased intracellular levels of antioxidant enzymes and inhibited apoptosis (147). A study on metabolic memory of mitochondrial oxidative damage found that in primary rat retinal endothelial cells (rRECs) cultured in high glucose, MMP and cyt-c levels decreased and ROS levels increased in the model group compared to the control group as the duration of high glucose culture increased, suggesting that metabolic memory of mitochondrial oxidative damage can lead to DR (148). Another study on Berberine (BBR) showed that BBR alleviated oxidative stress (inhibited cyt-c leakage and ROS production and increased antioxidant enzyme levels) in diabetic rats and high glucose-induced Müller cells by inhibiting the NF-κB signaling pathway, thereby preventing DR (149). Leakage of cyt-c and increased accumulation of Bax in mitochondria in STZ-induced diabetic rats and high glucose cultured retinal endothelial cells and pericytes, which were inhibited by SOD and its mimics (150). In another study, in addition to demonstrating the protective effect of manganese superoxide dismutase (MnSOD) on the retina, it was also demonstrated that complex III might be a more significant source of superoxide compared to complex I (151). It had been suggested that MTP-131 (a novel mitochondrial targeting peptide) alleviated H_2O_2-induced oxidative stress in RGC-5 (blocking MMP depolarization and cyt-c release, reducing ROS production and preventing apoptosis) (152). NaHS (donor of H_2S) blocked retinal abnormalities in diabetic rats and alleviated DR by inhibiting mitochondrial dysfunction and NF-κB activation (153).

In high glucose-induced and platelet-derived growth factor-induced retinal pigment epithelial cells, researchers found that SIRT3 knockdown led to epithelial-mesenchymal transition and migration of epithelial cells, which was alleviated by overexpression of SIRT3. Further studies revealed that the cause was knockdown of

SIRT3 leading to overproduction of mitochondrial ROS, suggesting the role for SIRT3 in inhibiting mitochondrial oxidative stress (154). In a vitro study, 670 nm photobiomodulation reduced high glucose-induced ROS production and maintained mitochondrial integrity in rat Müller glial cells (155). In an STZ-induced mouse experiment, STZ induced an increase in cytoplasmic and mitochondrial ROS, accompanied by lipid peroxidation and apoptosis, and a decrease in GSH and GSH-Px as well as optic nerve activity and vitamin A levels, which could be reversed by selenium and resveratrol (156). Some scientists investigated the mechanism of oxidative stress induced by high glucose in RGC-5, and concluded that high glucose induced ROS production, disrupted mitochondrial mechanisms (MMP, mtDNA and mitochondrial mass damage) and antioxidant mechanisms, and triggered the production of downstream inflammatory factors and neurodegenerative markers (157). A study on green tea (Camellia sinensis) and antioxidant vitamins showed that green tea and vitamins reduced retinal superoxide production and that green tea improved inhibition of ETC and complex III activity, but promoted tissue collagen matrix glyco-oxidation (150).

4.1.3 Diabetic peripheral neuropathy (DPN)

Phosphocreatine (PCr, a high-energy phosphate compound) prevented oxidative stress and promoted normalization of mitochondrial function *in vivo* and vitro experiments: PCr acted on complex I and complex II of the mitochondrial respiratory chain to increase cellular respiration and reduce ROS, and might be a potential drug for the treatment of diabetes-related neurodegenerative diseases (158). Salvianolic Acid A (SalA) inhibited high glucose-induced mitochondrial damage in Schwann RSC96 cells by modulating nuclear factor erythroid 2-related factor 2 (Nrf2): SalA scavenged mitochondrial ROS, reduced MMP, increased ATP production and upregulated OXPHOS-related gene expression; and alleviated abnormal glucolipid metabolism in KK-Ay mice, exerting peripheral neuroprotective effects (159). In contrast, high glucose induction led to abnormal changes on mitochondrial superoxide, MMP and neurosynaptic growth in Neuro2a cells, STZ-induced abnormalities in motor/nerve conduction and neuroblood supply in diabetic rats, and polydatin improved mitochondrial dysfunction and biogenesis *via* SIRT1/Nrf2 (160). Long chain fatty acids induced mitochondrial dysfunction of Schwann cells, while overexpression of long chain acyl CoA synthetase 1 improved mitochondrial coupling efficiency, reduced proton leakage, and improved mitochondrial function (161). Human neuroblastoma SH-SY5Y cells exposed to high glucose levels reduced neuropil numbers, downregulated uncoupling protein (UCP) 3, increased MMP and ROS levels, while insulin-like growth factor type 1 normalized these changes (162).

An *in vitro* study of quercetin showed that quercetin reduced high glucose-induced ROS production in RSC96 cells and improved mitochondrial morphological abnormalities and DNA damage, as well as peripheral nerve hypofunction in lesioned mice (163). In high glucose-induced Schwann cells, puerarin inhibited ROS production and mitochondrial depolarization and prevented apoptosis (164). Additionally, it had also been shown that Fuzi protected Schwann cells induced by high glucose, prevented excessive ROS production and apoptosis, and had neuroprotective effects (165).

4.2 mtDNA damage and recovery in diabetic microvascular complications

4.2.1 DKD

Increased urinary 8-OHdG was detected in DKD-sensitive DBA/2J mice and human DKD specimens and showed a correlation between glomerular endothelin-1 receptor type A expression and increased mtDNA damage (166). The combination of dietary fenugreek (Trigonella foenum-graecum) seeds and onion (Allium cepa) was effective in reducing STZ-induced oxidative stress, lowering triglyceride and total cholesterol levels, reducing 8-OHdG and DNA fragmentation, and eliminating mtDNA deletions (167). In addition, salidroside alleviated renal fibrosis and kidney damage in DKD mice, and promoted the increase of mtDNA copy number and mitochondrial biogenesis (168).

4.2.2 DR

In high glucose-induced retinal endothelial cells, researchers found increased damage to mtDNA and DNA repair mechanisms and decreased expression of genes responsible for encoding the ETC protein complex, however, overexpression of MnTBAP or MnSOD suppressed this phenomenon (169). Similarly, another study also pointed out that high glucose-induced mtDNA damage led to excessive ROS production and further promoted mtDNA damage, leading to a vicious cycle of oxidative stress (170). Hydrogen sulfide is an endogenous gastransmitter signaling molecule with antioxidant properties, and its donor GYY4137 exhibited antioxidant effects in STZ-induced diabetic mice by resisting mtDNA damage, promoting Cytb transcription, limiting ROS production and inhibiting increased mitochondrial membrane permeability (171). An interesting study found that mtDNA and its repair/replication mechanism were significantly associated with the course of DM: early mtDNA repair/replication enzymes increased compensatorily, and as the disease progressed the repair/replication mechanism was disrupted and the mtDNA copy number decreased significantly (172).

4.2.3 DPN

A study comparing differences in mtDNA and transcript levels between diabetic and PGC-1$\alpha^{(-/-)}$ diabetic mice found that PGC-1$\alpha^{(-/-)}$ exacerbated neurological abnormalities in diseased mice, promoted mtDNA damage and protein oxidation, and led to more severe mitochondrial degeneration, demonstrating that modulation of PGC-1α may be a strategy for treating DPN (173). TFAM overexpression upregulated mtDNA and total TFAM levels, prevented the reduction of mtDNA copy number and inhibited motor and sensory nerve conduction abnormalities in diseased mice (174). A study on the neurological evaluation of 125 Italian T2DM patients noted that mtDNA was reduced in T2DM patients, this result was more significant in DPN patients and was associated with the MIR499A gene polymorphism (175).

4.3 Inactivation and recovery of antioxidant defense systems in diabetic microvascular complications

4.3.1 DKD

CD38 inhibitor apigenin upregulated NAD/NADH ratio and SIRT3-mediated mitochondrial antioxidant enzyme activity, while knockdown of CD38 inhibited SIRT3 activity, suggesting a correlation between CD38 and SIRT3 in oxidative stress mechanisms (176). *In vitro* experiments using high glucose-induced glomerular mesangial cells revealed that high glucose stimulated ROS production, decreased SOD and GSH levels, increased NADPH oxidase activity and promoted an increase in apoptotic factors which was also verified in diabetic rats (177). Antioxidant peptide SS31 inhibited the reduction of MnSOD and CAT activity, inhibited NADPH oxidase and NF-κB p65 activity in db/db mice and high glucose induced HK-2 cells (178). It had also been shown that exercise increased the expression of SOD and reduced oxidative damage (179). In addition, the activity of antioxidant enzymes in the body changed with the duration of diabetic hyperglycemia. The mRNA expression and activity of heme oxygenase-1 (HO-1) and MnSOD increased, and GSH-Px activity increased during short-term hyperglycemia; as the disease progressed the mRNA expression and activity of both decreased, accompanied by an increase in MDA and a decrease in GSH levels (180). The use of fluorofenidone in db/db mice showed that fluorofenidone alleviated oxidative stress-induced renal injury by blocking RAGE/AGEs/NOX and PKC/NOX signaling, down-regulating NADPH oxidase and up-regulating GSH-Px and SOD (181). In another study using STZ to create a model of DM in rats, MDA, CAT and GSH-Px were significantly different compared with the control group and tempol treatment restored GSH-Px levels (182). Intervention with carnosine in H_2O_2-induced HK-2 cells concluded that carnosine increased total SOD activity, decreased NOX4 expression and ROS levels, and alleviated oxidative stress (183). The use of honokiol in BTBR ob/ob mice with T2DM resulted in the conclusion that honokiol ameliorated renal damage and maintained mitochondrial function by activating SIRT3 and thereby restoring SOD2 and PGC-1α expression (184). In STZ-induced diabetic rats, Rap1 significantly ameliorated mitochondrial dysfunction and oxidative stress injury in renal tubular cells, modulated C/EBP-β binding to the endogenous PGC-1α promoter, and the interaction of PGC-1α with CAT or SOD (185).

4.3.2 DR

Exendin-4 (a glucagon-like protein) increased GSH and magnesium superoxide dismutase levels, decreased NADPH oxidase levels, inhibited ROS production and cyt-c release, and prevented apoptosis in high glucose-induced adult human retinal pigment epithelial-19 cells by inhibiting p66Shc expression and activation (186). In a study on the relationship between retinal neuronal apoptosis and MnSOD in diabetic rats, it was noted that apoptosis increased in diabetic rats at 8 and 12 weeks, and the number of RGC cells decreased at 12 weeks, while MnSOD activity and mRNA levels decreased at 4, 8 and 12 weeks, indicating a close relationship between MnSOD and RGC apoptosis (187). Similarly, two other studies had shown that MnSOD overexpression inhibited the increase in 8-OHdG and nitrotyrosine levels, prevented the decrease in GSH and total antioxidant capacity caused by DR (188, 189). An interesting study explored the response of knockdown of the Sigma 1 receptor (σ1RKO) on primary retinal Müller glial cells, showing that SOD1, CAT and GPX1 expression and protein levels were reduced in the σ1RKO group, as well as GSH and GSH/GSSG ratios, demonstrating that the neuroprotective effects of σ1R are related to the inhibition of oxidative stress (190).

4.3.3 DPN

Aldose reductase inhibitors corrected neurological and metabolic abnormalities, restored GSH and ascorbic acid levels, and inhibited lipid peroxidation in diabetic rats (191). Berberine (BBR) increased Nrf-2-mediated antioxidant defense system, ameliorated mitochondrial damage and neurotransmission abnormalities in diabetic rats, and upregulated PGC-1α-mediated mitochondrial biogenesis in high glucose-induced N2A cells, demonstrating the important role of BBR in DPN treatment (192).

5 Abnormalities in metabolic pathways of oxidative stress

High glucose-induced activation of the AGE, PKC, polyol and hexosamine pathways, as well as the formation of ROS in the mitochondria and cytoplasm, contribute to increased ROS production, and promote mitochondrial dysfunction and induce oxidative stress, mediating cellular dysfunction and accelerating the disease process (32, 193–197) (Figure 4). We have previously addressed the formation of ROS in the cytoplasm and mitochondria, so the following section focuses on the other four metabolic pathways.

5.1 AGEs/RAGE pathway

Non-enzymatic glycosylation of proteins and other macromolecules caused by prolonged high glucose levels, resulting in a series of dehydration and fracture reactions leading to the production of AGEs, resulting in abnormal protein structure and function, and consequently abnormal physiological function (198). AGEs promote oxidative stress by impairing the ETC to promote ROS formation (199). At the same time, the production of ROS can in turn stimulate the production of AGEs, thus creating a vicious circle (200). In addition, AGEs mediate the activation of downstream inflammatory and fibrotic signaling pathways by binding to cell surface receptors (RAGE) (201–203).

5.2 The polyol pathway

High glucose environment promoted activation of the polyol pathway (204). Glucose is converted to sorbitol by aldose reductase (AR) and subsequently oxidised to fructose by sorbitol dehydrogenase (SDH), during which NADPH is consumed as an electron donor (205). However, the reduction of the antioxidant GSH is dependent

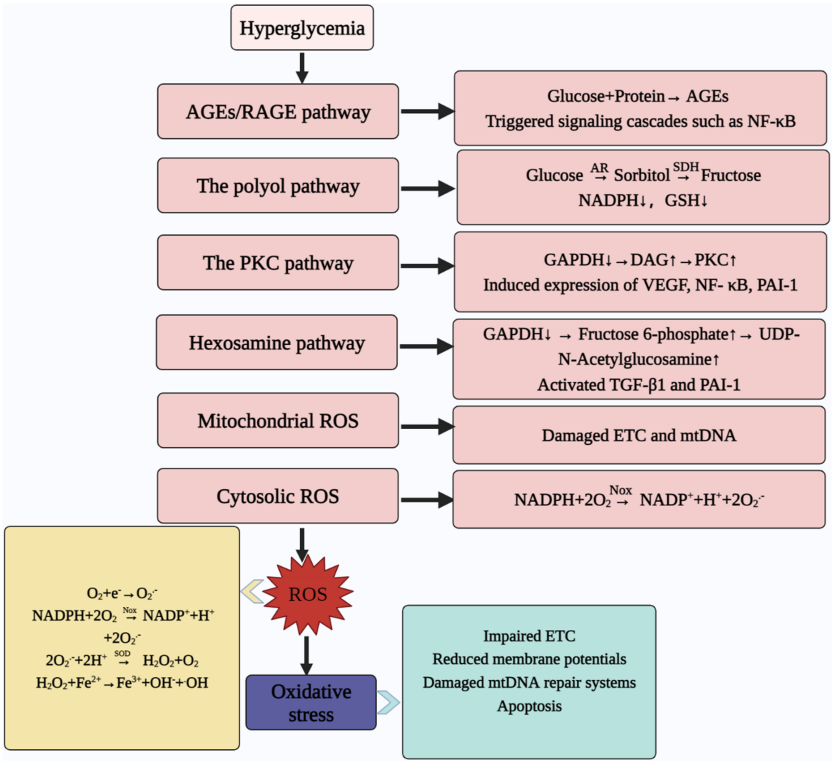


FIGURE 4
Abnormal metabolic pathways caused by hyperglycaemia. Hyperglycemia contributes to ROS production through the AGEs pathway, hexosamine pathway, PKC pathway, and polyol pathway; meanwhile, mitochondria and cytoplasm are also important sites for ROS production, which ultimately leads to oxidative stress. At the same time, oxidative stress can contribute to ETC abnormalities, reduce MMP, damage the mtDNA repair system and promote apoptosis. AGEs, advanced glycosylation end products; RAGE, the receptor for AGEs; NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells; AR, aldose reductase; SDH, sorbitol dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; GSH, reduced glutathione; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAG, diacylglycerol; PKC, protein kinase C; VEGF, vascular endothelial growth factor; PAI-1, plasminogen activator inhibitor-1; TGF-B1, transforming growth factor; ROS, reactive oxygen species; ETC, electron transfer chain; mtDNA, mitochondrial DNA; NOX, NADPH oxidases; $NADP^+$, nicotinamide adenine dinucleotide phosphate oxidized; O_2^-, superoxide anion; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; •OH: hydroxyl radical.

on NADPH, and the high glucose state accelerates the depletion of NADPH and reduces the antioxidant capacity of the body (205). At the same time, sorbitol can increase the osmotic pressure of cells, or act as a precursor substance for the formation of AGEs to promote the body's sensitivity to oxidative stress, leading to DPN or DR (23, 206, 207). In addition to this, some researchers verified the relationship between AR and NLRP3 inflammasome: AR inhibitors inhibited the activation of NLRP3 inflammasome, reduced the production of inflammatory factors and mitigated the production of ROS during oxidative stress. It proved that AR participated in the innate immune response induced by NLRP3 inflammasome (208, 209).

5.3 The PKC pathway

High glucose promotes increased glycolysis, leading to greater diacylglycerol (DAG) production, while inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) increases DAG activity and activates the PKC pathway (210). Activation of the PKC pathway is often accompanied by increased production of inflammatory factors and vascular endothelial growth factor (VEGF), and is closely associated with the development of diabetic complications (193, 211–215).

5.4 The hexosamine pathway

The hexosamine pathway is one of the pathways that promote the development of DM and its complications (195). Similarly, high glucose acts as a trigger switch for ROS production, resulting in the inhibition of GAPDH activity and the conversion of increased fructose-6-phosphate to the end product diphosphate uracil-N-acetylglucosamine (UDP-GlcNAc) (216). This is accompanied by an increase in ROS and fibrogenic factors downstream of the pathway, causing oxidative stress in mitochondria and is closely associated with thickening of the basement membrane of DKD (23, 217, 218).

6 Discussion

Oxidative stress is an imbalance in the redox state of the body, where excessive production of free radicals or damage to the antioxidant system, leads to a pathological outcome that is closely linked to the development of diseases such as cancer and metabolic disorders (219). ROS is a major component of free radicals, mainly produced in small amounts during OXPHOS in mitochondria, and

plays an important role in cell signaling, cell proliferation and antibacterial immunity (220, 221).

However, the prolonged and persistent hyperglycaemic state of DM leads to an increase in cellular respiratory substrates entering the mitochondria, with excess electron donors impairing ETC, contributing to ROS production, mediating the breakdown of the proton electrochemical gradient, impaired MMP, increased cyt-c leakage and inadequate ATP synthesis (56). Due to the lack of histone protection of mtDNA, the high mutability of the non-coding region and the restriction of its loop structure, ROS can further damage mtDNA, leading to a reduction in the copy number of mtDNA, damage the genes responsible for encoding some mitochondrial proteins and impair the function of mitochondria (87, 222, 223). At the same time, the increased ROS can damage the repair system of mtDNA, further deepening the damage to mtDNA and causing functional impairment of mitochondria (224, 225). In addition, the instability of ROS encourages cross-linking with macromolecular proteins, DNA and lipids, altering the structure and function of macromolecules and having toxic effects, further affecting cell function (226). However, ROS can also mediate the activation of downstream signaling pathways such as inflammation and fibrosis, leading to the progression of diabetic microvascular complications such as DKD and DPN (227–229).

Studies have shown that some herbal active ingredients (puerarin, polydatin, quercetin, etc.), vitamin C, vitamin E, α-lipoic acid are important antioxidant strategies (160, 163, 164, 230–233). Targeting mitochondria to overexpress catalase in mice extends lifespan and alleviates oxidative stress in diseases such as metabolic syndrome (234). A clinical trial of the drug elamipretide (a mitochondrial tetrapeptide that interacts with cardiolipin) showed that elamipretide significantly improved clinical symptoms and skeletal muscle performance in Barth syndrome (235). In addition, animal models have demonstrated that enzymatic antioxidants mimics (SOD mimics, GPX mimics and CAT mimics) can scavenge superoxide and inhibit oxidative stress (236–238). Recent studies have shown that bioadhesive hydrogel can promote oral wound healing in DM rats; novel nanoparticle can accelerate wound healing in DM and is an emerging and effective treatment strategy (239, 240). Some combinations of antioxidants have also been shown to have antioxidant effects (241–243). The relevant literatures state that the combinations of ferulic acid and metformin have been shown to improve DM (244). And the combinations of superoxide dismutase, α-lipoic acid, acetyl-L-carnitine, and vitamin B_{12} have been shown to improve sural nerve conduction velocity, amplitude and pain in patients with DPN (233). Therefore, antioxidants play a positive therapeutic role in the treatment of DM.

However, many antioxidants suffer from poor solubility, unstable storage and gastrointestinal degradation, thus limiting the use of oxidants in clinical practice (245). In recent years antioxidant drugs have mainly focused on animal studies and have not been adequately tested in clinical trials, therefore, poorly supported by clinical data. The few drugs that have been clinically studied have not yielded satisfactory results either, and achieving effective drug concentrations in the body is an important issue for modern science. In addition to this, mtDNA, a key structure involved in oxidative stress, is under-researched for drugs targeting mtDNA, leading to a lack of development of antioxidant drugs. We hope to be able to characterize mitochondrial dysfunction in the high glucose state and look forward to providing a bit of new ideas for future experimental studies.

7 Conclusion

Hyperglycemia causes redox imbalance and massive production of ROS leading to impairment of OXPHOS, mtDNA function, mitochondrial dysfunction and oxidation of macromolecules and in turn accelerates apoptosis and disease progression. Therefore, oxidative stress is an important mechanism that promotes the development of DM and its complications. Targeted development of antioxidants and the combination of multiple acting antioxidant components may be a strategy for the treatment of DM.

Author contributions

ZZ is responsible for writing and the conception of the article, QH is responsible for drawing and document sorting, and DZ is responsible for sorting out the ideas of the article. FL, XL, and WQ are responsible for controlling the overall quality of the article and revising the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Meta-analysis of the characteristic expression of circulating microRNA in type 2 diabetes mellitus with acute ischemic cerebrovascular disease

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Objective: To comprehensively evaluate the characteristics of the circulating microRNA expression profile in type 2 diabetic patients with acute ischemic cerebrovascular disease by systematic evaluation and meta-analysis.

Methods: The literatures up to March 2022 related to circulating microRNA and acute ischemic cerebrovascular disease in type 2 diabetes mellitus were searched and screened from multiple databases. The NOS quality assessment scale was used to evaluate methodological quality. Heterogeneity tests and statistical analyses of all data were performed by Stata 16.0. The differences in microRNA levels between groups were illustrated by the standardized mean difference (SMD) and 95% confidence interval (95% CI).

Results: A total of 49 studies on 12 circulating miRNAs were included in this study, including 486 cases of type 2 diabetes complicated with acute ischemic cerebrovascular disease and 855 controls. Compared with the control group (T2DM group), miR-200a, miR-144, and miR-503 were upregulated and positively correlated with acute ischemic cerebrovascular disease in type 2 diabetes mellitus patients. Their comprehensive SMD and 95% CI were 2.71 (1.64~3.77), 5.77 (4.28~7.26) and 0.73 (0.27~1.19), respectively. MiR-126 was downregulated and negatively correlated with acute ischemic cerebrovascular disease in type 2 diabetes mellitus patients, its comprehensive SMD and 95% CI were -3.64 (-5.56~-1.72).

Conclusion: In type 2 diabetes mellitus patients with acute ischemic cerebrovascular disease, the expression of serum miR-200a, miR-503, plasma and platelet miR-144 was upregulated and the expression of serum miR-126 was downregulated. It may have diagnostic value in the early identification of type 2 diabetes mellitus with acute ischemic cerebrovascular disease.

KEYWORDS

microRNA, type 2 diabetes mellitus, acute ischemic cerebrovascular disease, circulating, meta-analysis

1 Introduction

Type 2 diabetes mellitus (T2DM), a metabolic disease related to the increased risk of central nervous system diseases, has attracted increasing attention because it can lead to electrophysiological, structural, neurochemical and degenerative changes in the nervous system and seriously damage neural function (1, 2). Studies have shown that T2DM increases the risk of ischemic stroke by 3-4 times (3). The prognosis of T2DM patients with ischemic stroke is poor, and the mortality is high. Therefore, it is important to improve the prognosis of patients by increasing the early diagnosis rate of T2DM with acute ischemic cerebrovascular disease (AICVD) and taking effective intervention measures in time. At present, the methods used to diagnose T2DM with AICVD are limited, mainly including magnetic resonance imaging (MRI) and CT angiography (CTA). However, the implementation of these two examinations has certain limitations and contraindications for individual patients. Therefore, we hope to find an examination method that is easy to operate, has no side effects, is widely applicable and has objective diagnostic indicators as an effective means to identify AICVD early in T2DM.

MicroRNAs (miRNAs, miRs), small noncoding RNAs, are important regulators of many biological processes, such as cell growth, apoptosis, cell proliferation, embryonic development and tissue differentiation (4). They play key roles in mediating the physiological and pathogenic functions of diabetes and diabetic complications (5, 6). Many studies have shown that miRNAs can be used as a sensitive biomarker of secondary brain injury (7). In recent years, an increasing number of studies have shown that the specific expression of some miRNAs in blood plays an important role in the pathogenesis of AICVD in T2DM. Unfortunately, at present, there are few clinical studies on circulating miRNA and AICVD in T2DM, and the conclusions are still inconsistent. The characteristics of its expression profile in T2DM with AICVD have not been accurately evaluated.

The purpose of this study is to further clarify the characteristics of the circulating microRNA expression profile in T2DM with AICVD by reviewing the research results of literature, and in addition to provide evidence for circulating microRNA with potential diagnostic value in T2DM with AICVD.

2 Methods

2.1 Literature retrieval strategy

The PubMed, Embase, Cochrane Library, CNKI, Wanfang and Vip databases were searched to obtain relevant literature published up to March 2022. We used the keywords and subjects both in Chinese and English as follows: “diabetes mellitus or diabetes or T2DM”; “miRNA or microRNA”; “cerebrovascular disease or stroke or acute or cerebral infarction or cerebral ischemia”.

2.2 Selection criteria

The inclusion criteria were as follows: (1) the study must have the miRNA expression profile of a human model of T2DM with AICVD; (2) the patients with T2DM complicated with AICVD were the experimental group, and the healthy group, the patients with T2DM alone and the patients with AICVD alone were the control group. The general information of these groups, such as sex and age, was balanced and comparable; (3) the relative miRNA expression must be reported by miRNA microarray or qRT PCR; the miRNA detection methods and reagent sources in the literature were clear; (4) the sample size and source of the experimental group and the control group were clear; (5) the mean and standard deviation of miRNA expression in the experimental group and the control group, or the relevant data that could be used to calculate the above indicators, could be obtained; and (6) all patients with T2DM met the WHO diagnostic criteria, and all patients with AICVD were diagnosed by brain MRI. The exclusion criteria were as follows: (1) not meeting the inclusion criteria; (2) simple descriptive literature without a control group; (3) animal research and cell research; (4) overview, case report, abstract collection, meeting and letter; (5) repetitive literature; and (6) literature for which relevant data could not be obtained.

2.3 Data extraction and management

According to the inclusion and exclusion criteria, we strictly screened the literature and extracted the following data: the first author, the year of publication, the country of study, the source of samples, the list of up- and downregulated miRNAs, miRNA detection methods, the sample size, mean and standard deviation of the experimental group and the control group. If the mean and standard deviation cannot be obtained directly, we will obtain them through calculation. If the original data cannot be obtained directly, we will contact the author to obtain them. If different types of AICVD or different sample sources were mentioned in the same document, data extraction and corresponding analysis were carried out respectly.

2.4 Quality assessment

All included studies were case–control studies, so the Newcastle –Ottawa Scale (NOS) quality evaluation scale was used to assess the quality of the included studies. The scale includes three aspects: the selection method of the case group and control group, the comparability of the case group and control group, and the exposure evaluation method. The scoring range is 0~9 points, in which 5~9 points are high quality and 0~4 points are low quality.

2.5 Statistical analysis

Stata 16.0 was used to statistically analyze the expression of each miRNA. The heterogeneity of this study was tested by the Q test and I^2 test: if I^2<50%, a fixed effect model was used, and if I^2>50%, a random effect model was used. If the heterogeneity was still large, subgroup analysis and regression analysis were conducted to analyze the source of heterogeneity. The stability and reliability of the selected studies were discussed through sensitivity analysis. Finally, publication bias was evaluated by funnel chart and Egger’s test.

3 Results

3.1 Included studies and their characteristics

Figure 1 shows the selection process of the studies. There were 300 studies relevant to the search strategy, of which 39 were duplicates. A further 235 of these articles were excluded by browsing the titles and abstracts. After evaluating the full texts of the remaining studies, another 15 articles were excluded. Finally, 11 articles containing 486 cases and 855 controls were included in this meta-analysis (8–18). Details of the included studies are shown in Table S1.

3.2 Quality evaluation results

The NOS quality evaluation scale was used to assess the quality of 11 studies, as shown in Table 1. The total NOS score of all studies was between 6-9, and all the included studies were considered to be of high quality.

3.3 Results of the meta-analysis of the expression of each circulating miRNA in T2DM patients with AICVD

3.3.1 Analysis results of circulating miR-223

Meta-analysis results of circulating miR-223: I^2 was 99.14%. Because I^2 was greater than 50%, the comprehensive SMD and 95% CI obtained by using the random effect model was -1.38 (-3.55~0.80), P>0.05, which was not statistically significant, as shown in Figure 2A.

3.3.2 Analysis results of circulating miR-210

Meta-analysis results of the circulating miR-210: I^2 was 88.59%, and the comprehensive SMD and 95% CI obtained by using random effect model was -0.07 (-0.67~0.53), P>0.05, which was not statistically significant, as shown in Figure 2B.

3.3.3 Analysis results of circulating miR-200a

Meta-analysis results of circulating miR-200a: I^2 was 71.26%. The comprehensive SMD and 95% CI obtained by using the random effect model was 2.71 (1.64~3.77), P<0.05, which was statistically significant. The expression of miR-200a in the experimental group was upregulated compared with that in the control group, as shown in Figure 2C.

3.3.4 Analysis results of circulating miR-126

Meta-analysis results of circulating miR-126: I^2 was 97.18%. The random effect model was used to obtain the comprehensive SMD, and the 95% CI was -3.64 (-5.56~-1.72), P<0.05, which was statistically significant. The expression of miR-126 in the experimental group was downregulated compared with that in the control group, as shown in Figure 2D.

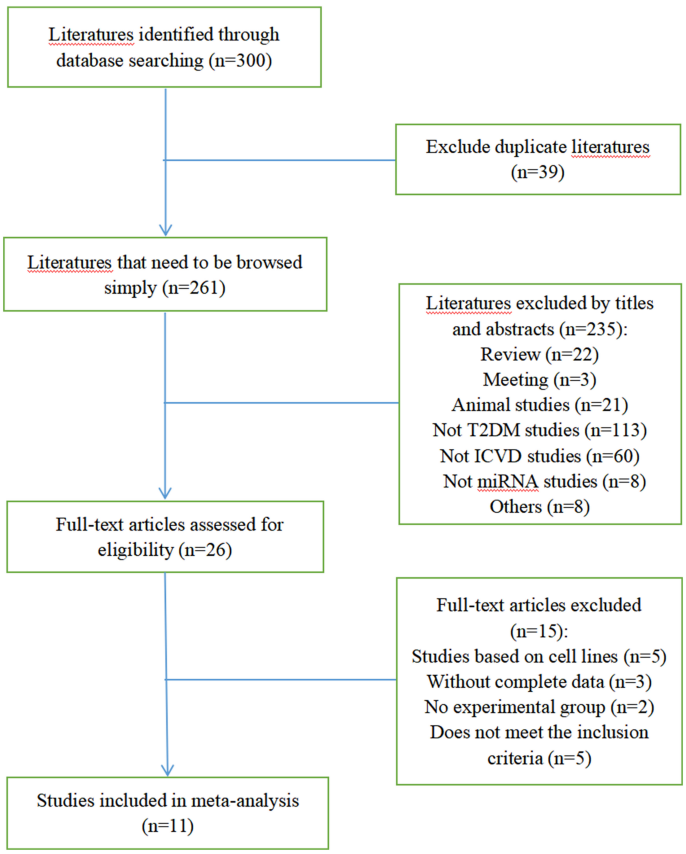


FIGURE 1
Literature screening flow chart.

TABLE 1 Quality evaluation of 11 included documents.

Study	Selection	Comparability	Exposure	Scores	Source of case samples	Number of cases
Fan Cai 2018	★★★	★★	★★★	8	Shanghai Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine	15
Saba Sheikhbahaei 2019	★★★★	★★	★★★	9	Alzahra hospital in Isfahan, Iran	15
Xiaqing Guo 2016	★★★	★	★★	6	Department of Neurology, Huaihe Hospital, Henan University	74
Zhen Liu 2020	★★★	★★	★★	7	Guangyuan Traditional Chinese Medicine Hospital	132
Dila Na 2021	★★★	★★	★★★	8	The Seventh Affiliated Hospital of Xinjiang Medical University	92
Yuming Long 2015	★★★	★★	★★★	8	Xiangya Hospital, Central South University	12
Kaishun Meng 2019	★★★	★★	★★	7	Endocrine Department of Sanya People's Hospital	27
Xiaomei Duan 2014	★★★	★★	★★★	8	Xiangya Hospital	6
Yuefu Jiang 2021	★★★	★	★★★	7	Affiliated Hospital of Southwest Medical University	15
Shuisheng Yang 2015	★★★	★★	★★★	8	Department of Endocrinology, Xiangya Hospital	58
Mauro Giordano 2020	★★	★★	★★	6	the Hospital of Marcianise, ASL Caserta, Italy	21/19

NOS uses the semi-quantitative principle of star system to evaluate the quality of literature, and the number of stars represents the score.

3.3.5 Analysis results of circulating miR-144

Meta-analysis results of circulating miR-144: I^2 was 91.41%. The comprehensive SMD and 95% CI obtained by using the random effect model was 5.77 (4.28~7.26), P<0.05, which was statistically significant. The expression of miR-144 in the experimental group was upregulated compared with that in the control group, as shown in Figure 2E.

3.3.6 Analysis results of circulating miR-146a

Meta-analysis results of circulating miR-146a: I^2 was 83.23%. The random effect model was used to obtain its comprehensive SMD, and the 95% CI was -1.61 (-2.87~-0.35), P<0.05, which was statistically significant. The expression of miR-146a in the experimental group was downregulated compared with that in the control group, as shown in Figure 2F.

3.3.7 Analysis results of circulating miR-195-5p

Meta-analysis results of circulating miR-195-5p: I^2 was 92.51%. The comprehensive SMD and 95% CI obtained by using the random effect model was 15.67 (9.60-21.74), P<0.05, which was statistically significant. The expression of miR-195-5p in the experimental group was upregulated compared with that in the control group, as shown in Figure 2G.

3.3.8 Analysis results of circulating miR-451a

Meta-analysis results of circulating miR-451a: I^2 was 97.43%. The comprehensive SMD and 95% CI obtained by using the random effect model was 10.83 (4.40~17.26), P<0.05, which was statistically significant. The expression of miR-451a in the experimental group was upregulated compared with that in the control group, as shown in Figure 2H.

3.3.9 Analysis results of circulating miR-503

Meta-analysis results of circulating miR-503: I^2 was 0.00%. The comprehensive SMD and 95% CI obtained by using a fixed effect model was 0.73 (0.27~1.19), P<0.05, which was statistically significant. The expression of miR-503 in the experimental group was upregulated compared with that in the control group, as shown in Figure 2I.

3.3.10 Analysis results of circulating miR-708-5p

Meta-analysis results of circulating miR-708-5p: I^2 was 93.85%. The random effect model was used to obtain its comprehensive SMD, and the 95% CI was -2.61 (-3.72~-1.50), P<0.05, which was statistically significant. The expression of miR-708-5p in the experimental group was downregulated compared with that in the control group, as shown in Figure 2J.

3.3.11 Analysis results of circulating miR-146b-3p and miR-368

We did not perform a meta-analysis on circulating miR-146b-3p and miR-368 because only one publication on them was included in this study. According to the literature results, the SMD and 95% CI of circulating miR-146b-3p was -7.56 (-9.60~-5.53), indicating that the expression of miR-146b-3p in the experimental group was downregulated compared with that in the healthy control group.

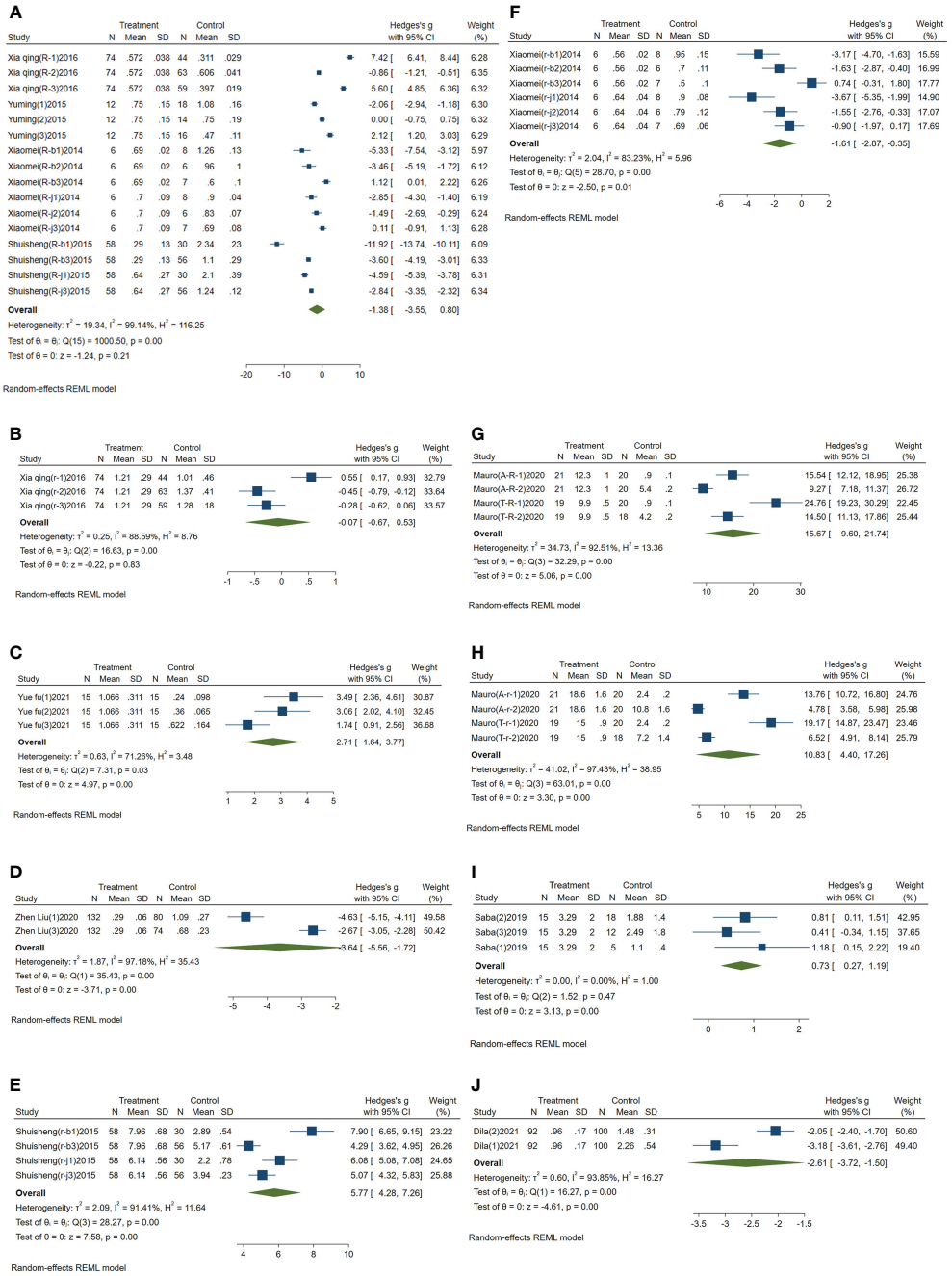


FIGURE 2
Forest plot of circulating miRNAs in type 2 diabetes mellitus with acute ischemic cerebrovascular disease. **(A)** Forest plot of circulating miR-223. **(B)** Forest plot of circulating miR-210. **(C)** Forest plot of circulating miR-200a. **(D)** Forest plot of circulating miR-126. **(E)** Forest plot of circulating miR-144. **(F)** Forest plot of circulating miR-146a. **(G)** Forest plot of circulating miR-195-5p. **(H)** Forest plot of circulating miR-451a. **(I)** Forest plot of circulating miR-503. **(J)** Forest plot of circulating miR-708-5p.

The SMD and 95% CI of circulating miR-368 was 4.07 (3.14~4.99), indicating that the expression of miR-368 in the experimental group was upregulated compared with that in the healthy control group.

3.4 Subgroup analysis

In this meta-analysis, the I^2 values of circulating miR-223, miR-210, miR-200a, miR-126, miR-144, miR-146a, miR-195-5p, miR-451a, and miR-708-5p were all greater than 50%, showing great heterogeneity. Therefore, they need to be analyzed in subgroups. However, due to the lack of publications on circulating miR-210, miR-200a, miR-126 and miR-708-5p, subgroup analysis was not performed.

3.4.1 Subgroup analysis of circulating miR-223

Subgroup analysis of circulating miR-223 was conducted according to the control group type and sample source. (1) According to the control group type (Figure 3A), the comprehensive SMD and 95% CI of circulating miR-223 expression

in the study of the classification of the experimental group and healthy control group was -3.19 (-8.22-1.83), and I^2 was 99.10%. The comprehensive SMD and 95% CI expression in the study of the classification of the experimental group and the AICVD group was -1.28 (-2.56-0.00), and I^2 was 89.28%. The comprehensive SMD and 95% CI expression in the study of the classification of the experimental group and the T2DM group was 0.41 (-2.31~3.13), and I^2 was 98.72%. The direction of circulating miR-223 expression in the experimental group was inconsistent with that in the healthy group and the simple T2DM group, and it was downregulated compared with that in the simple AICVD group. (2) According to the sample source (Figure 3B), the comprehensive SMD and 95% CI of circulating miR-223 expression derived from monocytes was 0.02 (-2.33-2.37), and I^2 was 95.69%. In the studies derived from plasma, the comprehensive SMD and 95% CI was 0.07 (-2.88~3.01), and I^2 was 99.25%. In the studies derived from platelets, the comprehensive SMD and 95% CI was -4.60 (-8.74~-0.46), and I^2 was 98.12%. This indicates that the direction of circulating miR-223 expression in the experimental group is inconsistent with that in the control group in the studies derived from monocytes and plasma, and it is downregulated in the studies derived from platelets compared with that in the control group.

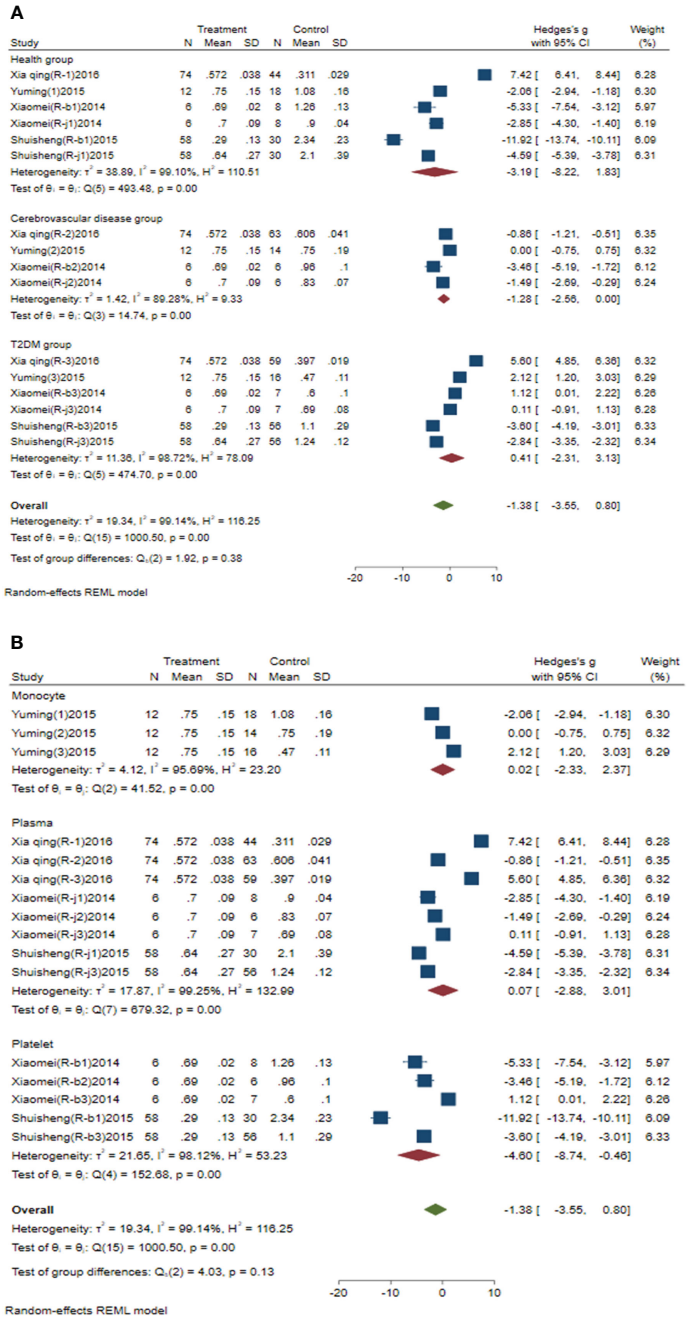


FIGURE 3
Forest plot of subgroup analysis of circulating miR-223 in type 2 diabetes mellitus with acute ischemic cerebrovascular disease. **(A)** Subgroup analysis of miR-223 by control group. **(B)** Subgroup analysis of miR-223 by sample source.

3.4.2 Subgroup analysis of circulating miR-144

Subgroup analysis of circulating miR-144 was conducted according to the control group type and sample source. (1) According to the control group type (Figure 4A), the comprehensive SMD and 95% CI of circulating miR-144 expression in the study of the classification of the experimental group and healthy control group was 6.95 (5.16~8.73), and I^2 was 80.00%. The comprehensive SMD and 95% CI expression in the study of the classification of the experimental group and the T2DM group was 4.66 (3.89~5.43), and I^2 was 57.54%. The expression of circulating miR-144 in the experimental group was upregulated compared with that in the healthy group and simple T2DM group. (2) According to the sample source (Figure 4B), the comprehensive SMD and 95% CI of circulating miR-144 expression derived from plasma was 5.52 (4.54~6.50), and I^2 was 59.66%. In the studies derived from platelets, the comprehensive SMD and 95% CI was 6.05 (2.51~9.59), and I^2 was 96.02%. This indicated that the expression of circulating miR-144 in the experimental group was upregulated compared with that in the control group in the studies derived from both plasma and platelets.

3.4.3 Subgroup analysis of circulating miR-146a

Subgroup analysis of circulating miR-146a was conducted according to the control group type and sample source. (1) According to the control group type (Figure 5A), the comprehensive SMD and 95% CI of circulating miR-146a expression in the study of the classification of experimental group and healthy group was -3.40 (-4.53~-2.26), and I^2 was 0.00%. The comprehensive SMD and 95% CI expression in the study of the classification of experimental group and the AICVD group was -1.59 (-2.46~-0.73), and I^2 was 0.00%. The comprehensive SMD and 95% CI expression in the study of the classification of experimental group and the T2DM group was -0.07 (-1.68~1.53), and I^2 was 78.14%. The direction of circulating miR-146a expression in the experimental group was downregulated compared with that in the healthy group and the simple AICVD group, and it was inconsistent compared with that in the simple T2DM group. (2) According to the sample source (Figure 5B), the comprehensive SMD and 95% CI of circulating miR-146a expression derived from plasma was -1.93 (-3.47~-0.38), and I^2 was 76.40%. In the studies derived from platelets, the comprehensive

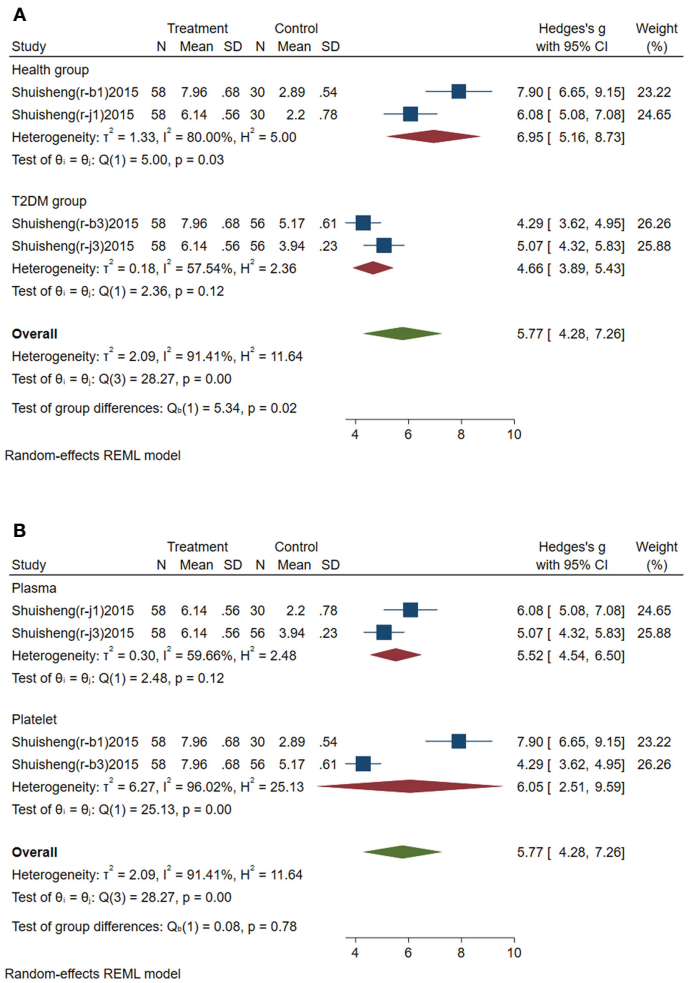


FIGURE 4
Forest plot of subgroup analysis of circulating miR-144 in type 2 diabetes mellitus with acute ischemic cerebrovascular disease. **(A)** Subgroup analysis of miR-144 by control group. **(B)** Subgroup analysis of miR-144 by sample source.

SMD and 95% CI was -1.30 (-3.53~0.93), and I^2 was 89.38%. This indicated that the direction of circulating miR-146a expression in the experimental group was downregulated compared with that in the control group in the studies derived from plasma, and it was inconsistent in the studies derived from platelets.

3.4.4 Subgroup analysis of circulating miR-195-5p

Subgroup analysis of circulating miR-195-5p was conducted according to the control group type and disease type. (1) According to the control group type (Figure 6A), the comprehensive SMD and 95% CI of circulating miR-195-5p expression in the study of the classification of the experimental group and healthy group was 19.88 (10.86~28.91), and I^2 was 87.08%. The comprehensive SMD and 95% CI expression in the study of the classification of the experimental group and the AICVD group was 11.71 (6.61~16.82), and I^2 was 85.02%. The expression of circulating miR-195-5p in the experimental group was upregulated compared with that in the healthy group and the simple AICVD group. (2) According to the disease type (Figure 6B), the comprehensive SMD and 95% CI of circulating miR-195-5p expression in the acute ischemic stroke (AIS) group was 12.25 (6.12~18.39), and I^2 was 89.34%. In the transient ischemic attack (TIA) group, the comprehensive SMD and 95% CI was 19.39 (9.34~29.43), and I^2 was 89.65%. This shows that the expression of circulating miR-195-5p in the experimental group was upregulated in the AIS and TIA groups compared with the control group.

3.4.5 Subgroup analysis of circulating miR-451a

Subgroup analysis of circulating miR-451a was conducted according to the control group type and disease type. (1) According to the control group type (Figure 7A), the comprehensive SMD and 95% CI of circulating miR-451a expression in the study of the classification of the experimental group and healthy group was 16.24 (10.96~21.52), and I^2 was 75.32%. The comprehensive SMD and 95% CI expression in the study of the classification of the experimental group and the AICVD group was 5.56 (3.86~7.26), and I^2 was 65.39%. The expression of circulating miR-451a in the experimental group was

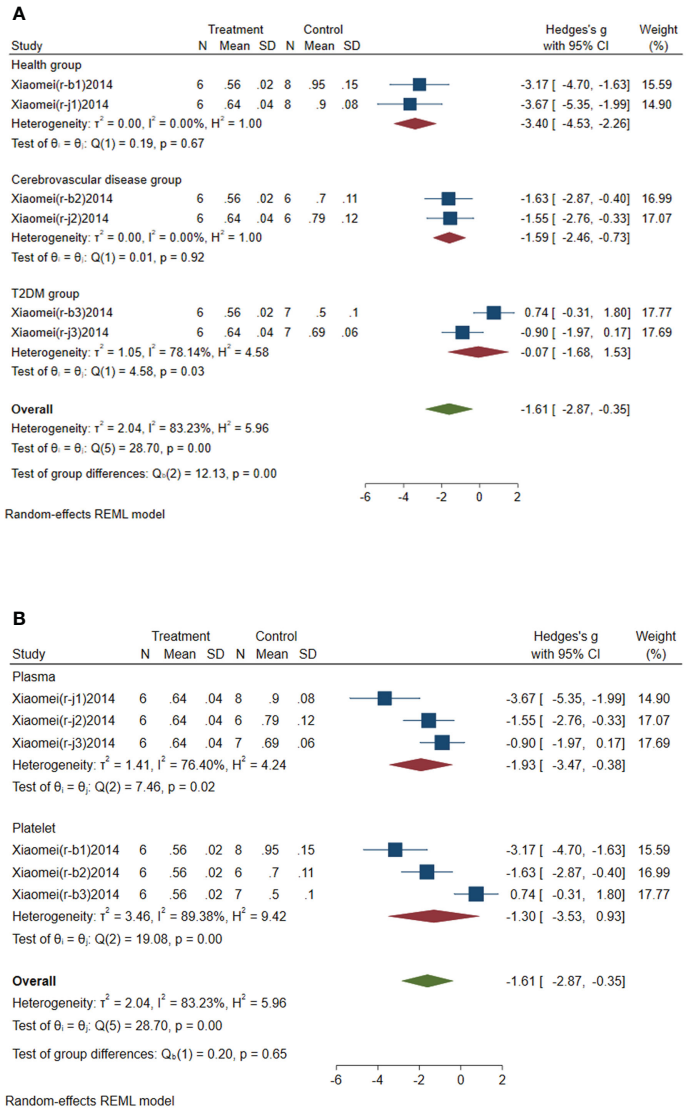


FIGURE 5
Forest plot of subgroup analysis of circulating miR-146a in type 2 diabetes mellitus with acute ischemic cerebrovascular disease. **(A)** Subgroup analysis of miR-146a by control group. **(B)** Subgroup analysis of miR-146a by sample source.

upregulated compared with that in the healthy group and the simple AICVD group. (2) According to the disease type (Figure 7B), the comprehensive SMD and 95% CI of circulating miR-451a expression in the AIS group was 9.16 (0.36~17.96), and I^2 was 95.56%. In the TIA group, the comprehensive SMD and 95% CI was 12.68 (0.29~25.07), and I^2 was 96.57%. This shows that the expression of circulating miR-451a in the experimental group was upregulated in the AIS and TIA groups compared with the control group.

3.5 Meta regression analysis

The expression levels of circulating miR-223, miR-144, miR-146a, miR-195-5p, miR-200a, miR-210, miR-503 and miR-451a were analyzed by meta-regression. Analysis showed that the miRNA regulation direction (P=0.004<0.05) was the main source of miR-223 heterogeneity. The control group type (P=0.003<0.05) was the main source of miR-146a heterogeneity. The main sources of miR-195-5p heterogeneity were the control group type (P=0.000<0.05) and disease type (P=0.000<0.05). The control group type (P=0.009<0.05) was the main source of miR-200a heterogeneity. The miRNA regulatory direction (P=0.000<0.05) was the main source of miR-210 heterogeneity. The control group type (P=0.000<0.05) was the main source of miR-451a heterogeneity. The source of heterogeneity of miR-144 and miR-503 was not found.

3.6 Sensitivity analysis

Sensitivity analyses of miR-223 (Figure 8A), miR-146a (Figure 8B), miR-144 (Figure 8C), miR-195-5p (Figure 8D), miR-200a (Figure 8E), miR-210 (Figure 8F), miR-503 (Figure 8G) and miR-451a (Figure 8H) were performed. In all sensitivity analyses, we found that the results of the remaining studies did not change significantly after excluding each study separately, indicating that the sensitivity of the studies we included was low and that the results were robust and credible.

3.7 Publication bias test

Stata16.0 was used to draw funnel plots. Due to the small number of other circulating miRNA studies, we only tested the publication bias of circulating miR-223, as shown in Figure 9, and obtained Egger's test result of P=0.0604>0.05. We believe that there was publication bias.

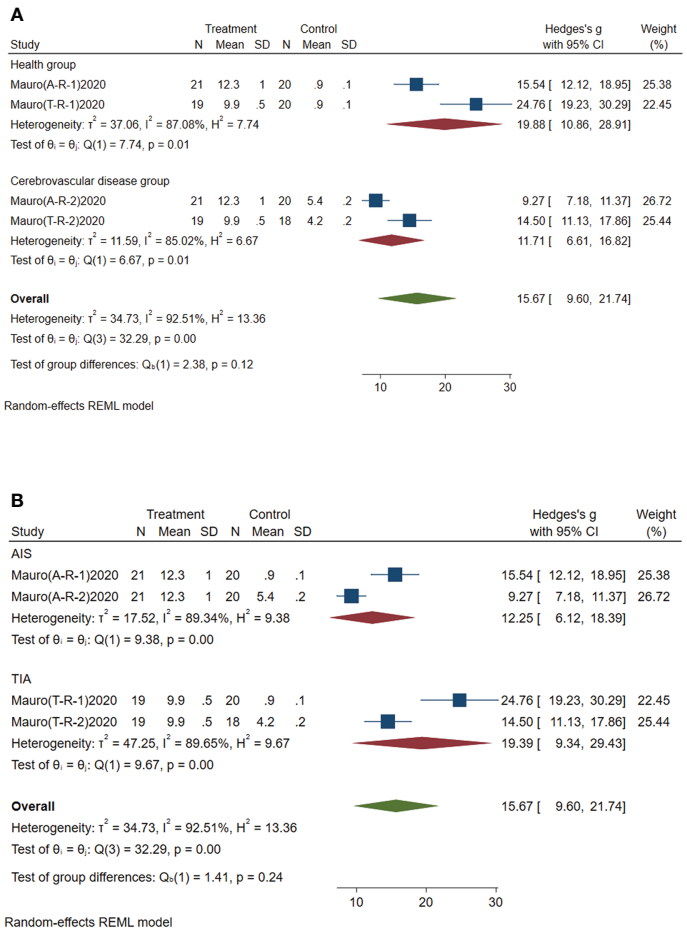


FIGURE 6
Forest plot of subgroup analysis of circulating miR-195-5p in type 2 diabetes mellitus with acute ischemic cerebrovascular disease. **(A)** Subgroup analysis of miR-195-5p by control group. **(B)** Subgroup analysis of miR-195-5p by disease type.

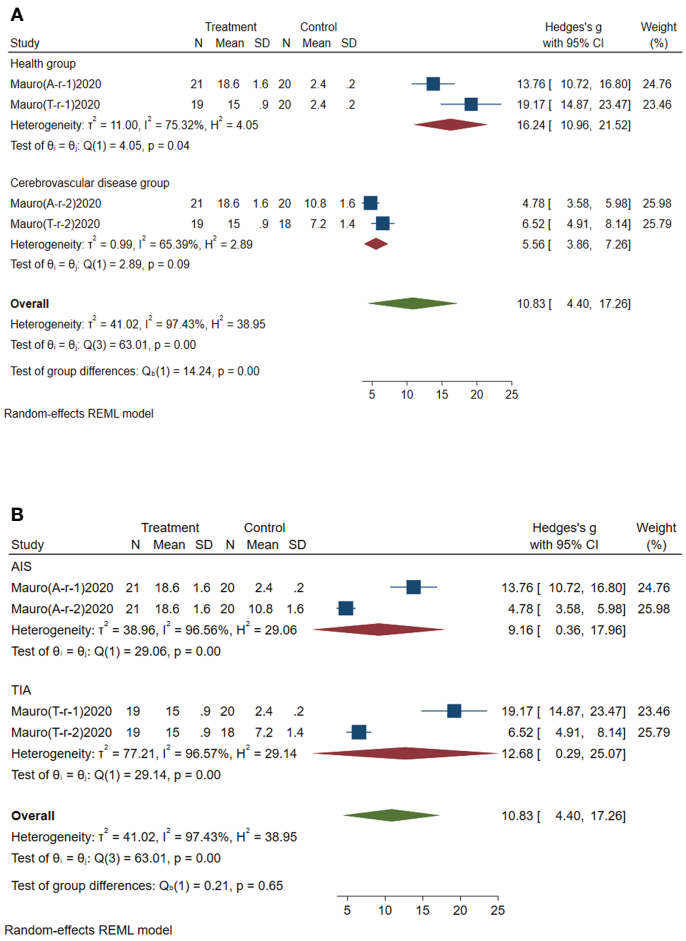


FIGURE 7
Forest plot of subgroup analysis of circulating miR-451a in type 2 diabetes mellitus with acute ischemic cerebrovascular disease. **(A)** Subgroup analysis of miR-451a by control group. **(B)** Subgroup analysis of miR-451a by disease type.

4 Discussion

AICVD is a common complication of T2DM and has a high mortality rate. In recent years, the incidence rate has been increasing yearly. MRI and CTA, as the current diagnostic tools for T2DM with AICVD, can only be carried out in hospitals due to their complex operation, which largely limits the early identification of T2DM with AICVD in asymptomatic people. In addition, MRI examination has certain restrictions for patients, such as metal products in the body, claustrophobia and patients who cannot cooperate with the examination cannot perform MRI examination. CTA, on the other hand, has X-ray radiation and is not suitable for pregnant women and young children. Because the contrast agent is excreted by the kidneys, it may cause kidney damage and may worsen kidney function in patients with renal insufficiency.With a large number of studies in recent years, miRNAs have been found to play an important role in diabetes and its related macroscopic and microvascular complications (19). And due to their stable expression properties, miRNAs can be detected in various biological fluids, such as blood, saliva, urine or aqueous humor (20–22). Detection of circulating miRNAs by blood collection is a clear source because collection is minimally invasive and blood samples can be taken regularly at the clinic, so miRNAs show promise as a biomarker for the diagnosis, prognosis, and clinical monitoring of T2DM with AICVD.

This study mainly performed meta-analysis of the expression characteristics of miR-223, miR-210, miR-200a, miR-126, miR-144, miR-146a, miR-146b-3p, miR-195-5p, miR-368, miR-451a, miR-503 and miR-708-5p in blood samples in T2DM with AICVD.

Through analysis, it was found that the expression of serum miR-200a in the experimental group was upregulated compared with that in the healthy group, the simple AICVD group and the simple T2DM group. Yuefu Jiang (16) demonstrated that miR-200a could exert antioxidant effects on the arterial endothelium in T2DM at the gene level. In addition, WanW et al. (23) showed that miR-200a plays an important role in the development of DR (diabetic retinopathy) by affecting the viability, apoptosis and cell migration of HRMECs and may be a potential therapeutic target by downregulating PDLIM1 in DR. Therefore, we believe that circulating miR-200a may play a protective role in T2DM and its complications and can be used as a valuable molecular marker for the early identification of AICVD in T2DM.

The expression of miR-144 from plasma and platelets in the experimental group was upregulated compared with that in the healthy group and simple T2DM group. Shuisheng Yang et al. (17) showed in their study that the expression of miR-144 in the plasma and platelets of patients with type 2 diabetes mellitus was increased, which downregulated IRS-1 through the IRS-1-PI3K-Akt signaling

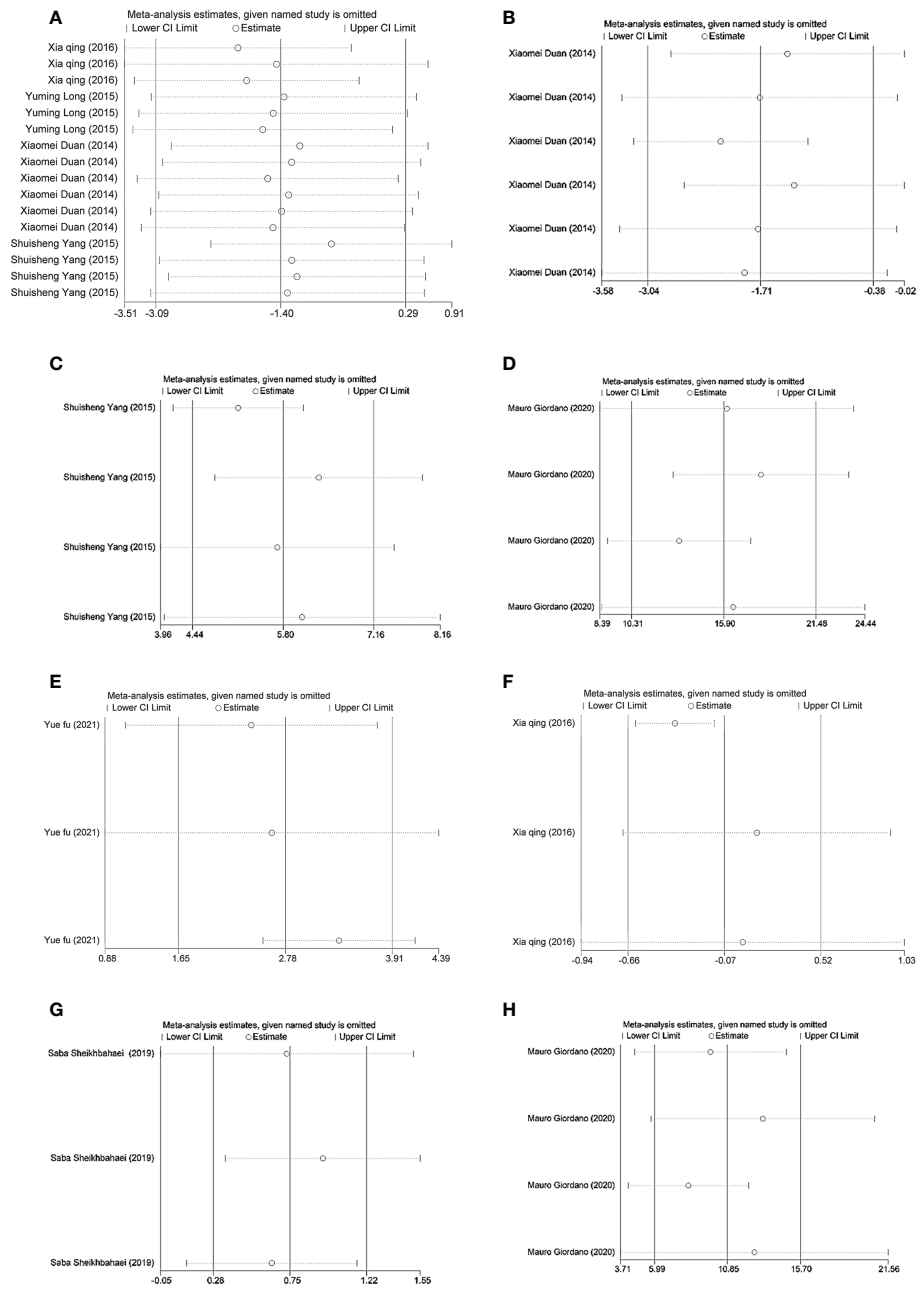


FIGURE 8
(A) Sensitivity analysis of miR-223. **(B)** Sensitivity analysis of miR-146a. **(C)** Sensitivity analysis of miR-144. **(D)** Sensitivity analysis of miR-195-5p. **(E)** Sensitivity analysis of miR-200a. **(F)** Sensitivity analysis of miR-210. **(G)** Sensitivity analysis of miR-503. **(H)** Sensitivity analysis of miR-451a.

pathway and then activated platelets, which increased the risk of ischemic stroke in patients with T2DM. Yu-Xiang Yan et al. (24) showed that miR-144 may play a role in the susceptibility of T2DM by changing the regulation of the stress response. In addition, Karolina et al. (25) demonstrated that peripheral blood miR-144 is a potential characteristic miRNA to distinguish IFG from T2DM. In conclusion, circulating miR-144 plays a role in the pathogenesis of T2DM and T2DM combined with AICVD and has specific expression. Therefore, we believe that it has potential diagnostic value in the early identification of T2DM combined with AICVD.

The expression of serum miR-503 was upregulated in the experimental group compared with the simple AICVD group, the simple T2DM group and the healthy group. Other studies have shown that miR-503 may inhibit the formation of neovascularization after diabetic ischemia. In addition, neutralizing miR-503 activity can also improve vascular healing and blood perfusion in ischemic limbs (26, 27). It has been demonstrated that miR-503 has an antiangiogenic effect in patients with T2DM and that miR-503 may increase the risk of stroke and disability in patients with diabetes mellitus by increasing inflammation and oxidative stress. Saba Sheikhbahaii (9) also showed that the differential expression of miR-503 among groups only occurred in the acute stage of ischemic cerebrovascular disease, and there was no significant difference in the expression of miR-503 among groups after 3

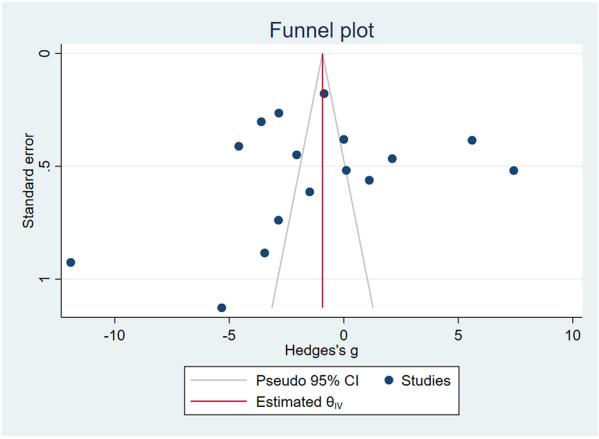


FIGURE 9
Funnel plot of circulating miR-223.

months. Therefore, miR-503 may be a potential diagnostic marker for AICVD in T2DM.

The expression of serum miR-126 in the experimental group was downregulated compared with that in the healthy group and simple T2DM group. Previous studies have shown that miR-126 is one of the most consistent miRs in diabetes (28, 29) and that it is mainly expressed in endothelial cells and plays a critical role in regulating endothelial cell function, angiogenesis, and vascular integrity (30, 31). Venkat et al. (32) concluded from mouse experiments that both T2DM and stroke significantly reduced the expression of miR-126, and the greatest reduction was observed in T2DM stroke mice. Venkat et al. also found that treatment with endothelial exosomes (EC-EXOs) in T2DM stroke mice significantly increased the expression of miR-126 in the serum and brain, improved neurological function and cognitive outcomes, and played an important role in the neurological recovery of T2DM stroke mice. Chen et al. (33) found in their study that T2DM significantly reduced the expression of miR-126 in the serum and brain tissue of T2DM mice. It was also demonstrated for the first time that human umbilical cord blood cell (HUCBC) treatment of stroke in T2DM mice increases serum, brain tissue, and brain endothelial cell miR-126 expression, thereby significantly decreasing BBB leakage and brain hemorrhage and increasing tight junction protein expression, promoting vascular and white matter remodeling, having antineuroinflammatory effects and improving functional outcomes after stroke. Based on the above studies on the diagnostic and therapeutic effects of miR-126 in T2DM mice with ischemic cerebrovascular disease, we speculate that circulating miR-126 has great value in the early identification and treatment of T2DM patients with ischemic cerebrovascular disease. Therefore, more relevant clinical studies can be carried out to confirm the diagnostic and therapeutic value of miR-126 in T2DM patients with AICVD.

In this study, the direction of circulating miR-223, miR-210 and miR-146a expression in the experimental group was inconsistent compared with that in the simple T2DM group, and there were still few related studies. Therefore, it is not considered a molecular marker for the early identification of T2DM complicated with AICVD. Alternatively, a large number of studies are still needed to explore its expression in T2DM complicated with AICVD.

In the included studies, the expression of circulating miR-195-5p and circulating miR-451a was upregulated in patients with T2DM complicated with AIS compared with healthy controls and patients with AIS alone. The expression of circulating miR-195-5p and circulating miR-451a was upregulated in patients with T2DM complicated with TIA compared with healthy controls and patients with TIA alone. The expression of circulating miR-708-5p in the experimental group was downregulated compared with that in the healthy group and the simple AICVD group. The expression of circulating miR-146b-3p in the experimental group was downregulated compared with that in the healthy group. The expression of circulating miR-368 in the experimental group was upregulated compared with that in the healthy group. However, there was no study on the differential expression of the above circulating miRNAs between the experimental group and the simple T2DM group in the literature we included. Therefore, it is not clear whether circulating miR-195-5p, miR-451a, miR-708-5p, miR-146b-3p and miR-368 can be used as valuable molecular markers for T2DM complicated with AICVD.

In this study, we analyzed and summarized the expression profile of miRNAs in T2DM with AICVD by synthesizing the existing findings. However, before translating miRNA profiles into clinical practice, studies using large, careful phenotypic cohorts and more technical standardization are necessary to identify miRNAs as effective and feasible markers for routine diagnosis and prognostic evaluation of T2DM with AICVD. On the one hand, there are few clinical studies on the specific expression of circulating miRNAs in T2DM with AICVD, and most of them are cross-sectional studies, and these results need to be further confirmed in large cohort studies whether they can be used as effective surrogates for current diagnostic tools. On the other hand, Baseline parameters, such as assessment of intra- and inter-individual variability, standardization of methods for all steps of sample collection and processing, RNA extraction, miRNA quantification, and analysis, will also need to be established to minimize interlaboratory biases. Vasu S et al. (34) pointed out that in different studies, the selection of endogenous miRNA controls may affect the results. In addition, such differences in small RNA input for cDNA conversion may introduce bias in the analysis. Small RNA sequencing avoids this bias by normalizing the expression to number of reads instead of the sample input. However, as small RNA sequencing is too expensive to perform on a routine basis. Absolute quantification of miRNA concentrations using synthetic miRNA mimics can help overcome these problems. Absolute quantification will also help in comparing different data sets and in correlation analysis. In summary, operational procedures should be standardized across studies for clinical use. We also found that miRNAs play a role in the pathogenesis of T2DM with AICVD. Improved understanding of the complex mechanisms underlying miRNAs dysregulation, and more well-designed studies utilizing prospective samples would facilitate the translation of these miRNAs to clinical treatment methods.

In addition, in our included studies, the detection of circulating miRNAs in the experimental group (T2DM with AICVD group) was performed after AICVD was diagnosed by brain MRI in patients with T2DM, and we could only speculate on the basis of the current

findings that they were also specifically expressed in T2DM patients who were about to develop AICVD. However, more clinical trials are needed to explore its value in assessing the risk of AICVD in patients with T2DM. For patients with abnormal expression of circulating miRNAs, further brain MRI is required to determine whether AICVD has occurred, and individualized intervention measures are taken in time to reduce the incidence and disability rate of AICVD.

In conclusion, we found that the expression of serum miR-200a, miR-503, plasma and platelet miR-144 was upregulated and the expression of serum miR-126 was downregulated in patients with T2DM complicated with AICVD. This may have diagnostic value in the early identification of T2DM complicated with AICVD. In addition, circulating miRNAs may play a role in the development of AICVD in patients with T2DM. More *in vivo* and *in vitro* studies are needed to further explore their roles and mechanisms to provide sufficient evidence-based medical evidence for clinical intervention of the disease.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

FH and LL wrote the manuscript and designed the study. XZ and GL analyzed the data. MC and ZL reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1129860/full#supplementary-material

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A clinical observation study on the effect of needle-free insulin syringe on blood glucose control and well-being index in patients with early-onset type 2 diabetes mellitus

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Objective: To explore the effect of using needle-free insulin syringe on blood sugar control and well-being index in patients with early-onset type 2 diabetes mellitus.

Methods: A total of 42 patients with early-onset type 2 diabetes mellitus treated with insulin aspart 30 injection in a stable condition in the Endocrinology Department of a tertiary hospital from January 2020 to July 2021 were randomly divided into two groups, one group received insulin pen injections followed by needle-free injections, and the other group received needle-free injections followed by insulin pen injections. Transient scanning glucose monitoring was performed during the last two weeks of each injection modality phase. Comparison of the two injection methods in terms of test indicators and differences in injection site pain scores, the number of red spots on the skin at the injection site and the number of bleeding spots on the skin at the injection site.

Results: The FBG of the needle-free injection group was lower than that of the Novo Pen group ($p<0.05$); the 2-hour postprandial blood glucose of the needle-free injection group was lower than that of the Novo Pen group, but there was no statistical significant difference. The amount of Insulin in the needle-free injector group was lower than that in the Novo pen group, but there was no statistical significant difference between the two groups. The WHO-5 score of the needle-free injector group was higher than that of the Novo Pen group($p<0.05$); the pain score at the injection site was lower than that of the Novo Pen group ($p<0.05$). The number of skin red spots using the needle-free syringe was more than that of the Novo pen group($p<0.05$); the number of skin bleeding at the site of injection was similar between the two injection methods.

Conclusion: Compared to traditional insulin pens, subcutaneous injection of premixed insulin using a needle-free syringe is effective in controlling fasting blood glucose in patients with early onset type 2 diabetes and is less painful at the injection site. In addition, blood glucose monitoring should be strengthened and insulin dosage should be adjusted in a timely manner.

KEYWORDS

Type 2 diabetes mellitus, early-onset, needle-free syringe, novo pen, transient scanning glucose monitoring system, well-being index

1 Introduction

Diabetes has become a global and serious social health problem. At present, there are about 425 million adults with diabetes worldwide, and it is estimated that by 2045, this number will grow to 629 million (1). The increased prevalence of early-onset type 2 diabetes is particularly pronounced in the rapidly growing diabetes population (2). Early-onset type 2 diabetes refers to type 2 diabetes diagnosed at age ≤ 40 years old (3). According to the data of the Global Diabetes Map (8th Edition) released by the International Diabetes Federation (IDF) in 2017, the top three countries and regions with the highest prevalence of early-onset type 2 diabetes are the Middle East and North Africa (4.1%), the United States. (4.0%) and China (3.6%). According to the latest epidemiological research results in China, the prevalence rate of early-onset type 2 diabetes is about 10% to 15% among diabetic patients who have been treated. Further studies (4, 5) have found that early-onset type 2 diabetes has more severe metabolic disorders, a higher risk of diabetic macrovascular disease and microvascular disease and a greater impact on life expectancy. Hence, critical attention must be given to patients with early-onset type 2 diabetes in order to strictly control blood sugar and reduce the occurrence of diabetic complications.

Patients with early-onset type 2 diabetes are more likely to use insulin to lower blood sugar, regardless of initial intensive insulin therapy or as the disease progresses, with the decline of pancreatic β-cell function. However, in the course of insulin therapy, problems such as pain, discomfort, fear, and adverse reactions caused by traditional needle injections will affect the acceptance and compliance of insulin therapy, and adversely affect the blood sugar control and quality of life of patients (6). "Guidelines for Diabetes Drug Injection Technology in China (2016 Edition)" recommends the use of needle-free syringes for insulin therapy in diabetic patients (7). Thus, the development of insulin injection devices prompts clinicians to re-examine the choice of hypoglycemic treatment strategies and injection devices.

Various researchers have conducted related studies to explore the differences in pharmacokinetics and clinical efficacy between needle-free injection of insulin and traditional subcutaneous injection (8, 9). For example, the "FREE study" conducted by Ji Linong in ten (10) research centers in China showed that, compared with needle-based insulin injection, needle-free insulin injection can better reduce a patients' glycosylated hemoglobin, lower the dosage of insulin required to achieve similar effect, less pain for patients, significantly lower incidence of adverse reactions such as induration at the injection site and higher patient contentment. At present, there is no clinical research on needle-free injection for early-onset type 2 diabetes. In-depth research on it will explore more effective insulin treatment methods and provide a better therapeutic approach in the treatment of early-onset type 2 diabetes.

The instantaneous scanning glucose monitoring system (flash glucose monitoring, FGM), uses advanced sensor-based technology whereby glucose data can be obtained through scanning. It is a tool for continuously recording 14-day blood glucose values and predicting the trend of blood glucose fluctuations, which can be used to evaluate and adopt treatment strategies to reduce blood glucose fluctuations (11). Based on the hospitals version of FGM data, this study took patients with early-onset T2DM as the research subjects, and compared the effect of needle-free injector and Novo pen subcutaneous insulin injection on blood sugar control and well-being index in patients with early-onset type 2 diabetes through a randomized cross-over study before and after self-control design. This was done in order to provide further clinical evidence in the treatment of early-onset type 2 diabetes using needle-free injection.

2 Subjects and methods

2.1 Subjects selection

The research subjects were selected between January 2020 to July 2021 in the the endocrinology department of a tertiary hospital in Shandong Province and were randomly divided into two groups. All the subjects were confirmed to be Type 2 diabetes patients.

2.2 Diagnostic criteria

2.2.1 Inclusion criteria

The following requirements had to be met for any subject to be included in the study:

1. Meet the 1999 WHO diabetes diagnostic criteria;
2. Aged between 18 and 40 years old;
3. using or be willing to use premixed insulin therapy;

4. Body Mass Index of $18Kg/m^2 \leqq BMI \leqq 32Kg/m^2$;
5. A hemoglobin A1c (HbA1c) in the range 7.5% - 11%;
6. Have read and signed the informed consent form.

2.2.2 Exclusion criteria

The following requirements had to be met for any subject to be rendered unfit or disqualified for the study:

1. Confusion or language barrier;
2. Patients with heart, lung, liver, kidney and other organ failure, acute cardiovascular and cerebrovascular diseases or severe sequelae;
3. Type 1 diabetes patients;
4. Individuals with psychiatric history;
5. People with combined acute and chronic severe diabetes complications;
6. Any patient with a history of insulin allergy;
7. Individuals with secondary diabetic complications such as acute pancreatitis, abnormal thyroid function, etc.;
8. Pregnancy and lactation mothers;
9. Cancer patients or people with history of autoimmune diseases;
10. Patients with irregular diet.

A total of 50 patients with type 2 diabetes mellitus and on premixed insulin regimens initially joined the study, among which two (2) patients fell off the flash glucose monitoring sensor, four (4) patients sought other treatment options midway, and two (2) patients did not complete the entire research process and dropped out. A total of forty-two (42) patients completed the study. The general information and metabolic status of the subjects before the intervention are shown in Table 1.

TABLE 1 General data and metabolic indexes of subjects before intervention (n=42).

Subject Parameters	Data
Male (number)	38
Female (number)	4
Age (years)	34.95 ± 4.62
Course of disease (years)	1.64 ± 1.71
BMI (Kg/m^2)	27.63 ± 3.67
Fasting blood glucose (mmol/L)	5.77 ± 1.41
2h postprandial blood glucose (mmol/L)	7.91 ± 1.57
HbA1c (%)	8.44 ± 3.51
TG (mmol/l)	2.84 ± 1.77
TC (mmol/l)	6.29 ± 2.11
HDL-C (mmol/l)	1.06 ± 1.34
LDL-C (mmol/l)	4.12 ± 2.15
SBP (mmHg)	127.21 ± 12.36
DBP (mmHg)	79.64 ± 9.72

2.3 Methods

2.3.1 Study design

This study adopted a prospective, randomized, open, cross-controlled design, see Figure 1. The overall intervention time was 8 weeks. Forty-two (42) patients were randomly divided into two groups with one group receiving insulin pen injections followed by needle-free injections, and the other group receiving needle-free injections followed by insulin pen injections. Before the intervention, the subjects were treated in the early stage, blood sugar was monitored and medications were adjusted for 2 weeks. This was done to ensure that the research was non-bias. Intervention Phase 1: After being discharged from the hospital, the two groups of subjects were given injections without needles and injections with needles, respectively, for three (3) weeks. Intervention Phase 2: three (3) weeks after being discharged from the hospital, the injection methods of the two groups were reversed for a further duration of three (3) weeks.

Among them, the needle-free injection used was a Beijing fast-comfort (QS-M) needle-free syringe; the syringe injection used was a Novo Nordisk Novo Pen ⑤ and a disposable insulin syringe of size 31G 5mm×0.25mm from Becton, Dickinson (BD) and company. Intervention Phase 1 and Intervention Phase 2 patients in both groups were treated with insulin aspart 30 (pen core/refill) manufactured by Novo Nordisk, Denmark. The lower abdomen was selected as the route of administration for the two different ways of insulin injection. Peripheral blood glucose was monitored with an Essence blood glucose meter, transient scanning glucose monitoring was performed two (2) weeks after the first and second intervention phases. Health records and management was established for all the patients and strengthened. During the study period, the same health guidance was given to all the subjects, including insulin injections, diet control, regular follow-up, exercise and outpatient review. This was done to ensure effective data gathering.

2.3.2 Comparison of various parameters

We did an evaluation and comparison of the effects of the two injection methods on fasting blood glucose (FBG), postprandial 2h blood glucose (2hPPG), time in range (TIR), World Health Organization-Five Well-Being Index (WHO-5), Insulin dosage [U/(kg·d)], pain score at the injection site, number of skin red spots at the injection site, number of skin bleeding at the injection site, times of hypoglycemia and time below range (TBR).

2.4 Statistical analysis

Using SPSS20.0 statistical software, the measurement data conforming to the normal distribution are expressed as ($\bar{x} \pm sd$). All comparisons between the two groups were first analyzed using a one-sample t test and did not conform to the normal distribution. As such, comparison between the two groups was performed by the non-parametric test where $p<0.05$ was considered statistically significant.

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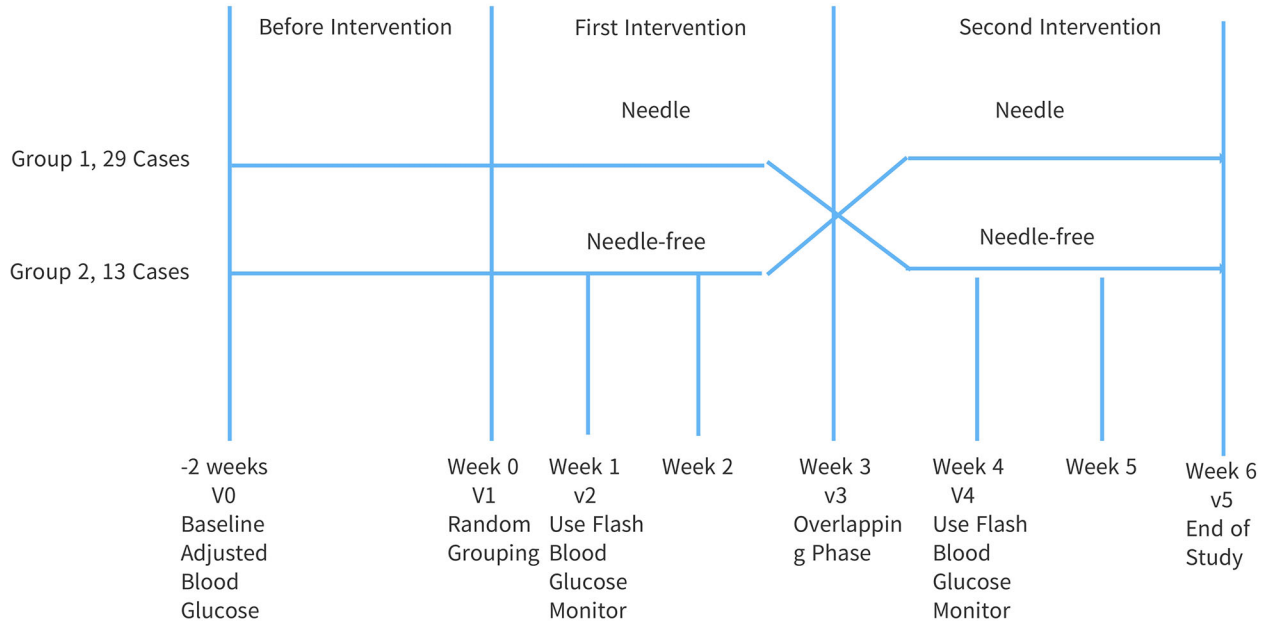


FIGURE 1
Flow chart of cross test.

3 Results

3.1 Comparison of the effects of the two injection methods on the dosage of FBG, 2hPPG, TIR, TBR and insulin

The patients in the two groups received subcutaneous insulin injection with Novo pen and needle-free syringe respectively. The fasting blood glucose (FBG) of the needle-free syringe was lower than that of the Novo pen group, and the difference was statistically significant ($p<0.05$); the 2h postprandial blood glucose (2hPPG) was lower in the needle-free group than that of the Novo pen group, but there was no statistically significant difference. In addition, no statistically significant difference was observed between the two groups for the TIR and TBR group. The amount of Insulin in the needle-free injector group was lower than that in the Novo pen group, but the difference was not statistically significant. A detailed account of the results is presented in Table 2.

3.2 Comparison of the effects of the two injection methods on the WHO-5, the pain score at the injection site, the number of red spots on the skin at the injection site and the number of bleedings at the injection site.

The WHO-5 score of patients using needle-free syringes was averagely higher than that of the Novo Pen group with a statistically significant difference ($p<0.05$). The pain score at the injection site was lower in the needle-free syringe group than that of the Novo Pen group, and the difference was statistically significant ($p<0.05$). The number of skin red spots using the needle-free syringe was more than that of the Novo pen group with a statistically significant difference ($p<0.05$). The number of skin bleeding was similar between the needle-free syringe group and the Novo Pen group. The data is presented in Table 3.

TABLE 2 Comparison of the effects of two the injection methods on blood glucose related indexes ($\bar{x}$ sd).

Group	Subjects (n)	FBG (mmol/L)	2h PPG (mmol/L)	TIR (%)	TBR (%)	Insulin Dosage [U/(kg·d)]
Needle-Injection Group	42	6.10 ± 1.74	7.94 ± 1.94	0.66 ± 0.19	0.13 ± 0.18	0.28 ± 0.08
Needle-free Injection Group	42	5.64 ± 1.59	7.67 ± 2.58	0.66 ± 0.17	0.12 ± 0.16	0.27 ± 0.28
T values		-2.338	0.887	-0.086	0.616	1.255
P values		0.024	0.380	0.932	0.542	0.217

TABLE 3 Comparison of the effects of the two injection methods on satisfaction indicators ($\bar{x}$ sd).

Group	Subjects (n)	WHO-5 score	Pain score	Skin red spots (times)	Skin bleeding (times)
Needle-Injection Group	42	73.67 ± 16.46	2.57 ± 1.93	0.19 ± 0.67	0.29 ± 0.64
Needle-free injection group	42	81.38 ± 15.70	1.83 ± 1.41	0.79 ± 1.22	0.45 ± 1.04
T values		4.387	4.109	2.909	2.011
P values		0.000	0.000	0.006	0.051

4 Discussion

Needle-free injectors use the principle of high-pressure jets, and mainly adopt transdermal dispersion drug delivery technology. Without the aid of needles, the liquid drug is ejected out from a small aperture at high speed (flow rate is generally greater than 100m/s) to reach the subcutaneous tissue with insulin distribution averagely higher than when a needle injection is used. This provides a very conducive area for maximal and complete absorption of insulin under the skin into bloodstream (12). In this study, a transient scanning glucose monitoring system was used to evaluate the effect of needle-free injection and needle injection within a 24-hour blood glucose fluctuations period in patients with early-onset type 2 diabetes mellitus. A very dynamic blood glucose data is attainable using the flash blood glucose (FBG) monitoring system, and the data obtained is beneficial in providing a more accurate trend and regularity of the changes in glucose occurring in a patient's body.

As a group that cannot be ignored, patients with early-onset type 2 diabetes often neglect the control of diabetes because they are busy with work and have the impression that they are in good health, thus causing an aggravation in the decrease of their β-cell function (13). Biphasic insulin aspart 30 is composed of 30% insulin aspart and 70% protamine insulin aspart, which can better meet the needs of patients for mealtime and basal insulin. The use of needle-free syringes to inject short-acting and rapid-acting insulin therapy can significantly shorten the time of onset of insulin, reverse the glycotoxic effect of hyperglycemia on β cells and significantly enhance the hypoglycemic effect (14). In the research conducted, the FBG of patients the patients using needle-free injectors was significantly lower than that of the Novo Pen group ($p<0.05$).

Time in range (TIR) refers to the time or percentage of glucose in the target range within 24 hours. It is a new indicator of blood glucose control that has attracted much attention in the field of diabetes. TIR can more intuitively and visually reflect the blood glucose fluctuation status of patients throughout the day (15). The WHO-5 scale was developed by the Danish scholar Bech.P and revised by the WHO Collaborative Center for Psychological Research (16, 17). It is the most widely used clinical scale to evaluate the well-being of diabetic patients. The scale can reflect the individual's mood or sense of well-being in the past two (2) weeks. It includes 5 items, each of which is divided into 6 levels, with a total score of 0-100 points. The higher the score, the stronger the sense of well-being (18). The score is graded as follows: ≤28 points for possible depression or low quality of life, 29-50 points for diminished sense of well-being and >50 points for happiness or contentment (19).

The WHO-5 scale was scored before the intervention, before the switch and at the end of the study. It must be noted that all the questions were willingly answered and filled in by the patients. The Numeric Rating Scale (NRS) is the most widely used unidimensional assessment scale (20). The system is based on a principle where a straight line is divided into ten (10) parts on average, and numbers from 0 to 10 is used at each point to determine the degree and aggravation of pain. The scaling is as follows: 0 indicates no pain; 1-3 indicates mild pain; 4-6 for moderate pain and 7-10 for severe pain. The patient is asked to circle the number that best represents their own level of pain. This evaluation form is more commonly used internationally. During the study, patients scored and recorded their own NRS scores after daily insulin injections. The number of skin red spots, skin bleeding and times of hypoglycemia were recorded by the patients and the data obtained is seen as fair as a consent form was signed.

A significant difference was observed in a meta-analysis (21) conducted on a subgroup of patients with diabetes mellitus who used insulin aspart 30. The fasting blood glucose (FBG) in the needle-free injector group was significantly lowered (MD = -1.31, 95%CI (-1.83, -0.78), $p<0.00001$) in comparison with the group that used the Novo pen. The results obtained is consistent with what we had in our report. In this study, there was no significant difference between the 2h postprandial blood glucose and TIR between the two groups, which may be attributed to the small sample size and short observation time.

The transdermal diffuse flow technology of needle-free injectors can increase the bioavailability of insulin (22) and help reduce the insulin dosage required to achieve similar therapeutic effect. As such, when changing to needle-free injectors, the insulin dose should be adjusted in time to avoid increasing the risk of hypoglycemia in patients. In this study, there is no statistically significant difference between the two groups of in terms of time below range (TBR), which is consistent with the previous research results that there is no difference in the incidence of hypoglycemia between needle-free injection and needle injection (23). In addition, it also shows the predictive effect of the scanning glucose monitoring system on the patient's glucose trend (24). In research conducted by Xie et al., the study found that when patients with type 2 diabetes change their insulin pens to needle-free injectors, regardless of the dosage form (fast-acting, short-acting, premixed) insulin, when the original insulin dose is maintained, the blood glucose levels of the patients dropped sharply within 0.5h, and hypoglycemia occurred frequently. In this regard, clinical therapists should strengthen the management of out-of-hospital diabetic patients who use needle-free injection for the first time, and conduct a comprehensive assessment based on the patient's blood sugar status and the body's absorption of insulin to avoid the

occurrence of hypoglycemia. In this study, the amount of insulin in the needle-free injector group was lower than that in the Novo pen group, but the difference was not statistically significant.

Needle-free injection is not painless and non-invasive, but minimally painful and minimally invasive. When the medical composition in the insulin injection penetrates the human epidermis, a small injection mark will be left, but since only the medicinal liquid enters the skin, and the injection depth is approximately 4-6mm with few nerve endings in this range, no obvious irritation will occur (25). Nonetheless, due to differences in individual physique, patients feel slightly different when injected. In this study, patients who used needle-free syringes had less pain at the injection site than needle-based injections, and the difference was statistically significant ($p<0.05$). The number of skin bleeding was similar between the two injections, but the number of red spots on the skin was more pronounce with the needle-free syringe than with the Novo pen, and the difference was statistically significant ($p<0.05$). The patients in this study had fewer daily injections, and the red spots or bleeding at the injection site disappeared after three (3) to five (5) days. There was a higher level of patient compliance and better injection experience among all the subjects by the end of the study.

Needle-free injection does not use needles, which not only reduces the patient's physical pain, but also eliminates the patient's psychological fear of injection needles. The WHO-5 scale can reflect an individual's mood or sense of well-being. In this study, the WHO-5 score of the needle-free injection group was higher than that of needle-based injection, and the difference was statistically significant ($p<0.05$). Patients were more willing to accept needle-free injection, so the well-being index was significantly improved. Studies have shown that the application of needle-free syringes can improve patients' depression as well as their quality of life (26). Although this study proved a point in helping determining which of the two injection methods is more ideal, it had certain limitations. The sample size of the study was mall and the observation time not long enough. For this reason, this study employed random grouping and cross-control design to reduce the difference between groups and in addition, enhance the feasibility of comparison between the two groups. In the future, the sample size will be increased, the observation time will be extended and further in-depth research will be conducted in order to gain a more comprehensive understanding of the advantages of needle-free injection in the administration of insulin.

In conclusion, compared with traditional insulin pens, the use of needle-free syringes and enhanced management can effectively reduce the fasting blood glucose of patients with early-onset type 2 diabetes mellitus treated with premixed insulin. To improve adherence to insulin therapy, critical attention should be paid to adjusting the insulin dose in time to aid in reducing the occurrence of hypoglycemia. Needle-free injection is more suitable for young patients and people who are Trypanophobia and it is particularly important to strengthen injection guidance among patients with early-onset type 2 diabetes mellitus.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, methodology, software, validation, formal analysis, writing—original draft preparation, XJ and QS; investigation, resources, data curation, visualization, CY, JH, and XLZ; supervision, project administration, and writing—review and editing, QG and XZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Development and validation of a nomogram for evaluating the incident risk of carotid atherosclerosis in patients with type 2 diabetes

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Introduction: The purpose of this study was to evaluate the clinical characteristics of carotid atherosclerotic disease in patients with type 2 diabetes mellitus, investigate its risk factors, and develop and validate an easy-to-use nomogram.

Methods: 1049 patients diagnosed with type 2 diabetes were enrolled and randomly assigned to the training and validation cohorts. Multivariate logistic regression analysis identified independent risk factors. A method combining least absolute shrinkage and selection operator with 10-fold cross-validation was used to screen for characteristic variables associated with carotid atherosclerosis. A nomogram was used to visually display the risk prediction model. Nomogram performance was evaluated using the C index, the area under the receiver operating characteristic curve, and calibration curves. Clinical utility was assessed by decision curve analysis.

Results: Age, nonalcoholic fatty liver disease, and OGTT3H were independent risk factors associated with carotid atherosclerosis in patients with diabetes. Age, nonalcoholic fatty liver disease, smoke, HDL-C, and LDL-C were characteristic variables used to develop the nomogram. The area under the curve for the discriminative power of the nomogram was 0.763 for the training cohort and 0.717 for the validation cohort. The calibration curves showed that the predicted probability matched the actual likelihood. The results of the decision curve analysis indicated that the nomograms were clinically useful.

Discussion: A new nomogram was developed and validated for assessing the incident risk of carotid atherosclerotic in patients with diabetes; this nomogram may act as a clinical tool to assist clinicians in making treatment recommendations.

KEYWORDS

diabetes, carotid atherosclerotic, predictive model, nomogram, diabetic complication

Introduction

Globally, diabetes mellitus (DM), a group of diseases defined by chronic hyperglycemia, is becoming more prevalent. According to the International Diabetes Federation, by 2021, 537 million people aged 20 to 79 will have diabetes worldwide (1). Diabetes causes many problems, with microvascular and macrovascular consequences accounting for a significant portion of the cost of treatment for type 2 diabetes mellitus (T2DM) (2). Based on a follow-up of deaths in 10 study centers worldwide, cardiovascular disease is the chief cause of death in diabetic patients, explaining 44% of deaths in patients with type 1 diabetes and 52% of deaths in T2DM (3).

Atherosclerosis is the main pathological cause of macroangiopathy in diabetic patients and is an independent risk element for cardiovascular disease. On the other hand, patients with atherosclerosis secondary to DM are particularly susceptible to Cardiovascular and cerebrovascular diseases (CCVD) or other vascular diseases, which will significantly reduce their quality of life (4–6). As living standards improve, CCVD events are becoming younger, and mortality and disability are increasing (7). Therefore, to reduce cardiovascular and cerebrovascular diseases and their complications, early identification of patients with atherosclerotic cardiovascular disease and effective interventions are important (7). The early involved vessels in atherosclerotic lesions are carotid arteries (8). Carotid atherosclerosis disease (CAD) is associated with the severity of ischemic cardiovascular disease and also acts as a window into the status of other vascular atherosclerosis in the body (9, 10). Guoqing Huang et al. established CAD risk prediction models based on age, body mass index (BMI), diastolic blood pressure (DBP), DM, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transpeptidase (GGT) for the normal population (11). Fengqi Guo et al. showed that the occurrence of CAD in the T2DM population is closely related to gender, age, hypertension, lipids, diabetic retinopathy, and other factors (12). We did not find the development of a clinical risk prediction model based on this special population by searching existing literature reports. Therefore, in this study, we aimed to create a practical nomogram for predicting the risk of CAD in adults with T2DM. The nomogram is a common visual presentation tool for disease risk prediction models that is simple and easy to use (13). In addition, a clear understanding of the important risk factors for CAD will provide a foundation for preventing and delaying serious complications.

Abbreviations: AUC, Area under the ROC curve; CI, Confidence interval; DCA, Decision curve analysis; ROC, Receiver operating characteristic; NAFLD, nonalcoholic fatty liver; FHD, family history of diabetes; CAD, carotid atherosclerotic disease; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, urea nitrogen; CREA, creatinine; UA, blood uric acid; Cys-c, cystatin C; CHOL, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; GSP, glycated serum protein; OGTT, oral glucose tolerance test; HbA1c, glycated hemoglobin; IRT, insulin-releasing test.

Materials and methods

Study population

In this retrospective cross-sectional study, we collected 1049 patients with T2DM hospitalized at Zhongnan Hospital of Wuhan University from January 2015 to May 2021 according to inclusion and exclusion criteria. Those with severe missing information (more than 20% of the total) were excluded, and those with less missing information (less than 20% of the total) were filled by multiple interpolations. Patients were divided into a training cohort and a validation cohort by setting seeds in R. The inclusion criteria for participants were T2DM diagnosis based on the World Health Organization (WHO) guidelines (1999) (14). The main exclusion criteria were as follows: (1) Patients with severe infection, acute myocardial infarction, acute cerebral infarction, severe trauma, malignant tumor, surgery, and another stress state; (2) those taking lipid-lowering drugs; (3) those diagnosed with diabetes during pregnancy; (4) those without complete ultrasound measurements of the carotid artery.

The study met the ethical standards of the Declaration of Helsinki (2013) and was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (ethical approval code: 2022167K). The flow chart of the participants is shown in Figure 1.

Collection of clinical data

The clinical data were obtained from medical records. Personal history (gender, age, height, body weight; smoking or drinking habits; high blood pressure, and family history of diabetes (FHD)), laboratory serological indexes (liver function, renal function, blood fat, oral glucose tolerance test (OGTT), insulin-releasing test (IRT), glycosylated hemoglobin (HbA1c), and glycosylated serum protein (GSP)), and laboratory data (carotid color ultrasonography, and abdominal ultrasonography) were obtained.

Body mass index (BMI) (kg/m2) was calculated using the formula: body mass index (BMI) (kg/m2) = height (kg)/ weight2 (m2).

Evaluation of carotid atherosclerotic disease, OGTT test and IRT test

Color Doppler ultrasound (GE Healthcare, Milwaukee, Wisconsin) was used as the diagnostic method for CAD. Experienced sonographers were blinded to all clinical information performed in the evaluation. Carotid intima-media thickness (IMT) and carotid plaque were recorded during the ultrasound examination; IMT was defined as the distance between the leading edge of the first and second echo lines of the distant arterial wall; the presence of CAP was defined as a local increase in thickness of 0.5 mm or 50% of the surrounding IMT value (15).

Subjects underwent standard OGTT and IRT tests the following morning after fasting (8-10 hours). After fasting venous blood

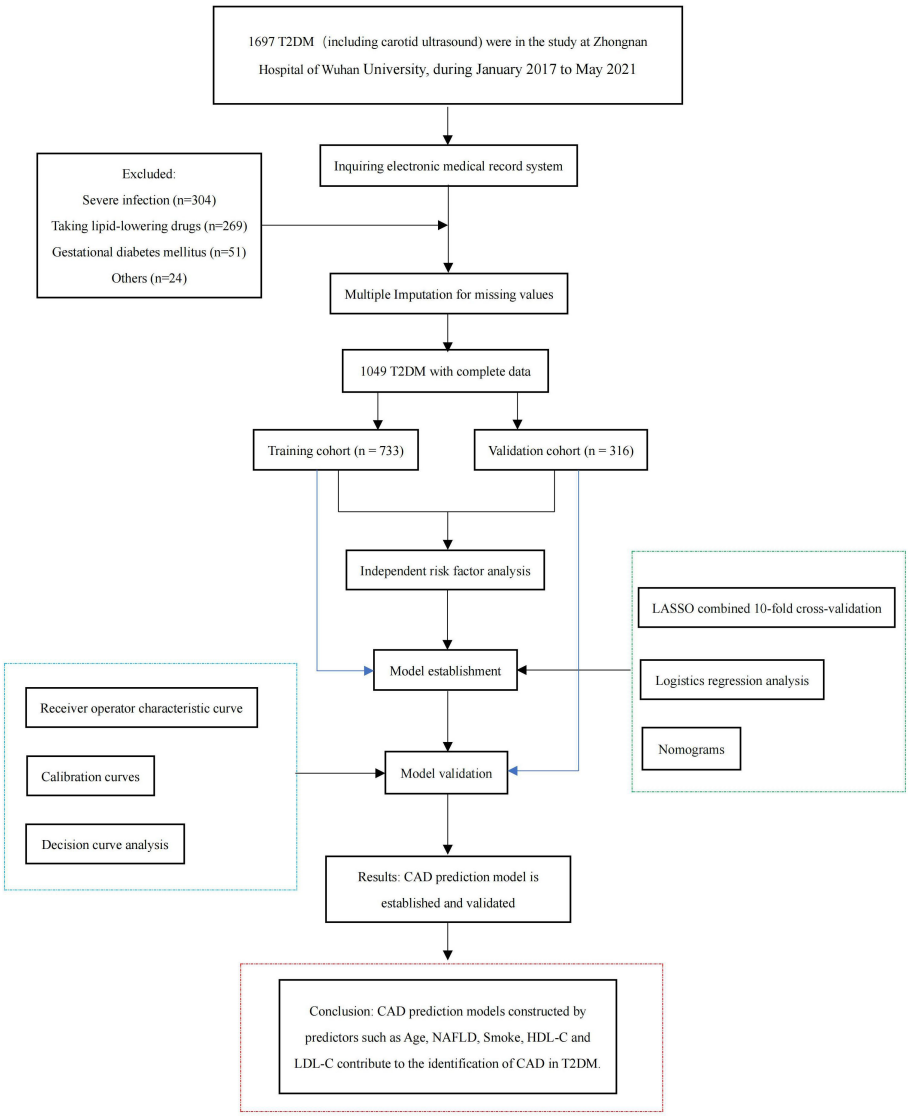


FIGURE 1
Flowchart of the participants.

collection for determination of fasting glucose (OGTT0H) and fasting insulin (IRT0H), patients were instructed to take 75 g of anhydrous glucose within 5 minutes, and blood samples were collected from the anterior elbow vein at intervals of 30, 60, 120 and 180 minutes for determination of plasma glucose concentrations (OGTT0.5H, OGTT1H, OGTT2H, OGTT3H) and serum insulin concentrations (IRT0.5H, IRT1H, IRT2H, IRT3H).

Statistical analysis

The Kolmogorov-Smirnov test was used for testing the normality distribution of continuous data. Continuous data that conformed to normal distribution were expressed as "mean ± standard deviation (x ± s) ", and t-test was used for comparison between two groups; continuous data that did not conform to normal distribution were expressed as "median (lower quartile, upper quartile) " and Mann-Whitney U test was used for comparison between groups. The frequency of dichotomous variables was performed by $\chi 2$ analysis and was expressed as "frequency (proportion) ". The independent risk factors were identified using multivariate logistic regression analyses. First, Least Absolute Shrinkage and Selection Operator (LASSO) regression was performed using the glmnet package to screen out relevant variables. Then, a multivariate logistic regression analysis was performed on the selected variables. Nomogram was built *via* the rms and regplot packages. Sensitivity and specificity were defined by receiver operating characteristic (ROC) curves drawn by the pROC software package. The calibration curves were drawn using the rms package. The rmda package was used for decision curve analysis (DCA). SPSS 18.0 (SPSS Inc, Chicago, IL, USA) and R version 4.0.3 (https://www.rproject.org/) were used for statistical analysis, and $P < 0.05$ or $P < 0.1$ was considered statistically significant.

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Results

Clinical characteristics of the patients with T2DM

Patients were randomly divided into a training cohort (n = 733) and a validation cohort (n = 316) according to the 7:3 ratio by setting seeds in R (16). The patients' laboratory examination findings and clinical characteristics are shown in Table 1. Most patients with T2DM were male (63% and 67%, respectively). The mean age of the training cohort was 56 (48, 65) years, and that of the validation cohort was 56.5 (50, 65) years. Except for the aspartate aminotransferase (AST), there were no significant differences between the other characteristics, indicating that random grouping does not produce bias. The results of the Univariate analysis are shown in Table 2. The mean ages of the no-CAD group (HC) and the CAD group were 53 (43,60) years and 61 (54,68) years, respectively. The two groups differed in age, NAFLD, BMI, liver function, renal function, lipids, and blood pressure.

TABLE 1 The Clinical characteristics of patients in the training and validation cohorts.

Parameter	Training cohort (n = 733)	Validation cohort (n = 316)	*p* value
Gender (%)			0.189
male	459 (63)	212 (67)	
female	274 (37)	104 (33)	
Age (years)	56 (48, 65)	56.5 (50, 65)	0.338
NAFLD (%)			0.803
No	440 (60)	193 (61)	
Yes	293 (40)	123 (39)	
Smoke (%)			0.759
No	512 (70)	217 (69)	
Yes	221 (30)	99 (31)	
Drink (%)			0.324
No	500 (68)	205 (65)	
Yes	233 (32)	111 (35)	
FHD (%)			0.313
No	712 (97)	311 (98)	
Yes	21 (3)	5 (2)	
CAD (%)			0.913
No	390 (53)	170 (54)	
Yes	343 (47)	146 (46)	
SBP(mmHg)	128 (114, 139)	126 (113, 138)	0.179
DBP(mmHg)	75 (70, 84)	75.5 (70, 83)	0.679
BMI(kg/m^2)	24.98 (22.84, 27.1)	24.77 (22.58, 27.04)	0.352
ALT(U/L)	22 (15, 33)	21 (15, 30)	0.125
AST(U/L)	20 (16, 26)	19 (15, 24)	**0.033**
ALP(U/L)	83 (69, 100)	83 (68, 104)	0.577
BUN (mmol/L)	5.3 (4.5, 6.3)	5.4 (4.4, 6.3)	0.913
CREA(μmol/L)	64 (53.8, 75.4)	63.85 (54, 74)	0.943
UA(μmol/L)	332.6 (269.4, 405.1)	332.8 (269.72, 399.87)	0.813
Cys-c (mg/L)	0.86 (0.73, 1)	0.85 (0.71, 1.01)	0.662
CHOL (mmol/L)	4.68 (3.89, 5.42)	4.61 (3.93, 5.62)	0.656
TG (mmol/L)	1.64 (1.14, 2.58)	1.73 (1.22, 2.73)	0.228
HDL-C(mmol/L)	0.99 (0.82, 1.19)	0.98 (0.84, 1.15)	0.543
LDL-C(mmol/L)	2.69 (2.03, 3.27)	2.66 (2.13, 3.29)	0.506
GSP(μmol/L)	409.9 (323.2, 512.4)	402.85 (325.48, 491.57)	0.427
HbA1c(%)	8.85 (7.4, 10.57)	8.55 (7.1, 10.43)	0.233
OGTT0H(mmol/L)	9.46 (7.39, 12.48)	9.14 (7.31, 12.03)	0.543
OGTT0.5H(mmol/L)	13.14 (10.62, 16.03)	13.06 (10.77, 15.96)	0.634
OGTT1H(mmol/L)	16.9 (13.89, 20.17)	16.73 (13.72, 20.29)	0.695
OGTT2H(mmol/L)	18.86 ± 5.42	18.99 ± 5.62	0.721
OGTT3H(mmol/L)	16.55 ± 5.90	16.76 ± 6.35	0.626
IRT0H(uIU/ml)	11 (6.32, 16.4)	9.95 (5.94, 15.22)	0.061
IRT0.5H(uIU/ml)	17.5 (10.7, 28.9)	16.6 (10.5, 26.52)	0.369
IRT1H(uIU/ml)	26.1 (15.1, 43.2)	25.65 (14.2, 39.51)	0.284
IRT2H(uIU/ml)	33.3 (18.7, 55)	32.05 (18.78, 50.31)	0.360
IRT3H(uIU/ml)	28.6 (15.7, 48.4)	26.3 (15.7, 45.37)	0.256

*p-value indicates statistically significant.
n, number of patients; NAFLD, nonalcoholic fatty liver; FHD, family history of diabetes; CAD, carotid atherosclerotic disease; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, urea nitrogen; CREA, creatinine; UA, blood uric acid; Cys-c, cystatin C; CHOL, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; GSP, glycated serum protein; OGTT, oral glucose tolerance test; HbA1c, glycated hemoglobin; IRT, insulin-releasing test.

Independent risk factors

Based on univariate analysis (Table 2), we selected candidate variables with P<0.1 for inclusion in the multivariate logistic regression analysis. Independent risk factors associated with CAD in T2DM were finally identified, including age, NAFLD, and OGTT3H (Table 3).

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TABLE 2 Univariate analysis of carotid atherosclerosis.

Parameter	Overall	HC	CAD	P value
N	1049	560	489	
Gender (%)				0.768
Male	671 (64)	361(64)	310(63)	
Female	378 (36)	199(36)	179(37)	
Age	56(49,65)	53(43,60)	61(54,68)	**0.000**
NAFLD (%)				**0.065**
No	633(60)	353(63)	280(57)	
Yes	416(40)	207(37)	209(43)	
Smoke (%)				0.474
No	729(69)	395(71)	334(68)	
Yes	320(31)	165(29)	155(32)	
Drink (%)				0.440
No	705(67)	370(66)	335(69)	
Yes	344(33)	190(34)	154(31)	
FHD (%)				0.805
No	1023(98)	545(97)	478(98)	
Yes	26(2)	15(3)	11(2)	
SBP (mmHg)	127(114,139)	126(113,138)	130(115,141)	**0.011**
DBP (mmHg)	75(70,84)	76(70,85)	75(70,83)	**0.033**
BMI(kg/m^2)	24.91(22.77,27.08)	25.14(22.96,27.11)	24.69(22.49,26.99)	**0.028**
ALT(U/L)	22(15,32)	22.5(16,35)	20(15,29)	**0.001**
AST(U/L)	19(16,25)	20(16,26)	19(15,25)	0.127
ALP(U/L)	83(68,101)	82(69,99)	83(68,103)	0.305
BUN (mmol/L)	5.3(4.5,6.3)	5.2(4.3,6.18)	5.5(4.5,6.5)	**0.001**
CREA(μmol/L)	64(54,75)	63.8(53,74)	64.4(54,77)	**0.091**
UA(μmol/L)	332.6(269.5,404.5)	337.55(272.58,411.55)	325.8(266.5,390.7)	0.148
Cys-c(mg/L)	0.85(0.72,1)	0.81(0.7,0.97)	0.88(0.75,1.04)	**0.000**
CHOL (mmol/L)	4.64(3.91,5.49)	4.69(3.95,5.46)	4.57(3.83,5.49)	0.473
TG (mmol/L)	1.67(1.15,2.61)	1.73(1.17,2.76)	1.61(1.13,2.38)	**0.014**
HDL-C (mmol/L)	0.99(0.83,1.18)	0.98(0.82,1.19)	1(0.84,1.16)	0.512
LDL-C (mmol/L)	2.68(2.06,3.28)	2.67(2.05,3.24)	2.69(2.07,3.32)	0.293
GSP(μmol/L)	407.3(324,506.5)	403(322.78,506.85)	410.7(325.1,505.8)	0.777
HbA1c (%)	8.8(7.32,10.5)	8.6(7.2,10.65)	8.9(7.4,10.3)	0.680
OGTT0H (mmol/L)	9.38(7.37,12.41)	9.29(7.21,12.46)	9.47(7.42,12.36)	0.743
OGTT0.5H (mmol/L)	13.13(10.64,16.03)	13.28(10.51,16.34)	12.99(10.78,15.72)	0.578
OGTT1H (mmol/L)	16.83(13.87,20.21)	16.86(13.61,20.58)	16.78(14.38,19.95)	0.898
OGTT2H (mmol/L)	18.9 ± 5.48	18.65 ± 5.58	19.18 ± 5.35	0.116
OGTT3H (mmol/L)	16.61 ± 6.04	16.19 ± 6.05	17.1 ± 5.99	**0.015**
IRT0H(uIU/ml)	10.7(6.19,16.1)	10.1(6.03,15.93)	11.43(6.58,16.2)	0.067

(Continued)

TABLE 2 Continued

Parameter	Overall	HC	CAD	P value
IRT0.5H(uIU/ml)	17.1(10.7,28)	16.6(10.2,27.8)	17.8(11.3,28.1)	0.204
IRT1H(uIU/ml)	25.8(14.8,42.2)	24.7(13.97,41.21)	27.5(15.8,44.1)	0.100
IRT2H(uIU/ml)	32.83(18.7,53.9)	32.3(17.4,50.82)	34.1(20.7,56.7)	**0.052**
IRT3H(uIU/ml)	28.1(15.7,47.51)	26.85(14.78,44.82)	29.1(17.2,50.7)	**0.050**

*p-value indicates statistically significant. The bold shows that P values are meaningful.
n, number of patients; NAFLD, nonalcoholic fatty liver; FHD, family history of diabetes; CAD, carotid atherosclerotic disease; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; BUN, urea nitrogen; CREA, creatinine; UA, blood uric acid; Cys-c, cystatin C; CHOL, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; GSP, glycated serum protein; OGTT, oral glucose tolerance test; HbA1c, glycated hemoglobin; IRT, insulin-releasing test.

Construction of predictive models

A total of 733 patients in the training cohort were included in the LASSO regression analysis, and 13 non-zero characteristic variables were screened (Figure 2). Next, to further develop predictive models for CAD, the aforementioned indicators were included in multivariate logistic regression analysis; we selected age, NAFLD, smoking, HDL-C, and LDL-C for model construction (Table 4).

Development of the nomogram to predict carotid atherosclerosis

A nomogram for predicting CAD in T2DM was created based on the results of a multivariate logistic regression model (Figure 3). The results showed the highest risk scores (100) for age (≥90 years of age). Visualization of risk factors for CAD in T2DM can predict the risk of individual CAD. First, each unique CAD risk factor was projected upward to the first row of the scale to obtain a score for each element; then, the scores for the five risk factors were summed to get a total score. The higher the total score, the higher the CAD risk of the individual.

Validation of the nomogram to predict carotid atherosclerosis

The ROC curve was used to assess the predictive accuracy of the nomograms. The results showed that the area under the ROC curve for the training cohort was 0.763 (95% CI = 0.73–0.80), and that of the validation cohort was 0.717 (95% CI = 0.66–0.77). The C- index of the training and validation cohorts were 0.76 and 0.72. The abovementioned results indicate that the nomogram has an excellent predictive effect on CAD (Figure 4). Next, a calibration curve was used to evaluate the deviation between the nomogram's predicted results and actual values. The predicted results showed good agreement for both the training and validation cohorts

TABLE 3 Multivariate logistic regression analysis.

Parameter	Coefficients	Odds ratio (95% CI)	P value
Age	0.073	1.076(1.061–1.092)	**0.000**
NAFLD	0.474	1.607(1.204–2.151)	**0.001**
SBP	0.005	1.005(0.995–1.016)	0.310
DBP	-0.002	0.998(0.981–1.016)	0.837
BMI	-0.037	0.964(0.923–1.006)	0.096
ALT	-0.002	0.998(0.992–1.004)	0.567
BUN	-0.039	0.962(0.876–1.056)	0.413
CREA	0.003	1.003(0.995–1.011)	0.509
Cys-c	0.116	1.133(0.662–1.871)	0.624
TG	-0.003	0.997(0.933–1.061)	0.929
OGTT3H	0.026	1.026(1.001–1.053)	**0.045**
IRT2H	0.002	1.002(0.996–1.008)	0.515
IRT3H	-0.002	0.998(0.991–1.004)	0.442

*p-value indicates statistically significant. The bold shows that P values are meaningful.
NAFLD, nonalcoholic fatty liver; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; BMI, body mass index; ALT, alanine aminotransferase; BUN, urea nitrogen; CREA, creatinine; Cys-c, cystatin C; TG, triglyceride; OGTT, oral glucose tolerance test; IRT, insulin-releasing test.

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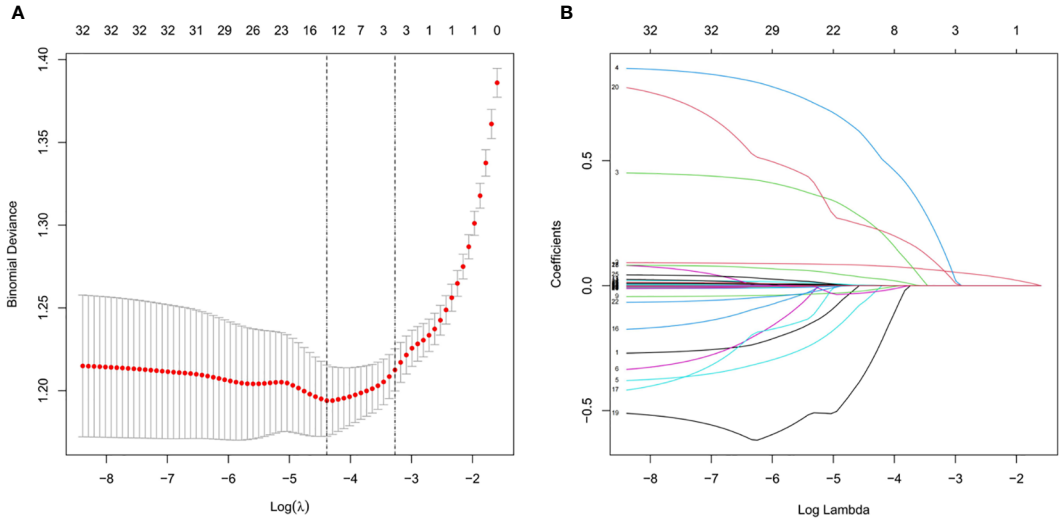


FIGURE 2
Clinical index selection using LASSO regression analysis. **(A)** Log(lambda) and partial likelihood deviations are shown, and the dotted line shown at the smallest log(lambda) represents the best predictor. **(B)** LASSO coefficients for the 32 clinical indicators. Non-zero coefficients were determined based on the best logarithm (lambda).

(Figure 5). DCA curves were then used to assess the clinical usefulness of the nomograms. The results showed that in the training cohort, using this nomogram to predict CAD risk was more useful than the all-intervention or no-intervention methods if the threshold probabilities for patients and physicians were >2% and <76%, respectively. The decision curves show that in the validation cohort, CAD risk prediction with our nomogram is more informative than the all-intervention or no-intervention scheme when the threshold probabilities for patients and physicians are >3% and <80%, respectively (Figure 6). These results suggest that nomograms have excellent predictive power for CAD.

Discussion

The nomogram model is a reliable statistical tool and is widely used with diagnostic prediction models of diabetic complications (13, 17). It uses a very intuitive graphical representation, which interprets risk models very simply and easily (18, 19). In this study, a CAD risk prediction model for patients with T2DM was developed using nomograms. The model was validated by the ROC curve, calibration curve, and C index, and the predicted and observed values were found to be in general agreement, indicating that the nomogram prediction model in this study is reliable. DCA showed that the nomogram has clinical applications. In the model's process, five risk factors, age, fatty liver, smoking, high-density lipoprotein, and low-density lipoprotein, were screened and identified as risks of CAD in T2DM. According to the nomogram model, age was the largest risk factor among the five factors, followed by LDL-C and HDL-C, fatty liver, and smoking history. In addition, age, NAFLD, and OGTT3H were identified as independent risk factors for CAD in T2DM.

Most risk assessment methods for the development of CAD in T2DM include risk factor analysis. A study conducted in 2020 reported that age, gender, history of hypertension, coronary artery disease, and diabetes are risk factors for carotid atherosclerotic plaque formation (20). The risk factors for CAD in T2DM are similar to common risk factors for stroke in the Chinese population (21). Moreover, advanced age, male, lower education, hypertension, diabetes, passive smoking, and high LDL-C levels are independent risk factors for early atherosclerosis (22). From the results of these studies, several risk prediction models have been designed to assess the risk of carotid atherosclerosis disease in T2DM. However,

TABLE 4 Multivariate logistic regression analysis.

Parameter	Coefficients	Odds ratio (95% CI)	P value
Age	0.093	1.097(1.078–1.117)	**0.000**
NAFLD	0.457	1.579(1.104–2.268)	**0.013**
Smoke	0.900	2.459(1.581–3.863)	**0.000**
HDL-C	-0.792	0.453(0.233–0.859)	**0.017**
LDL-C	0.316	1.371(1.128–1.673)	**0.002**

*p-value indicates statistically significant. The bold shows that P values are meaningful.
NAFLD, nonalcoholic fatty liver; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein.

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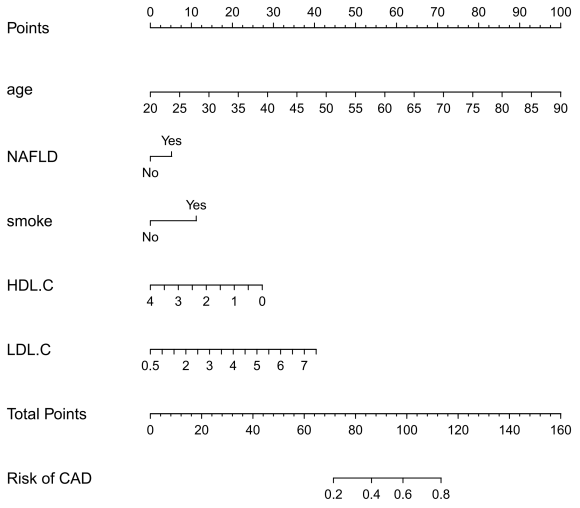


FIGURE 3
Nomogram: A Nomogram was created to determine the incidence of carotid atherosclerosis. A total score was obtained from the values of each index, and the CAD risk rate corresponding to the total score was the predicted rate of the Nomogram.

limitations of these studies, such as individualized differences in populations, have led to a lack of simple and intuitive tools to facilitate the use of these models, so few of them have been applied in clinical practice. In our study, we developed the first nomogram describing CAD risk factors in T2DM. We performed an internal validation with the area under the ROC curves of 0.763 and 0.717 for the training and validation cohorts, respectively. The decision curves of the training cohort showed that using this nomogram to predict CAD risk in the present study was more favorable than all-patient intervention scenarios or no-intervention scenarios if the threshold probabilities for patients and physicians were 2% and 76%, respectively. Also, the DCA of the validation cohort was shown to be clinically useful.

The nomogram developed in this study allowed direct prediction and visual analysis of factors that greatly affect the risk of CAD in T2DM. Moreover, age is an unavoidable factor in many chronic diseases. According to a cohort study including 318,083 patients with T2DM from Sweden, age is greatly associated with T2DM with cardiovascular and mortality risk, which is consistent with the findings of our study (23). The possible mechanism for this is that the physiological and endocrine functions of the body diminish with age, leading to atherosclerosis; furthermore, older people tend to undergo physiological and structural changes in their blood vessels, thus reducing nitric oxide utilization and leading to increased production of angiotensin (24). Meanwhile, advanced age is an immutable risk factor for diabetes (25). Advanced age leads to the aging of pancreatic β-cells, resulting in defective insulin secretion and decreased glucose sensitivity (26); hyperglycemia is a risk factor for atherosclerosis (27). Persistent hyperglycemia causes changes in most cells in the vascular tissue, which accelerates atherosclerosis (28). Therefore, advanced age further affects blood glucose and aggravates atherosclerosis.

Our study also showed that NAFLD is strongly associated with the development of CAD in patients with T2DM. A study involving 8020 patients showed that persistent NAFLD was associated with an increased risk of developing subclinical CAD (29). Also, NAFLD is greatly associated with diabetes (30), further contributing to the development of CAD. The possible mechanism is that both NAFLD and T2DM are associated with a state of systemic hypo-inflammation, which may encourage atherosclerosis through the secretion of various cytokines such as interleukin-6, interleukin-1, tumor necrosis factor-α, and acute phase proteins (C-reactive protein, fibrinogen, and fetal protein-A) (31).

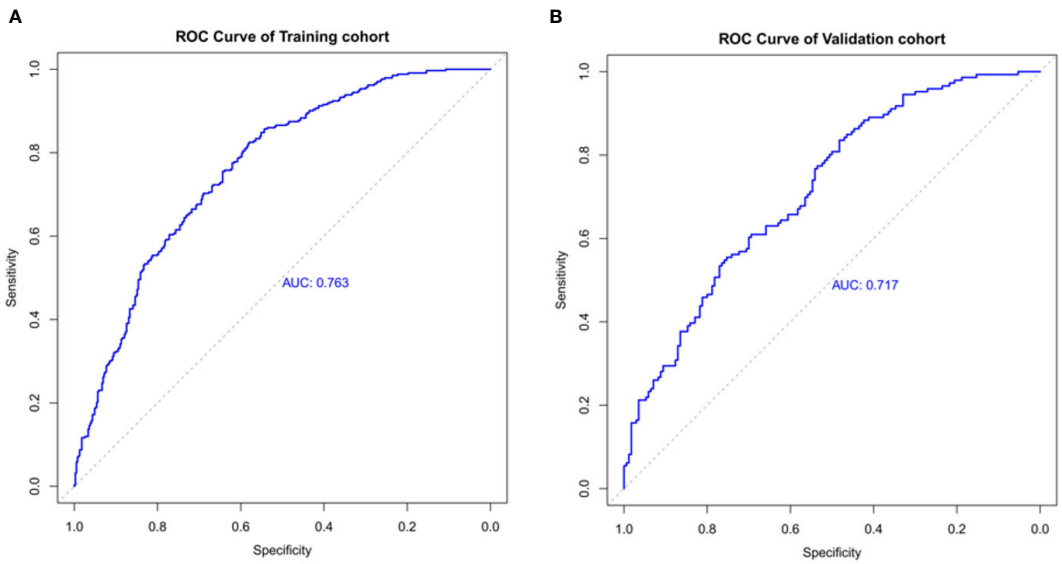


FIGURE 4
The nomogram model predicted the receiver operating characteristic curve of carotid atherosclerosis in patients with T2DM. **(A)** The area under the curve of the training cohort is 0.763. **(B)** The area under the curve of the validation cohort is 0.717.

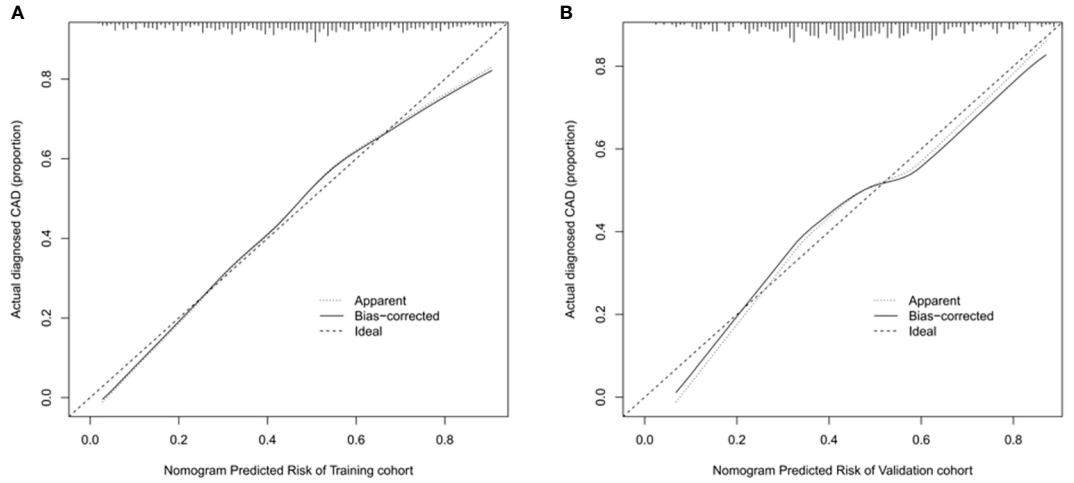


FIGURE 5
Calibration curve of nomogram model for predicting carotid atherosclerosis in patients with T2DM. **(A)** In the training cohort, B = 1000 repetitions, boot mean absolute error = 0.021, n = 733. **(B)** In the validation cohort, B = 1000 repetitions, boot mean absolute error = 0.032, n = 316. The X-axis represents the predicted risk of carotid atherosclerosis; Y-axis represents the actual diagnosed carotid atherosclerosis. Y-axis represents actually diagnosed carotid atherosclerosis. The diagonal dotted line represents the perfect prediction by the ideal model. The solid line represents the performance of the nomogram; the closer to the diagonal dotted line, the better the prediction.

Smoking is still a major health hazard that significantly affects the morbidity and mortality of the cardiovascular disease. All stages of atherosclerosis are affected by it (32). The increased risk of cardiovascular disease in patients with diabetes is partly associated with a high prevalence of other cardiovascular disease risk factors. Therefore, the management of modifiable cardiovascular disease risk factors can minimize the risk of vascular complications in patients with diabetes (33).

The pathological process leading to atherosclerosis is usually associated with elevated LDL-C concentrations, which alter cell permeability and progressively affect the arterial wall (34). HDL-C may reduce the risk of cardiovascular events (35, 36). In addition, a large body of preclinical and mechanistic evidence suggests that HDL has an antidiabetic function and improves glycemic control by increasing insulin sensitivity and β-cell function (37). This is consistent with our study.

Therefore, it was essential to apply five risk predictors in our model. Despite the good performance of our nomogram, the study has some possible limitations. First, it was a retrospective study. The data collected did not provide information on other risk factors for

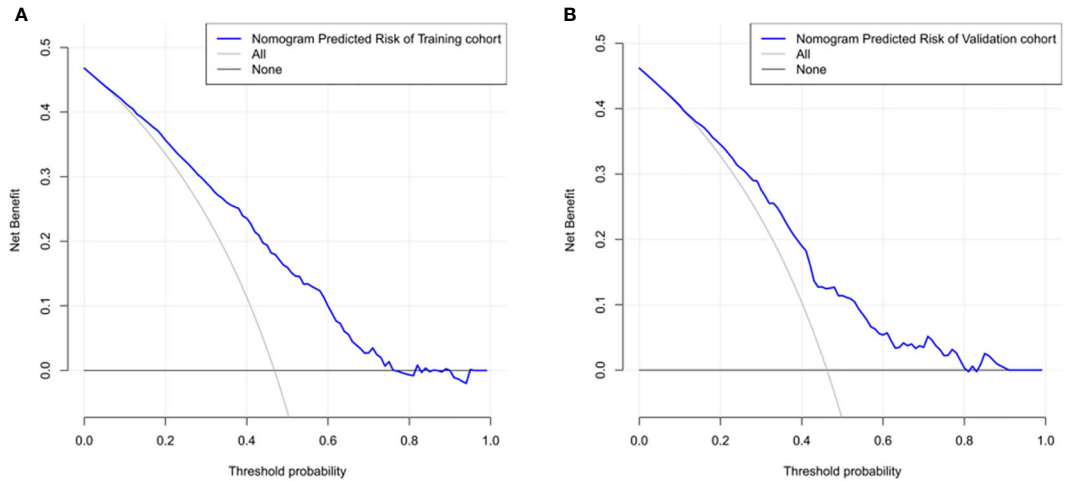


FIGURE 6
The nomogram model predicted the decision curve of carotid atherosclerosis in patients with T2DM. The y-axis measures the net return. The dotted line represents the CAD risk nomogram. The thin solid line represents the assumption that all patients are CAD. The thick solid line represents the assumption that no patients are CAD. **(A)** Decision curves for the training cohort show that using this nomogram predicts more benefit for CAD risk than intervening with the all-patient scenario or the no-intervention scenario if the threshold probabilities for patients and physicians are >2% and <76%, respectively. **(B)** Decision curves for the validation cohort show that using this nomogram to predict CAD risk adds more benefit than intervening with an all-patient regimen or a no-intervention regimen if the threshold probabilities for patients and physicians are >3% and <80%, respectively.

diabetes, such as lifestyle factors, including chronic high-sugar diet or lack of exercise. Second, only internal validation was used. Third, all patients were from a single center with a limited sample size. Differences in ethnicity were not taken into account, which is not a good representation of the whole population. Finally, our collection had data with some missing values, which could introduce selection bias if participants with incomplete records from the full case study were completely excluded from model building. Therefore, we used multiple interpolations to replace missing values in the analysis. In the future, it is hoped that our study can be a collaborative effort of multiple centers to collect as many variables as possible to continuously test and modify predictive models in clinical practice.

In conclusion, we identified three CAD associated independent risk factors in T2DM, including age, NAFLD, and OGTT3H. Meanwhile, we screened and visualized five clinical indicators closely associated with the occurrence of CAD in T2DM using LASSO and logistics regression, which can be used as a clinical tool for clinicians to perform personalized screening by validating their model accuracy and good clinical usefulness.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were approved by by the Ethics Committee of the Zhongnan Hospital of Wuhan University (ethical approval code: 2022167K). Written informed consent from the participants was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

XF wrote the manuscript, performed data analysis, and prepared Figures 1-5; LR and YXi collected the data and prepared Tables 1-2; YXu designed the entire study, provided financial support, and supervised the whole process. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1131430/full#supplementary-material

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Quantitative study of 3T MRI qDixon-WIP applied in pancreatic fat infiltration in patients with type 2 diabetes mellitus

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Objective: To investigate the application value of 3T MRI qDixon-WIP technique in the quantitative measurement of pancreatic fat content in patients with type 2 diabetes mellitus (T2DM).

Methods: The 3T MRI qDixon-WIP sequence was used to scan the livers and the pancreas of 47 T2DM patients (experimental group) and 48 healthy volunteers (control group). Pancreatic fat fraction (PFF), hepatic fat fraction (HFF), Body mass index (BMI) ratio of pancreatic volume to body surface area (PVI) were measured. Total cholesterol (TC), subcutaneous fat area (SA), triglyceride (TG), abdominal visceral fat area (VA), high density lipoprotein (HDL-c), fasting blood glucose (FPC) and low-density lipoprotein (LDL-c) were collected. The relationship between the experimental group and the control group and between PFF and other indicators was compared. The differences of PFF between the control group and different disease course subgroups were also explored.

Results: There was no significant difference in BMI between the experimental group and the control group (P=0.231). PVI, SA, VA, PFF and HFF had statistical differences (P<0.05). In the experimental group, PFF was highly positively correlated with HFF (r=0.964, P<0.001), it was moderately positively correlated with TG and abdominal fat area (r=0.676, 0.591, P<0.001), and it was weakly positively correlated with subcutaneous fat area (r=0.321, P=0.033). And it had no correlation with FPC, PVI, HDL-c, TC and LDL-c (P>0.05). There were statistical differences in PFF between the control group and the patients with different course of T2DM (P<0.05). There was no significant difference in PFF between T2DM patients with a disease course ≤1 year and those with a disease course <5 years (P>0.05). There were significant differences in PFF between the groups with a disease course of 1-5 years and those with a disease course of more than 5 years (P<0.001).

Conclusion: PVI of T2DM patients is lower than normal, but SA, VA, PFF, HFF are higher than normal. The degree of pancreatic fat accumulation in T2DM patients with long disease course was higher than that in patients with short disease course. The qDixon-WIP sequence can provide an important reference for clinical quantitative evaluation of fat content in T2DM patients.

KEYWORDS

multi-echo Dixon, magnetic resonance imaging, pancreatic fat infiltration, type 2 diabetes, quantitative study

1 Introduction

Type 2 diabetes mellitus(T2DM) is the most common type of diabetes mellitus (1, 2). Pancreatic fat infiltration may play an important role in the occurrence and development of T2DM (3, 4). The degree of lipid infiltration in the pancreas is closely related to abnormal lipid metabolism. With β-cell dysfunction and defective insulin secretion, lipid oxidation and lipolysis are inhibited, which leads to the increase of lipid deposition in the pancreas. The increased degree of pancreatic fat infiltration promotes the development of T2DM (5–7). Therefore, monitoring pancreatic fat content in T2DM patients may provide a certain reference for clinical evaluation of efficacy and disease progression.

Although pancreatic biopsy is the "golden standard" for the quantitative determination of pancreatic fat content, due to the fact that this method only provides small tissue samples, the final measured pancreatic fat content may vary with the different range and degree of pancreatic fat infiltration. Moreover, its invasiveness and poor patient compliance limit the regular detection of pancreatic fat content in T2DM patients. the pancreas is a retroperitoneal organ surrounded by abundant blood vessels and intestines, which makes puncture more difficult (8, 9).

In recent years, multi-echo dixon technology based on Magnetic resonance image (MRI), which is safe, non-invasive and has good tissue resolution, has been confirmed in various organs including the pancreas in terms of tissue fat quantification (10–13). The early two-point Dixon technique could only quantify the adipogenic variation below 50% (14), which was greatly affected by the non-uniformity of the main magnetic field and the attenuation effect of T1 and T2* (15). Three-point Dixon technique can collect one more in-phase echo signal on the basis of two-point method, which can correct T2* attenuation to a certain extent. However, the obtained organ fat fraction is susceptible to various confounding factors, and its accuracy and repeatability are not enough to be a reliable index of fat quantification (16). 6 Echo Dixon (qDixon) technology, compared with the earlier Dixon technology, effectively corrects the errors caused by the magnetic field inhomogeneity and T2* attenuation, making the quantitative results more accurate. The fat distribution map can not only directly measure the fat content quantitatively, but also fully reflect the fat distribution (17).

The purpose of this study was to investigate the value of 3T qDixon technique in the quantitative determination of pancreatic fat content in T2DM patients, and to provide reference for the early diagnosis, clinical treatment, disease progression and efficacy evaluation of pancreatic changes in T2DM patients by comparing the relationship between relevant indicators.

2 Materials and methods

2.1 Research objects

A total of 95 volunteers were recruited from April 1, 2019 to June 30, 2022, including 36 females (17 T2DM patients, 19 normal controls) and 59 males (30 T2DM patients, 29 normal controls). T2DM Patients ranged from 32 to 71 years old (51.32 ± 10.60). The normal control group ranged from 31 to 68 years old (51.28 ± 8.91). Inclusion criteria: (1) patients diagnosed with T2DM and healthy volunteers with similar age to T2DM patients (± 3 years old) and no related diseases. Exclusion criteria: (1) patients unable to participate in MRI examination due to contraindications or other reasons; (2) patients with liver and pancreatic tumors; (3) patients after splenectomy; (4) patients with abnormal metabolic function or metabolic diseases excluding T2DM; (5) patients with hepatitis virus or hepatitis B, and liver iron deposition; (6) patients with liver trauma or patients receiving a liver transplant; (7) patients with pancreatic inflammation and alcoholics; (8) Patients with a history of drug therapy for the the pancreas (Sulfonamides, azathioprine, glucocorticoids, thiazide diuretics) and liver (Platinum agents, antibiotics, alkylating agents, antipsychotics, anti-tuberculosis drugs, and anti-tumor drugs) within six months. This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the hospital Ethics Committee (NO.2022-E460-01).

2.2 Instruments and methods

MRI scans were performed on all subjects by the same operator with 10 years of extensive MRI scanning experience. Abdominal axial scan was performed at the end of breath using a 3.0T MRI scanner Siemens 3T MRI scanner (Prisma, Siemens Healthcare, Erlangen, Germany). qDdixon-WIP sequence scanning parameters: echo time (TE): 1.26, 2.60, 3.94, 5.28, 6.62, 7.96ms; repetition time (TR): 9.25ms;

slice thickness: 3.5mm, matrix: 160×120; bandwidth: 1040Hz/Pixel; field of view: 380mm×313.5mm; scanning time: about 18s.

2.3 Image processing

Data measurements were performed by two radiologists who were familiar with image post-processing and had more than 5 years of experience in abdominal diagnosis. Measurement process: The Region of interest (ROI) was delineated independently on the fat content (FF) diagram of qDixon-WIP sequence, and the fat fraction was directly measured (fat fraction =10%× the mean measured by software). For liver, the intrahepatic sink area was avoided as far as possible. Four ROIs (liver S2/3, S4, S5/8, S6/7) were selected (Figures 1A, B) to measure liver fat fraction, each ROI was about 0.4 ~ 0.6cm2, and the corresponding Goodness of fit was measured (Figures 2A, B). Average values were taken (<5% indicates good accuracy). For the pancreas, three ROIs (head, body and tail of the pancreas) were selected (Figures 3A–C) to measure pancreatic fat fraction, each ROI was about 0.1-0.2 cm^2, and Goodness of fit was also measured (Figures 4A–C), and average values were taken. The images were uploaded to Ziostation workstation (Ziostation2 Version 2.4.0.2), and the "3D standard and Viewer" functions in the workstation were used for image processing: The whole the pancreas was manually delineated, and the pancreatic volume was automatically calculated by the software (Figure 5A), and visceral fat area (VA) and subcutaneous fat area (SA) were measured in the experimental and control groups via the umbilical plane (Figure 5B). For pancreatic volume, in order to exclude the influence of height, weight and other factors among individuals, pancreatic volume to body surface area (PVI) was obtained by conversion (male: body surface area [m^2] = 0.0057 × height [cm] + 0.0121 × weight [kg] + 0.0882; Female: body surface area [m^2] = 0.0073 × height [cm] + 0.0127 × weight [kg] - 0.2106; Pancreatic volume per unit body surface area: PVI [cm^3/m^2]= pancreatic volume cm^3/body surface area m^2) (18). All measurement data were taken from the mean values measured by two doctors. The patient's clinical data was queried through HIS system of our institution; Height and weight were measured on the day of MRI scan.

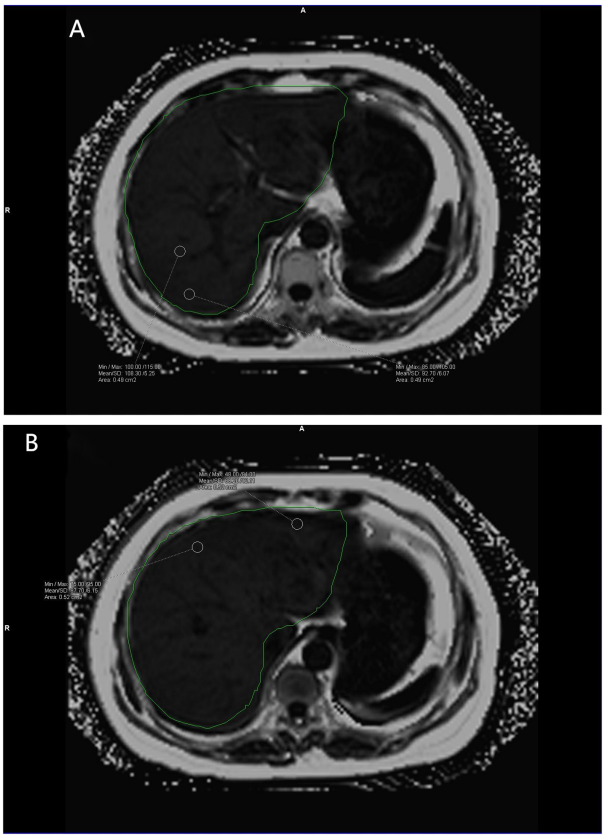


FIGURE 1
(A, B) show the liver fat fraction maps of volunteers. Mean fat fraction =10%× (108.30 + 92.70+68.20+87.70)/4 = 8.92 (two decimal places reserved). The green area is the liver region automatically delineated by the software.

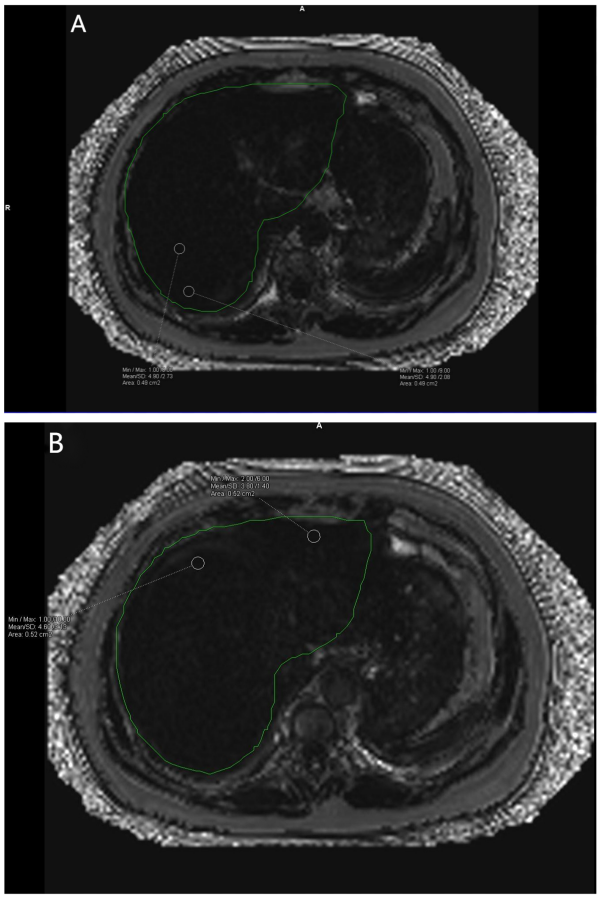


FIGURE 2
(A, B) show the corresponding Goodness of fit plots for liver fat fraction. A Goodness of fit average = (4.90% + 4.90% + 4.60% + 3.80%)/4 = 4.55%. The green area is the liver region automatically delineated by the software.

2.4 Statistical methods

SPSS22.0 software was used for statistical analysis. Kolmogorov-Smirnov(K) method was used to test the normal distribution of the data. Measurement data with normal distribution were represented as

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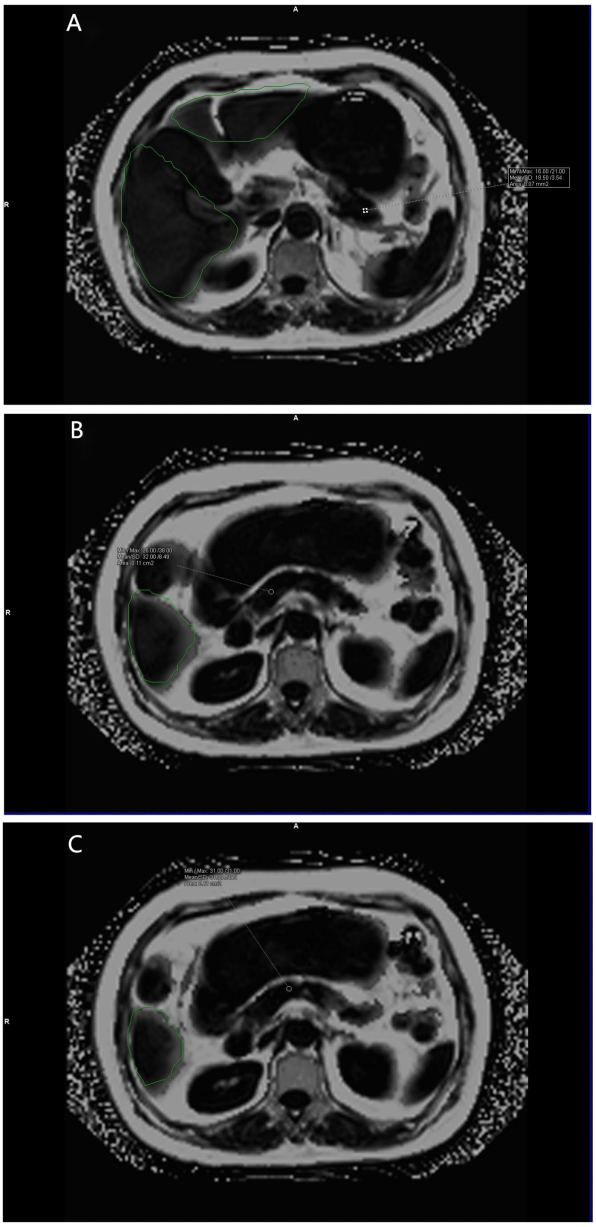


FIGURE 3
(A–C) show the pancreatic fat fraction maps of the volunteers. Mean pancreatic fat fraction =10%× (18.50 + 32.00+31.00)/3 = 2.72 (keep two decimal places). The green area is the liver region automatically delineated by the software.

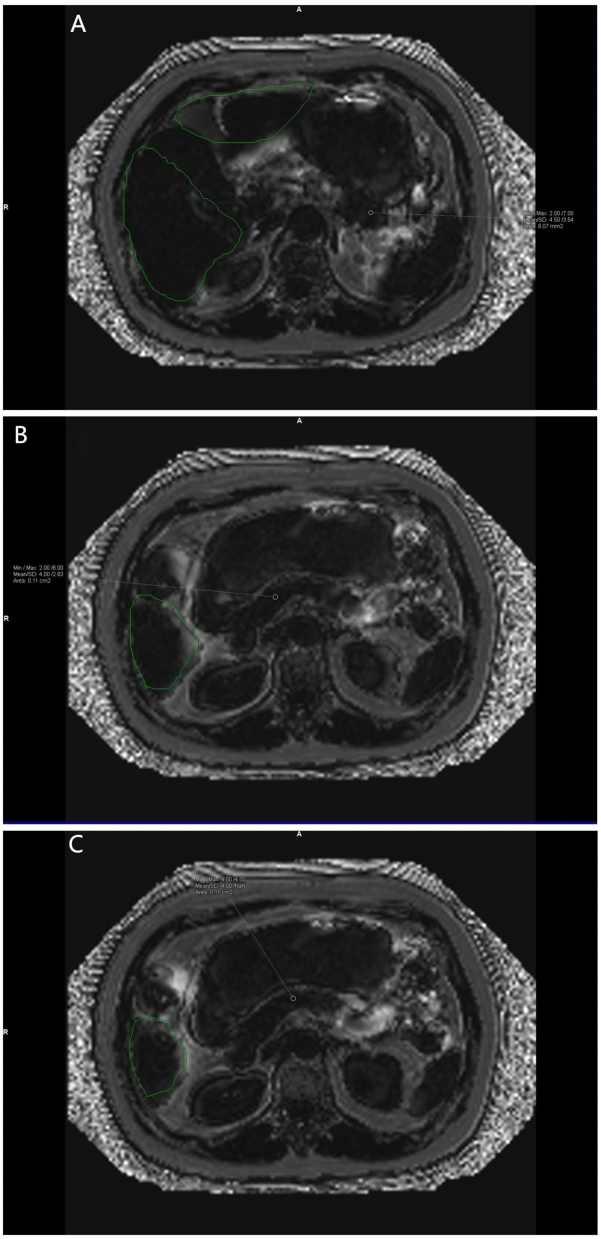


FIGURE 4
(A–C) show the corresponding Goodness of fit plots for pancreatic fat fraction. Goodness of fi mean = (4.5%+4.0%+4.0%)/3 = 4.17% (keep two decimal places). The green area is the area automatically delineated by the software.

mean ± standard deviation (M). Measurement data with non-normal distribution were expressed as median, and quartile. Pearson chi-square test was used to compare the differences in gender composition. The independent sample t test (normal distribution) or Mann-Whitney U test (non-normal distribution) was used to compare the Pancreatic fat fraction (PFF), SA, VA and PVI between the experimental group and the control group. Pearson (normal distribution) or Spearman (non-normal distribution) correlation analysis was used to evaluate the correlation between the measured PFF and Hepatic fat component (HFF), PVI, SA, VA and clinical indicators in T2DM patients. The threshold for significance was set at 0.05.

3 Results

3.1 Consistency test for determination of pancreatic fat

The PFF, HFF, SA, VA and PVI of the experimental group and the control group were measured by two doctors (A and B) at different times. The Intraclass correlation coefficient (ICC) consistency test showed that the measured results were consistent between the groups (Table 1). It can be considered that the data measured by different doctors were highly consistent with intra-observer and inter-observer.

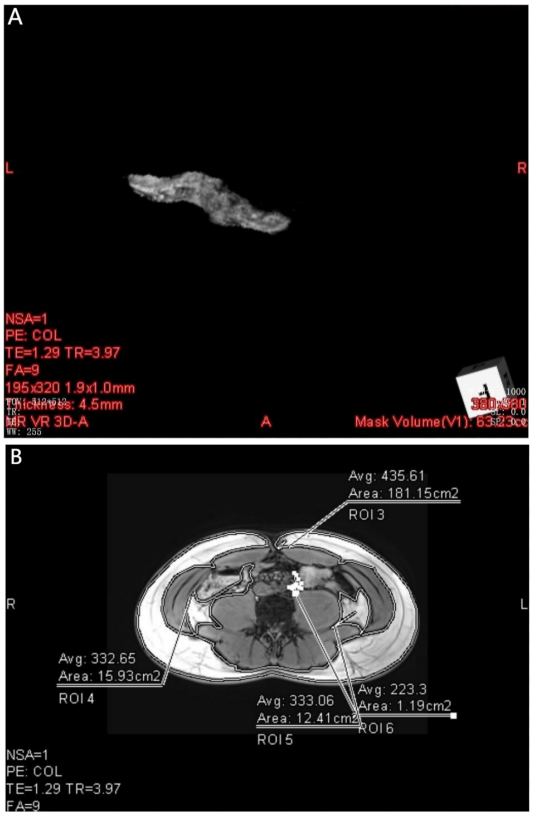


FIGURE 5
(A) shows the pancreatic Volume map of the volunteer the pancreas obtained by 3D standard processing, and the lower right corner of the figure shows the pancreatic volume Mask Volume(V1):63.23 cc. **(B)** shows the subcutaneous fat area(ROI 3 = 181.15cm^2) and abdominal visceral fat area(ROI 4 = 15.93cm^2, ROI 5 = 12.41cm^2, ROI 6 = 1.91cm^2) of volunteers after processing with Viewer.

3.2 Clinical parameter processing and normality test of measurement data

The normality test showed that BMI, PVI, SA, VA, TC, TG, HDL-c in the experimental group and BMI, PVI, SA, VA, PFF and HFF in the normal control group were all normal distribution (P>0.05). In the experimental group, PFF, HFF, FPC and LDL-c showed non-normal distribution (P<0.05) (Table S1).

3.3 Comparison and analysis results of related fat mass and parameters between T2DM patients and control group

There was no significant difference in age and gender distribution between the experimental group and the normal control group (P>0.05). The other indicators were BMI, PVI, SA, VA, PFF and HFF. There was no significant difference in BMI between the experimental group and the control group (P>0.05). There were statistical differences in PVI, SA, VA, PFF, and HFF between the two groups (P<0.05) (Table 2). PVI of T2DM patients was lower than that of control group, while SA, VA, PFF and HFF were higher than those of control group.

3.4 Correlation analysis between fat-related measurements and clinical indicators in T2DM patients

PFF was positively correlated with HFF in the experimental group (r=0.964, P<0.001). It was moderately positively correlated with TG, VA and Disease course (r=0.676, 0.591, 0.615, P<0.001), and weakly positively correlated with SA (r=0.321, P=0.033). There was no

TABLE 1 Consistency test of PVI, subcutaneous fat area, abdominal fat area, PFF% and HFF% measured values between the experimental group and the control group by two doctors.

Group	Measurement index	Doctor A1	Doctor A2	Doctor B	Consistency coefficient (and 95% credibility Interval)	
					A1 and A2	A1 and B
experimental	PVI (cm^3/m^2)	31.55 ± 1.79	31.58 ± 1.76	31.29 ± 1.73	0.993 (0.987~0.996)	0.917 (0.845~0.956)
	SA (cm^2)	126.67 ± 44.70	126.65 ± 43.71	133.10 ± 45.05	1.000 (1.000~1.000)	0.957 (0.916~0.978)
	VA (cm^2)	82.93 ± 26.48	82.94 ± 26.52	84.93 ± 22.59	1.000 (1.000~1.000)	0.925 (0.889~0.963)
	PFF (%)	3.70 (2.70~5.40)	3.70 (2.50~5.50)	3.08 (2.34~5.90)	0.991 (0.984~0.995)	0.936 (0.893~0.971)
	HFF (%)	6.03 (3.975~8.15)	6.00 (4.00~8.50)	5.36 (3.48~8.42)	0.994 (0.990~0.997)	0.949 (0.913~0.972)
control	PVI (cm^3/m^2)	33.68 ± 1.76	33.67 ± 1.76	34.48 ± 2.49	0.998 (0.996~0.999)	0.923 (0.861~0.958)
	SA (cm^2)	108.56 ± 36.40	108.54 ± 36.41	107.95 ± 46.24	1.000 (1.000~1.000)	0.944 (0.891~0.975)
	VA (cm^2)	37.31 ± 10.77	37.33 ± 10.76	38.52 ± 12.24	1.000 (1.000~1.000)	0.910 (0.829~0.945)
	PFF (%)	1.76 ± 0.66	1.79 ± 0.63	1.67 ± 0.69	0.992 (0.986~0.996)	0.950 (0.923~0.987)
	HFF (%)	3.37 ± 1.47	3.41 ± 1.49	3.13 ± 1782	0.999 (0.998~0.999)	0.933 (0.901~0.970)

PVI, ratio of pancreatic volume to body surface area; SA, subcutaneous fat area; VA, visceral fat area; PFF, pancreatic fat fraction; HFF, hepatic fat fraction.

TABLE 2 Age, PFF, HFF, BMI, PVI and other indicators of T2DM patients and control group.

Evaluating indicator	T2DM	control group	T/X^2	P
BMI(kg/m^2)	23.04 ± 2.826	23.68 ± 2.327	-1.206	0.231
PVI(cm^3/m^2)	31.27 ± 1.761	34.01 ± 2.487	-6.220	<0.001
SA(cm^2)	133.07 ± 44.91	108.44 ± 18.39	3.377	0.001
VA(cm^2)	84.72 ± 22.30	37.91 ± 12.057	12.307	<0.001
PFF(%)	3.10(2.31~5.84)	1.73 ± 0.697	-6.209	<0.001
HFF(%)	5.50(3.48~8.42)	3.24 ± 1.617	-4.682	0.016
Age(year)	51.32 ± 10.604	51.28 ± 8.907	-0.024	0.981
gender	F=17,M=30	F=19,M=29	0.118	0.883
n	47	48	–	–

BMI, body mass index; PVI, patio of pancreatic volume to body surface area; SA, subcutaneous fat area; VA, visceral fat area; PFF, pancreatic fat fraction; HFF, hepatic fat fraction; n, example number.

significant correlation with FPC, TC, PVI, HDL-c, LDL-c (r=0.385, 0.236, -0.163, -0.168, -0.002; P=0.194, 0.437, 0.292, 0.276, 0.987) (Table 3 and Figure 6). The non-standardized linear regression equation constructed with PFF as the dependent variable and the other indicators as the independent variables is: PFF=10.287 +0.284HFF-0.255PVI-0.329TG+0.758Disease course(According to the inspection level of 0.05, only HFF, PVI, TG and Disease course were included in the regression equation) (Table 4). The standardization coefficients of HFF, PVI, TG and Disease course are 0.637, -0.233, -0.18 and 0.303 (Table 4).

TABLE 3 PFF, HFF, abdominal wall, abdominal fat area and related clinical parameters in T2DM patients.

Clinical data	T2DM
FPC (mmol/L)	7.73 (5.61~9.83)
TC (mmol/L)	5.02 ± 1.20
TG (mmol/L)	1.83 ± 0.958
LDL-c (mmol/L)	2.86 (2.34~4.02)
HDL-c (mmol/L)	1.10 ± 0.296
PVI (cm^3/m^2)	31.27 ± 1.76
SA (cm^2)	133.07 ± 44.91
VA (cm^2)	84.72 ± 22.30
PFF (%)	3.10 (2.31~5.84)
HFF (%)	5.50 (3.48~8.42)
Age (year)	51.32 ± 10.604
gender	F=17,M=30
n	47

FPC, fasting blood glucose; TC, total cholesterol; TG, triglyceride; LDL-c, low density lipoprotein; HDL-c, high density lipoprotein; PVI, patio of pancreatic volume to body surface area; SA, subcutaneous fat area; VA, visceral fat area; PFF, pancreatic fat fraction; HFF, hepatic fat fraction.

3.5 Comparison and analysis of PFF values between experimental group and control group in patients with different course of disease

The measurement results of PFF values in the control group and the experimental group with different course of disease are shown in Table 5. The results of comparison between groups are shown in Table S2. The PFF of the control group and the experimental group were statistically different (P<0.05), and the pancreatic fat content of the control group was lower than that of the experimental group. There was no statistically significant difference in PFF between patients with less than one year of disease course and those with one to five years of disease course in the experimental group (P>0.05), which could not indicate that the PFF of patients with one to five years of disease course was higher than that of patients with one year of disease course. The PFF of patients with less than 1 year and 1 to 5 years of disease course was statistically different from that of patients with more than 5 years of disease course (P<0.05), which could be considered that the PFF of patients with less than 1 year and 1 to 5 years of disease course was less than that of patients with more than 5 years of disease course.

4 Discussion

For ectopic fat accumulation in T2DM, ectopic lipid deposition can promote its development and plays an important role in its progression (19, 20). Studies have reported that pancreatic fatty infiltration is associated with insulin resistance, and the incidence of diabetes in people with pancreatic fatty infiltration is significantly higher than the other people (21, 22). At present, MRI-based fat quantification technology can identify small changes in fat content, quantify fat and monitor steatosis, making it play an increasingly important role in the assessment of pancreatic fat content (10).

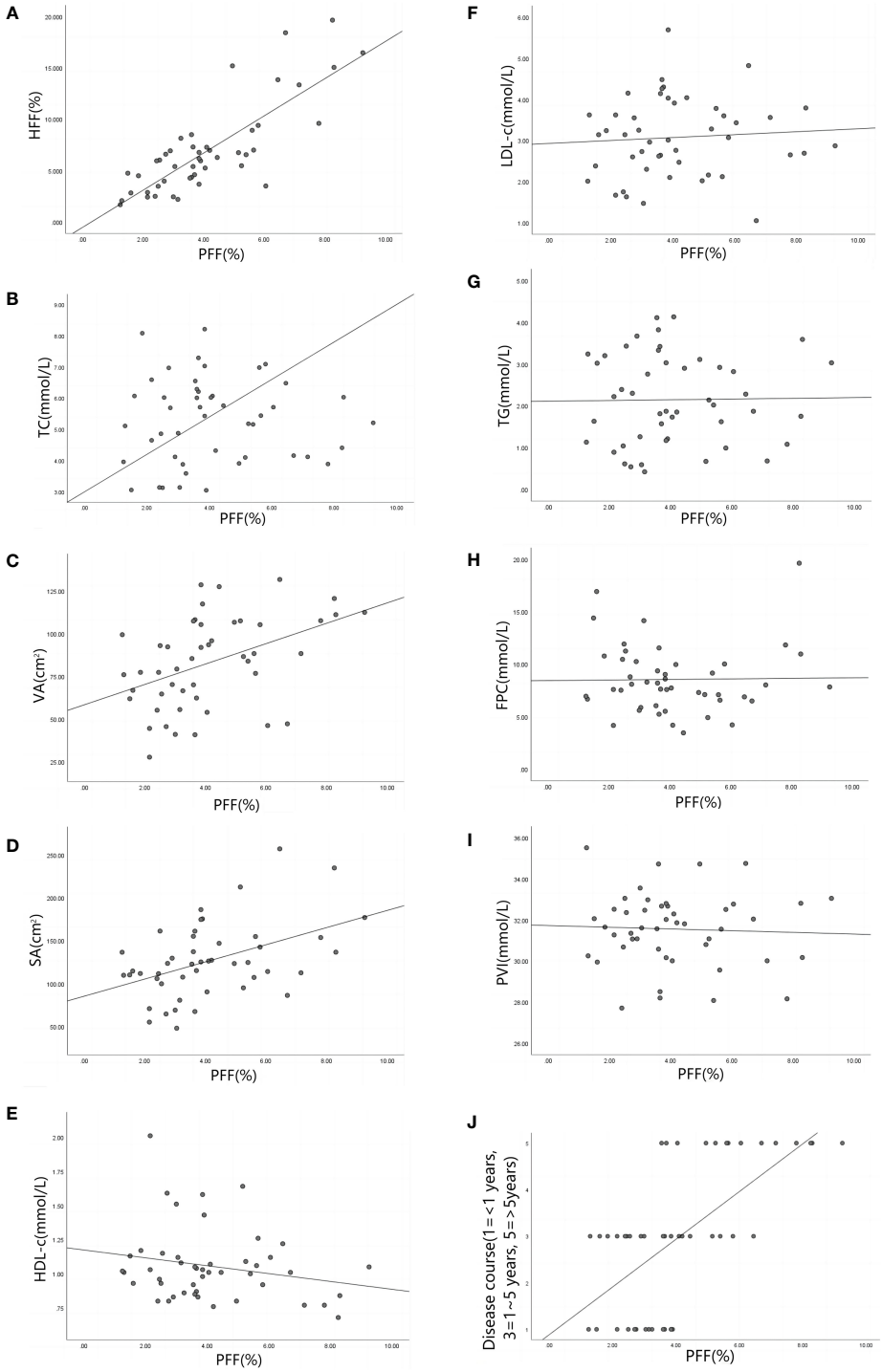


FIGURE 6
(A–J) are scatter plots of PFF and HFF(r=0.964, P<0.001), TC(r=0.236, P=0.437),VA(r=0.591, P<0.001), SA (r=0.321, P=0.033), HDL-c(r=-0.168, P=0.276), LDL-c(r=-0.002, P= 0.987),TG(r=0.676, P<0.001), FPC(r=0.385, P=0.194), PVI(r=-0.163, P=0.292) and Disease course(r=0.615, P<0.001) respectively.
Note: pancreatic fat fraction (PFF), hepatic fat fraction (HFF), total cholesterol (TC), visceral fat area (VA), subcutaneous fat area (SA), high density lipoprotein (HDL-c), low density lipoprotein (LDL-c), triglyceride (TG), fasting blood glucose (FPC), patio of pancreatic volume to body surface area (PVI).

In this study, 3.0T MRI qDIXon-WIP sequence was used to quantify pancreatic fat, which improved the solution to the problem of inverse calculation of water image and fat image in qDixon image. Under the condition of good consistency of gender, age and BMI matching between the experimental group and the control group, the HFF, PFF and intraperitoneal and external fat contents of the experimental group were higher than those of the control group, which reflects that there is a certain connection between abnormal fat metabolism and ectopic fat deposition. The accumulation of lipids in the the pancreas can lead to the blockage of signaling pathways and insulin resistance, thus leading to the release of inflammatory adipokines, and ultimately aggravating the deposition of fat in the

TABLE 4 Linear regression relationship between PFF and different indicators in T2DM patients.

Index of correlation	Non-standardized coefficients	Standardization coefficient	t value	P value
Constant	10.287	-	2.122	0.041
AGE (years)	-0.036	-0.181	-1.712	0.096
BMI (kg/m^2)	-0.05	-0.072	-0.917	0.366
HFF (%)	0.284	0.637	6.194	<0.001
PVI (cm^3/m^2)	-0.255	-0.233	-2.22	0.033
SA (cm^2)	0.003	0.068	0.468	0.643
VA (cm^2)	0.006	0.084	0.616	0.542
FPC (mmol/L)	-0.033	-0.056	-0.617	0.541
HDL-c (mmol/L)	0.709	0.096	0.956	0.346
LDL-c (mmol/L)	0.207	0.101	0.998	0.325
TC (mmol/L)	0.012	0.009	0.079	0.938
TG (mmol/L)	-0.329	-0.18	-2.074	0.046
Disease course (<1 years, 1~5 years, >5years)	0.758	0.303	3.117	0.004

BMI, body mass index; HFF, hepatic fat fraction; PVI, patio of pancreatic volume to body surface area; SA, subcutaneous fat area; VA, visceral fat area; FPC, fasting blood glucose; HDL-c, high density lipoprotein; LDL-c, low density lipoprotein; TC, total cholesterol; TG, triglyceride.

abdominal organs (17). However, abnormal glucose metabolism (decreased insulin secretion or insulin resistance) will lead to weakened liver cells' ability to metabolize fat, resulting in increased ectopic fat deposition (17, 20–22).

In this study, the PFF value of T2DM group was almost 2 times that of the normal control group (Table 2), which is similar to the study conducted by Tushuizen et al (23). However, in this study, the data in T2DM group conformed to the normal distribution and the patient sample size was sufficient. In the experimental group, HFF and PFF of patients showed a strong positive correlation, suggesting that liver fat deposition was closely related to pancreatic fat deposition, which was similar to the research results of van Geenen (24). Some of the differences in results may be related to assessment methods (ultrasound, CT, magnetic resonance), individual differences (psychological factors, diet, exercise, BMI, etc.), measurement methods (delineation of areas of interest, uneven distribution of fat deposits in the the pancreas) and other factors. T2DM patients have abnormal metabolism, which will cause the increase of TG. When the TG in the body is supersaturation in adipose tissue, lipids will be accumulated in non-fatty organs, such as the pancreas, etc., and pancreatic fat infiltration will promote the progression of T2DM and the increase of TG (5). In the experimental group, the moderate positive correlation between PFF and TG indicates that they have a close relationship. The study of Hu and Yamazaki showed that abdominal fat accumulation and abdominal fat deposition were related to diabetes and other risk factors (25, 26). The study of Yu, Van and Anderson showed that SA and VA in T2DM patients were also related to T2DM: intra-abdominal fat decreased the inhibitory effect of insulin on lipolysis by increasing gluconogenesis and insulin sensitivity (27, 28). In this study, PFF was moderately positively correlated with abdominal fat and weakly positively correlated with subcutaneous fat area, which also reflected that intra-abdominal and extra-abdominal fat deposition were related factors for pancreatic fat infiltration. Some studies also pointed out that there was a significant correlation between abdominal fat distribution and older patients (29), and the different course of T2DM patients led to certain differences in results. In addition, this study also compared the correlation between PFF and FPC, PVI, HDL-c, TC and LDL-c, and the results indicated that there was no significant correlation. The constructed linear regression equation points out that among the relevant indicators in this study, HFF, PVI, TG and Disease course have greater contribution to PFF, that is, these four factors are closely related to PFF.

The patients in the experimental group were divided into three groups according to the course of disease: course of disease ≤1 year, 1 year < course of disease < 5 years, and course of disease ≥5 years. The

TABLE 5 Kolmogorov-Smirnov(K) test of PFF values of T2DM patients and control volunteers with different disease stages.

Group	PFF (%)	Statistical variables	sample capacity	P value
control group	1.73 ± 0.697	0.098	48	0.200
disease course ≤1 year	2.28 (1.99~3.45)	0.289	14	0.002
disease course of 1-5 years	2.75 (2.35~4.40)	0.241	19	0.005
disease course > 5 years	6.46 ± 1.914	0.142	14	0.200

PFF, pancreatic fat fraction.

results suggest that pancreatic fat accumulation is higher in patients with long course of T2DM than in those with short course of T2DM. According to the linear regression analysis, the standardized regression coefficient for Disease course was 0.303, which points out the degree of fat accumulation is higher in those with long-standing diabetes. And as mentioned above, insulin resistance causes ectopic fat deposition, and pancreatic fat also accumulates in the progression of T2DM. The results of this study may partly explain that pancreatic fat infiltration is a gradual accumulation process in patients with long disease course, but the degree of fat accumulation is slower in patients with short disease course.

Limitations of this study: (1) Due to the small sample size, further sample expansion is needed to improve the reliability of the experimental results. (2) Due to the age distribution characteristics of the diabetic population, the age of T2DM patients included in this study ranged from 32 to 71 years old, and the corresponding age of normal control population was matched, and the data obtained had certain bias. (3) In this study, T2DM patients were randomly sampled, and subgroup analysis of patients with different clinical interventions was not performed. The degree of pancreatic fat infiltration is likely to be different in patients with different interventions. This study can further focus on the relationship between pancreatic fat deposition and T2DM intervention.

In this study, the qDixon-WIP sequence was used to conduct clinical experiments. The results showed that: (1) PVI decreased, while SA, VA, PFF and HFF increased in T2DM patients. (2) PFF was positively correlated with HFF, TG, abdominal fat area and subcutaneous fat area in T2DM patients. (3) The degree of pancreatic fat accumulation in patients with long course of disease was higher than that in patients with short course of disease. This sequence can be used in clinical research to quantitatively measure pancreatic fat content with good repeatability, which can provide reference for clinical assessment of pancreatic fat to achieve real-time monitoring of the occurrence and progression of diseases.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: The datasets generated during and/or analysed during the current study are not publicly available, but are available from the corresponding author on reasonable request. Requests to access these datasets should be directed to LiLing Long, cjr.longliling@vip.163.com.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (NO.2022-E460-01). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Material preparation and data collection were performed by JY, FX, TL, SL and QF. Data analysis were performed by JY, FX. The first draft of the manuscript was written by JY, FX and BL and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version. LL contributed to the study conception and design.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1140111/full#supplementary-material

SUPPLEMENTARY TABLE 1
Kolmogorov-Smirnov (K) test of normal distribution of experimental data.

SUPPLEMENTARY TABLE 2
Test results of PFF difference between T2DM patients and control group volunteers with different disease course.

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Assessment of the association between genetic factors regulating thyroid function and microvascular complications in diabetes: A two-sample Mendelian randomization study in the European population

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Background: Observational studies have identified a possible link between thyroid function and diabetic microangiopathy, specifically in diabetic kidney disease (DKD) and diabetic retinopathy (DR). However, it is unclear whether this association reflects a causal relationship.

Objective: To assess the potential direct effect of thyroid characteristics on DKD and DR based on Mendelian randomization (MR).

Methods: We conducted an MR study using genetic variants as an instrument associated with thyroid function to examine the causal effects on DKD and DR. The study included the analysis of 4 exposure factors associated with thyroid hormone regulation and 5 outcomes. Genomewide significant variants were used as instruments for standardized freethyroxine (FT4) and thyroid-stimulating hormone (TSH) levels within the reference range, standardized free triiodothyronine (FT3):FT4 ratio, and standardized thyroid peroxidase antibody (TPOAB) levels. The primary outcomes were DKD and DR events, and secondary outcomes were estimated glomerular filtration rate (eGFR), urinary albumin-to-creatinine ratio (ACR) in diabetes, and proliferative diabetic retinopathy (PDR). Satisfying the 3 MR core assumptions, the inverse-variance weighted technique was used as the primary analysis, and sensitivity analysis was performed using MR-Egger, weighted median, and MR pleiotropy residual sum and outlier techniques.

Results: All outcome and exposure instruments were selected from publicly available GWAS data conducted in European populations. In inverse-variance weighted random-effects MR, gene-based TSH with in the reference range was associated with DKD (OR 1.44; 95%CI 1.04, 2.41; P = 0.033) and eGFR (β: -0.031; 95%CI: -0.063, -0.001; P = 0.047). Gene-based increased FT3:FT4 ratio,

decreased FT4 with in the reference range were associated with increased ACR with inverse-variance weighted random-effects β of 0.178 (95%CI: 0.004, 0.353; P = 0.046) and -0.078 (95%CI: -0.142, -0.014; P = 0.017), respectively, and robust to tests of horizontal pleiotropy. However, all thyroid hormone instruments were not associated with DR and PDR at the genetic level.

Conclusion: In diabetic patients, an elevated TSH within the reference range was linked to a greater risk of DKD and decreased eGFR. Similarly, decreased FT4 and an increased FT3:FT4 ratio within the reference range were associated with increased ACR in diabetic patients. However, gene-based thyroid hormones were not associated with DR, indicating a possible pathway involving the thyroid-islet-renal axis. However, larger population studies are needed to further validate this conclusion.

KEYWORDS

thyroid function, diabetic kidney disease, diabetic retinopathy, Mendelian randomization, kidney function

Introduction

Diabetic Kidney Disease (DKD) and Diabetic Retinopathy (DR) are among the most crucial microvascular lesions in diabetes, and frequently occur concomitantly (1). The growing global prevalence of diabetes is also leading to an increase in the population affected by DKD and DR. DKD is a major contributor to End-stage Renal Disease (ESRD), with an estimated 91% of all new cases of diabetes-related ESRD attributed to Type 2 Diabetes Mellitus (T2DM) according to the US Renal Data System (2). In developed countries, nearly 40% of DKD patients eventually require dialysis (3). DR is a leading cause of blindness among adults, and it is projected that over 200 million people worldwide will develop DR by 2040 (4). The complex chain reaction initiated by diabetes may be attributed to the buildup of Advanced Glycation End Products (AGEs) and increased erythrocyte adhesion to endothelial cells, which is a key pathogenic mechanism of the vascular complications associated with T2DM (5, 6). There may also be potential associations with other factors, including thyroid function.

Thyroid-related diseases and diabetes mellitus are two of the most significant metabolic disorders that have a well-documented association (7–9). The presence of thyroid hormone receptors in the vascular endothelial tissue means that alterations in circulating thyroid hormone levels can contribute to the development and progression of vascular disease (10). Hence, the influence of thyroid function on diabetic microvascular complications is attracting increased attention. The regulation of thyroid function is intricate and involves multiple components, including the pituitary and hypothalamus, feedback mechanisms, and the thyroid's own characteristics and functions. The pituitary gland produces and releases thyroid-stimulating hormone (TSH), which stimulates the release of thyroxine from the thyroid gland. Thyroxine circulates in the body in a balanced state between its isolated protein-bound form and the bioavailable free form, referred to as free thyroxine (FT4). In both the thyroid and peripheral tissues, FT4 is converted to the active form of triiodothyronine (FT3), which can be assessed by the FT3:FT4 ratio in the circulation (11). Thyroid peroxidase antibodies (TPOAB), a biomarker of autoimmune thyroid disease, is a sensitive indicator of thyroid function and diverse physiological responses (12). Observational cross-sectional studies have revealed an independent association between the presence of subclinical hypothyroidism (SCH) and DKD (13). The results showed that DKD was negatively correlated with levels of free triiodothyronine (FT3) and free thyroxine (FT4), and positively correlated with thyroid-stimulating hormone (TSH) levels. In addition, low to normal levels of thyroid hormones were associated with the presence of massive albuminuria, and TSH and FT3 were found to be potential predictors of DKD (14–16). The hypothalamic-pituitary-thyroid axis plays a critical role in retinal development and increases retinal vascular density (17, 18). A recent study conducted in China explored the relationship between FT3 levels and DR in patients with T2DM who have normal thyroid function. Results showed a negative association between FT3 levels and DR in these patients (19). This observation was further supported by the finding that treatment of T2DM patients with thyroid hormones was associated with improvement in retinopathy (20). DKD and DR are interdependent risk factors for one another, with evidence suggesting a shared relationship with thyroid function (21–23). However, despite being influenced by common factors, current population-based clinical studies lack sufficient evidence to establish a direct causal link between thyroid function and the development of DKD and DR. Further research is needed to fully understand the underlying mechanisms and establish a clear relationship between these conditions.

Mendelian randomization (MR) is an analytical approach that leverages genetic variation to assess the causal relationship between independent and outcome variables in observational studies. This method is considered to be less susceptible to confounding or reverse causality compared to traditional observational analyses (24). The premise of MR is that if 7thyroid hormone levels have a direct impact on the development of DKD and DR, then genetic variants affecting thyroid function should also be associated with DKD and DR, with the magnitude of this association being consistent with the observed relationships.

The aim of this study was to investigate the potential causal relationship between thyroid function and DKD and DR using two-sample MR analysis. To complement the findings of MR, we conducted a further analysis using estimated glomerular filtration rate (eGFR) and urinary albumin to creatinine ratio (ACR) in patients with DM as secondary outcomes. The purpose of this complementary analysis was to provide additional evidence on the causal effect of thyroid function on DKD. Additionally, our study aimed to explore the causal effect of thyroid hormones on proliferative diabetic retinopathy (PDR), with the intention of identifying the need for increased protection of thyroid function in patients with severe DR lesions.

Methods

Data sources

In a genome-wide association study (GWAS) of thyroid traits among individuals of European ancestry, instrument-exposure associations were identified for FT4, FT3:FT4 ratio, TSH, and TPOAB based on single nucleotide polymorphisms (SNPs). Only SNPs that reached genome-wide significance levels ($p < 5 \times 10^{-8}$) were considered in this European population. To avoid linkage disequilibrium reactions and the potential double counting of similar genes within a certain range, screening conditions were set at $R^2 < 0.001$ and kb = 10,000. A GWAS of reference range FT4 levels was conducted using data from 72,167 European subjects, as published by Teumer et al. The study identified 31 genetic loci with significant associations with reference range FT4 levels, implicating a role for these loci in thyroid development, physiological function and transport of thyroid hormones, as well as metabolism of these hormones (25). In a study conducted by Panicker et al., GWAS data for the FT3:FT4 ratio was obtained. The results revealed that a SNP, rs2235544, located in the DIO1 gene was significantly associated with the FT3:FT4 ratio at a genome-wide level. The researchers found that the presence of the C allele at this SNP locus was associated with an increase in the activity of deiodinase 1, leading to an elevated FT3:FT4 ratio. This elevated ratio was found to affect the physiological function of the thyroid gland (26). Genetic susceptibility loci associated with TSH levels in the reference range were derived from two studies that identified a total of 61 susceptibility loci associated with TSH levels in the reference range, and these loci were strongly associated with the development of thyroid cancer and goitre (27, 28). Medici et al. and Schultheiss et al. conducted two GWAS studies that identified a total of five susceptibility loci associated with TPOAB that predicted which TPOAB positivity predisposed to the development of clinical thyroid dysfunction (29, 30). The studies were granted ethical clearance by the institutional review board. Table 1 displays the characteristics of the results from the Genome-Wide Association Study (GWAS).

The outcome measures were drawn from publicly available genetic association studies conducted in European populations. The primary outcomes were DKD and DR, and the raw data was obtained from the Finngen database (r8) (31), which included patients with all types of diabetes. The genetic association study cohort for DKD consisted of 3,676 cases and 283,456 controls, while the cohort for DR consisted of 8,942 cases and 283,545 controls. Secondary outcomes included eGFR and ACR in individuals with diabetes and PDR. The genetic association study data for eGFR in individuals with diabetes was obtained from the study by Pattaro et al. published in 2016, which consisted of 39 studies and a total of 55,114 individuals with diabetes. eGFR was calculated using the four-variable Modification of Diet in Renal Disease Study Equation (32). The genetic association study data for ACR in 5,825 individuals with diabetes and 46061 controls was obtained from the study by Teumer et al. published in 2016, and ACR was calculated as the ratio of urinary albumin to urinary creatinine to account for variations in urine concentration (33). The genetic association study cohort for PDR was also obtained from the Finngen database, consisting of 8,383 cases and 329,756 controls (31).

TABLE 1 The characteristics of GWAS studies on the outcomes.

Outcomes	Consortium	Sample size	Ethnicity	Web source
DKD	Finngen	3,676 cases and 283,456 controls	European	https://www.finngen.fi/en/access_results
DR	Finngen	8,942 cases and 283,545 controls	European	https://www.finngen.fi/en/access_results
PDR	Finngen	8,383 cases and 329,756 controls	European	https://www.finngen.fi/en/access_results
eGFR in diabetes	–	55,114 individuals	European	PMID: 26831199
ACR in diabetes	CKDgen	5,825 cases and 46061 controls	European	http://ckdgen.imbi.uni-freiburg.de/ PMID: 26631737

DKD, diabetic kidney disease; DR, diabetic retinopathy; PDR, proliferative diabetic retinopathy; eGFR, estimated glomerular filtration rate; ACR, urinary albumin-to-creatinine ratio.

Mendelian randomization analysis

In this study, the MR analysis tool was utilized to determine the causal relationship between thyroid function and various outcome indicators. SNPs were used as instrumental variables to estimate the causal effect. To ensure the validity of the results, three core assumptions were made: first, the genetic variations were associated with exposure factors; second, the genetic variations were independent of confounding factors; and third, the genetic variations only had an effect on the outcome through the exposure and not through any other pathways (Figure 1). To obtain the primary overall instrumental estimate, an Inverse-Variance Weighted Fixed-Effects MR method was employed, which considered all genetic variants as valid instruments without any pleiotropy. The individual instrumental estimates and their standard errors were then combined using the Inverse-Variance Weighted (IVW) method to produce the final MR results (34). In order to mitigate the impact of horizontal pleiotropy, where genetic variation has a significant effect on results *via* pathways other than exposure, the current study utilized three statistical methods: IVW random-effects (IVW-RE), weighted median (WM), and MR-Egger methods (35, 36). To ensure robust results, a range of sensitivity analyses were conducted, including heterogeneity tests, horizontal pleiotropy tests, funnel plot analysis, and a leave-1-variant-out analysis using the IVW-RE method, where one variable was excluded from the analysis in each iteration. The individual instrumental variables were analyzed using the Instrumental Variable Ratio (Wald) estimator.

In this study, the strength of all instruments was evaluated using the F statistic (calculated as $F = \beta^2_{exposure}/SE^2_{exposure}$). This approach was taken to ensure that weak instrumental variables do not influence the results of the causal estimation. The results showed that there were no weak instrumental variables in the study, as the F-statistic ranges for FT4, TSH, TPOAB and FT3:FT4ratio tools were 29-394, 29-576, 10-19, and 21, respectively. To check for the presence of pleiotropy, several sensitivity analyses were performed. The Q statistics (Cochran's Q for IVW and Rücker's Q for MR-Egger) were calculated to assess heterogeneity in individual causal effects, with p-values less than 0.05 indicating the presence of heterogeneity (37). The MR-Egger intercept term was used to evaluate horizontal pleiotropy, and a deviation from zero indicates directional pleiotropy. The slope of the MR-Egger regression was used to provide a valid MR estimate in the presence of horizontal multiplicity (38, 39). A complementary weighted median method was also employed, which assumes that at least 50% of the inverse-variance is valid and ranks the inverse of the weighted variance of MR estimates for each inverse-variance (34). The MR pleiotropy residual sum and outlier (MR-PRESSO) outlier test was performed to correct for horizontal pleiotropy *via* outlier removal (40). The effect values are expressed as β when the ending variable is a continuous variable and as odds ratio (OR) when it is a dichotomous variable.

Results

MR estimates of causal effects of thyroid function on DKD

Figure 2 demonstrates the MR estimation between thyroid function and DKD. After conditional screening and MR-PRESSO test, we identified 16 of 31 SNPs for FT4 within the reference range, 1 of 1 SNP for FT3:FT4 ratio (the DIO1, rs2235544), 39 of 61SNPs for TSH within the reference range, 4 of 5 SNPs for TPOAB concentration. Genetically predicted TSH was associated with DKD with an IVW-RE OR of 1.44 (95% CI 1.04-2.41; P = 0.033) (Figure 2, eTable 2, eFigure 2A, B in the Supplement) with similar and a more significant result in IVW-FE (OR=1.44, 95% CI, 1.10-1.89; P = 0.009), and the results were similar with the MR-Egger and WM analyses. We did not find a significant risk association between FT4 (OR=0.83, 95% CI 0.67-1.03; P = 0.093) and TPOAB (OR=1.17, 95% CI 0.57-2.38; P = 0.672) in the reference range and DKD (Figure 2, eTable 1, 3, eFigure 1A, B, 3A, B in the Supplement). A genetically predicted 1 SD–increase in FT3:FT4

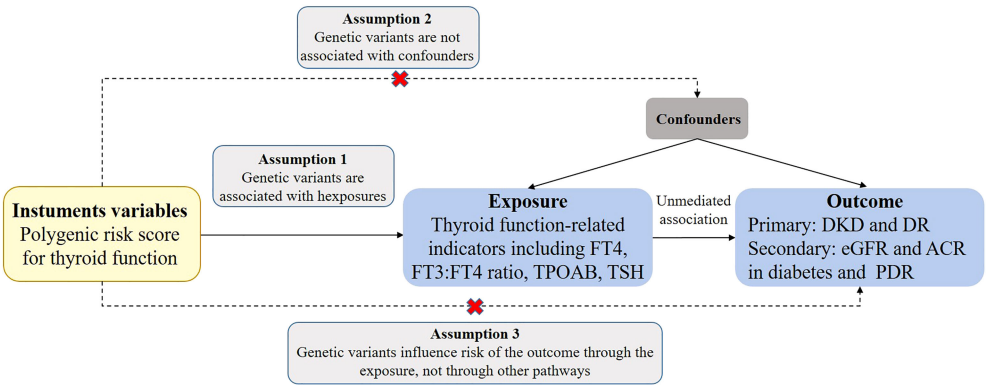


FIGURE 1
Assumptions of a Mendelian randomization analysis for thyroid function and risk of DKD and DR. Broken lines represent potential pleiotropic or direct causal effects between variables that would violate Mendelian randomization assumptions. FT4, free thyroxine; FT3, free triiodothyronine; TSH, thyroid-stimulating hormone; TPOAB, thyroid peroxidase antibodies; DKD, diabetic kidney disease; DR, diabetic retinopathy; PDR, proliferative diabetic retinopathy; eGFR, estimated glomerular filtration rate; ACR, urinary albumin-to-creatinine ratio.

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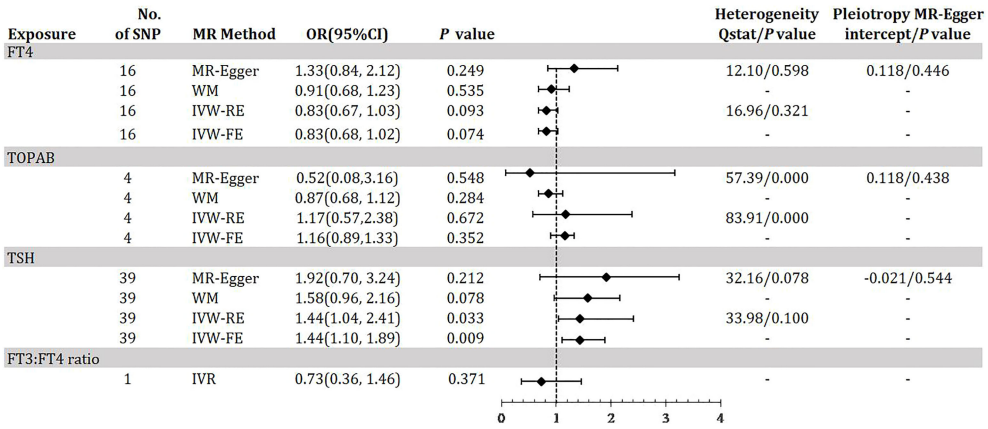


FIGURE 2
Odds ratio for association of genetically predicted thyroid function with DKD. FT4, free thyroxine; FT4, free thyroxine; FT3, free triiodothyronine; TSH, thyroid-stimulating hormone; TPOAB, thyroid peroxidase antibodies; DKD, diabetic kidney disease; CI, confidence internal; OR, odds ratio; IVW-FE, inverse-variance weighted fixed-effects MR; IVW-RE, inverse-variance weighted random-effects MR; MR, mendelian randomization; WM, weighted median; IVR, instrumental variable ratio (Wald) estimator; SNP, single-nucleotide polymorphism. *P* value for heterogeneity based on Cochran's Q statistic for IVW, and Rücker's Q for MR-Egger.

ratio by the C allele was not associated with increased DKD with an OR of 0.73 (95% CI 0.36-1.46; P =0.371) (Figure 2, eTable 1 in the Supplement). There was some evidence of heterogeneity based on Q-statistic (Q-value $_{IVW}$ = 83.91, P-value = 0.000; Q-value $_{MR\text{-}Egger}$ = 57.39, P-value = 0.00) for the TPOAB analysis. Consequently, weights were penalized for the IVW method. The FT4 and TSH variants were distributed symmetrically about the combined effect size in the funnel plot (eFigure 1C, 2C in the Supplement), and MR-Egger did not show evidence of horizontal pleiotropy (FT4: P $_{for\ MR\text{-}Egger}$ = 0.446; TSH: P $_{for\ MR\text{-}Egger}$ = 0.544) (Figure 2). Because TPOAB has fewer relevant instrumental variables, the causal effects of its funnel plot are not symmetric (eFigure 3C in the Supplement). However, the MR-Egger intercept that we performed did not show evidence of horizontal pleiotropy (P $_{for\ MR\text{-}Egger}$ = 0.438) (Figure 2). These results show that no directional pleiotropic effects are present in our study. The leave-one-out test did not identify any thyroid function-related variants that had a strong effect on the overall results (eFigure 1D,2D, 3 in the Supplement).

MR estimates of causal effects of thyroid function on DR

Figure 3 demonstrates the MR estimation between thyroid function and DR. After conditional screening and MR-PRESSO test, we identified 16 of 31 SNPs for FT4within the reference range, 1of1SNP forFT3:FT4 ratio (the DIO1, rs2235544), 37 of 61SNPs for TSH within the reference range, 4 of 5 SNPs for TPOAB concentration. We did not find any statistically

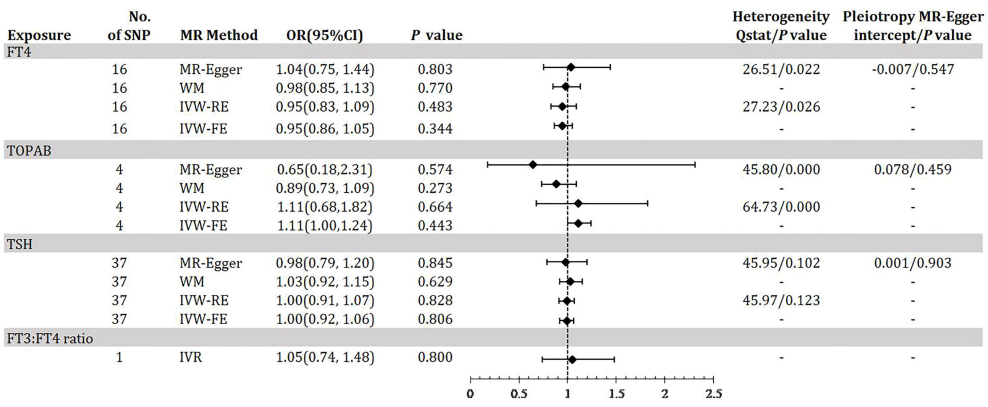


FIGURE 3
Odds ratio for association of genetically predicted thyroid function with DR. FT4, free thyroxine; FT4, free thyroxine; FT3, free triiodothyronine; TSH, thyroid-stimulating hormone; TPOAB, thyroid peroxidase antibodies; DR, diabetic retinopathy; CI, confidence internal; OR, odds ratio; IVW-FE, inverse-variance weighted fixed-effects MR; IVW-RE, inverse-variance weighted random-effects MR; MR, mendelian randomization; WM, weighted median; IVR, instrumental variable ratio (Wald) estimator; SNP, single-nucleotide polymorphism. *P* value for heterogeneity based on Cochran's Q statistic for IVW, and Rücker's Q for MR-Egger.

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significant genetic risk association between thyroid function-related instruments and DR by IVW-RE (FT4: OR=0.95, 95% CI 0.83-1.09; P=0.483; TPOAB: OR=1.11, 95% CI 0.68-1.82; P=0.664; TSH: OR=1.00, 95% CI 0.91-1.07; P=0.828; Figure 3, eTable 4-6, eFigure 4-6, A, B in the Supplement), which is similar to the results of IVW-FE, MR-Egger and WM analyses. A genetically predicted 1 SD–decrease in FT3:FT4 ratio by the C allele was not associated with increased DR with an OR of 1.05 (95% CI, 0.74-1.48; P =0.800) (Figure 2, eTable 4 in the Supplement). There was some evidence of heterogeneity in the analysis regarding FT4 (Q-value $_{IVW}$ = 27.23, P-value = 0.026; Q-value $_{MR\text{-}Egger}$ = 26.51, P-value = 0.022) and TPOAB (Q-value $_{IVW}$ = 64.73, P-value = 0.000; Q-value $_{MR\text{-}Egger}$ = 45.80, P-value = 0.000) according to Q-statistics. The FT4 and TSH variants were distributed symmetrically about the combined effect size in the funnel plot (eFigure 4C, e5C in the Supplement), and MR-Egger did not show evidence of horizontal pleiotropy (FT4: P $_{for\ MR\text{-}Egger}$ = 0.547; TSH: P $_{for\ MR\text{-}Egger}$ = 0.459) (Figure 3). Because TPOAB has fewer relevant instrumental variables, the causal effects of its funnel plot are not symmetric (eFigure 6C in the Supplement). However, the MR-Egger intercept that we performed did not show evidence of horizontal pleiotropy (P $_{for\ MR\text{-}Egger}$ = 0.903) (Figure 3). These results show that no directional pleiotropic effects are present in DR study. The leave-one-out test did not identify any thyroid function-related variants that had a strong effect on the overall results (eFigure 4D, 5D, 6D in the Supplement).

MR estimates of causal effects of thyroid function on eGFR and ACR in diabetes

Figure 4 shows the MR estimation between thyroid function and renal impairment indicators eGFR and ACR in diabetic patients to further reflect the genetic association with DKD. After linkage disequilibrium screening and MR-PRESSO assay, we identified a total of 10 out of 31 SNPs for FT4 within the reference range, 1 SNP for FT3:FT4 ratio (DIO1, rs2235544), 19 out of 61 SNPs for TSH in the reference range, and 4 SNPs for TPOAB concentration. We found that IVW-RE genetically predicted TSH was negatively correlated with eGFR, i.e., for 1-sd increase in TSH in the reference range, eGFR decreased by 0.031 (95% CI -0.063, -0.001; P=0.047), a result that was more significant in IVW-FE (Effect: -0.031, 95% CI -0.057, - 0.005; P=0.018) was more significant and similar to the results of WM analysis (Figure 4, eTable 8, eFigure 8A, B in the Supplement). No significant association between other thyroid function predictors and eGFR was found (eTable 7,9, 7 A-B, 9 A-B in the Supplement). The IVW-RE OR for ACR per SD of FT4 within the reference range was -0.078 (95% CI -0.142, -0.014; P = 0.017) (Figure 4, eFigure 10 A-B in the Supplement). Results were similar for IVW-FE (P = 0.015), MR-Egger (P = 0.073) and WM (P = 0.043) analysis. Notably, we found that a 1 SD increase in the FT3:FT4 ratio of the C allele was associated with an increase in ACR with an effect of 0.178 (95% CI, 0.004-0.353; P=0.046), a result consistent with the trend in FT4

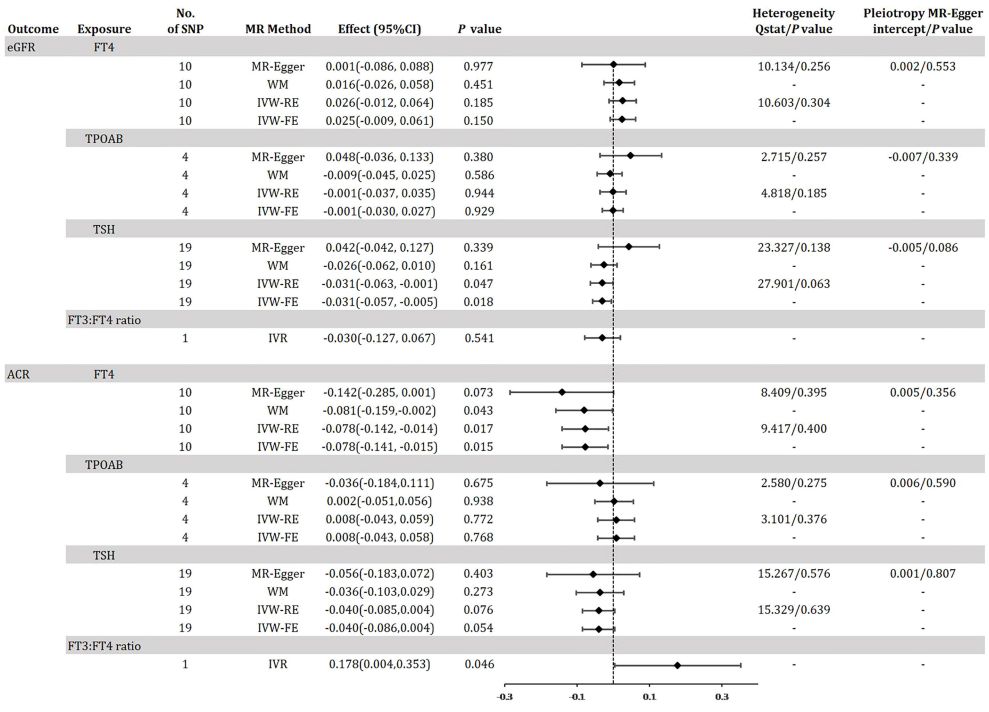


FIGURE 4
Odds ratio for association of genetically predicted thyroid function with eGFR and ACR in diabetes. FT4, free thyroxine; FT4, free thyroxine; FT3, free triiodothyronine; TSH, thyroid-stimulating hormone; TPOAB, thyroid peroxidase antibodies; eGFR, estimated glomerular filtration rate; ACR, urinary albumin-to-creatinine ratio; CI, confidence internal; OR, odds ratio; IVW-FE, inverse-variance weighted fixed-effects MR; IVW-RE, inverse-variance weighted random-effects MR; MR, mendelian randomization; WM, weighted median; IVR, instrumental variable ratio (Wald) estimator; SNP, single-nucleotide polymorphism. *P* value for heterogeneity based on Cochran's Q statistic for IVW, and Rücker's Q for MR-Egger.

results (eTable 10 in the Supplement). There was no evidence of heterogeneity based on Q-statistic for analyses of all thyroid function indicators ($P_{for\ het} > 0.05$). In our study, we found no evidence of pleiotropy, i.e., the *P* values for the pleiotropy of FT4, TSH and TPOAB were all greater than 0.05(eFigure 7-12C in the Supplement). The leave-one-out test did not identify any thyroid function-related variants that had a strong effect on the overall results (eFigure 7-12D in the Supplement).

MR estimates of causal effects of thyroid function on PDR

Figure 5 demonstrates the MR estimation between thyroid function and PDR. We did not find any statistically significant genetic risk association between thyroid function-related instruments and DR (eTable13-15, eFigure 13-15A, B in the Supplement). The MR-Egger intercept that we performed did not show evidence of horizontal pleiotropy (C of eFigure 13-15 in the Supplement), and the leave-one-out test did not identify any thyroid function-related variants that had a strong effect on the overall results (D of eFigure 13-15 in the Supplement). These results are similar to the MR analysis of DR.

Discussion

The current study, to the best of our knowledge, is the first to assess the relationship between thyroid function and diabetic microvascular complications, including DKD and DR, using MR analysis. Using publicly available GWAS data from European populations, we have made a novel finding that there is a potential genetic influence on TSH levels in the normal range that is associated with DKD, a hypothesis that was supported by the evidence in the eGFR. Furthermore, we examined the risk association between genetically determined thyroid function and ACR, and found that both FT4 and FT3:FT4 ratio may be genetic factors that are implicated in ACR. However, the combined genetic effect of all thyroid function indices did not provide strong evidence for a direct association with DR and PDR.

The thyroid-islet-renal axis represents a range of phenotypes that are dependent on phenotypes upstream of the axis and also on negative feedback mechanisms downstream. Both insulin and thyroid hormones are affected by autoimmune pathology, are part of the metabolic syndrome, and affect cellular metabolism. The pathophysiological association between diabetes and thyroid dysfunction is thought to be the result of the interaction of various biochemical, genetic and hormonal dysfunctions (41). As the most important microvascular complication of diabetes, the association between DKD and thyroid function is increasingly being demonstrated. We derived the presence of a genetic-based effect of thyroxine on DKD through multiple MR stratification analyses, based on unique and shared genetic tools, which is qualitatively different from extant epidemiological studies. Our study found that TSH levels in the reference range were positively associated with DKD risk and negatively associated with eGFR, i.e., elevated TSH may increase the risk of developing DKD as well as decrease eGFR. This result was confirmed in several observational studies. Renal function is directly correlated with thyroid function, as reflected by the positive correlation between TSH and serum creatinine and the negative correlation with eGFR (42, 43). Even when thyroid function is within the normal range, patients with DKD have higher TSH levels than diabetic patients without DKD (44, 45). A recent study reconfirmed that levothyroxine treatment reduced urinary albumin excretion in patients with early DKD with mildly elevated TSH levels and positive serum TPOAB (46) In addition, our study identified a risk association between FT3/FT4 and FT4 and ACR, and although this result was not statistically significant in DKD events and eGFR, the effect values showed a reliable and consistent trend. FT4 SNPs were derived from associations between

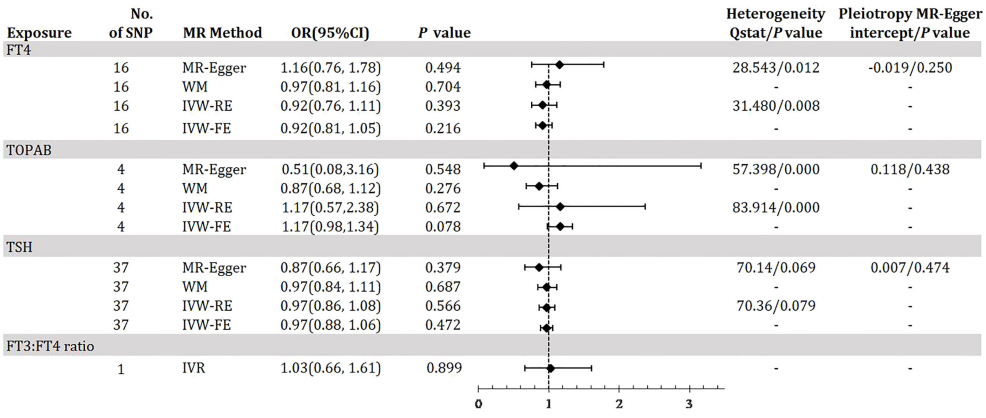


FIGURE 5
Odds ratio for association of genetically predicted thyroid function with PDR. FT4, free thyroxine; FT4, free thyroxine; FT3, free triiodothyronine; TSH, thyroid-stimulating hormone; TPOAB, thyroid peroxidase antibodies; PDR, proliferative diabetic retinopathy; CI, confidence internal; OR, odds ratio; IVW-FE, inverse-variance weighted fixed-effects MR; IVW-RE, inverse-variance weighted random-effects MR; MR, mendelian randomization; WM, weighted median; IVR, instrumental variable ratio (Wald) estimator; SNP, single-nucleotide polymorphism. *P* value for heterogeneity based on Cochran's Q statistic for IVW, and Rücker's Q for MR-Egger.

individuals with FT4 levels in the reference range and no evidence of thyroid disease. In contrast, TSH instrumentation within the reference range is associated with hypothyroidism and hyperthyroidism. These differences may emphasize that normal variation in TSH and thyroid function drives the association of instrumentation with DKD. Another possible explanation is that although ACR and eGFR are the most commonly used clinical tools to assess chronic kidney disease, one study found no correlation between serum creatinine and ACR in patients with T2DM (47), so the association between thyroid function and them is informative but not determinative for the risk of DKD events. Clinical evidence found reduced FT4 and elevated TSH in the DKD population compared to non-DKD patients, and hypothyroidism was associated with increased ACR or reduced eGFR in patients with T2DM, and hypothyroid patients with T2DM exhibited higher ACR and urinary transferrin excretion (48), which is consistent with our results. The relationship between FT3:FT4 and DKD is unclear, but studies are currently being conducted in other areas related to diabetes. For example, a recent report from southern China found that low FT3/FT4 was associated with a poor prognosis of acute myocardial infarction in T2DM patients with normal thyroid function (49). Another MR study showed that genetically based high FT3/FT4 was associated with an increased risk of atrial fibrillation (12). In addition, a Belgian report showed that FT3:FT4 in late pregnancy was positively associated with gestational diabetes, adverse pregnancy outcomes and poor metabolic profile in the early postpartum period (7).

In our present study no correlation between thyroid hormones and DR was found, despite our stratification of DR. There is conflicting evidence regarding the association between DR and thyroid function (50–52), with some studies suggesting no significant association while others indicating a possible link. A decrease in thyroid hormone or SCH may increase the probability of DR, PDR, and diabetic macular edema (53–55). Lin et al. first retrospectively found that high TSH serum levels were associated with an increased prevalence of DR in diabetic patients, and then found *in vitro* that high glucose stimulated apoptosis and mitochondrial dysfunction in human peripapillary cells, which could be attributed to co-stimulation of glucose and high TSH (56). These studies supporting the association of thyroid function with DR are based on Asian populations, whereas our study was conducted in a European population. Clinical observational studies are difficult to control for confounders and multiple biases making it difficult to derive a direct causal association between exposure and outcome, therefore the relationship between thyroid function and DR needs to be further demonstrated in larger well-designed trials.

The pathogenesis of abnormal thyroid function is associated with endothelial dysfunction, hyperlipidemia and atherosclerosis (57–61), which can increase the risk of diabetes and its complications (62, 63). Diabetes often leads to hyperlipidemia, which increases the risk of atherosclerotic vascular disease, characterized by arterial lesions affected from the intima, usually preceded by accumulation of lipids and complex sugars, hemorrhage and thrombosis, followed by fibrous tissue proliferation and calcium deposition, as well as progressive chemosis and calcification of the arterial middle layer, leading to thickening and sclerosis of the arterial wall and narrowing of the lumen (64, 65). DKD and DR are common result of hyperglycemia-induced accumulation of AGEs, which is inextricably linked to microangiopathy (66, 67). Abnormal thyroid hormone secretion not only directly disrupts endothelial function, but also exacerbates the damage to endothelial cells by the hyperglycemic state, thus contributing to the development of DKD and DR. In addition, abnormal thyroid hormone secretion decreases endothelial nitric oxide availability, which further promotes microvascular damage in diabetic patients (68, 69). Thus, thyroid hormones protect the endothelium of diabetic microvessels from degeneration, which may be reliable evidence to support our main results.

This study exhibits several strengths that warrant investigation. Firstly, the research employed MR methods to evaluate gene-based causality of FT4, TSH, TPOAB, and FT3:FT4 in DKD and DR. MR analysis provides a robust estimate of causality by minimizing reverse causal effects or confounding factors. Secondly, the study used eGFR and ACR as indicators for DKD evaluation, which strengthens the causal relationship between thyroid function and renal function in diabetic patients. Thirdly, the two-sample MR approach was utilized to assess the genetic association between thyroid function and diabetic microvascular complications from various perspectives. Fourthly, the study used multiple sensitivity analyses, such as the simple median, weighted median, and MR-Egger methods, to ensure consistent and robust causal estimation. Finally, the aggregated statistics of the GWAS were collected from European populations, providing a larger sample size than epidemiological studies, and suggesting a more reliable cause-and-effect relationship. Despite meeting the 3 core assumptions, there are still limitations to our MR study. Unobserved pleiotropy, beyond vertical pleiotropy, may exist. Additionally, the lack of an available FT3 instrument limits causal evidence for a genetic association with DKD and DR. Finally, race-based findings may limit generalizability to other populations. Further clinical studies with larger samples are needed to validate these issues.

In conclusion, our study provides direct evidence supporting that genetically based high TSH levels are associated with low eGFR and high DKD risk in diabetic patients, and that ACR in diabetic patients is negatively correlated with FT4 in the reference range and positively correlated with FT3:FT4. We found no genetic evidence of thyroid function associated with DR. These findings suggest that maintaining normal thyroid function and regulation of thyroid hormone secretion may be effective in preventing microvascular complications in diabetes, particularly DKD. Further larger population-based studies are necessary to investigate the causal relationship between thyroid function and diabetic microangiopathy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

HL and ML conceived and designed the study. SD and AD performed the initial data source acquisition. HL examined the data and performed the data analysis. SZ made methodological recommendations for the article. HL wrote the initial manuscript and MZ checked the manuscript and approved the final manuscript. All listed authors made substantial contributions to the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1126339/full#supplementary-material

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The concordance and discordance of diabetic kidney disease and retinopathy in patients with type 2 diabetes mellitus: A cross-sectional study of 26,809 patients from 5 primary hospitals in China

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Introduction: Diabetic kidney disease (DKD) and diabetic retinopathy (DR) share similar pathophysiological mechanisms. However, signs of DKD may be present at diagnosis of diabetes without retinopathy. Risk factors for the development of DKD and DR may not be identical.

Methods: This study aimed to evaluate the concordance and discordance between DKD and DR by investigating the distribution of DKD and DR in patients with type 2 diabetes mellitus from 5 Chinese cities. A total of 26,809 patients were involved in this study. The clinical characteristics were compared among patients based on the presence of DKD and DR. Logistic regression models were used to analyze the independent risk factors of DKD and DR.

Results: The prevalence of DKD and DR was 32.3% and 34.6%, respectively. Among eligible patients, 1,752 patients without DR had an increased urinary albumin-to-creatinine ratio (ACR) or reduced estimated glomerular filtration rate (eGFR), and 1,483 patients with DR had no DKD. The positive predictive value of DR for DKD was 47.4% and negative predictive value was 67.1%. Elder age, male gender, a longer duration of disease, higher values of waist circumference and HbA1c were associated with both DR and DKD. A lower educational level was associated with DR. Higher BP and TG would predict increased prevalence of DKD.

Conclusions: DKD and DR shared many risk factors, but a significant discordance was present in patients with type 2 diabetes mellitus. DKD was more strongly associated with blood pressure and triglycerides than DR.

KEYWORDS

diabetic kidney disease, diabetic retinopathy, type 2 diabetes mellitus, metbolic syndrome, discordance

1 Introduction

Diabetic kidney disease (DKD) affects 20-40% of patients with diabetes (1, 2). The prevalence of DKD or chronic kidney disease (CKD) in Chinese patients with diabetes is increasing (3, 4) as type 2 diabetes mellitus becomes an epidemic disease. DKD is diagnosed based on the presence of albuminuria and/or the reduced estimated glomerular filtration rate (eGFR < 60 mL/min/1.73m^2) in the absence of signs or symptoms of other kidney diseases. Previous studies suggested that DKD might not solely develop from microalbuminuria to macroalbuminuria to azotemia as Mogensen proposed (5, 6). The reduced eGFR without albuminuria has been frequently reported in patients with type 1 diabetes mellitus and type 2 diabetes mellitus (7).

Diabetic retinopathy (DR), another microvascular complication, is supposed to share similar pathophysiological mechanisms with DKD, and the two are frequently found simultaneously. El-Asrar et al. reported that type 1 diabetes mellitus patients with DR were 13.39 times more likely to develop DKD than those without DR (8). Results of a meta-analysis showed that patients with DR were nearly 4 times more likely to be complicated by DKD. Patients with DKD were twice more likely to be diagnosed as DR (9). DR was typically used as an indicator of DKD in the differential diagnosis (10). However, discordance of DKD and DR was also discussed. Signs of DKD may be present in the time of diagnosis or in type 2 diabetes mellitus patients without retinopathy (11). It was reported that risk factors for the development of DKD and DR might not be identical. Japanese scholars demonstrated that systolic blood pressure (SBP) variability was an independent predictor for the development and progression of DKD, rather than DR, in type 2 diabetes mellitus patients (12). Genetic data revealed that the DR-related single nucleotide polymorphisms did not have an individual or cumulative genetic effect on the risk of DKD, eGFR status or end-stage renal disease (ESRD) outcomes of type 2 diabetes mellitus patients in Taiwan (13). The most important evidence originates from a series of randomized controlled trials published in recent years (i.e., new classes of antidiabetic drugs have different preventive effects on DKD and DR) (14). A meta-analysis showed that hypoglycemic medicine glucagon-like peptide 1 receptor agonists (GLP-1RA) reduced the risk of kidney disease progression by 18% (hazard ratio (HR), 0.82, 95% confidence interval (CI): 0.75-0.89, P<0.001), while sodium-glucose cotransporter 2 (SGLT2) inhibitors reduced the mentioned risk by 38% (HR, 0.62, 95%CI, 0.58-0.67, P<0.001) (15). The preventive effects of GLP-1RA and SGLT2 inhibitors on DR in humans have not yet been reported (14). Taken together, the concordance and discordance of DKD and DR in patients with type 2 diabetes mellitus exist and need to be further elaborated.

The present study aimed to investigate the concordance and discordance between DKD and DR, as well as the relevant risk factors.

2 Methods

2.1 Study subjects

Patients with type 2 diabetes mellitus who were admitted to Ruijing diabetes hospital chains (China) were enrolled in this study. Five hospitals from Beijing, Lanzhou, Harbin, Chengdu, and Taiyuan were included. The data were collected continuously from March 2016 to December 2021. The inclusion criteria were as follows: diagnosis of type 2 diabetes mellitus was based on the diagnostic criteria presented by the World Health Organization (WHO) in 1999 (16), and patients who aged 18 - 80 years old. Those patients who had severe heart (New York Heart Association III/IV), liver (severe hepatic impairment or liver failure), lung (conditions that may predispose to hypoxemia), or renal diseases (primary nephrotic syndrome, glomerulonephritis, obstructive renovascular disease, nephrectomy, renal transplant, etc.), and those were pregnant, or had been diagnosed with type 1 diabetes mellitus, special type of diabetes or gestational diabetes were excluded. This study was approved by the Ethics Committee of Tsinghua Changgung Hospital (Beijing, China; Approval No. [2016] 004). The flowchart of screening patients was shown in Figure 1.

2.2 Data collection

Patients' data were collected at the first visit in each hospital through face-to-face interviews, including demographic data, educational level, smoking status, individual medical history (hypertension, dyslipidemia, and cardiovascular disease), and family history of diabetes mellitus. Blood samples were collected after an overnight (10-14 h) fasting, and the laboratory tests were conducted in the local hospital, including liver function, renal function, fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), and lipid profiles (low-density lipoprotein cholesterol (LDL-C), high-density

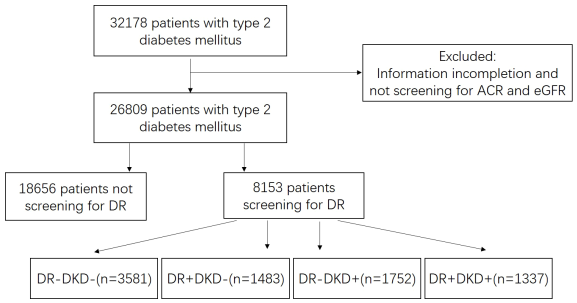


FIGURE 1
Flowchart of screening patients. ACR, urinary albumin-to-creatinine ratio. EGFR, estimated glomerular filtration rate. DR, diabetic retinopathy. "+" means positive, and "-" means negative.

lipoprotein cholesterol (HDL-C), triglyceride (TG)). HbA1c was measured by high-performance liquid chromatography using the ADAMS A1c, HA-8180T analyzer (Array, Tokyo, Japan) or MQ-2000 PT Balk analyzer (Huazhong, Shanghai, China), which was the second-level reference for glycosylated hemoglobin of International Clinical Chemistry Committee. Blood lipids, liver functions and kidney functions were assessed by automated analysis (AU5800; Beckman Coulter Inc., Brea, CA, USA). Urinary albumin was determined using a DADE BEHRING BN II analyzer (Siemens, Munich, Germany) by nephelometry (N antiserum to Human Albumin Assay, Dade Behring). Urinary creatinine concentration was measured *via* a Hitachi 7600 analyzer (Hitachi, Tokyo, Japan) using the sarcosine oxidase-PAP method. The urinary albumin-to-creatinine ratio (ACR) was computed and was reported in milligrams per gram (mg/g). Retinopathy status was assessed by fundus photography (TRC-NW100 camera; Nikon, Tokyo, Japan), and all images were graded by an experienced ophthalmologist. Diagnostic criteria of DR were based on the worse eye according to international clinical diabetic retinopathy and diabetic macular edema disease severity scales published in 2002 (17). All the laboratories participated in the quality control program as requested by the authority. All data were automatically downloaded from hospital information system.

DKD was defined as elevated urinary ACR (≥30 mg/g), or reduced eGFR (<60 mL/min/1.73 m^2), or both, for longer than 3 months, excluding clinically significant renal diseases through medical history and laboratory results, in accordance with the current guidelines of Kidney Disease: Improving Global Outcomes (KDIGO) (18, 19). The eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) study formula (20) as follows: 186 × [serum creatinine (mg/dL)] - 1.154×(age) - 0.203 × (0.742 if female). The diagnosis of albuminuria was divided into three stages according to ACR (ACR < 30 mg/g was defined as non-albuminuria, 30 mg/g ≤ ACR < 300 mg/g as microalbuminuria, and ACR ≥ 300 mg/g as macroalbuminuria).

Diagnosis of metabolic syndrome was made by presence of any three or more of the following (21): 1. Abdominal obesity (central obesity): waist circumstance ≥90 cm in men or ≥85cm in women. 2. Hyperglycaemia: FPG ≥ 6.1 mmol/L or OGTT 2hPG ≥ 7.8 mmol/L and/or confirmed diabetes that is under treatment. 3. Hypertension: blood pressure ≥130/85 mmHg and/or diagnosed and on antihypertensive therapy. 4. Fasting TG ≥ 1.70 mmol/L. 5. Fasting HDL-C < 1.04 mmol/L.

2.3 Statistical analysis

SPSS 23.0 software (IBM, Armonk, NY, USA) was used for data analysis. Normally distributed data were expressed as the mean ± standard deviation (SD), and abnormally distributed data were expressed as median (interquartile range). The χ^2 test was used to compare the clinical categorical variables among different groups. Logistic regression models were established to analyze the independent risk factors of DKD and DR. Risk factors included age (every 10 years), gender (female as 0, male as 1), duration of disease (every 5 years), educational level (junior school or below as 1, high school or above as 2), body mass index (BMI, < 24 kg/m^2 as 1, ≥24 kg/m^2 as 2), waist circumference (every 10 cm), smoking history (never as 0, with smoking history as 1), HbA1c (< 7% as 1, 7% ~ 9% as 2, ≥ 9% as 3), systolic blood pressure (SBP, <140 mmHg as 1, 140mmHg ~ 160 mmHg as 2, ≥160 mmHg as 3), LDL-C (< 2.6 mmol/L as 1, 2.6 mmol/L ~ 3.3 mmol/L as 2, ≥ 3.3 mol/L as 3), TG (< 1.7 mmol/L as 1, 1.7 mmol/L ~ 5.0 mmol/L as 2, ≥5.0mol/L as 3), and DR (absent as 0, non-proliferative retinopathy (NPDR) as 1, and proliferative retinopathy (PDR) as 2). P <0.05 indicated statistical significance.

3 Results

3.1 Clinical characteristics of patients with type 2 diabetes mellitus

A total of 26,809 patients with type 2 diabetes mellitus were involved in this study. There were 14,813 (55.3%) male patients and 11,996 (44.7%) female patients. The average age, duration of disease, BMI, and HbA1c were 59.2 ± 10.7 years old, 8.6 ± 6.9 years, 25.2 ± 3.4 kg/m^2, and 8.6 ± 2.1% (70 mmol/mol), respectively.

Data of ACR and eGFR were available for all patients. There were 18,875 (70.4%) patients with eGFR ≥ 90mL/min/1.73m^2, 6,685 (24.9%) patients with eGFR equal to 60-90 mL/min/1.73m^2, 1,053 (3.9%) patients with eGFR equal to 30-60 mL/min/1.73m^2, and 196 (0.7%) patients with eGFR < 30 mL/min/1.73m^2. The majority of patients had normal albuminuria level (69.1%), and 23.5% and 7.4% of them had microalbuminuria or macroalbuminuria, respectively. According to the latest diagnostic criteria for DKD, there were 8,660 (32.3%) patients with eGFR < 60 mL/min/1.73m^2 and/or ACR ≥ 30 mg/g, including 384 (1.4%) patients without albuminuria (eGFR < 60 mL/min/1.73m^2 and ACR < 30 mg/g), 7,411 (27.6%) patients with eGFR ≥ 60 mL/min/1.73m^2 and ACR ≥ 30 mg/g, and 865 (3.2%) patients with eGFR < 60 mL/min/1.73m^2 and ACR ≥ 30 mg/g.

Among 8,153 patients who were screened for retinopathy status, there were 2,820 (34.6%) patients who were diagnosed with DR, including 2,592 patients with NPDR and 228 patients with PDR. Comparison between DKD negative (n = 5064) with DKD positive

(n = 3089) and DR negative (n = 5333) with DR positive (n = 2820) were made in those people (Table 1). Patients with DKD were elder, had longer duration of disease, higher values of BMI, waist circumference, HbA1c, BP, LDL-C, TG, and higher proportion of metabolic syndrome than those with DKD-negative. Similar significant clinical indicators were observed in patients with DR compared to those without, except for BMI and TG.

There were 3,581 patients with DR-negative and DKD-negative, 1,483 patients with DR-positive and DKD-negative, 1,752 patients with DR-negative and DKD-positive, and 1,307 patients with DR-positive and ACR-positive. Patients' clinical characteristics in the four groups are shown in Table 2. For patients with DR-positive and DKD-positive, they had the longest duration of disease, the highest HbA1c, BP, and TG level, the lowest eGFR, and the highest ACR. DR was more frequent in patients with DKD, while it was not an indicator of DKD. The positive predictive value (PPV) of DR for DKD was 47.4% and negative predictive value (NPV) was 67.1%.

3.2 Concordance and discordance between DR and DKD

Logistic regression models were established to estimate risk factors for DKD and DR, respectively. Elder age, male gender, a longer duration of disease, higher values of waist circumference and a higher HbA1c level were associated with both DKD and DR. A lower educational level was associated with DR. Higher BP and TG would predict increased prevalence of DKD (Table 3).

Risk factors for diabetes mellitus complicated by an increased ACR versus a reduced eGFR and NPDR versus DR were shown in supplemental materials (Supplementary Tables 1, 2). They shared most risk factors.

4 Discussion

It was found that 32.3% and 34.6% of Chinese patients with type 2 diabetes mellitus were complicated by DKD and DR, respectively. Hospital-based investigation and the longer duration of diabetes may be explanation for the difference with that reported previously (22).

As we know, both DR and DKD are microvascular complications of diabetes mellitus. Evidence-based medical research showed that lowering blood glucose and blood pressure reduced the incidence rates of DKD and DR (23). Because these two complication are tightly correlated, DR is often used in clinical practice to differentiate DKD from other CKDs (24). However, retinopathy was absent in 56.7% of patients with DKD in this study. In contrast, 52.6% of patients with retinopathy did not have DKD.

TABLE 1 Characteristics of different groups of patients with DKD negative, DKD positive, DR negative and DR positive.

	All (n=8153)	DKD negative (n=5064)	DKD positive (n=3089)	*P*	DR negative (n=5333)	DR positive (n=2820)	*P*
Age (years)	59.45 ± 10.51	58.94 ± 10.30	60.28 ± 10.81	<0.001	58.95 ± 10.94	60.39 ± 9.60	<0.001
Duration of disease (years)	8.80 ± 6.89	8.12 ± 6.53	9.93 ± 7.30	<0.001	7.97 ± 6.64	10.39 ± 7.07	<0.001
BMI (kg/m^2)	25.24 ± 3.38	25.09 ± 3.30	25.48 ± 3.50	<0.001	25.18 ± 3.38	25.34 ± 3.39	0.055
Waist circumference (cm)	90.14 ± 9.45	89.62 ± 9.12	90.99 ± 9.82	<0.001	89.86 ± 9.40	90.69 ± 9.52	<0.001
Fasting blood glucose	10.86 ± 4.11	10.38 ± 3.92	11.62 ± 4.29	<0.001	10.69 ± 4.07	11.21 ± 4.18	0.001
HbA1c (%)	8.82 ± 2.14	8.63 ± 2.09	9.15 ± 2.18	<0.001	8.74 ± 2.17	8.98 ± 2.07	<0.001
SBP (mmHg)	134.45 ± 18.42	131.78 ± 16.99	138.82 ± 19.79	<0.001	133.71 ± 18.29	135.85 ± 18.59	<0.001
DBP (mmHg)	80.51 ± 11.11	79.51 ± 10.63	82.16 ± 11.66	<0.001	80.21 ± 11.02	81.10 ± 11.25	0.001
LDL-c (mmol/L)	2.73 (2.17,3.33)	2.72 (2.17,3.30)	2.76 (2.17,3.38)	<0.001	2.72 (2.16,3.30)	2.77 (2.20,3.37)	0.003
TG (mmol/L)	1.70 (1.20,2.49)	1.62 (1.17,2.34)	1.81 (1.30,2.70)	<0.001	1.70 (1.20,2.50)	1.70 (1.21,2.47)	0.249
HDL-c (mmol/L)	1.34 (1.15,1.56)	1.34 (1.16,1.56)	1.34 (1.14,1.57)	0.683	1.34 (1.16,1.57)	1.33 (1.15,1.56)	0.342
Smoking history	10.7%	10.2%	11.5%	0.148	10.2%	11.6%	0.086
Utilization rate of ACEI/ARB	25.7%	21.7%	32.3%	<0.001	22.0%	32.7%	<0.001
Rate of HbA1c <7%	21.1%	24.0%	16.5%	<0.001	23.2%	17.2%	<0.001
Rate of BP < 130/80 mmHg	22.2%	25.4%	17.1%	<0.001	23.1%	20.6%	0.008
Rate of LDL-c < 2.6 mmol/l	43.6%	44.5%	42%	0.029	44.2%	42.4%	0.130
eGFR (mL/min/1.73m^2)	95.53 ± 19.71	98.80 ± 16.93	90.18 ± 22.57	<0.001	96.60 ± 20.17	93.52 ± 18.65	<0.001
ACR (mg/g)	16.90 (5.90,61.92)	8.00 (3.65,15.00)	106.71 (48.60,305.40)	<0.001	14.10 (5.22,14.10)	24.48 (7.93,133.18)	<0.001
Proportion of metabolic syndrome	71.9%	68.4%	77.7%	<0.001	70.7%	74.1%	0.001

DKD negative: ACR < 30mg/g and eGFR > 60 mL/min/1.73m^2; DKD positive: ACR < 30mg/g or eGFR > 60 mL/min/1.73m^2; DR negative: normal fundus; DR positive: non-proliferative retinopathy (NPDR) and proliferative retinopathy (PDR).

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TABLE 2 Characteristics of different groups of patients with DR-DKD-, DR-DKD+, DR+DKD- and DR+DKD+.

	DR-DKD- (n=3581)	DR+DKD- (n=1483)	DR-DKD+ (n=1752)	DR+DKD+ (n=1337)	*P*
Age (years)	58.43±10.63	60.17±9.35*	60.02±11.47*	60.63±9.86*	<0.001
Duration of disease (years)	7.53±6.36	9.53±6.74*	8.85±7.11*#	11.35±7.31*#†	<0.001
BMI (kg/m^2)	25.05±3.31	25.17±3.27	25.45±3.49*#	25.52±3.51*#	<0.001
Waist circumference (cm)	89.40±9.20	90.17±9.10*	90.79±9.72*	91.25±9.94*#	<0.001
Fasting blood glucose	10.33±3.93	10.50±3.91	11.36±4.24*#	12.00±4.33*#†	<0.001
HbA1c (%)	8.60±2.14	8.69±1.97	9.04±2.21*#	9.30±2.14*#†	<0.001
SBP (mmHg)	131.51±17.00	132.45±16.88	138.21±19.89*#	139.61±19.65*#†	<0.001
DBP (mmHg)	79.30±10.60	80.01±10.69*	82.05±11.62*#	82.30±11.72*#	<0.001
LDL-c (mmol/L)	2.70(2.17,3.28)	2.75(2.19,3.33)	2.75(2.14,3.34) *	2.80(2.21,3.41) *#	<0.001
TG (mmol/L)	1.63(1.17,2.36)	1.60(1.17,2.30)	1.82(1.27,2.77) *#	1.80(1.30,2.61) *#†	<0.001
HDL-c (mmol/L)	1.34(1.16,1.57)	1.33(1.16,1.55)	1.34(1.15,1.57)	1.34(1.14,1.57)	0.803
Smoking history	10.1%	10.5%	10.4%	12.9%*#†	0.040
Utilization rate of ACEI/ARB	19.5%	27.0%*	27.2%*	39.0%*#†	<0.001
Rate of HbA1c <7%	25.5%	20.4%*	18.6%*	13.8%*#†	<0.001
Rate of BP < 130/80 mmHg	25.9%	24.1%	17.4%*#	16.7%*#	<0.001
Rate of LDL-c < 2.6 mmol/l	45.0%	43.2%	42.4%	41.5%*	0.094
eGFR (mL/min/1.73m^2)	99.27±18.33	97.67±12.89*	91.14±22.54*#	88.92±22.57*#†	<0.001
ACR (mg/g)	7.70(3.50,15.00)	8.50(4.00,15.40)	85.40(44.86,249.15) *#	146.70(57.90,374.13) *#†	<0.001
Proportion of metabolic syndrome	67.7%	70.0%	76.9%*#	78.7%*#	<0.001

* compared with patients of DR-DKD-, P < 0.05. # compared with patients of DR+DKD-, P < 0.05. † compared with patients of DR-DKD-, P < 0.05.

The discordance was 39.7% (DR-negative and DKD-positive plus DR-positive and DKD-negative) in the present study. A similar finding was reported in an Italian study, the discordance between DR and DKD was 36.6% (25). Interestingly, data from a real-world study revealed that there was no significant difference in albumin excretion rate between the presence and absence of DR in the whole population (26).

The estimated PPV of DR for DKD was 47.4% in this study, and the NPV of DR for DKD was 67.1%. This PPV was lower than the reports from KDIGO, i.e., the PPV of retinopathy for typical

TABLE 3 Comparison of predictors for DKD and DR in all patients (n = 7409).

	DKD (n = 2783)			DR (n = 2525)		
	β	OR(95% confidence interval)	*P*	β	OR(95% confidence interval)	*P*
Age (years)	0.108	1.114(1.06,1.17)	<0.001	0.060	1.062(1.01,1.117)	0.018
Gender (M vs F)	0.107	1.113(1.002,1.236)	0.045	0.163	1.177(1.058,1.31)	0.003
Duration (years)	0.222	1.248(1.177,1.324)	<0.001	0.373	1.452(1.367,1.543)	<0.001
Educational level	-0.043	0.958(0.867,1.058)	0.399	-0.477	0.621(0.562,0.686)	<0.001
BMI (kg/m^2)	0.021	1.021(0.911,1.145)	0.720	0.012	1.012(0.901,1.136)	0.845
Waist circumference (cm)	0.080	1.084(1.023,1.148)	0.006	0.061	1.063(1.003,1.127)	0.040
Smoking history	0.140	1.15(0.976,1.355)	0.096	0.102	1.108(0.938,1.308)	0.229
HbA1c (%)	0.325	1.384(1.297,1.476)	<0.001	0.211	1.235(1.157,1.318)	<0.001
SBP (mmHg)	0.474	1.606(1.483,1.739)	<0.001	0.071	1.073(0.989,1.165)	0.090
LDL-c (mmol/L)	0.045	1.046(0.985,1.111)	0.146	0.053	1.054(0.992,1.121)	0.091
TG (mmol/L)	0.316	1.371(1.26,1.491)	<0.001	-0.048	0.953(0.874,1.039)	0.277

diabetic glomerulopathy ranged from 67% to 100% in patients with macroalbuminuria, and the NPV had a broader range of 20-84%. For microalbuminuria, PPVs were lower at around 45%, while NPVs were close to 100% (27). The prevalence of DKD was about 60% in patients with type 2 diabetes mellitus with advanced DR (28). A meta-analysis demonstrated that the pooled sensitivity and specificity of DR to predict DKD were 0.65 and 0.75, respectively, while PDR had a low sensitivity (0.25) and high specificity (0.98) for predicting DKD, respectively (10). Taken together, these results suggested that DR was not sensitive enough to predict DKD but it was good indicator to confirm DKD.

Elder age, male gender, a longer duration of disease, a higher value of waist circumference and a higher HbA1c level were correlated with both DKD and DR. These findings were also confirmed in other previous studies (29, 30). Although DKD and DR share similar mechanisms, numerous studies suggested that DKD and DR may differ in some way. Firstly, a noticeable proportion of patients with type 2 diabetes mellitus had DKD or DR alone (25). Secondly, a new classification of diabetes had been proposed by a Swedish group according to GAD antibody, BMI, age at onset, HbA1c level, homeostatic model assessment-β (HOMA-β), and HOMA of insulin resistance (HOMA-IR). Among them, cluster 3 (characterized by severe insulin resistance) was related to a higher incidence of kidney disease and cardiovascular disease, while cluster 2 (characterized by severe insulin deficiency) was associated with a higher incidence of DR (31). More importantly, SGLT2 inhibitors and GLP1-RA, two new classes of hypoglycemic drug, exerted outstanding renal but not retinal protective effects (32). Therefore, it was reasonable to assume that DKD and DR would be associated with different risk factors and pathogeneses.

Metabolic syndrome was called insulin resistance syndrome. In the present study, the proportion of metabolic syndrome was much higher in patients with DKD than those in DKD negative group. The higher level of SBP and triglyceride were independently associated with DKD but not DR. These were consistent with the new classification according to cluster analysis (31), i.e., patients with severe insulin resistance were more likely to be complicated by kidney disease. A study demonstrated that the visceral adiposity index was found to be strongly associated with the prevalence of DKD, while it was not associated with the prevalence of DR in Chinese subjects (33). Taken together, these data suggested that improving insulin resistance as well as controlling metabolic syndrome in patients with type 2 diabetes mellitus may be much more important in the prevention of DKD, as compared to in the prevention of DR.

We found that patients with DR were associated with lower educational levels. It was reported that patients with higher educational level may be prone to internalize health information and hence change their life-style, which could explain for lower DR rate in those patients (34).

The present study had several limitations. Firstly, the cross-sectional nature of this study precluded exploration of any cause-effect relationship. Secondly, concomitantly treatment affected the measurement of HbA1c, SBP, triglycerides and other biologic parameters. Thirdly, DKD was diagnosed based only on the clinical characteristics without renal biopsy, so that DKD might be over diagnosed. False positive of increased ACR due to poor blood glucose control may also be a concern. Last but not least, the proportion of screening of DR in patients with proteinuria was 36.2%, while it was 27.8% in patients without proteinuria. Patients with albuminuria were more likely to screen their retinopathy status, which might lead to selection bias. More detailed and comprehensive screening of DR are needed for Chinese patients with diabetes. The strength of this study was its large sample size. All participants were from 5 cities in China, and the large sample size might promote the generalization of the findings. The concordance and discordance between DR and DKD were discussed, and the corresponding strategies were put forward for the prevention of DKD.

5 Conclusion

The discordance was significant between retinopathy and DKD in type 2 diabetes. DKD was associated with a higher level of components of the metabolic syndrome, DR was more in patients with lower educational level. Further studies are required to discriminate their differences in the development and prevention of DR and DKD.

Guarantor statement

JX is the guarantor of this work and had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Tsinghua Changgung Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZL conceptualized the study, interpreted the analyses, wrote the initial manuscript, and reviewed and revised the manuscript. XL collected data, contributed intellectually to the research topics, and critically reviewed the scientific content of the manuscript. YW analyzed the data, designed and supervised the statistical analysis, and reviewed and revised the manuscript. YS, QL, JG, WF, and CL collected data, reviewed and revised the manuscript. CC and WZ conceptualized the study, supervised the statistical analysis, and reviewed and revised the manuscript. JX conceptualized the study, coordinated and supervised data collection, acquired funding for the

study, and critically reviewed the manuscript for important intellectual content. JX is the guarantor of this research and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1133290/full#supplementary-material

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Investigation of risk factors associated with impaired glucose regulation: Using the momentum equation to assess the impact of risk factors on community residents

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Objective: To identify risk factors for impaired glucose regulation (IGR) and assess their impact on community residents, this study used a questionnaire to conduct cross-sectional surveys and analysis.

Methods: Overall, 774 residents of an urban community in northern China (Jian city) participated in this study. Trained investigators conducted surveys using questionnaires. Based on their medical history, respondents were divided into three glucose status groups as follows: normal (NGT), IGR, and diabetes mellitus (DM). Statistical analysis of survey data was performed using SPSS v. 22.0.

Results: Age, hypertension, family history of diabetes (FHD), dyslipidemia, obesity, and cardiovascular and cerebral disease (CVD) were positively correlated with IGR in men and women. IGR was negatively correlated with a sedentary lifestyle in men and positively correlated with being overweight in women. The number of type 2 diabetes mellitus (T2D) risk factors per subject was positively correlated with age in the NGT group. Glucose status deteriorated with increasing age and the number of risk factors. FHD was the strongest risk factor in both men and women.

Conclusions: Prevention of IGR includes weight control, physical activity, and prevention of hypertension and dyslipidemia, especially in subjects with FHD.

KEYWORDS

type 2 diabetes mellitus, impaired glucose regulation, risk factors, general population, hypertension

1 Introduction

Type 2 diabetes mellitus (T2D) increases the risk of microvascular and macrovascular complications, and as a result, puts a great economic burden on patients and society. T2D has become an important public health issue worldwide, especially in developing countries (1, 2). Previous studies have indicated that interventions in populations at high risk of developing T2D can effectively prevent diabetes progression (3–8). Unfortunately, despite these efforts, investigations in recent decades have shown that T2D prevalence has gradually increased, as has the prevalence of prediabetes, including impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) (9–13). In China, the incidence of T2D and prediabetes among adults in 2010 was 11.6% and 50.1%, respectively (12). A population-based cross-sectional survey published in 2017 (14) used oral glucose tolerance test (OGTT) data from 43,846 adults aged 20 years or older from the 2007-2008 Chinese Diabetes and Metabolic Disorder Study and resulted in 2,801 newly diagnosed cases of type 2 diabetes. Of these newly diagnosed patients, 654 (23.3%) were aged <43 years and thus had early-onset DM. Early-onset diabetes in China accounts for a higher proportion of new-onset diabetes, suggesting that diabetes in young people has reached pandemic levels. The reasons underlying for this increase are complicated. T2D is the result of interactions between multiple factors including genetic, environmental, lifestyle, nutrition, economic and social factors (15). Most previous studies on T2D prevention have focused on IGT patients, in whom regulation glucose was abnormal at the time of the study (3, 4, 16). Some studies have shown that after a 6-year lifestyle intervention, 73% of people with IGT developed T2D 23 years later (17).

In recent years, IGR has been established as an independent risk factor for acute coronary events (18). Preventive measures from clinical trials cannot eliminate the risk factors or stop the onset and progression of T2D in IGR patients (2, 15). Individuals with normal glucose regulation may also be at risk for T2D (19).

Since IGR is the first stage of T2D, prevention of IGR should be the first stage of T2D prevention, and special attention should be given to risk factors in the general population before glucose regulation in these individuals becomes abnormal. In this study, we conducted a cross-sectional survey to assess the prevalence and ranking of risk factors for IGR and T2D in people with NGR.

There are many risk factors that increase the risk of IGR. Because their prevalence in the community varies, so does their impact on community populations. To evaluate the impact of a risk factor on the population, it is better to consider both the relevance of the risk factor and its incidence in the population. In our study, we used the product of a risk factor's odds ratio (OR) and its prevalence in the community which we termed "risk momentum" (RM), to assess the impact of each risk factor on the community.

2 Methods

2.1 Ethical approval

Informed written consent was obtained from all participants prior to enrolment.

2.2 Study population

The study population was recruited from an urban residential community (Jian city) in China, from December 2013 to June 2014. The sampling frame consisted of the population living in the community ≥ 2 years and aged ≥15 years. Simple randomization was performed to select participants. A total of 774 subjects participated in the study.

2.3 Questionnaire and measurements

The questionnaire on risk factors for T2D (Table 1) was designed based on the Guidelines for Prevention and Treatment of Type 2 Diabetes in China (2010 Edition) (20). Trained investigators performed the survey using a published questionnaire (Table 1). Subjects were divided into three glucose regulation groups according to their medical history and health records: the diabetic group (the DM group=0, fasting plasma glucose (FPG) ≥7.0 mmol/L and/or 2 hours postprandial blood glucose (P2hBG) ≥11.1 mmol/L, or diagnosed as having T2D by a doctor); the impaired glucose regulation group (the IGR group=1, 6.1 mmol/L≤ FPG <7.0 mmol/L and/or 7.8 mmol/L≤ P2hBG <11.1 mmol/L, or diagnosed as having IGR by a doctor); and the normal group (the NGT group=2, FPG <6.1 mmol/L and P2HBG <7.8 mmol/L). Participants were required to answer the questionnaire based on their latest health reports.

Body mass index (BMI) was calculated by dividing body weight (kg) by the square of height (m). Blood pressure was measured three times in the sitting position with at least 5 minutes of rest before the measurement, and their average was used in the analysis. Past medical history (hypertension, HP; gestational diabetes mellitus, GDM; dyslipidemia; cardiovascular and cerebral disease, CVD; glucocorticoid-induced diabetes, GID; polycystic ovary syndrome, PCOS; and severe mental illness/long-term use of antidepressants, SMLUA) was used based on the previous diagnosis.

2.4 Statistical analysis

Statistical analyses were performed using SPSS v. 22.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were summarized and presented as the mean ± standard deviation (SD), and nonparametric variables were presented as numbers and

TABLE 1 Questionnaire on risk factors for type 2 diabetes mellitus (T2D).

1. History of impaired glucose regulation (Glucose status) (diabetes = 2, yes = 1, no = 0)
2. Age ≥ 45 years (yes = 1, no = 0)
3. Overweight status and obesity (body mass index (BMI) ≥28 kg/m^2 was regarded as obese, BMI ≥24 kg/m^2 was regarded as overweight, and BMI 18–24 kg/m^2 was regarded as normal) (obesity = 2, overweight = 1, normal = 0)
4. History of T2D in the immediate family (FHD) (yes = 1, no = 0)
5. History of macrosomia (infant birth weight ≥4 kg) (yes = 1, no = 0)
6. History of gestational diabetes mellitus (GDM) (yes = 1, no = 0)
7. History of hypertension (HP, systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥ 90 mmHg or current antihypertensive treatment) (yes = 1, no = 0)
8. History of dyslipidemia or current lipid-lowering therapy (yes = 1, no = 0)
9. History of cardiovascular and cerebral disease (CVD) (yes = 1, no = 0);
10. History of glucocorticoid-induced diabetes (GID) (yes = 1, no = 0)
11. History of polycystic ovary syndrome (PCOS) (yes = 1, no = 0)
12. Severe mental disease/long-term use of antidepressants (SMLUA) (yes = 1, no = 0)
13. Sedentary lifestyle, defined as no or very little physical activity during work, housework, transportation, and leisure time. The standard was medium-intensity physical activity of <30 min per day most days of the week (yes = 1, no = 0).

percentages. Only risk factors with an incidence of ≥10% were included in the correlation analysis. Bivariate correlation analyses were performed to identify factors associated with IGR. Factors identified as significant from bivariate analyses were included in a binary logistic regression analysis, and odd ratios with 95% confidence intervals and *p*-values were reported. A bivariate analysis was used to analyze the correlation between the following: the number of T2D risk factors (excluding age) and age (Pearson test); the number of T2D risk factors (excluding age) and glucose status (Spearman test); and glucose status and age (Spearman test). The significance level was set at p <0.05, and all analyses were two-sided. The product of a risk factor's OR value and its prevalence in the community was calculated as RM.

3 Results

3.1 General information

Of the 774 participants, the NGT group comprised 208 men (aged 47.13 ± 15.61 years) and 381 women (aged 45.92 ± 16.13 years), the IGR group comprised 35 men (aged 60.89 ± 11.44 years) and 48 women (aged 59.27 ± 13.66 years), and the DM group comprised 41 men (aged 62.29 ± 10.08 years) and 61 women (aged 64.03 ± 8.94 years). The prevalence of T2D was 14.4% in men and 12.4% in women. The prevalence of IGR was 12.3% in men and 9.8% in women.

3.2 Prevalence of risk factors

The prevalence of T2D risk factors in different groups is shown in Table 2. The prevalence of T2D risk factors was higher in the DM and IGR groups than in the NGT group. The prevalence of overweight was the highest among all the factors in both men and women.

3.3 Number of risk factors in each group

Excluding age as a factor, the number of T2D risk factors per person in each group was as follows: DM men, 2.85 ± 1.49 factors and DM women, 3.87 ± 1.52 factors; IGR men, 3.71 ± 1.27 factors and IGR women, 3.81 ± 1.42 factors; NGT men, 1.62 ± 1.37 factors and NGT women, 1.56 ± 1.32 factors. A significant difference in the number of T2D risk factors per person was observed between the NGT group and the other two groups (p <0.01), but there was no significant difference between the IGR and DM groups (p >0.05). A bivariate Spearman correlation analysis showed that glucose status was negatively correlated with the number of T2D risk factors (excluding age) per person (men, R = −0.435, p <0.01; women, R = −0.461, p <0.01).

3.4 Age

A bivariate Spearman correlation analysis showed that glucose status was negatively correlated with age (R= −0.412, p <0.01). The number of T2D risk factors excluding age showed a positive correlation with age in the NGT group using the bivariate Pearson correlation analysis. In the NGT group, Pearson's correlation coefficient was 0.32 (p <0.01) for men and 0.487 (p <0.01) for women. In the IGR group, Pearson's correlation coefficient was −0.128 (p >0.05) for men and 0.208 (p >0.05) for women. In the DM group, Pearson's correlation coefficient was 0.249 (p >0.05) for men and 0.184 (p >0.05) for women.

TABLE 2 Rank of prevalence of T2D risk factors (on the basis of NGT men group).

T2D Risk Factor	DM		IGR		NGT	
	MAN (%)	WOMAN (%)	MAN (%)	WOMAN (%)	MAN (%)	WOMAN (%)
Overweight	46.3	45.9	48.6	47.9	41.8*†	33.3*†
Sedentary Lifestyle	29.3	39.3	20.0	31.3	33.7	31.5
Hypertension	48.8	75.4	60.0	56.3	24.5*†	20.5*†
FHD	31.7	49.2	48.6	43.8	20.7*	16.3*†
Dyslipidemia	61.0	49.2	51.4	33.3	17.8*†	11.8*†
Obesity	26.8	34.4	22.9	29.2	15.4*†	14.7*†
CVD	39.0	59.0	42.9	47.9	13.5*†	17.3*†
Macrosomia	9.8	21.3	11.4	6.3	7.2	11.3
PCOS	0.0	4.9	0.0	0.0	0.0	0.8
GDM	0.0	1.6	0.0	4.2	0.0	0.3
GID	2.4	1.6	0.0	4.2	0.0	0.5
SMLUA	0.0	4.9	0.0	2.1	0.0	0.3

T2D, type 2 diabetes mellitus; FHD, family history of diabetes; CVD, cardiovascular and cerebral disease; PCOS, polycystic ovary syndrome; GDM, gestational diabetes mellitus; GID, glucocorticoid-induced diabetes; SMLUA, severe mental disease/long-term use of antidepressants; DM, diabetes mellitus group; IGR, impaired glucose regulation group; NGT, normal group. *Compared with IGR, p <0.05; †compared with DM, p <0.05.

3.5 Risk factors for IGR

All variables with a prevalence of 10% or greater were subjected to bivariate correlation analysis to assess the strength of their association with IGR. Eight variables, including FHD, dyslipidemia, hypertension, CVD, overweight and obesity, age, macrosomia, and sedentary lifestyle, were added to a binary logistic regression model for further analysis (Table 3). A sedentary lifestyle showed a negative association with IGR, and overweight was not correlated with IGR in men, in contrast with these variables in women. FHD was the strongest risk factor for IGR in both men and women.

3.6 Risk momentum: The product of (OR-1) * the prevalence of risk factors for IGR

In men, FHD had the largest RM, followed by hyperlipidemia and hypertension. In women, FHD also had the largest RM,

TABLE 3 Binary logistic regression analysis of risk factors for impaired glucose regulation among men and women.

	Men				Women			
Variable	OR	95% of C.I.	*p* value	Product (OR-1)*Prevalence	OR	95% of C.I.	*p* value	Product (OR-1)*Prevalence
Age	1.052	1.047-1.057	0.000		1.043	1.038-1.048	0.000	
Overweight	1.046	0.922-1.186	0.484	1.92	2.140	1.921-2.384	0.000	37.96
Sedentary lifestyle	0.538	0.471-0.614	0.000	-15.57	1.515	1.374-1.670	0.000	16.23
Hypertension	1.848	1.650-2.070	0.000	20.78	1.552	1.406-1.713	0.000	11.32
FHD	3.342	2.982-3.746	0.000	48.48	3.553	3.243-3.893	0.000	41.61
Dyslipidemia	2.949	2.631-3.305	0.000	34.70	1.620	1.473-1.781	0.000	7.32
Obesity	1.374	1.170-1.613	0.000	5.77	2.033	1.803-2.293	0.000	15.19
CVD	1.569	1.388-1.774	0.000	7.68	1.415	1.283-1.561	0.000	7.18
Constant	0.003		0.000		0.004		0.000	

Odds ratio (OR), 95% confidence interval (C.I.) and significance (p value).
FHD, family history of diabetes; CVD, cardiovascular and cerebral disease.

followed by an overweight, sedentary lifestyle and obesity (Table 3; Figure 1).

4 Discussion

Our survey revealed that age, hypertension, FHD, dyslipidemia, obesity, and CVD were positively correlated with IGR, and a sedentary lifestyle was negatively correlated with IGR in men. In women, age, overweight, sedentary lifestyle, hypertension, FHD, dyslipidemia, obesity, and CVD were positively correlated with IGR. FHD had the strongest correlation with IGR in both men and women. After excluding age as a factor, the number of T2D risk factors was positively correlated with age in the NGT group. Glucose status deteriorated with increasing age and risk factors in the NGT group. FHD had the largest RM in both men and women.

We used IGR prevention as a starting point for T2D prevention. Therefore, we assessed the prevalence of T2D-related risk factors in community residents and identified risk factors associated with IGR to inform IGR prevention strategies. Our data are also consistent with earlier observations suggesting that FHD, obesity, hypertension, dyslipidemia, sedentary lifestyle, and age are correlated with IGR (12, 21).

In our study, FHD had the strongest correlation with IGR both in men and women. The correlation of genetic factors with T2D is known (22, 23). Research has shown that 40% of first-degree relatives of T2D patients develop diabetes, compared with only 6% in the general population (24). Furthermore, monozygotic twins have higher concordance rates (96%) than dizygotic twins in some but not all twin studies (24, 25). The effect of family history may include not only genetic factors but also family aggregation. In addition to genetic factors, family members have similar lifestyles, which could lead to similar T2D risks. The results of a meta-analysis suggest that spousal diabetes can be a diabetc risk factor (26). Although the incidence of FHD was not the highest, the RM of FHD was the largest in both men and women. This finding suggests that FHD might be the most dangerous risk factor for IGR in the community.

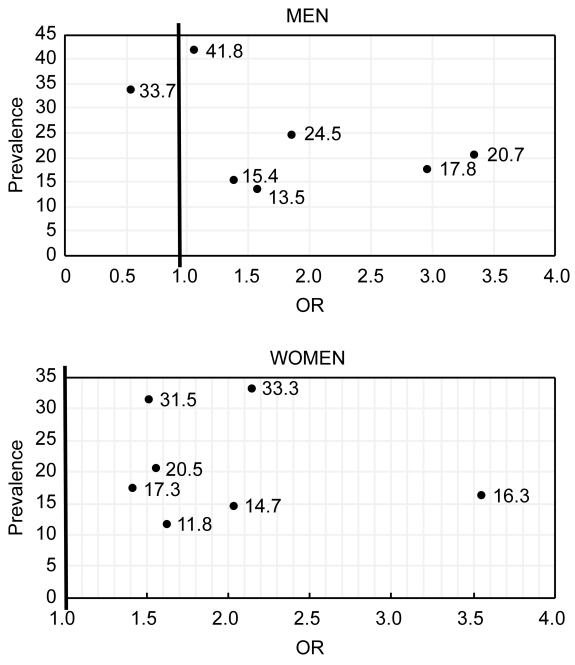


FIGURE 1
The relationship between the prevalence of risk factors and IGR incidence in men (Ain diagram) and women (top diagram).

We found that being overweight was the most prevalent risk factor in the NGT group. When the prevalence of obesity was considered in addition to the prevalence of overweight, 50% of NGT subjects were overweight. Obesity was significantly correlated with IGR in men and women, but overweight was correlated with IGR only in women. The RM was 37.96 for overweight and 15.19 for obesity in women. This suggests that the criteria for overweight should be defined separately for each gender. Other studies have shown that obesity/overweight plays important role in the progression of DM through adipose tissue factors (27–29). Being overweight (BMI 25-29.9 kg/m2) is significantly correlated with prediabetes (21). A high BMI also correlates with other T2D risk factors (28).

Sedentary lifestyle was the second most prevalent risk factor in the NGT group for both men and women. Other studies have shown sedentary lifestyle to be correlated with diabetes (30). In an investigation on Australian males aged 45 years and older, George ES found that time spent sitting was associated with diabetes, independent of BMI (31). In the present investigation, we found the prevalence of a sedentary lifestyle was not significantly different among the three glucose status groups. Contrary to previous studies (32), we found sedentary lifestyle was positively correlated with IGR in men. This finding might be because doctors generally advise people with IGR to manage their diet and increase physical activity, which could lead to people with IGR performing more physical activity than those without IGR. Moreover, it was well documented that people with IGR can manage the prevalence of T2D *via* diet modification (33). Our findings also indicated that sedentary lifestyles are prevalent throughout the community. The RM of a sedentary lifestyle was the third largest in women. Thus, addressing and reversing this unhealthy lifestyle trend is important.

In this study, the number of T2D risk factors per person was positively correlated with age, and glucose regulation deteriorated with age. Sandovici et al. (34) found changes in gene expression that were linked to T2D in rats. In a 10-year longitudinal study of 10,000 adults in Korea, Ohn et al. (19), found that all participants developed some degree of insulin resistance with age. In the present study, we found that once abnormalities of glucose regulation were present, regardless of IGR or DM, the number of risk factors lost its relevance with age, suggesting that prevention of IGR should begin at a young age.

Dyslipidemia, hypertension and CVD were significantly correlated with IGR in our study. Dyslipidemia had the second largest RM, and hypertension had the third largest RM in men,

whereas these factors had the fifth and sixth largest RMs in women. Diabetes and IGR are often accompanied by hypertension, dyslipidemia, and CVD. Hypertension, dyslipidemia and, hyperglycemia are important risk factors for mortality and disease associated with CVD (28, 31, 35). CVD can be considered an indicator of IGR as well as a result of IGR. However, no clear evidence exists as to whether there is a causal relationship between hyperglycemia and dyslipidemia or hypertension, and the underlying connections remain to be determined. Some lifestyle intervention studies have found that T2D incidence decreased with decreasing BMI, blood pressure, and lipid levels (3, 7, 9).

Notably, in our survey, the prevalence of diabetes and prediabetes in the community differed from the latest national survey data (DM, 10.7%; pre-DM, 35.7%) (36). As the incidence of risk factors in different regions may differ, strategies and priorities for the prevention of diabetes and IGR may vary from region to region. Because this was a cross-sectional study, the correlation analysis could not fully account for the long-term effects of risk factors on human physiology. A large longitudinal study is required to validate our findings. In this study, self-reported health information was accepted. Because most Chinese adults conduct general medical examinations only annually, some medical information might have changed between the most recent health examinations and the time when they participated in this study. For instance, patients with latent IGR or T2D might have been missed, which could reduce the effectiveness of our statistical analysis.

5 Conclusion

In conclusion, among the analyzed risk factors FHD had the strongest correlation with IGR. Overweight and obesity are the most common risk factors for IGR, and FHD has the largest RM. IGR should be addressed at a young age, especially in those with FHD. The best means of preventing IGR include weight control, prevention of hypertension and dyslipidemia, and consistent physical activity.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Jinan Central Hospital Affiliated to Shandong University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MG: Concept. ZW: Review of data. SW: Correction and revision. JW: Revision of papers. QJ: Conceptualize and writing. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Antibiotic-laden bone cement for diabetic foot infected wounds: A systematic review and meta-analysis

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Objective: A large body of literature has demonstrated the significant efficacy of antibiotic bone cement in treating infected diabetic foot wounds, but there is less corresponding evidence-based medical evidence. Therefore, this article provides a meta-analysis of the effectiveness of antibiotic bone cement in treating infected diabetic foot wounds to provide a reference basis for clinical treatment.

Methods: PubMed, Embase, Cochrane library, Scoup, China Knowledge Network (CNKI), Wanfang database, and the ClinicalTrials.gov were searched, and the search time was from the establishment of the database to October 2022, and two investigators independently. Two investigators independently screened eligible studies, evaluated the quality of the literature using the Cochrane Evaluation Manual, and performed statistical analysis of the data using RevMan 5.3 software.

Results: A total of nine randomized controlled studies (n=532) were included and, compared with the control group, antibiotic bone cement treatment reduced the time to wound healing (MD=-7.30 95% CI [-10.38, -4.23]), length of hospital stay (MD=-6.32, 95% CI [-10.15, -2.48]), time to bacterial conversion of the wound (MD=-5.15, 95% CI [-7.15,-2.19]), and the number of procedures (MD=-2.35, 95% CI [-3.68, -1.02]).

Conclusion: Antibiotic bone cement has significant advantages over traditional treatment of diabetic foot wound infection and is worthy of clinical promotion and application.

Systematic review registration: PROSPERO identifier, CDR 362293.

KEYWORDS

bone cement, diabetic foot, infection, Antibiotics, meta-analysis, clinical efficacy

Introduction

With the continuous improvement of socioeconomic and living standards, the global prevalence of diabetes is increasing rapidly and is expected to increase to 7.7% globally in 2030 (1). Diabetic foot infection (DFI) is one of the common complications of diabetic foot. The prevalence rate of diabetic foot is up to 25% (2, 3), of which the mortality rate is up to 12%. More than half of amputees are expected to die within 5 years. The mortality rate is higher than that of most cancers, posing a serious threat to patients' health (4).

Current clinical practice guidelines on the treatment of DFIs guide essentially the same pattern of negative pressure closed drainage therapy, debridement and dressing changes, hematologic reconstruction, wound dressing, and education of patients and families (5–7). However, DFI is currently difficult to treat and the wound takes a long time to heal, often leading to extended hospital stays and increased hospital costs, adding to the patient's burden.

As a unique bone repair material, antibiotic bone cement can release high concentrations of antibiotics locally to lower the risk of systemic toxicity and accomplish the goal of preventing and treating infection. According to studies, the use of antimicrobial bone cement in the treatment of DFI wounds has also yielded positive therapeutic outcomes. However, there is still no relevant evidence-based medical evidence to confirm this, so our article systematically evaluates the clinical efficacy of antibiotic bone cement in the treatment of diabetic foot to provide a reference basis for the future clinical treatment of diabetic foot.

Methods

Search strategy and selection criteria

This systematic review and meta-analysis are reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (8) Statement and was registered in the International Prospective Register of Systematic Reviews (number CDR 362293).

The Embase, PubMed, Cochrane, Scoup, CNKI, CBM, and ClinicalTrials.gov were searched for relevant studies published between the database inception date and 11 October 2022. We applied no language restrictions. We used the following combined text and MeSH terms: "Diabetic Foot." The complete search used for PubMed was: ("Diabetic Foot"[Mesh]) OR ((((Foot, Diabetic[Title/Abstract]) OR (Diabetic Feet[Title/Abstract])) OR (Feet, Diabetic[Title/Abstract])) OR (Foot Ulcer, Diabetic[Title/Abstract])). We considered all potentially eligible studies for review, irrespective of the primary outcome or language. We also conducted a manual search using the reference lists of key articles published in English (Supplementary materials).

Study selection and data extraction

Inclusion criteria

(1) meeting the management of diabetic foot: a clinical practice guideline by the Society for Vascular Surgery in collaboration with the American Podiatric Medical Association and the Society for Vascular Medicine (9); (2) meeting the criteria for the diagnosis and treatment of diabetic foot infection published by the American Diabetes Association (ADA) infection (10); (3) the study is a randomized clinical trial.

Exclusion criteria

(1) Basic studies; (2) Conference reports and reviews; (3) Repeatedly published literature; (4) Literature with incomplete data; (5) Patients with non-diabetic foot infections; combined hematologic disorders other than anemia; and mental disorders were included in the literature.

Screening and selection of literature

Two independent investigators (DTT, HJ) reviewed study titles and abstracts, and studies that satisfied the inclusion criteria were retrieved for full-text assessment. Trials selected for detailed analysis and data extraction were analyzed by two investigators (DTT and HJ); disagreements were resolved by a third investigator (SZM).

We extracted the following data from each selected study: total number of participants, age, sex, trial duration, and treatment modality. Two independent reviewers (DTT, HJ) assessed the risk of bias according to the PRISMA recommendations.

Outcome indicators

The main outcome indicators were wound healing time, length of hospital stay, time for bacterial culture Negative-conversing, and number of surgeries.

Patient and public involvement

Patients and/or the public were not involved in the design, conduct, reporting, or dissemination plan of this study.

Quality evaluation of included studies

The quality of the included articles was assessed by the Cochrane RoB 2.0 tool (11, 12), which included the following: (1) risk of bias arising from the randomization process; (2) risk of bias due to deviations from the intended interventions (effect of assignment to intervention); (3) missing outcome data; (4) risk of bias in the measurement of the outcome; and (5) risk of bias in the selection of the reported result. Each study was assessed as having a "low risk of bias," "some concerns," or "high risk of bias" according to the probability of bias.

Data synthesis

All data processing and statistical analyses were performed using Review Manager (RevMan) software version 5.3 (http://

www.ims.cochrane.org/revman/). Heterogeneity between studies was evaluated by the Q statistic and the I (2) statistic. When I (2) < 50%, p > 0.05, indicating little or no heterogeneity, a fixed effects model was used; when I (2) > 50%, p < 0.05, indicating significant heterogeneity, a random effects model was used. Subgroup analysis was also conducted, with a sample size cut-off of n=50, to compare the combined effect results of small and large sample subgroups and to explore sources of heterogeneity. Publication bias was assessed using funnel plots, sensitivity analyses were conducted using the sequential omission of a single study from the total studies and evaluating the influence of each study on the pooled effect estimates, and subgroup analyses were conducted based on the general characteristics of the study population and sample size.

Results

Screening and selection of literature

We identified 224 studies, downloaded the full texts to read, and excluded 11, of which, nine (with data for 532 participants) were included in our analysis (Figure 1), all of which were randomized controlled studies. The nine trials (13–21)were all published between 2016 and 2022.

Design characteristics

A total of 532 patients with diabetic foot were included in nine randomized controlled trials (13–21), with 266 patients in the intervention group and 266 in the control group. The subjects included in the study were aged 31-88 years. All had diabetic foot with a Wagner classification of 1-5. In the intervention group, cefoperazone bone cement was used in 13 patients, gentamicin bone cement in six patients, and vancomycin bone cement in 27 patients in the study by Xin Liu et al.[13]. The remaining eight studies were treated with vancomycin bone cement and the control group was treated with VSD. The baseline characteristics of the study participants and design characteristics are presented in Table 1. The quality of the literature is assessed in Figures 2, 3.

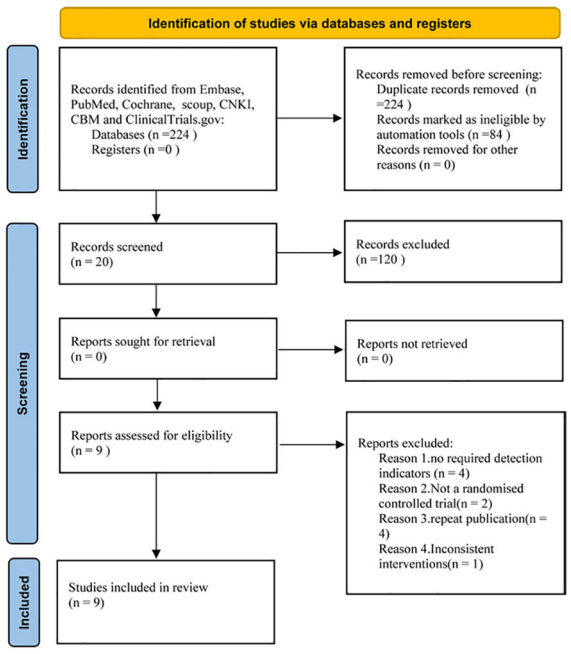


FIGURE 1
Flowchart of the meta-analysis.

Meta-analysis results

Wound healing time

Compared with those of the control group, wound healing time was shortened (MD=-7.30 95% CI[-10.38, -4.23]), As the meta-analysis showed a high degree of heterogeneity with Tau^2 = 12.91, I^2 = 81%, p<0.001, a random effects model was used for the combined analysis (Figure 4). Subgroup analysis according to sample size showed significantly lower heterogeneity in both the subgroup with sample size < 50 (-8.95[-17.98,0.09], I^2 = 91%, p<0.001) and the subgroup with sample size ≥ 50 (-5.92[-7.29,-4.56], I^2 = 0%, p=0.52). The antibiotic bone cement treatment of diabetic foot was shorter than the control group in terms of wound healing time in all cases (Figure 5).

Length of hospital stay

A total of six papers reported the length of hospital stay and the results showed that the length of hospital stay was shorter for antibiotic bone cement for diabetic foot than the control group (MD=-6.32, 95% CI [-10.15,-2.48]) and there was heterogeneity in the data (Tau^2 = 19.78, I^2 = 89%, P < 0.001) (Figure 6). Subgroup analysis according to the sample size showed significantly lower heterogeneity in both the subgroup with sample size <50 (-8.96 [-19.42,1.49], I2 = 94%, p<0.001) and the subgroup with sample size ≥ 50 (-6.32[-10.15,-2.48], I2 = 37%, p=0.2). The length of stay was shorter for antibiotic bone cement for diabetic foot than the control group (Figure 7).

Time for bacterial culture negative-conversing

A total of four papers reported bacterial culture turnaround times, which showed shorter bacterial culture turnaround times in diabetic foot treated with antibiotic bone cement than in the control group (MD=-5.15,95% CI [-7.15,-2.19]) and there was heterogeneity in the data (Tau^2 = 4.58, I^2 = 89%, P < 0.001); therefore, a random effects model was used to calculate the combined effect size (Figure 8).

Number of surgeries

A total of six publications reported the number of procedures and showed that the number of procedures for diabetic foot treated with antibiotic bone cement was less than the control group (MD=-2.35,95% CI [-3.68, -1.02]) and there was heterogeneity in the data

TABLE 1 General characteristics of included literature.

Name	Year	Parallel design	Age	Number of patients	Man/ female	Treatment modality		Intervention time (day)	Wagner classification for diabetic foot	Diabetic foot wound area	Diabetic foot duration (week)	Observed indicators
						Control group	Test group					
Ailian Liu (13)	2021	Yes	43-75	66	39/27	VSD	Vancomycin Bone Cement	N/A	NR	10-75	1-6	1
Min Xiong (14)	2020	Yes	41-88	80	37/43	VSD	Vancomycin Bone Cement	47	2-4	8-40	N/A	1,2,3,4
Xin Liu (15)	2016	Yes	35-72	46	23/23	VSD	Vancomycin Bone Cement; Cefoperazone Bone Cement; Gentamicin Bone Cement	56	2-4	8-20	4.63-6.63	1,2,4
Zhe Chen (16)	2022	Yes	46-78	90	48/42	VSD	Vancomycin Bone Cement	38	3-5	N/A	30-102.86	2,3,4
Suling Zhang (17)	2020	Yes	51.6-82	60	44/16	VSD	Vancomycin Bone Cement	84	>2	N/A	N/A	1
Hongjun Huang (18)	2019	Yes	53-79	36	N/A	VSD	Vancomycin Bone Cement	39	3-4	6-77	1-5.86	1,2,4
Xiaoguang Zhang (19)	2022	Yes	41-83	88	45/43	VSD	Vancomycin Bone Cement	15	1-4	5-35	52.14-156.43	1,2,3,4
He Lv (20)	2021	Yes	66-81	32	17/15	VSD	Vancomycin Bone Cement	41	2-4	N/A	48.51-159.92	1,2,3,4
Feng Yang (21)	2021	Yes	31-84	34	N/A	VSD	Vancomycin Bone Cement	84	2-4	N/A	N/A	1

VSD, vacuum sealing drainage; Observed indicators: 1, wound healing time; 2, length of hospital stay; 3, Time for bacterial culture Negative-conversing; 4, number of surgeries; N/A means not reported.

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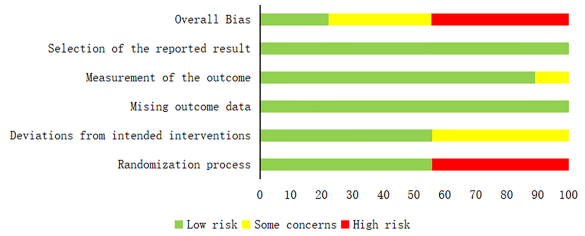


FIGURE 2
Risk of bias graph.

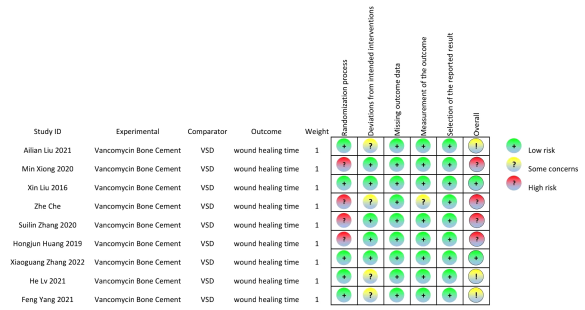


FIGURE 3
Risk of bias summary.

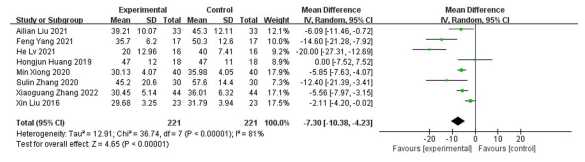


FIGURE 4
Forest plot of wound healing time.

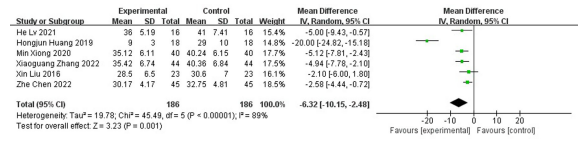


FIGURE 5
Subgroup analysis of wound healing time.

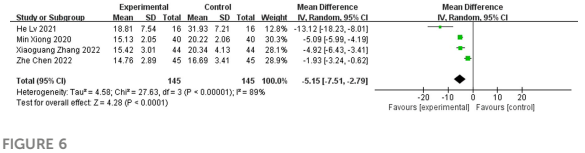


FIGURE 6
Forest plot of length of hospital stay.

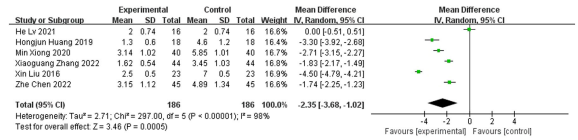


FIGURE 7
Subgroup analysis of length of hospital stay.

(Tau^2 = 2.71, I^2 = 89%, P < 0.001); therefore, a random effects model was used to calculate the combined effect size (Figure 9).

Discussion

Our results show that antibiotic bone cement treatment reduced wound healing time, length of hospital stay, time to negative bacterial culture of wound secretions, and the number of procedures compared with other diabetic foot treatments, and the results provide support for antibiotic bone cement as a treatment for diabetic foot infections.

Patients with DFI have low resistance and skin tissue regeneration capacity, resulting in slow wound healing, and these open wounds are susceptible to invasion by pathogenic bacteria, resulting in serious infections (22). Patients with DFI are treated with systemic antibiotics against infection, but the long-term application of antibiotics will produce adverse effects and bacterial resistance (23). Sun Shujuan et al. (24) investigated and analyzed the pathogenic bacteria of diabetic foot in Beijing and showed that multi-drug-resistant bacteria accounted for 16.9%. Due to the long trauma healing time, long hospital stay, and high medical costs of DFI patients, it not only imposes a heavy burden on clinical care but also causes increased anxiety and depression in patients, which affects treatment outcomes and quality of life (25, 26). In addition, routine debridement and surgical treatment for DFI may cause improper wound management, resulting in longer healing times, easy recurrence of ulcers, increased risk of metastatic ulcers, amputation, and death (27, 28).

As shown in Figures 4, 6, antibiotic bone cement for DFI reduced wound healing time and length of hospital stay compared to the controls, which is consistent with other results reported in the literature (13, 29). This may be due to the fact that when the bone cement covers the wound, it produces a 1-2 mm thick biofilm, which becomes an induced membrane and is capable of secreting relevant cytokines to promote wound healing, such as transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), etc. The secreted cytokines also have angiogenic and potential osteogenic properties (30–32). Most patients with DFI have pathological manifestations of small blood vessels and capillary blockages at the ends of the limbs, and the formation of IM promotes fresh angiogenesis, which, in turn, improves blood flow to the extremities, facilitating wound healing and shortening the patient's hospital stay (33–37).

Our meta-analysis research showed that antibiotic bone cement treatment shortened the bacterial turnaround time of the trauma and reduced the number of procedures compared with conventional debridement combined with negative pressure closure and drainage treatment. Due to poor blood flow to the

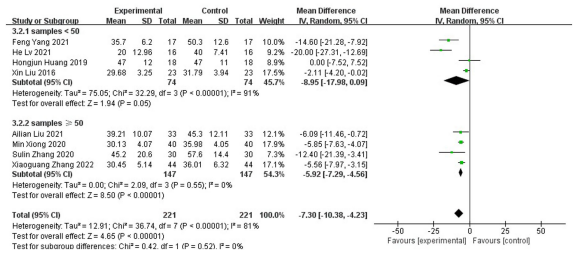


FIGURE 8
Forest plot of Time for bacterial culture Negative-conversing.

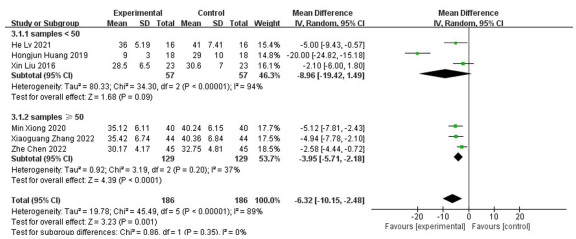


FIGURE 9
Forest plot of number of surgeries.

foot and reduced peripheral perfusion in patients with DFI, intravenous systemic antibiotics reduce delivery to bone or soft tissues and limit their efficacy, thus not achieving effective bactericidal concentrations at the site of the lesion. Antibiotic bone cement has eluting properties, releasing a much higher concentration of antibiotics than systemically applied antibiotics, with an efficiency of 81%, effectively preventing the emergence of drug-resistant strains (38). The bone cement gradually creates a local sterile environment with a slow and continuous local release of antibiotics, which can act directly on the lesion area and, thus, kill the bacteria, shortening the time until bacterial transformation of the wound (39, 40). In addition, the drug released locally rarely enters the systemic circulatory system, thus reducing the side effects of antibiotics (41). The simultaneous application of antibiotic bone cement treatment is easy to perform, with short operative times, easy post-operative care and dressing changes, and lower overall treatment costs.

Vancomycin is a common additive to antibiotic bone cement, releasing topical vancomycin at a concentration of approximately 0.5-2.0 μg/mL to meet the minimum inhibitory concentration requirements (42). In assessing the breadth of response to the dynamic release of vancomycin, it was found that the plateau period for vancomycin release could be >10 days (43–45). Vancomycin has a broad spectrum of sensitive bacteria and kills the most common pathogenic microorganisms (46). In the intervention group, the wound was covered with vancomycin using bone cement as a carrier, and the infection was effectively controlled, facilitating wound healing.

Our study also has some shortcomings. First, the sample size of the included literature was small, with four papers having a sample size of fewer than 50 cases. Second, the different lengths of interventions and inconsistent outcome indicators in the included literature may cause some bias in the study results and increase the heterogeneity of the Meta-analysis results. Finally, the number of high-quality literature included is limited, and more multicenter, large sample randomized controlled clinical trials are needed.

In summary, antibiotic bone cement is effective in treating DFI wounds, saving medical resources and costs, and it is worth promoting its use in clinical practice. In the future, more multicenter and large sample size randomized controlled clinical trials should be conducted to increase the in-depth discussion on the induction of film formation by trauma bone cement in different periods of the diabetic foot and the most suitable types of trauma for antibiotic bone cement, to comprehensively evaluate the effectiveness of antibiotic bone cement in the treatment of diabetic foot and provide a more rigorous and objective reference basis for the treatment of diabetic foot in the clinic in the future.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

TD and QH contributed equally to the design of the study. TD, QH, and ZS collected the data and performed the qualitative analysis. QH performed the quantitative analysis. TD, QH, and ZS drafted the manuscript. TD, QH, and ZS participated in the discussion and interpretation of the data. All authors approved this final version for publication. ZS acted as guarantor, assumed full responsibility for the completion of the article, had access to all data, and controlled the decision to publish.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Postoperative short-term mortality between insulin-treated and non-insulin-treated patients with diabetes after non-cardiac surgery: a systematic review and meta-analysis

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Background: Diabetes mellitus is an independent risk factor for postoperative complications. It has been reported that insulin-treated diabetes is associated with increased postoperative mortality compared to non-insulin-treated diabetes after cardiac surgery; however, it is unclear whether this finding is applicable to non-cardiac surgery.

Objective: We aimed to assess the effects of insulin-treated and non-insulin-treated diabetes on short-term mortality after non-cardiac surgery.

Methods: Our study was a systematic review and meta-analysis of observational studies. PubMed, CENTRAL, EMBASE, and ISI Web of Science databases were searched from inception to February 22, 2021. Cohort or case-control studies that provided information on postoperative short-term mortality in insulin-treated diabetic and non-insulin-treated diabetic patients were included. We pooled the data with a random-effects model. The Grading of Recommendations, Assessment, Development, and Evaluation system was used to rate the quality of evidence.

Results: Twenty-two cohort studies involving 208,214 participants were included. Our study suggested that insulin-treated diabetic patients was associated with a higher risk of 30-day mortality than non-insulin-treated diabetic patients [19 studies with 197,704 patients, risk ratio (RR) 1.305; 95% confidence interval (CI), 1.127 to 1.511; $p < 0.001$]. The studies were rated as very low quality. The new pooled result only slightly changed after seven simulated missing studies were added using the trim-and-fill method (RR, 1.260; 95% CI, 1.076–1.476; $p = 0.004$). Our results also showed no significant difference between insulin-treated diabetes and non-insulin-treated diabetes regarding in-hospital mortality (two studies with 9,032 patients, RR, 0.970; 95% CI, 0.584–1.611; $p = 0.905$).

Conclusion: Very-low-quality evidence suggests that insulin-treated diabetes was associated with increased 30-day mortality after non-cardiac surgery. However, this finding is non-definitive because of the influence of confounding factors.

Systematic review registration: https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42021246752, identifier: CRD42021246752.

KEYWORDS

postoperative mortality, diabetes mellitus, hypoglycemic regimens, non-cardiac surgery, insulin

1. Introduction

Diabetes comprises a group of metabolic disorders that is characterized by hyperglycemia, and it is a rapidly growing health problem worldwide, affecting the quality of life; diabetes had the ninth highest global mortality rate in 2010 (1–3). The International Diabetes Federation estimated that the prevalence of diabetes among adult women and men was 8.4 and 8.9%, respectively, in 2017. It is estimated the prevalence of diabetes in men and women would have increased to 9.9% by 2045 (4). The proportion of patients with diabetes who were undergoing cardiac surgery and non-cardiac surgery increased from 12.3% in 1995 to 21.2% in 2009 (5), and from 20.3% in 2004 to 25.4% in 2013 (6), respectively.

Compared to patients without diabetes, those with diabetes have a higher risk of postoperative complications, including postoperative pneumonia, wound complications, delayed wound healing, unplanned readmission, unplanned reoperation, and extended length of hospital stay (7–14). Diabetes can be divided into insulin-treated diabetes and non-insulin-treated diabetes according to the treatment regimen (15). Insulin-treated diabetes is not necessarily type 1 diabetes; it may also be type 2 diabetes that cannot be controlled with oral hypoglycemic drugs (16). Therefore, insulin-treated diabetes may be an indicator of the degree of progression of type 2 diabetes.

Meta-studies have shown that patients with insulin-treated diabetes have a higher short-term postoperative mortality risk compared to those with non-insulin-treated diabetes during cardiac surgery (17, 18). However, no such meta-analysis has been performed for non-cardiac surgery. According to a report, diabetes treated with insulin alone was an independent risk factor for death 30 days after surgery compared with diabetes treated with oral hypoglycemic drugs alone (19). The Revised Cardiac Risk Index included insulin-treated diabetes as a predictor of cardiac risk after non-cardiac surgery (20). Notably, although studies have reported higher short-term postoperative mortality in insulin-treated diabetes than in non-insulin-treated diabetes, there was no correlation between insulin-treated diabetes and postoperative mortality in multivariate logistic regression analysis (21–23). Another study also showed that in elderly patients with coronary artery disease or heart failure, insulin exposure 3 months before surgery was not associated with 30-day mortality after non-cardiac surgery (24). One study held the opposite view (25); among patients with coronary artery disease, diabetes treated with oral hypoglycemic agents was associated with a higher 2-year all-cause mortality than diabetes treated with insulin after non-cardiac surgery (25). To date, there is disagreement regarding postoperative mortality between insulin-treated diabetes and non-insulin-treated diabetes in non-cardiac surgery. Insulin-treated diabetes has been associated with more diabetes-related comorbidities and coexisting medical conditions than non-insulin-treated diabetes, and diabetes-related comorbidities and coexisting medical conditions were independent risk factors for death 30 days after non-cardiac surgery (19, 22, 26).

Therefore, we hypothesized that in non-cardiac surgery, insulin-treated diabetes would not be associated with postoperative mortality compared with non-insulin-treated diabetes after controlling for confounding factors. Patients with insulin-treated diabetes and those with non-insulin-treated diabetes were grouped according to long-term hypoglycemic regimens before admission. Considering that the hypoglycemic regimens of patients with diabetes may change after surgery, we determined the outcome as short-term postoperative mortality, including 30-day mortality and in-hospital mortality. In summary, we performed a meta-analysis of observational studies that assessed the effect of insulin-treated diabetes and non-insulin-treated diabetes on short-term mortality after non-cardiac surgery.

2. Methods

This study was conducted according to the Meta-analyses of Observational Studies in Epidemiology (MOOSE) (27). This study was registered with PROSPERO (CRD42021246752).

2.1. Data sources and search strategy

We comprehensively searched PubMed, EMBASE, CENTRAL (Cochrane Library), and Web of Science databases from inception to February 22, 2021. The retrieval strategies for each database are described in Supplementary Digital Content 1 (see Text document, Supplementary Digital Content 1, which demonstrates search strategies). The reference lists of relevant reviews were also identified.

2.2. Inclusion and exclusion criteria

We included studies that met each of the following criteria: (1) cohort or case-control studies; (2) inclusion of patients with diabetes who were undergoing non-cardiac surgery; (3) availability of information on 30-day mortality or in-hospital mortality regarding insulin-treated diabetes and non-insulin-treated diabetes; (4) insulin-treated diabetes and non-insulin-treated diabetes grouped according to long-term hypoglycemic regimens before admission; (5) insulin-treated diabetes referred to patients with diabetes who were receiving long-term insulin treatment before admission, including a combination of insulin and non-insulin therapy; and (6) non-insulin-treated diabetes referred to patients with diabetes who were receiving long-term non-insulin treatment before admission, excluding those who received only diet or lifestyle modifications.

We excluded studies that met any of the following criteria: (1) patients were not grouped according to pre-admission diabetes treatment regimens; (2) after contacting the authors three times, the relative risk (RR) of death and its 95% confidence interval (CI) or the odds ratio (OR) and its 95% CI or the number of deaths were not available; and (3) duplicate publications, comprising duplicate publications in different languages, duplicate publications of the same data in the same database, and articles published using the same data in the same research. Duplicate publications in different languages were translated into English using online translation software and were included as one study. For articles that collected the same data from the same database and led

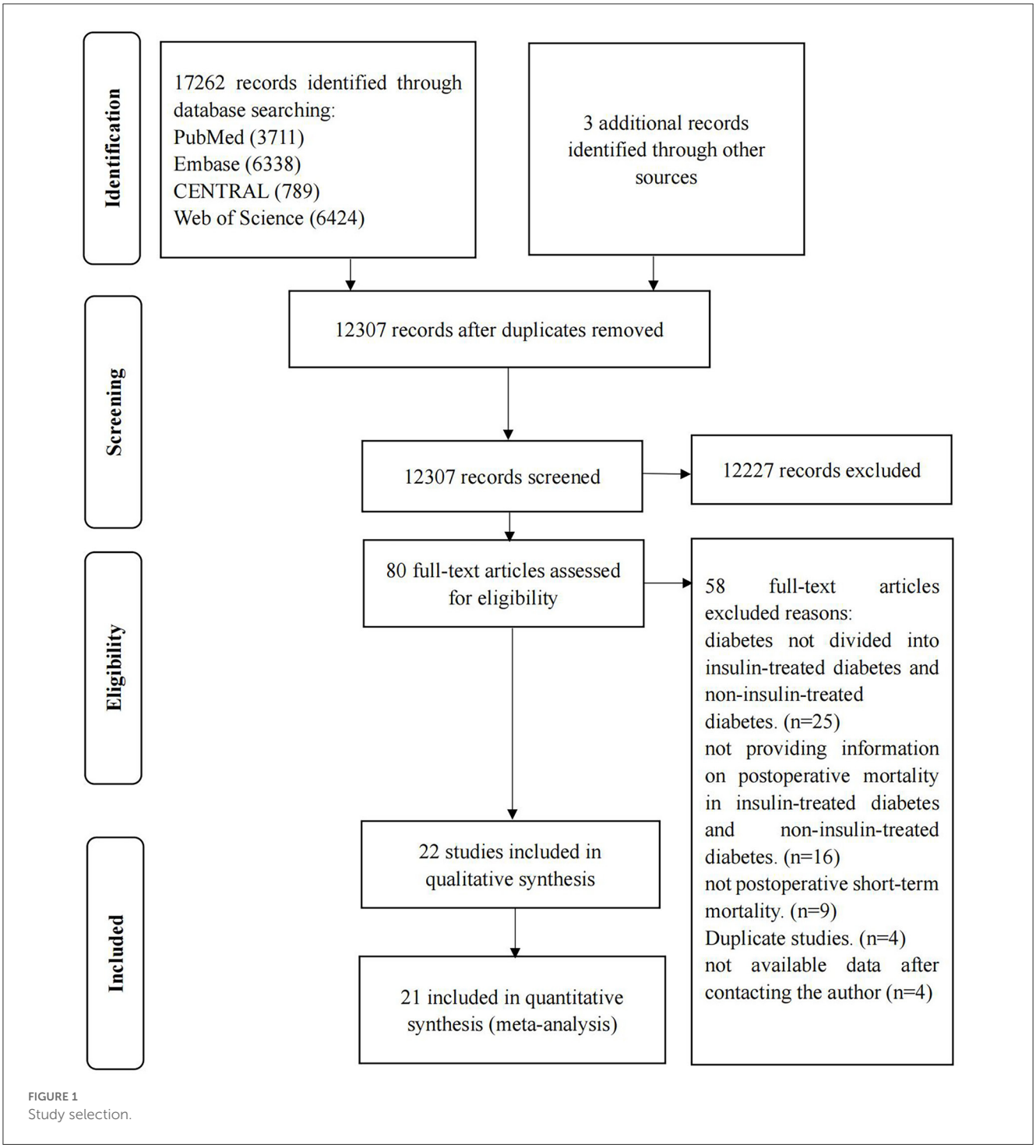


FIGURE 1
Study selection.

to repeated publications, priority was given to studies that had controlled for confounding factors. If the effect size adjusted for confounding factors was unavailable, then the latest published study was selected. For articles published using the same data in the same study, all articles were comprehensively analyzed and included as one study.

There were no language restrictions on search strategies. Non-English articles were translated into English using an online translation software.

2.3. Study selection

Two reviewers (RS and JJ) independently screened the titles and abstracts after using document management software to remove duplicate articles. Full-text analyses of studies that met the inclusion criteria were conducted. Finally, the included studies were determined based on the inclusion and exclusion criteria. Any differences were resolved through discussion.

2.4. Data extraction

Regarding the studies to be included, RS and JJ extracted the following data from the studies: the first author, publication year, study type, surgery type, demographic information of patients with insulin-treated diabetes and non-insulin-treated diabetes, RR or OR of postoperative mortality, and adjusted factors. Any differences were resolved through discussion. We extracted the death outcomes of different surgical types reported in a study.

2.5. Quality assessment

The Newcastle-Ottawa Scale (NOS) was used to evaluate the quality of the studies (28). A study with a score of ≥7 points was considered high quality (29, 30). In the comparability score criteria, complications and comorbidities of patients with diabetes at admission were considered the most important confounding factors. Age and American Society of Anesthesiologists (ASA) scores were considered the second most important confounding factors.

2.6. Data analyses

In the present study, 30-day mortality was the primary outcome, and hospital mortality was the secondary outcome. We chose the adjusted RR or OR of death to pool the data. Since the 30-day and hospital mortality after non-cardiac surgery are very low, the OR can be considered an approximate estimate of RR. Therefore, for studies in which the adjusted RR was unavailable, the adjusted OR was extracted. For studies in which neither the adjusted RR nor OR was available, we directly extracted or calculated the crude RR or OR to pool data. Although one study reported the outcome of interest, the number of deaths in both cohorts was zero. Since the RR or OR of this study could not be calculated, the data of this study was not pooled with the data of other studies. However, clinical heterogeneity was unavoidable. Therefore, a random-effects model using the DerSimonian-Laird method was used to pool the data.

A standard chi-squared test with a significance level of $\alpha = 0.1$ and the I^2 statistic were used to assess the magnitude of heterogeneity. $P \leq 0.1$ or $I^2 \geq 50\%$ was considered to indicate significant heterogeneity. The source of heterogeneity was explored using subgroup and sensitivity analyses. We conducted subgroup analysis by age, surgery type, RR/OR type, NOS comparability score, and mortality type. Sensitivity analysis was performed by restricting the meta-analysis to studies with a NOS score of ≥7 points and studies that controlled for complications and comorbidities of patients with diabetes based on the design or analysis. We excluded studies one by one to assess the impact of a single study. We used the funnel plot, Begg's test, and Egger's test to evaluate publication bias. If publication bias seemed present, we used the trim-and-fill method to describe its potential impact (31). All statistical analyses were performed with STATA version 15.0. The Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach was used to rate the level of evidence (32).

3. Results

A total of 17,265 studies were retrieved, and 22 studies were eventually included (15, 16, 19, 22, 23, 33–49). Details of the screening process are shown in Figure 1. The excluded articles through full-text evaluation and the reasons for exclusion can be found in Supplemental Digitary Content 2 (see Text document, Supplementary Digital Content 2, which demonstrates excluded studies).

3.1. Characteristics of the included studies

Twenty-two studies were included, involving 208,214 patients with diabetes, of which 70,806 were insulin-treated and 137,408 were non-insulin-treated. The average age of participants in 15 studies was over 60 years (15, 19, 22, 33–35, 38–41, 43–46, 49), while the average age of participants in four studies was <60 years (37, 42, 47, 48). We were unable to determine the average age in three studies (16, 23, 36). The type of surgery in one study was breast reconstruction surgery; therefore, only female patients were included (37). Another study was based on the Department of Veterans Affairs Patient Treatment File database; therefore, 99% of the study population was male (33). In the remaining studies, the proportion of men ranged from 29.94 to 68.6%. Two studies in the study population only included patients with type 2 diabetes (19, 35). Detailed characteristics of the study are presented in Table 1.

The NOS scores of the studies ranged from 4 to 7 points. Most of them scored five points. Only two studies were rated as high quality (38, 47). The details of the NOS scores are shown in Table 2. Regarding the comparability score, only three studies controlled for the most important confounding factor (36, 38, 47), and only three studies controlled for the second most important confounding factor (23, 38, 47).

3.2. Thirty-day mortality

The 20 included studies provided information on the outcome of 30-day mortality. Because four studies provided death outcomes for different types of surgery (23, 36, 37, 39), there were 24 groups of postoperative death data. Because the death outcomes of insulin-treated diabetes and non-insulin-treated diabetes in one study were both zero events (37), the data from this study could not be combined with data from other studies. Finally, 22 sets of data (19 studies, 206,736 participants) were pooled. Among these 22 sets, five were the adjusted OR, and the remaining 17 were the crude RR. A random-effects model was used to pool all the data, and a significant difference was found (RR, 1.305; 95% CI, 1.127–1.511; $p < 0.001$; Figure 2), suggesting that insulin-treated diabetes had a higher risk of 30-day mortality compared to non-insulin-treated diabetes. The quality of evidence was rated as very low. The details of the GRADE summary of the

TABLE 1 Characteristics of the included trials.

References	Number of ITDM	Number of NITDM	Total	Age ≥ 60 years	Male (%)	RR	Lower limit of 95% CI	Upper limit of 95% CI	RR type	Study type	Surgery type	Data source	Year of data collection	Country	ITDM definition	NITDM definition	Types of diabetes	Mortality type	Adjusted factor in the analysis
Axelrod et al. (33)	3,306	4,565	7,871	≥60	99	1.16	0.85	1.57	Crude OR	Retrospective cohort	Major vascular surgery	Department of veterans affairs patient treatment file database	1997–1999	United States	Treated with insulin	Treated with oral hypoglycemic agents or diet	Both	Hospital mortality	None
Bolliger et al. (19)	189	171	360	≥60	67.22	2.94	0.977	8.846	Crude RR	Prospective cohort	Major non-cardiac surgery	A Swiss university hospital and a Swiss tertiary-care center	2005–2009	Switzerland	Treated with insulin, including a combination of insulin and oral hypoglycaemics	Oral hypoglycaemics alone	Type 2	30-day mortality	None
Wallaert et al. (34)	620	541	1,161	≥60	67.13	0.667	0.327	1.361	Crude RR	Retrospective cohort	Lower-extremity bypass surgery	Vascular Study Group of New England	2003–2010	United States	Reliance on insulin administration at baseline to control diabetes	Patients who were diabetic, but did not rely on insulin	Both	Hospital mortality	None
Bakker et al. (35)	87	242	329	≥60	68.6	3.709	0.847	16.24	Crude RR	Retrospective cohort	Vascular surgery	Department of Vascular Surgery of the Erasmus university Medical Center	2002–2011	The Netherlands.	Treated with insulin	Treated without insulin	Type 2	30-day mortality	None
Serio et al. (36)[a]	15,050	20,747	35,797	NA	NA					Retrospective cohort					Insulin dependent	Dependent on oral hypoglycaemics	Both	30-day mortality	

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TABLE 1 (Continued)

References	Number of ITDM	Number of NITDM	Total	Age >= 60 years	Male (%)	RR	Lower limit of 95% CI	Upper limit of 95% CI	RR type	Study type	Surgery type	Data source	Year of data collection	Country	ITDM definition	NITDM definition	Types of diabetes	Mortality type	Adjusted factor in the analysis
Serio (vascular surgery)[a]	NA	NA	NA	NA	NA	1.06	0.83	1.35	Adjusted OR		Vascular surgery	MSQC	2007–2011	United States					Anesthesia technique, male gender, ventilator dependent, COPD, cardiac risk factors, on dialysis, steroid use, 10% weight loss, sepsis, emergent, ASA class, wound classification
Serio (general surgery)[a]	NA	NA	NA	NA	NA	1.18	1	1.39	Adjusted OR		General surgery	MSQC	2007–2011	United States					Anesthesia technique, male gender, ventilator dependent, COPD, cardiac risk factors, on dialysis, Steroid use, 10% weight loss, sepsis, emergent, ASA class, wound classification

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TABLE 1 (Continued)

References	Number of ITDM	Number of NITDM	Total	Age ≥ 60 years	Male (%)	RR	Lower limit of 95% CI	Upper limit of 95% CI	RR type	Study type	Surgery type	Data source	Year of data collection	Country	ITDM definition	NITDM definition	Types of diabetes	Mortality type	Adjusted factor in the analysis
Qin et al. (37)[b]	377	1,101	1,478	<60	0					Retrospective cohort					Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	
Qin (prosthetic breast reconstruction[b]	283	815	1,098	<60	0	NA	NA	NA	NA		Prosthetic breast reconstruction	ACS-NSQIP	2005–2012	United States					None
Qin (autologous breast reconstruction)[b]	94	286	380	<60	0	NA	NA	NA	NA		Autologous breast reconstruction	ACS-NSQIP	2005–2012	United States					None
Golinvaux et al. (15)	787	1,650	2,437	≥60	45.78	2.935	0.935	9.219	Crude RR	Retrospective cohort	Elective lumbar fusion	ACS-NSQIP	2005–2012	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Haltmeier et al. (38)	2,280	2,280	4,560	≥60	49.6	1.177	0.998	1.388	Crude RR	Retrospective cohort	Emergency abdominal surgery	ACS-NSQIP	2005–2009	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Qin et al. (39)[c]	2,481	5,252	7,733	≥60	38.29					Retrospective cohort					Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	

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TABLE 1 (Continued)

References	Number of ITDM	Number of NITDM	Total	Age ≥ 60 years	Male (%)	RR	Lower limit of 95% CI	Upper limit of 95% CI	RR type	Study type	Surgery type	Data source	Year of data collection	Country	ITDM definition	NITDM definition	Types of diabetes	Mortality type	Adjusted factor in the analysis
Fu et al. (40)	295	691	986	≥60	45.45	4.685	0.426	51.465	Crude RR	Retrospective cohort	Total shoulder arthroplasty	ACS-NSQIP	2011–2014	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Qin (Open Ventral hernia repair)[c]	2,348	4,882	7,230	≥60	38.56	0.882	0.473	1.645	Crude RR		Open ventral hernia repair	ACS-NSQIP	2005–2012	United States					None
Qin (laparoscopic ventral hernia repair)[c]	133	370	503	<60	34.4	0.923	0.038	22.517	Crude RR		Laparoscopic Ventral hernia repair	ACS-NSQIP	2005–2012	United States					None
Patterson et al. (41)	91	153	244	≥60	29.94	3.363	0.309	36.566	Crude RR	Retrospective cohort	Proximal humerus fractures	ACS-NSQIP	2005–2014	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Phan et al. (42)	171	270	441	<60	51.25	4.727	0.194	115.369	Crude RR	Retrospective cohort	Anterior cervical discectomy and fusion	ACS-NSQIP	2005–2012	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Webb et al. (43)	4,881	15,367	20,248	≥60	40.85	1.296	0.538	3.124	Crude RR	Retrospective cohort	Total knee arthroplasty	ACS-NSQIP	2005–2014	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None

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TABLE 1 (Continued)

References	Number of ITDM	Number of NITDM	Total	Age ≥ 60 years	Male (%)	RR	Lower limit of 95% CI	Upper limit of 95% CI	RR type	Study type	Surgery type	Data source	Year of data collection	Country	ITDM definition	NITDM definition	Types of diabetes	Mortality type	Adjusted factor in the analysis
Di Capua et al. (44)	250	540	790	≥60	45.79	0.617	0.129	2.95	Crude RR	Retrospective cohort	Elective adult spinal deformity surgery	ACS-NSQIP	2010–2014	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Stepan et al. (45)	1,772	2,679	4,451	≥60	42.42	3.402	1.049	11.029	Crude RR	Retrospective cohort	Upper extremity surgery	ACS-NSQIP	2005–2015	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Pothof et al. (46)	1,919	3,118	5,037	≥60	62.16	1.937	1.154	3.252	Crude RR	Retrospective cohort	Carotid endarterectomy	ACS-NSQIP	2011–2015	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Traven et al. (16)	2,484	5,332	7816	NA	56.89	2.147	0.622	7.408	Crude RR	Retrospective cohort	Shoulder arthroscopy	ACS-NSQIP	2005–2016	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None

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TABLE 1 (Continued)

References	Number of ITDM	Number of NITDM	Total	Age ≥ 60 years	Male (%)	RR	Lower limit of 95% CI	Upper limit of 95% CI	RR type	Study type	Surgery type	Data source	Year of data collection	Country	ITDM definition	NITDM definition	Types of diabetes	Mortality type	Adjusted factor in the analysis
Gu et al. (22)	975	1,890	2,865	≥60	44.86	10.752	0.501	250	Adjusted OR	Retrospective cohort	Revision total knee arthroplasty	ACS-NSQIP	2007–2016	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	NA
Leonard-Murali et al. (23)[d]	29,361	60,012	89,373	NA	NA					Retrospective cohort					Requiring daily insulin therapy	Requiring therapy with a noninsulin anti-diabetic agent	Both	30-day mortality	
Leonard-Murali (laparoscopic sleeve gastrectomy)[d]	16,342	40,414	56,756	NA	NA	1.23	0.73	2.08	Adjusted OR		Laparoscopic sleeve gastrectomy	MBSAQIP	2015–2017	United States					Age, sex, race, ASA class, and BMI
Leonard-Murali (laparoscopic Roux-en-Y gastric bypass)[d]	13,019	19,598	32,617	NA	NA	1.33	0.78	2.25	Adjusted OR		Laparoscopic Roux-en-Y gastric bypass	MBSAQIP	2015–2017	United States					Age, sex, race, ASA class, and BMI
Mamidi et al. (47)	211	379	590	<60	32.39	5.377	0.22	131.419	Crude RR	Retrospective cohort	Tonsillectomy	ACS-NSQIP	2005–2018	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None

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TABLE 1 (Continued)

References	Number of ITDM	Number of NITDM	Total	Age ≥ 60 years	Male (%)	RR	Lower limit of 95% CI	Upper limit of 95% CI	RR type	Study type	Surgery type	Data source	Year of data collection	Country	ITDM definition	NITDM definition	Types of diabetes	Mortality type	Adjusted factor in the analysis
Selemon et al. (48)	334	700	1,034	<60	47.16	0.466	0.101	2.144	Crude RR	Retrospective cohort	Aseptic revision total hip arthroplasty	ACS-NSQIP	2006–2016	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None
Kebaish et al. (49)	2,885	9,728	12,613	≥60	50.96	2.698	1.066	6.828	Crude RR	Retrospective cohort	Total hip arthroplasty	ACS-NSQIP	2012–2016	United States	Treated with insulin, including a combination of insulin and non-insulin pharmacologic methods	Noninsulin pharmacologic methods, excluding diabetes controlled by diet and/or lifestyle	Both	30-day mortality	None

ITDM, insulin-treated diabetes mellitus; NITDM, non-insulin-treated diabetes mellitus; NA, not available; RR, relative risk; CI, confidence interval; OR, odds ratio; ACS-NSQIP, American College of Surgeons National Surgical Quality Improvement Program Database; MBSAQIP, Metabolic and Bariatric Surgery Accreditation and Quality Improvement Program database; MSQC, Michigan Surgical Quality Collaborative; COPD, chronic obstructive pulmonary disease; ASA, American Society of Anesthesiologists; BMI, body mass index.

[a]Serio et al. (36) provided mortality information for vascular surgery and general surgery, respectively.

[b]Qin et al. (37) provided mortality information for prosthetic breast reconstruction and autologous breast reconstruction, respectively.

[c]Qin et al. (38) provided mortality information for open ventral hernia repair and laparoscopic ventral hernia repair, respectively.

[d]Leonard-Murali et al. (23) provided mortality information for laparoscopic sleeve gastrectomy and laparoscopic Roux-en-Y gastric bypass, respectively.

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TABLE 2 Newcastle-Ottawa Scale (cohort studies).

References	Selection				Comparability		Outcome			Quality
	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the basis of the design or analysis (the most important factor)	Comparability of cohorts on the basis of the design or analysis (the second factor)	Assessment of outcome	Was follow-up long enough for outcomes to occur	Adequacy of follow up of cohorts	
Axelrod et al. (33)	*	–	*	–	–	–	*	*	–	4
Bolliger et al. (19)	–	*	*	*	–	–	*	*	*	6
Wallaert et al. (34)	*	–	*	–	–	–	*	*	–	4
Bakker et al. (35)	–	–	*	–	–	–	*	*	–	3
Serio et al. (36)	*	*	*	–	*	–	*	*	–	6
Golinvaux et al. (15)	*	*	*	–	–	–	*	*	–	5
Qin et al. (37)	*	*	*	–	–	–	*	*	–	5
Haltmeier et al. (38)	*	*	*	–	*	*	*	*	–	7
Qin et al. (39)	*	*	*	–	–	–	*	*	–	5
Fu et al. (40)	*	*	*	–	–	–	*	*	–	5
Patterson et al. (41)	*	*	*	–	–	–	*	*	–	5
Phan et al. (42)	*	*	*	–	–	–	*	*	–	5
Webb et al. (43)	*	*	*	–	–	–	*	*	–	5
Di Capua et al. (44)	*	*	*	–	–	–	*	*	–	5
Stepan et al. (45)	*	*	*	–	–	–	*	*	–	5
Pothof et al. (46)	*	*	*	–	–	–	*	*	–	5
Traven et al. (16)	*	*	*	–	–	–	*	*	–	5
Gu et al. (22)	*	*	*	–	–	–	*	*	–	5
Leonard-Murali et al. (23)	*	*	*	–	–	*	*	*	–	6
Mamidi et al. (47)	*	*	*	–	*	*	*	*	–	7
Selemon et al. (48)	*	*	*	–	–	–	*	*	–	5
Kebaish et al. (49)	*	*	*	–	–	–	*	*	*	6

*The scale uses a star system, with a maximum of score of 9 stars, and includes 3 categories: selection, comparability, and outcome. Asterisk represents one point.

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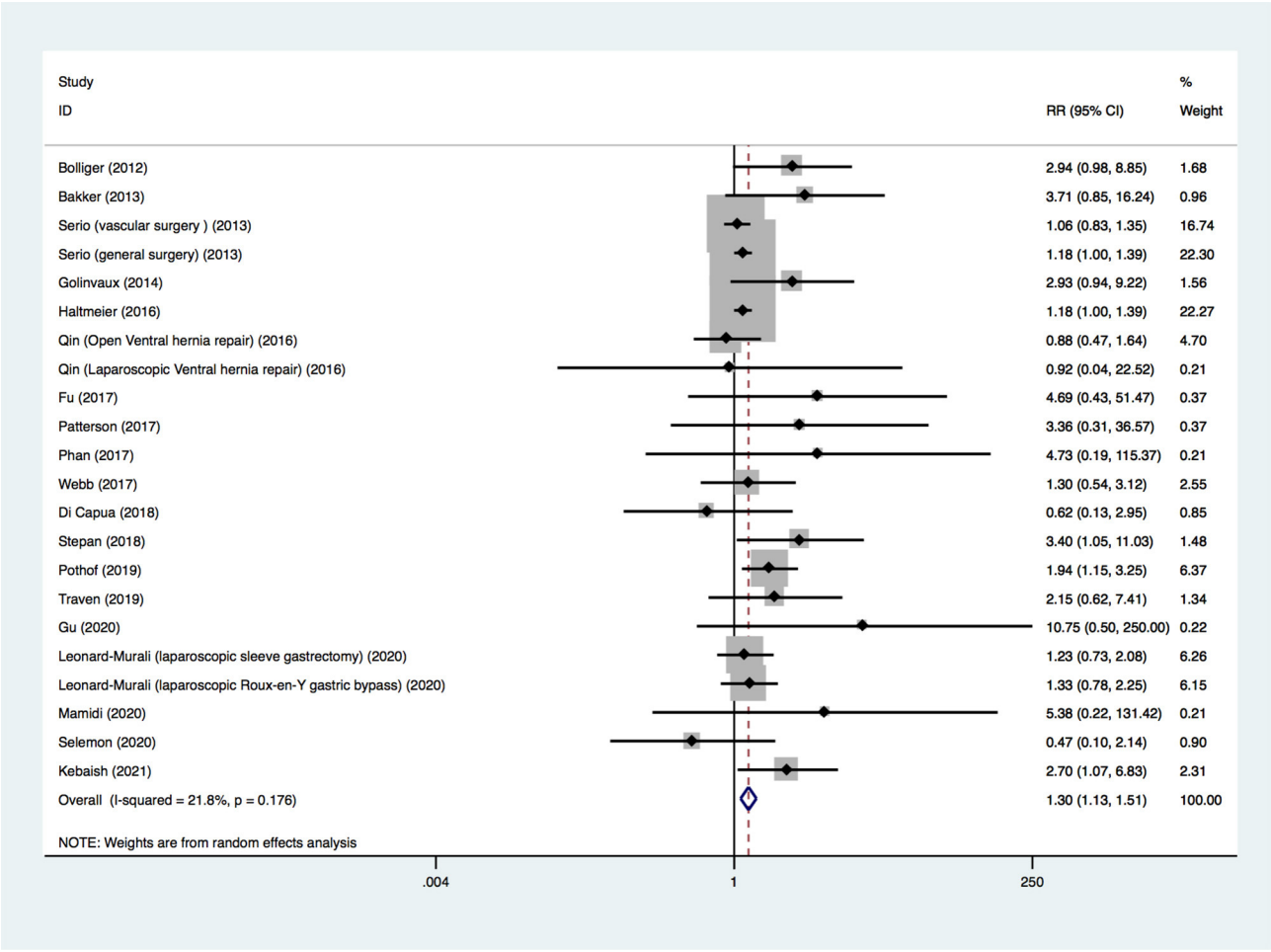


FIGURE 2
Forest plot for risk of 30-day mortality.

findings are shown in Supplementary Digital Content 3 (see Table, Supplementary Digital Content 3, which demonstrates GRADE).

We conducted subgroup analyses by the age of participants, type of surgery, RR/OR type, and comparability score of NOS (Supplemental Digital Content 4). In the subgroups of aged $\geq$ 60 years, orthopedic surgery, general surgery, upper extremity surgery, crude RR, adjusted OR, comparability score of 0-point, 1-point and 2-point, the results showed a higher risk of 30-day mortality of insulin-treated diabetes compared to non-insulin-treated diabetes. In the subgroups of aged $<$ 60 years, vascular surgery, ventral hernia repair, bariatric surgery, emergency abdominal surgery, tonsillectomy, major non-cardiac surgery, the results showed a similar 30-day mortality between insulin-treated diabetes and non-insulin-treated diabetes.

The details of the subgroup analysis are presented in Table 3.

We also conducted sensitivity analysis. Limiting the analysis to studies with a NOS score $\geq$ 7, the results were consistent with the original analysis (RR, 1.182; 95% CI, 1.002–1.393; p = 0.047). Limiting the analysis to the studies that controlled for complications and comorbidities of patients with diabetes based on the design or analysis, the results were consistent with the original analysis (RR, 1.157; 95% CI, 1.042–1.286; p = 0.006). The included studies were excluded one by one, and no single study had a noticeable influence on the total combined effect size (see Text document, Supplementary Digital Content 4, which demonstrates sensitivity analysis).

The publication bias was evaluated. The funnel plot appeared asymmetrical (see Text document, Supplementary Digital Content 5, which demonstrates funnel plot). Significant publication bias was detected using Egger's test (bias = 0.88; 95% CI, 0.31–1.44; p = 0.004). After using the trim-and-fill method, the new pooled effect size from the random-effects model was similar to the primary result (RR, 1.260; 95% CI, 1.076–1.476; p = 0.004). A new funnel plot after adding seven simulated missing studies is presented in Supplementary Digital Content 6 (see Text document, Supplementary Digital Content 6, which demonstrates new funnel plot).

3.3. Hospital mortality

Two included studies provided information on the outcome of hospital mortality. A random-effects model

TABLE 3 Results of subgroup and sensitivity analyses for risk of 30-day mortality.

	No. of studies	No. of data	No. of patients	Heterogeneity	Pooled risk ratio (95% CI)	Significance
Age of participants						
<60 yr	4[a]	4[a]	25,68	I-squared = 0.0%, P = 0.402	0.975 (0.301, 3.162)	p = 0.967
≥60 yr	13[a]	13[a]	62,150	I-squared = 41.3%, P= 0.059	1.716 (1.244, 2.366)	p = 0.001
Unknown	3[b,c]	5[b,c]	132,986	I-squared = 0.0%, P = 0.769	1.164 (1.025, 1.322)	p = 0.019
Type of surgery						
Orthopedic surgery	10	10	49,474	I-squared = 3.7%, P = 0.406	1.827 (1.165, 2.865)	p = 0.009
Vascular surgery	3[b]	3[b]	[d]	I-squared = 69.3%, P = 0.038	1.560 (0.861, 2.827)	p = 0.143
Ventral hernia repair	1[a]	2[a]	7,733	I-squared = 0.0%, P= 0.978	0.883 (0.479, 1.629)	p = 0.691
Bariatric surgery	1[c]	2[c]	89,373	I-squared = 0.0%, P = 0.837	1.278 (0.881, 1.855)	p = 0.196
Emergency abdominal surgery	1	1	4,560	–	1.177 (0.998, 1.388)	p = 0.053
Tonsillectomy	1	1	590	–	5.377 (0.220, 131.419)	p = 0.302
Major non-cardiac surgery	1	1	360	–	2.940 (0.977, 8.847)	p = 0.055
General surgery	1[b]	1[b]	[e]	–	1.180 (1.001, 1.391)	p = 0.049
Upper extremity surgery	1	1	4,451	–	3.402 (1.049, 11.031)	p = 0.041
RR/OR type						
Crude RR	16[a]	17[a]	69,669	I-squared = 28.8%, P = 0.129	1.614 (1.218, 2.138)	p = 0.001
Adjusted OR	3	5[b,c]	128,035	I-squared = 0.0%, P = 0.584	1.161 (1.021, 1.319)	p = 0.022
Comparability score of NOS						
0 point	15[a]	16[a]	67,384	I-squared = 13.8%, P = 0.296	1.802 (1.317, 2.466)	p < 0.001
1 point	2[b,c]	4[b,c]	125,170	I-squared = 0.0%, P = 0.832	1.156 (1.017, 1.314)	p = 0.026
2 points	2	2	5,150	I-squared = 0.0%, P = 0.352	1.182 (1.002, 1.393)	p = 0.047

[a]Qin et al. (39) provided mortality information for open ventral hernia repair and laparoscopic ventral hernia repair, respectively.
[b]Serio et al. (36) provided mortality information for vascular surgery and general surgery, respectively.
[c]Leonard-Murali et al. (23) provided mortality information for laparoscopic sleeve gastrectomy and laparoscopic Roux-en-Y gastric bypass, respectively.
[d]Serio et al. (36) did not provide demographic information for vascular surgery.
[e]Serio et al. (36) did not provide demographic information for general surgery.

was used to pool the data, and no significant difference was found (RR, 0.970; 95% CI, 0.584–1.611; p = 0.905; Figure 3) between insulin-treated diabetes and non-insulin-treated diabetes, suggesting that the two groups had a similar hospital mortality. The quality of evidence was rated as very low. The details of the GRADE summary of the findings are shown in Supplementary Digital Content 3 (see Table, Supplementary Digital Content 3, which demonstrates GRADE).

As only two studies were included in the outcome, we did not conduct subgroup analysis, sensitivity analysis or evaluate publication bias.

4. Discussion

Our study demonstrated that compared with non-insulin-treated diabetes, insulin-treated diabetes was associated with a

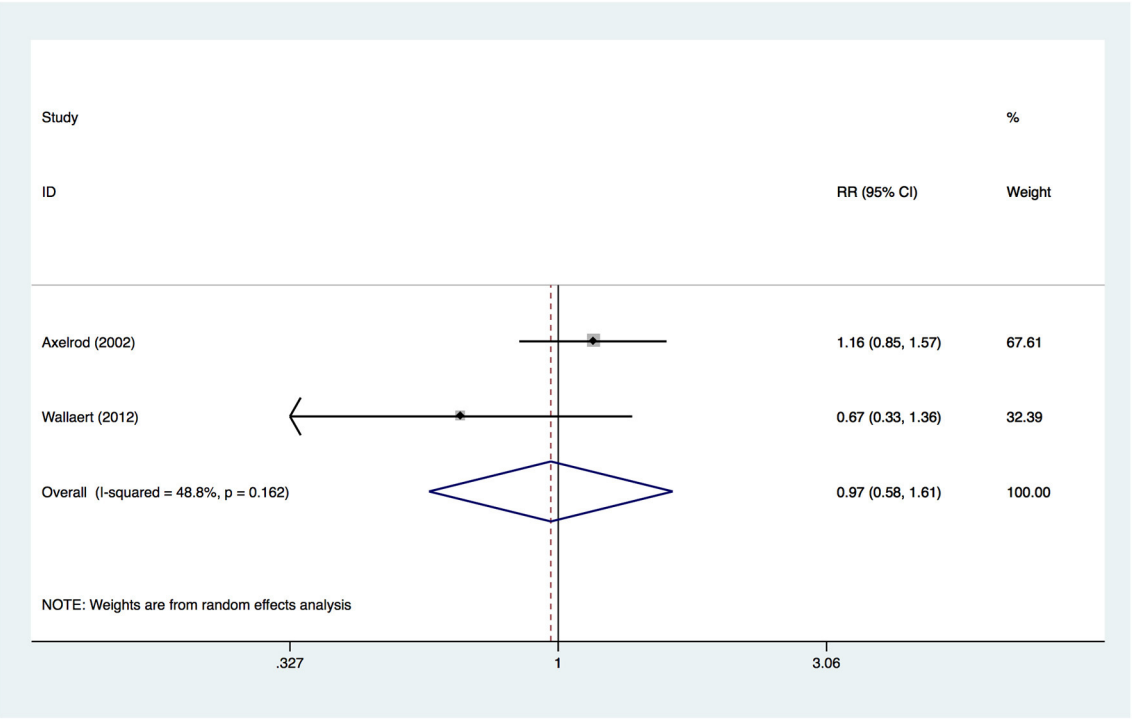


FIGURE 3
Forest plot for risk of in-hospital mortality.

higher risk of 30-day mortality, but a similar risk of in-hospital mortality after surgery. The quality of the evidence was rated as very low.

4.1. Comparison with the published literature

Our findings are consistent with two previously published systematic reviews of cardiac surgery that found that insulin-treated diabetes had a significantly higher risk of short-term mortality (<1 year) after percutaneous coronary intervention (18) and a significantly higher risk of short-term mortality (≤30 days) after coronary artery bypass surgery compared with non-insulin-treated diabetes (17).

Although all the included studies provided postoperative death outcomes for insulin-treated diabetes and non-insulin-treated diabetes, only seven studies specifically compared postoperative mortality outcomes between insulin-treated and non-insulin-treated diabetes (19, 22, 23, 33, 36, 38, 47). Only one study found that insulin-treated diabetes was associated with increased postoperative mortality compared to non-insulin-treated diabetes (19). However, in this study, the relationship only held for insulin-only diabetes, and did not include diabetes treated both with and without insulin.

It has also been reported that neither diabetes nor insulin exposure is an independent risk factor for death at 30 days after non-cardiac surgery in 65-year-old patients with coronary artery disease or heart failure (24). Among studies with longer follow-up times after non-cardiac surgery, some studies found insulin-treated diabetes to be an independent risk factor for postoperative death compared with non-insulin-treated diabetes (50, 51), while one study found no association (52). One study found that oral hypoglycemic therapy, but not insulin therapy, was associated with non-cardiac mortality at 2 years in patients at coronary risk (25).

4.2. Strengths

Our study had several strengths. First, to our knowledge, it is the first systematic review comparing short-term postoperative mortality between insulin-treated diabetes and non-insulin-treated diabetes in non-cardiac surgery. Second, we screened 17,265 articles and finally included 22 studies that comprised 208,214 patients with diabetes; thus, our study comprised a notably large number of patients. Third, no significant heterogeneity was found in our study ($I^2 = 22.5\%$, $p = 0.159$). Although there was significant publication bias, the new pooled result was only slightly changed after adding the simulated missing study using the trim-and-fill method, suggesting that the effect of publication bias on the result might be small. Fourth, of the studies we included, only two included type 2 diabetes (19, 35). The remaining 21 studies did not clarify the type of diabetes. The patients were grouped based on prehospital hypoglycemic regimens. This shows that our conclusions are practical.

4.3. Implications for clinicians, policy, and research

Our study showed that insulin-treated diabetes and non-insulin-treated diabetes had a similar risk of in-hospital mortality after surgery. However, only two studies were included in the outcome, thus, the findings may be not reliable. Our study also showed that in non-cardiac surgery, the 30-day mortality of insulin-treated patients with diabetes was higher than that of non-insulin-treated diabetes patients. This suggests that prehospital hypoglycemic regimens might be one of the bases for the preoperative risk stratification of patients with diabetes. However, it must be noted that because of the generally low quality of the originally included studies, this conclusion may be unreliable. In particular, only three studies controlled the bias of comorbidities and complications of patients with diabetes in the study design or data analysis. Only one of the originally included studies found that type 2 diabetes treated with insulin alone was an independent risk factor for short-term postoperative death compared with diabetes treated with oral hypoglycemic agents alone. However, the results of the study might be explained by its inclusion of more patients with a history of myocardial infarction, more patients with a history of coronary artery bypass graft, more patients with a history of heart failure, and a higher ASA grade in the insulin-only group. Surprisingly, insulin-treated diabetes was still associated with increased postoperative mortality but with a reduced effect size in our sensitivity analyses that included only studies that controlled for comorbidities and complications and only high-quality studies. This suggests on the one hand that confounders caused an overestimation of the effect size, and on the other hand, long-term prehospital hypoglycemic regimens might indeed influence short-term postoperative mortality. This might be related to the long-term cardiovascular protective effects of oral hypoglycemic agents (53–55).

In published models predicting postoperative cardiac complications for non-cardiac surgery, the Revised Cardiac Risk Index included preoperative treatment with insulin as a risk factor (20), while the Geriatric-Sensitive Perioperative Cardiac Risk Index included diabetes as a risk factor (56). Although it is still not clear whether the hypoglycemic regimen or the severity of the patient's condition leads to increased short-term postoperative mortality, our current results still tended to stratify diabetes patients according to prehospital hypoglycemic regimens and assign insulin-treated diabetes with a higher risk level.

Sensitivity and subgroup analyses with adjusted effect sizes showed that insulin-treated diabetes was an independent risk factor. However, in the subgroup analysis of surgical types, only the subgroup of orthopedic surgery and vascular surgery could be further analyzed, while the number of studies on other surgical types was too small to be further analyzed. In the vascular surgery subgroup, insulin-treated diabetes was not associated with increased 30-day mortality. This suggests that there is a need to further distinguish the risks of long-term hypoglycemic regimens between different surgical types. There was no association between insulin-treated diabetes and 30-day mortality in the subgroup of age < 60 years. This indicates that our results may not be applicable to all populations. It is also possible that the severity of diabetes might be similar in patients undergoing vascular surgery or in patients aged < 60 years. However, our current research was unable to answer this question; thus, well-designed and high-quality research are required to answer these questions in the future.

5. Limitation

The most notable limitation of our study is that the quality of most of the originally included studies was not high. Of the 22 studies, only two were rated as high-quality studies. Diabetes-related complications and comorbidities were independent risk factors for non-cardiac postoperative 30-day mortality (26). Age and ASA grade were also listed as risk factors for major adverse cardiovascular events and mortality within 30 days after surgery (56–58). Therefore, we regarded diabetes-related complications and comorbidities as the most important confounding factors, while age and ASA grade were the second most important confounding factors. Of the 22 studies included, only three controlled for diabetes-related complications and comorbidities, and only three controlled for age and ASA classification. The evidence quality rating was very low. This showed that the results obtained are uncertain.

In the subgroup analysis, the results of the vascular surgery and age < 60 subgroups were not consistent with the original analysis. Thus, our study conclusions may not be applicable to all surgery types and populations.

6. Conclusion

Our study showed that insulin-treated diabetes was associated with an increased risk of 30-day mortality after non-cardiac surgery compared with non-insulin-treated diabetes. The quality of the evidence was rated as very low. As it is difficult to rule out the influence of confounding factors, we believe that the results obtained are not reliable. Well-designed, high-quality studies that controls for important confounding factors is needed to investigate the impact of prehospital hypoglycemic regimens on short-term mortality after non-cardiac surgery.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

JJ and SW: methodology and writing—original draft preparation. RS and SL: data curation and writing—review and editing. YZ and ZZ: methodology and writing—editing. JB: data curation and funding acquisition. AL: funding acquisition and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1142490/full#supplementary-material

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Evaluation of efficacy and safety of glucokinase activators—a systematic review and meta-analysis

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Aims: Glucokinase activators (GKAs) promote the activity of glucokinase (GK) and is under development for the treatment of diabetes. The efficacy and safety of GKAs require evaluation.

Methods: This meta-analysis included randomized controlled trials (RCTs) with a duration of at least 12 weeks conducted in patients with diabetes. The primary objective of this meta-analysis was the difference of hemoglobin A1c (HbA1c) change from baseline to study end between GKA groups and placebo groups. Risk of hypoglycemia and laboratory indicators were also evaluated. Weighted mean differences (WMDs) and 95% confidence intervals (CIs) were calculated for the continuous outcomes, and odds ratios (ORs) and 95% CI were calculated for the risk of hypoglycemia.

Results: Data from 13 RCTs with 2,748 participants treated with GKAs and 2,681 control participants were analyzed. In type 2 diabetes, the level of HbA1c decreased greater in patients with GKA treatment compared with placebo (WMD = -0.339%, 95% CI -0.524 to -0.154%, P < 0.001). The OR comparing GKA versus placebo was 1.448 for risk of hypoglycemia (95% CI 0.808 to 2.596, P = 0.214). The WMD comparing GKA versus placebo was 0.322 mmol/L for triglyceride (TG) levels (95% CI 0.136 to 0.508 mmol/L, P = 0.001). When stratified by drug type, selectivity, and study duration, a significant difference was found between groups. In type 1 diabetes, the result of HbA1c change and lipid indicators showed no significant difference between the TPP399 group and the placebo group.

Conclusions: In patients with type 2 diabetes, GKA treatment was associated with a better glycemic control but a significant elevation in TG concentration in general. The efficacy and safety varied with drug type and selectivity.

Systematic review registration: International Prospective Register of Systematic Reviews, identifier CRD42022378342.

KEYWORDS

glucokinase activators, efficacy, safety, diabetes, hypoglycemia

Introduction

Diabetes is a complex, chronic illness requiring continuous medical care. In 2021, approximately 537 million people were living with diabetes worldwide, of whom 90%–95% were diagnosed with type 2 diabetes and 1.2 million were diagnosed with type 1 diabetes (1). The management of these populations is particularly challenging. Despite that novel drug therapeutic strategies acting with different mechanisms of actions have been developed continuously, unmet needs still exist in clinical practice.

Glucokinase (GK) is an enzyme that functions as a glucose sensor, which plays a critical role in glucose homeostasis. GK regulates glucose-stimulated insulin secretion (GSIS) and intrinsic glucagon release in pancreatic cells. In the liver, GK promotes hepatic glucose uptake, glycogen synthesis, and storage (2, 3). Based on the hypothesis that control over GK activity might affect glucose homeostasis, GK activators (GKAs) emerged as a distinct glucose-lowering option in the management of diabetes. Recently, one GKA was approved for treatment of type 2 diabetes in China (4) and several are in the late stage of clinical development.

GKAs are small molecules that bind to an allosteric site on GK with effects of promoting GK activation. These GKAs can be classified according to the site of action: pancreatic and hepatic dual-acting GKAs and hepatoselective GKAs (5). So far, several GKAs have been developed and tested for clinical application purposes, whereas different efficacies and safeties were shown in these agents. The main concerns with use of GKAs focused on the increased risk of worsening of dyslipidemia, hypoglycemia, and liver damage (5, 6). In a randomized controlled trial (RCT), MK-0941 was found to be associated with hemoglobin A1c (HbA1c) reduction of -0.5% to -0.8% and a significant increased incidence of hypoglycemia and hypertriglyceridemia by 30 weeks in patients with treatment of insulin (7). Two RCTs evaluating AZD1656 demonstrated different results in HbA1c reduction, while an increased risk of hypertriglyceridemia was observed (8, 9). However, recently phase 3 RCTs conducted in drug-naïve or metformin-treated type 2 diabetes patients showed that dorzagliatin could improve HbA1c for a duration of 24 weeks without significant increased risk of hypoglycemia. However, a consistent increased level of liver enzymes, serum triglyceride, and serum uric acid (SUA) was observed in the study groups that received dorzagliatin treatment (10, 11). Due to the inconsistencies shown in clinical trials with regard to efficacies and side effects, it is feasible and necessary to make a comprehensive meta-analysis to evaluate the efficacy and safety of GKA treatment.

Materials and methods

Systematic review protocol

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement was used to conduct this meta-analysis (12). The study protocol is available in the International Prospective Register of Systematic Reviews (registration no. CRD42022378342)

Data sources and search strategy

We searched electronic databases including PubMed, EMBASE, and the Cochrane Central Register of Controlled Trials last updated on 01/12/2022. Two investigators (HW and WY) independently searched for clinicals trials of GKAs. The search terms were as follows: RCTs; GK; GKA; piragliatin; RO4389620; dorzagliatin; HMS5552; globalagliatin; SY-004; LY2608204; MK-0941; AZD1656; PB-201; PF-04937319; PF-04991532; AMG 151; ARRY-403; TTP399; GKI-399; AZD 6370; TMG123. The search strategies were as follows: (1) RCTs and GK; (2) RCTs and GKA; (3) every GKA was added respectively as the collateral searching term addition to RCTs. The detail of the search strategy is shown in Supplementary Table 1.

Selection criteria and data extraction

The criteria for including the studies were as follows: (1) RCTs conducted in diabetes patients (both type 1 diabetes and type 2 diabetes); (2) RCTs with GKAs and placebo control in different treatment arms; (3) since the primary outcome of this meta-analysis was to evaluate the efficacy of GKAs, RCTs with study duration ≥12 weeks were selected; (4) RCTs with available data of glucose control with or without safety outcomes. The exclusion criteria were as follows: (1) non-RCTs; (2) RCTs with study duration<12 weeks; (3) RCTs that did not report efficacy or safety outcomes.

We removed the duplicates and screened the remaining articles at the title and abstract levels according to the predetermined inclusion and exclusion criteria for possible inclusions. The process of search and selection was performed by two independent blinded investigators (HW and WY) with Covidence software. If either investigator considered a study potentially eligible, we further obtained and screened the full text. We invited a third investigator (CL) to join the discussion and resolved discrepancies by consensus.

Two investigators (WY and HW) independently performed the data extraction from each publication using a standardized form: publication data, baseline characteristics of the study population (sample size, sex, age, body mass index [BMI], baseline body weight), baseline laboratory indicators (HbA1c, fasting plasma glucose [FPG], postprandial plasma glucose [PPG], fasting insulin [FINS], homeostasis model assessment of insulin resistance [HOMA-IR], homeostasis model assessment-β [HOMA-β], total cholesterol [TCHO], triglyceride [TG], low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C], alanine transaminase [ALT], aspartate aminotransferase [AST], serum uric acid [SUA], estimated glomerular filtration rate [eGFR]), incidence of hypoglycemia, duration of follow-up, description of the GKA and control interventions, and changes of

laboratory indicators from baseline to study end point. Disagreements or discrepancies were resolved by discussion between the two investigators and a third investigator (XC).

The PICOTS (population, interventions, comparators, outcomes, timing, and setting) criteria of this meta-analysis were summarized as follows (Table 1):

Assessment of methodological quality

Two investigators (WY and HW) independently assessed the quality of each included studies using a modified Cochrane risk-of-bias instrument that includes response options of "definitely or probably yes" (assigned as low risk of bias) or "definitely or probably no" (assigned as high risk of bias) (13). Each study is judged on seven items: sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessors, incomplete outcome data, selective outcome reporting, and other sources of bias.

Data synthesis and analysis

Weighted mean differences (WMDs) and 95% confidence intervals (CIs) were calculated for continuous measures. We used mean changes from baseline and standard deviations (SDs) extracted from published data when reported. We calculated mean changes from baseline by subtracting baseline means from outcome means and calculated change SDs from baseline by using methods outlined in the Cochrane Handbook when authors reported results as measures before and after intervention (14). When SDs were missing, we estimated them from standard errors or confidence intervals. Odds ratios (ORs) and 95% CI were calculated for odds of hypoglycemia. For studies with multiple intervention arms, we selected the groups relevant to the purpose of our study. Then, we combined groups to create a single pairwise comparison recommended by the Cochrane Handbook (14).

Between-study heterogeneity was assessed using the Q test and I (2) statistic, with significance set at $P < 0.05$; heterogeneity was considered low, moderate, substantial, or considerable for estimated I (2) values of 0%–40%, 30%–60%, 50%–90%, and 75%–100%, respectively. The random-effect model was used in this meta-analysis. To interpret the level of heterogeneity, we also calculated prediction intervals, as these intervals could reflect the variation in treatment effects over different settings, including what effect is to be expected in patients in the future.

Meta-regression analysis was performed to evaluate the association between change in HbA1c level, risk of hypoglycemia, change in TG concentration, and clinical features. Potential confounding factors, including age, sex, BMI, and duration of diabetes, were adjusted by using multivariable meta-regression analysis. A P value <0.05 was considered statistically significant.

Funnel plot and Egger's test were used to assess publication bias. Statistical analyses were conducted with Review Manager, version 5.4 (Nordic Cochrane Centre, Copenhagen, Denmark), and STATA statistical software package, version 17 (Stata Corp, College Station, TX, United States).

Results

Overall analysis

In total, 13 placebo-controlled studies were included in this meta-analysis, among which are two RCTs with treatment of AZD 1656 in type 2 diabetes, three RCTs with treatment of dorzagliatin in type 2 diabetes, two RCTs with treatment of MK-0941 in type 2 diabetes, two studies with treatment of PB-201 in type 2 diabetes, two studies with treatment of PF-04991532 in type 2 diabetes, one study with treatment of TPP399 in type 2 diabetes, and one study with treatment of TPP399 in type 1 diabetes (including two separate parts) (Figure 1). The baseline characteristics and risk-of-bias evaluation for the included studies are summarized in Table S2 and Table S3. The overall risk of bias was low. Funnel plots and Egger's test showed even distributions (P = 0.416) (Figure S1).

Type 2 diabetes

Efficacy results

In type 2 diabetes patients, compared with the placebo group, GKA treatment was associated with a greater reduction in the level of HbA1c (WMD = -0.339%, 95% CI -0.524 to -0.154%, $P < 0.001$; predictive interval -1.03 to 0.35; $I^2 = 84.5\%$). Greater reductions in HbA1c were observed in the AZD 1656 subgroup (WMD = -0.431%, 95% CI -0.754 to -0.107%, P = 0.009; $I^2 = 65.1\%$) and dorzagliatin subgroup (WMD = -0.575%, 95% CI -0.719 to -0.430%, $P < 0.001$; $I^2 = 36.5\%$), whereas no significant HbA1c change differences were observed in the MK-0941 subgroup (WMD = -0.480%, 95% CI -0.972 to 0.012%, P = 0.056; $I^2 = 54.4\%$), PB-201 subgroup (WMD = -0.121%, 95% CI -0.267 to 0.026%, P = 0.106; $I^2 = 0.0\%$), PF-04991532 subgroup (WMD = 0.078, 95% CI -0.372 to 0.529, P = 0.734, $I^2 = 82.9\%$), or TTP399 subgroup (WMD = -0.562, 95% CI -1.247 to 0.124, P = 0.108, $I^2 = 58.4\%$) (Figure 2). As for duration of follow-up, greater HbA1c reductions were observed in RCTs with longer follow-up (stratified by 12 and 24

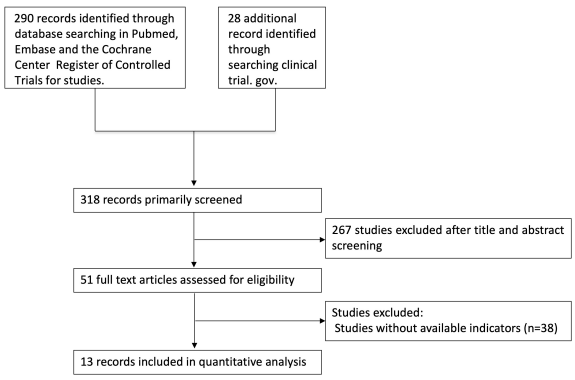


FIGURE 1
Flow diagram of the included studies.

weeks, respectively) (Figures S2A, B). In terms of selectivity, dual-acting GKAs (WMD = -0.410%, 95% CI -0.578 to -0.242%, P < 0.001; I^2 = 78.2%) and hepatoselective GKAs (WMD = -0.183%, 95% CI -0.638 to 0.271%, P = 0.429; I^2 = 81.7%) showed a difference in the effects on HbA1c (Figure S2C).

Compared with the placebo group, the level of FPG was decreased slightly in patients with GKA treatment (WMD = -0.302 mmol/L, 95% CI -0.566 to -0.037 mmol/L, P = 0.025; predictive interval -1.14 to 0.54; I^2 = 63.3%), which was mainly driven by the AZD 1656 subgroup (WMD = -0.522 mmol/L, 95% CI -0.927 to -0.116 mmol/L, P = 0.012; I^2 = 0.0%) (Figure 3). The study-duration analysis indicated that greater reductions in FPG were observed in RCTs with follow-up longer than 12 weeks (WMD = -0.422 mmol/L, 95% CI -0.775 to -0.070 mmol/L, P = 0.019; I^2 = 59.4%) (Figures S3A, B). Similarly with the results of HbA1c, greater changes in FPG were found in RCTs with dual-acting GKAs (WMD = -0.346 mmol/L, 95% CI -0.660 to -0.031 mmol/L, P = 0.031; I^2 = 72.8%) (Figure S3C).

Four studies reported the change of PPG from baseline, among which are three RCTs with treatment of dorzagliatin and one RCT with treatment of MK-0941. Compared with the placebo group, GKA treatment was associated with greater reduction in the level of PPG (WMD = -2.360 mmol/L, 95% CI -2.715 to -2.006 mmol/L, P < 0.001; predictive interval -3.14 to -1.58; I^2 = 0.0%). The sensitivity analysis by drug type and study duration showed consistent results (Figures S4A–C).

When compared with the placebo group, GKA treatment was associated with a greater decrease in FINS (WMD = -0.964 μIU/ml, 95% CI -1.317 to -0.611 μIU/ml, P < 0.001; predictive interval -1.58 to -0.34; I^2 = 9.0%) (Table S4). In addition, compared with the placebo group, a greater decrease in HOMA-IR (WMD = -0.077, 95% CI -0.133 to -0.022, P = 0.006; predictive interval -0.44 to 0.28; I^2 = 0.0%) and a greater increase in HOMA-β (WMD = 2.681, 95% CI 1.141 to 4.221, P = 0.001; I^2 = 0.0%) were revealed in the GKA (all with dorzagliatin treatment) group (Table S4).

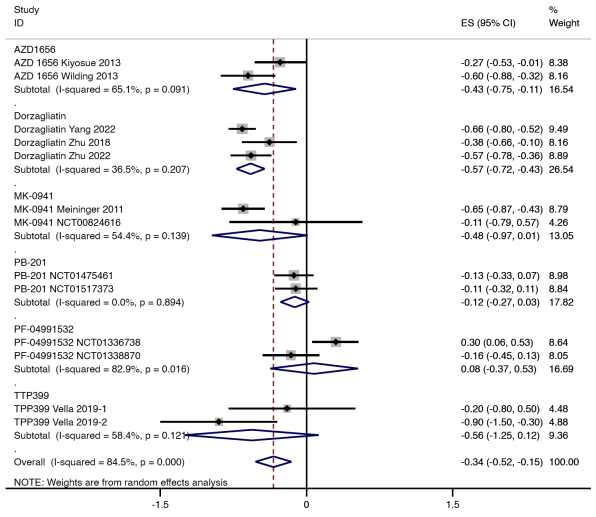


FIGURE 2
HbA1c change from baseline with GKA treatment versus placebo in type 2 diabetes. HbA1c, hemoglobin A1c; GKA, glucokinase activator; ES, effect size; CI, confidential interval.

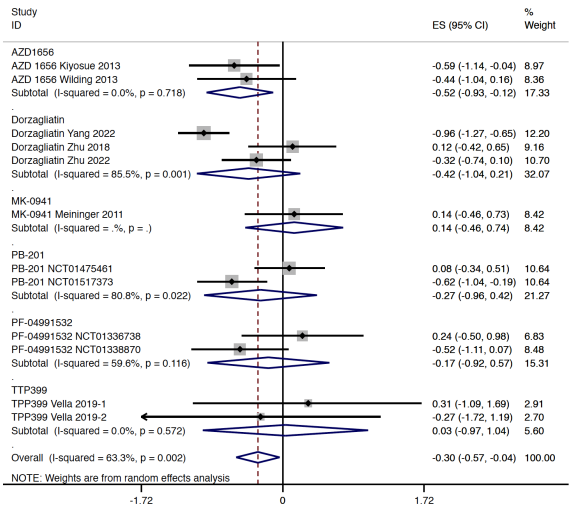


FIGURE 3
FPG change from baseline with GKA treatment versus placebo in type 2 diabetes. FPG, fasting plasma glucose; GKA, glucokinase activator; ES, effect size; CI, confidential interval.

Safety results

In type 2 diabetes, results from this meta-analysis indicated that GKA treatment showed a similar risk of hypoglycemia when compared with the placebo treatment (OR = 1.448, 95% CI 0.808 to 2.596, P = 0.214; predictive interval 0.39 to 5.32; I^2 = 29.3%). Among the investigated agents, the AZD 1656 subgroup (OR = 18.100, 95% CI 2.438 to 134.406, P = 0.005; I^2 = 0.0%) was found to be associated with increased risk of hypoglycemia (Figure 4). Compared with the placebo group, treatment of dorzagliatin (OR = 4.241, 95% CI 0.752 to 23.915, P = 0.102; I^2 = 0.0%), MK-0941 (OR = 1.361, 95% CI 0.938 to 1.976, P = 0.105; I^2 = 0.0%), TPP399 (OR = 0.256, 95% CI 0.037 to 1.795, P = 0.170; I^2 = 40.7%), PB-201 (OR = 1.165, 95% CI 0.323 to 4.199, P = 0.815), and PF-04991532 (OR = 1.368, 95% CI 0.223 to 8.387, P = 0.735; I^2 = 0.0%) showed a similar risk of hypoglycemia in type 2 diabetes (Figure 4). The sensitivity analyses by study duration showed consistent results (Figures S5A, B). As for selectivity, increased risk of hypoglycemia was only observed in the dual-acting GKA group (OR = 1.884, 95% CI 1.023 to 3.469, P = 0.042; I^2 = 24.0%) (Figure S5C).

In total, four studies reported available data regarding the change of blood lipid and uric acid concentration in type 2 diabetes. Compared with the placebo group, GKA treatment was associated with a greater elevation in TG concentration (WMD = 0.322 mmol/L, 95% CI 0.136 to 0.508 mmol/L, P = 0.001; predictive interval -0.22 to 0.86; I^2 = 52.8%). The drug-type analysis indicated that the significant increase in TG level was observed in the dorzagliatin subgroup (WMD = 0.450 mmol/L, 95% CI 0.334 to 0.566 mmol/L, P < 0.001; I^2 = 12.3%), but not in the AZD 1656 subgroup (WMD = 0.240 mmol/L, 95% CI -0.195 to 0.675 mmol/L, P = 0.280; I^2 = 0.0%) or TTP399 subgroup (WMD = -0.074 mmol/L,

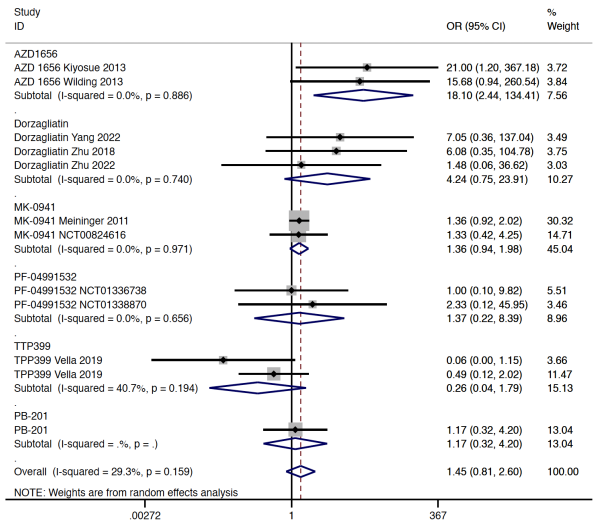


FIGURE 4
Incidence of hypoglycemia with GKA treatment versus placebo in type 2 diabetes. GKA, glucokinase activator; OR, odd ratio; CI, confidential interval.

95% CI -0.461 to 0.313 mmol/L, P = 0.707; I^2 = 0.0%) (Figure 5). In addition, the change of TG concentration from baseline was significantly greater in RCTs with dual-acting GKAs (WMD = 0.439 mmol/L, 95% CI 0.334 to 0.544 mmol/L, P < 0.001; I^2 = 0.0%), when compared with the placebo group (Figure S6). In terms of other lipid indicators, one RCT with treatment of TPP399 and two RCTs with treatment of dorzagliatin reported available data in type 2 diabetes. Compared with the placebo group, GKA treatment was associated with a slight elevation of TCHO level (WMD = 0.136 mmol/L, 95% CI 0.057 to 0.214 mmol/L, P = 0.001; I^2 = 0.0%). Increased SUA levels (WMD = 29.07 μmol/L, 95% CI 18.11 to 40.03 μmol/L, P < 0.001; I^2 = 52.7%) were observed in the GKA group (all were RCTs with dorzagliatin treatment) compared with placebo users (Table S5).

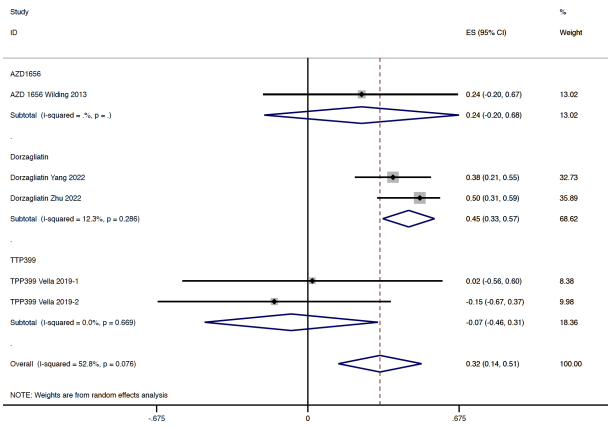


FIGURE 5
TG change from baseline with GKA treatment versus placebo in type 2 diabetes. TG, triglyceride; GKA, glucokinase activator; ES, effect size; CI, confidential interval.

In terms of liver safety, significant elevations in ALT (WMD = 4.146 U/L, 95% CI 2.339 to 5.988 U/L, P < 0.001; I^2 = 38.0%) and AST (WMD = 4.26 U/L, 95% CI 3.574 to 5.477 U/L, P < 0.001; I^2 = 0.0%) were observed after treatment with GKA as compared with placebo (Figures S7,S8). The change in LDL-C, HDL-C, and eGFR from baseline showed no significant difference between the GKA group and placebo group (Table S5).

Type 1 diabetes

Efficacy results

In type 1 diabetes, HbA1c was evaluated in one RCT with two separate parts, whereas the aggregated result of HbA1c change showed no significant difference between the TPP399 group and placebo group (WMD = -0.366%, 95% CI -0.800 to -0.068%, P = 0.098; I^2 = 60%) (Figure S9).

Safety results

In type 1 diabetes, TG concentration change from baseline showed no significant difference between the TTP399 treatment and placebo groups (WMD = 0.036 mmol/L, 95% CI -0.355 to 0.427 mmol/L, P = 0.857; I^2 = 75.8%). Results of TCHO change, LDL-C change, or HDL-C change from baseline showed no significant difference between the TPP399 group and placebo group (Table S6).

Associated factors with the GKA efficacy and safety

In type 2 diabetes, meta-regression analysis results showed that covariates such as age, gender, duration of diabetes, BMI, baseline HbA1c, FPG change, TG change from baseline, and FPG change from baseline was associated with the change of HbA1c in the GKA group when compared with the placebo group (Table S7). The above covariates and change of HbA1c were not associated with the risk of hypoglycemia (Table S8) or change of TG concentration (Table S9) in the GKA group when compared with the placebo group.

Discussion

This is the first meta-analysis systematically evaluating efficacy and safety of GKA treatment. In the current study, we found that treatment with GKA showed greater reduction in HbA1c, FPG, and PPG when compared with the placebo group. The sensitivity analysis demonstrated a difference when stratified by drug type, selectivity, and study duration. Regarding the safety profiles, risk of hypoglycemia showed no significant difference between the GKA group and placebo group, whereas a significant elevation in TG concentration was observed in GKA users. In addition, levels of ALT, AST, and SUA appeared to be associated with treatment with GKA in some studies, especially in studies with dorzagliatin.

GK is the enzyme catalyzing glucose phosphorylation, which expressed primarily in beta cells and hepatocytes. In the pancreas, GK establishes the threshold for GSIS, whereas in the liver, it regulates glucose uptake and controls glycogen synthesis (2, 3). However, the research and development process of GKA is

TABLE 1 The PICOTS for the evaluation of efficacy and safety of glucokinase activators.

Population	Patients with type 2 diabetes patients or type 1 diabetes
Interventions	Glucokinase activators, including piragliatin; RO4389620; dorzagliatin; HMS5552; globalagliatin; SY-004; LY2608204; MK-0941; AZD1656; PB-201; PF-04937319; PF-04991532; AMG 151; ARRY-403; TTP399; GKI-399; AZD 6370; and TMG123.
Comparisons	Placebo
Outcomes	Primary outcome: The difference of HbA1c change from baseline between the GKA group and placebo group. Secondary outcomes: (i) The difference of FPG, PPG, FINS, HOMA-IR, HOMA-β, TCHO, TG, LDL-C, HDL-C, ALT, AST, SUA, and eGFR change from baseline between the GKA group and placebo group. (ii) The risk of hypoglycemia comparing the GKA group with the placebo group.
Time	Published before 01/12/2022
Study design	Randomized controlled trials

PICOTS, population, interventions, comparators, outcomes, timing, and setting; GKA, glucokinase activator; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; PPG, postprandial plasma glucose; FINS, fasting insulin; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment -β; TCHO, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; ALT, alanine transaminase; AST, aspartate aminotransferase; SUA, serum uric acid; eGFR, estimated glomerular filtration rate.

challenging; only a minority have reached the clinical trial stage. According to the pooled results in this meta-analysis, GKA treatment demonstrated an improved effect on glycemic control in type 2 diabetes, with a placebo-adjusted HbA1c reduction ranging from -0.12% to -0.57%, except for PF-04991532. Among these GKAs, dorzagliatin and TTP399 800 mg once daily appeared to be superior in HbA1c improvement. The placebo-adjusted FPG decreased slightly ranged from -0.17 to -0.52 mmol/L, whereas the placebo-adjusted PPG reduction ranged from -1.88 to -2.47 mmol/L. It seems that the improvement observed in HbA1c was primarily derived from the change in PPG. The efficacy results indicated that GKA treatment only showed a slight improvement in glycemia control. It is of note that results from the previous UK Prospective Diabetes Study (UKPDS) indicated that the minimum clinically important differences (MCIDs) for HbA1c in type 2 diabetes was 1%, which bring on improvement of clinical complications (15). Therefore, does the slight improvement in glycemia control observed in GKA treatment bring benefits for type 2 diabetes patients? Large cardiovascular outcomes trials (CVOTs) are needed to answer this question. In addition, head-to-head studies directly comparing the efficacy of GKAs and other hypoglycemic agents are expected in the future studies.

In this meta-analysis, we observed a significant decrease in FINS level with treatment of GKAs when compared with placebo. Among the included studies, the concentration of FINS declined significantly with treatment of AZD1656 and PB-201, whereas no significant difference in FINS was found in treatment of dorzagliatin or TPP399 when compared with placebo. The pooled analysis from dorzagliatin showed favorable effects in both HOMA-IR and HOMA-β. Unfortunately, results regarding HOMA-IR and HOMA-β were not available for other GKA agents in the included studies. Additionally, a previous study indicated that activation of GK was associated with increased level of glucagon-like peptide-1 (GLP-1) (5), whereas few included studies provide relevant results. More studies with thorough evaluation of islet cell function, insulin resistance, and effects on incretins would provide comprehensive insight into the mechanism of action of GKAs.

GKAs could be classified into full-acting activator and hepatoselective activator (5). The original intension for developing hepatoselective activator was to reduce the potential pancreatic GK overactivating-related adverse effects. Of note, significant improvement in HbA1c and FPG was not observed in hepatoselective GKAs from this meta-analysis, including TTP399 and PF-04991532. A lack of effectiveness was the prominent obstacle in the previous development process of PF-04991532. Meanwhile, in the two-dose arms of TTP399 conducted in type 2 diabetes, significant glycemic improvement was only observed in the high-dose group of 800 mg once daily when compared with placebo. Therefore, the overall effect size of hepatoselective GKAs on glycemic control was weakened by the PF-04991532 and TPP399 400 mg once daily groups. Whether the pancreas and liver dual-acting activator play a stronger effect on glycemic control than the hepatoselective activator is still unknown. The result from the TPP399 800 mg once daily arm, after all, demonstrated a significant effectiveness on glycemic control with a good safety profile during the 6-month treatment (16). More evidence is expected from clinical trials evaluating the efficacy and safety of hepatoselective activator.

Currently, one of the most concerned issues regarding GKAs was the attenuated efficacy over time, which was observed in previous studies (7, 9). The possible explanation for the observed lack of sustained efficacy for MK-0941 was that participants with long duration of diabetes were included, who had severe beta cell impairment. It was explained that GKA was unable to exert a pancreatic effect on this kind of patients. For the lack of sustained efficacy of AZD 1656, it was speculated that the glucolipotoxicity from chronic ongoing activation of the GK in the pancreatic beta cell led to a reduction in glucose responsive islets (17–19).

According to our analysis, no attenuated efficacy over time was found in the changes of HbA1c. Studies with a longer duration of follow-up are further needed to fully elucidate this issue.

Increased risk of hypoglycemia, which was associated with the GK overstimulation and subsequent disruption of the GSIS threshold (5, 17, 18), is a pronounced adverse effect that hindered the development of GKAs. According to this meta-analysis, the overall risk of hypoglycemia was similar when compared with placebo, whereas the dual-activator subgroup showed an significant increased risk of hypoglycemia. Conversely, compared with placebo, the risk of hypoglycemia was similar with the treatment of hepatoselective activators TTP399 and PF-04991532. This finding was consistent with the mechanism proposed above exactly. Effectively balancing efficacy and risk of hypoglycemia remains to be an essential issue to be solved.

The occurrence of dyslipidemia was another concern regarding GKAs. The activation of GK was required for the initial step of the hepatic lipogenic pathway, and GK mRNA expression was associated with markers of *de novo* lipogenesis and liver triglyceride content in humans. Based on these physiological pathways, it had been speculated that the inappropriate activation of liver GK could result in elevations of TG concentrations (5, 19). For patients with diabetes who were already at higher risk of cardiovascular complication, dyslipidemia was an undesired adverse effect. More than that, hepatic steatosis, steatohepatitis, and ultimate cirrhosis induced by chronic elevation of TG should also be considered (19). According to this meta-analysis, the pooled TG level was demonstrated to be increased significantly in the dorzagliatin group when compared with placebo. Furthermore, estimation of laboratory indicators suggested an elevation in ALT, AST, and SUA in patients receiving dorzagliatin. There is a high likelihood that the elevation of ALT, AST, and SUA is the side effect of dorzagliatin as evidenced by the fact that when placebo-treated study subjects were switched to dorzagliatin treatment in the clinical trials, ALT, AST, and SUA all increased to the same level as that in dorzagliatin treatment groups (20). Were the unfavorable changes in these metabolic parameters transient or continuous? Based on the current limited evidence, the answer was not available. However, previous genome-wide association studies might provide some clues. These studies revealed that glucokinase regulator protein (GCKR) single-nucleotide polymorphisms (SNPs) were associated with the elevated level of SUA and the increased risk of gout (21–25). Further investigations are needed to elucidate the clinical implications of those unfavorable changes and the underlying mechanism.

In this meta-analysis, we also synthesized the two parts of SimpliciT1 Study performed in type 1 diabetes. In patients with type 1 diabetes, hepatic exposure to insulin was subnormal, resulting in less GK expression and subsequent compromised liver uptake and metabolism of glucose (26). Additionally, restoring GK expression in the liver improved glycemic control in streptozotocin-induced type 1 diabetic rats (27). In this context, the SimpliciT1 Study was conducted to determine the efficacy and safety of TTP399 in type 1 diabetes (28). The pooled HbA1c change from baseline was not statistically significant with TPP399 treatment when compared with placebo. However, participants assigned to TPP399 in both parts achieved better glycemic control with numeric reduction in insulin doses. Importantly, TPP399 treatment was found to be associated with a reduced risk of hypoglycemia and a reduced risk of ketosis. Given the above observations, TTP399 appeared to be a promising adjunct therapy to insulin in the treatment of type 1 diabetes and merited more extensive evaluations.

This meta-analysis also has some limitations. First, we combined data from trials that varied in study duration, interventions, and baseline characteristics. However, we used the random-effect model for statistical analysis and performed multiple sensitivity analyses to control the heterogeneity. Second, some included RCTs were dose-finding studies containing diverse doses. Nevertheless, as a class of agents under exploration and development, the optimal dose still could not be determined for some GKAs. Thus, the deviations in results brought by the non-optimal doses could not be avoided in the current meta-analysis. Third, the complete follow-up information could not be obtained from the published data in part of the included studies, leading to reporting bias to the pooled analysis. Fourth, the study duration of included studies was relatively short, weakening the power to examine the long-term efficacy and safety. Fifth, limited computable laboratory indicators hindered the in-depth evaluation of metabolic and safety issues.

In conclusion, according to the results of this meta-analysis, GKA treatment was associated with a better glycemic control. Regarding the safety profiles, risk of hypoglycemia showed no significant difference between the GKA group and placebo group, whereas a significant elevation in TG concentration was observed in GKA users. The efficacy and safety of GKA treatment varied with drug type and selectivity. More studies with a longer follow-up duration and larger sample size are still needed to provide solid evidence to evaluate the long-term efficacy and safety of GKAs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

LJ and XC designed the study. WY and HW conducted the data collection. WY, XC, CL, and RJ conducted the statistical analysis. WY drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1175198/full#supplementary-material

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Aflibercept for long-term treatment of diabetic macular edema and proliferative diabetic retinopathy: a meta-analysis

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Purpose: This meta-analysis compared the long-term (12 months or 24 months) efficacy and safety of intravitreal aflibercept injection (IAI) for diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR).

Methods: We selected 16 randomized controlled trials (RCTs) performed after 2015 that had a minimum of 12 months and up to 24 months of treatment and conducted a meta-analysis with Review Manager version 5.3. Visual acuity (VA), central subfield thickness (CST) and adverse events were the outcomes selected for evaluation from the eligible studies.

Results: Based on 16 RCTs, we evaluated a total of 7125 patients. For PDR and severe DME with poor baseline vision, after a minimum of 12 months and up to 24 months of treatment, the aflibercept treatment group obtained better VA improvement than the focal/grid laser photocoagulation treatment group (MD=13.30; 95%CI: 13.01~13.58; P<0.001) or other treatments (ranibizumab, focal/grid laser photocoagulation, PRP, et al.) group (MD=1.10; 95%CI: 1.05~1.16; P<0.001). In addition, the aflibercept treatment group got higher CST reduction than the focal/grid laser photocoagulation treatment (MD=-33.76; 95%CI: -45.53 ~ -21.99; P<0.001) or other treatments (ranibizumab, focal/grid laser photocoagulation, et al.) group (MD=-33.76; 95%CI: -45.53 ~ -21.99; P<0.001). There was no significant difference in the overall incidence of ocular and non-ocular adverse events in each treatment group.

Conclusions: This meta-analysis showed that the advantages of IAI are obvious in the management of DME and PDR with poor baseline vision for long-term observation (a minimum of 12 months and up to 24 months) with both VA improvement and CST reduction. Applied IAI separately trended to be more effective than panretinal photocoagulation separately in VA improvement for

PDR. More parameters should be required to assess functional and anatomic outcomes.

KEYWORDS

diabetic macular edema, proliferative diabetic retinopathy, aflibercept, meta-analysis, anti-vascular endothelial growth factor, focal/grid laser photocoagulation, panretinal photocoagulation

1 Introduction

Diabetic retinopathy (DR) has become an increasingly common microvascular complication of diabetes that affects visual health. Proliferative diabetic retinopathy (PDR) and severe diabetic macular edema (DME), especially center-involved DME (CI-DME), have become the most common causes of visual loss among working population (1–3).

PDR and DME have been managed by panretinal laser photocoagulation (PRP) and focal/grid laser photocoagulation for the past 40 years (4, 5). Vascular endothelial growth factor (VEGF), as a "real killer" that seriously threatens vision, plays an important role in the improvement of diabetic retinal vascular permeability and is an important contributor to both vascular leakage and new blood vessel growth (6, 7). To date, many researchers have conducted systematic reviews and standard meta-analyses of anti-VEGF treatments which have been recognized as novel approaches for visual impairment of DR (8, 9). However, these existing meta-analysis did not include direct and indirect comparisons of the long-term observation (a minimum of 12 months and up to 24 months) of aflibercept and focal/grid laser photocoagulation or other treatments respectively in patients with DME, or afliberceptand panretinal photocoagulation (PRP) in patients with PDR. Therefore, we conducted a meta-analysis that included randomized controlled trials (RCTs) reported after 2015. This report compared the therapeutic effects of intravitreal injections of aflibercept with that of focal/grid laser photocoagulation (patients with DME), PRP (patients with PDR) or other treatments, evaluated the efficacy and safety of intravitreal aflibercept injection (IAI) in the management of DME and PDR for long-term observation (a minimum of 12 months and up to 24 months).

2 Materials and methods

2.1 Search strategy

We systematically searched and identified relevant trials and literature from the PubMed, Embase, and Web of Science databases, as well as the Cochrane Central Register of Controlled Trials, and the publication time is until January 2023. The scope of the search was restricted to both English languages. The following key search points and medical keywords were used: *diabetic retinopathy, randomized controlled trials, aflibercept, anti-vascular endothelial growth factor, focal/grid laser photocoagulation, panretinal photocoagulation, diabetic macular edema, and proliferative diabetic retinopathy.* Retrospective research, reviews,case reports, letters and surveys were excluded. Visual acuity (VA), central subfield thickness (CST), and adverse events were the focus of our meta-analysis. Only anonymous online public data were used in the research, without the active participation of patients and informed consent.

2.2 Eligibility of studies

The RCTs that meet the following criteria were considered eligible: (1) participants over 18 years of age with type 1 or 2 diabetes; (2) participants with DME or PDR; (3) published number of patients, age, gender, and intervention details; (4) treatments of interest were intravitreal injection of aflibercept 2.0mg compared with other treatment schemes, including ranibizumab 0.3mg/0.5mg, dexamethasone 0.7mg, brolucizumab 6.0 mg, faricimab 6.0 mg, focal/grid laser photocoagulation and PDR; the treatments determined by individual researchers could be proactive (fixed), reactive (pro re nata, PRN), or proactive/reactive (treat and extend, T&E); (5) the follow-up time of these study were 12 months or more; (6) studies that provided main outcomes evaluation parameters as mean ± SD: mean change in best-corrected visual acuity (BCVA) [measure in Early Treatment Diabetic Retinopathy Study (ETDRS) letters), mean change in CST, and adverse events; (7) all included studies should be compliant with the Declaration of Helsinki and written informed consent from enrolled patients; (8) if the same research subjects were reported in different publications, only the most recent and authoritative publications with available data for targeted outcomes was included.

The exclusion criteria were as follows: (1) retrospective studies and review articles; (2) unpublished data were not adopted; (3) participants only suffered from non-proliferative diabetic retinopathy; (4) no comparison was made between aflibercept and other treatment schemes; (5) RCTs with too short follow-up time.

2.3 Data extraction and quality assessment

The assessment of the full-text articles and data extraction of each study was independently conducted by two authors. In case of disagreement between two authors, the third author assessed again.

Assessment contents included: publication metrics (name of the first author, year of publication, location and study design, etc.), the information of the participants (diagnosis, sample size, demographic characteristics, clinical characteristics, criteria of inclusion and exclusion), the information on intervention (options/frequency of treatment, dosage of medicine, duration of follow-up), and the main information on outcomes (BCVA, CST and adverse events).

Two authors independently assessed the risk of bias of the included RCTs, including random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective outcome reporting, and other bias (10). The divergences were resolved through full discussion, with the assistance of a third author if necessary.

2.4 Statistical analysis

Review Manager 5.3 was used for statistical analysis. The visual evaluation parameter was BCVA, the anatomical evaluation parameter was CST, and safety indicators included systemic or ocular adverse events during the injection treatment. The fixed effect model was used for data processing (11). Continuous outcomes were estimated using the mean difference (MD) and 95% credible intervals (CIs). Dichotomous outcomes were estimated using the risk difference (RD) and 95% CIs. Forest plots were used to summarize the weighted estimates.

3 Results

3.1 Literature search

We performed a preliminary literature search through all databases and retrieved 4430 articles. The literature selection process and reasons for exclusion are summarized in Figure 1. First, we excluded 2395 articles with nonconforming title and 851 articles with nonconforming abstracts, and then 1184 potential relevant articles were screened out. Next, we excluded 992 articles according to the article type, including review (n=899), case reports (n=53), letters (n=32), surveys (n=8). Then there were 192 articles qualified for full-text assessment. Moreover, 179 articles were excluded by study design, including retrospective study (n=87), irrelevant population (n=24), irrelevant intervention schemes (n=37), irrelevant comparison objects (n=9), no extracted results (n=3), incomplete published data (n=12), repetitive research (n=7). Ultimately, 13 articles (16 RCTs) were included in this meta-analysis.

3.2 Characteristics of the included studies

Table 1 summarizes the basic characteristics of the 16 RCTs in the 13 articles included in this meta-analysis. The study sample sizes ranged from 42 to 951 patients. The characteristics of the patients with DME or PDR were similar among the trials. The follow-up duration ranged from 12 months to 24 months. The dose of aflibercept was 2.0 mg in the aflibercept treatment groups in all included studies (12–24). Other treatments include focal/grid laser photocoagulation, PRP, vitrectomy with PRP, and intravitreal injection of ranibizumab 0.3mg/0.5mg, dexamethasone 0.7mg, brolucizumab 6.0 mg, faricimab 6.0 mg.

3.3 Risk of bias

Figure 2 showed the risk of bias graph and summary for each included study. Fifteen studies had random sequence generation and allocation concealment. Regarding blinding of participants and personnel, 12 studies were assessed as low risk and 2 studies as high risk. Regarding blinding of outcome assessment, 11 studies were assessed as low risk and one study as high risk. Regarding incomplete outcome data, 14 studies were assessed as low risk and 2 studies as high risk. Regarding selective outcome reporting, 10 studies were assessed as low risk and 5 studies as high risk. Other studies were rated as having unclear risks.

3.4 Effects of interventions

3.4.1 Visual acuity

Because BCVA is the main visual index to judge the curative effect and progress, and CST is an important anatomical index to judge the degree of macular edema, we analyzed the data of BCVA and CST. Among these RCTs we have included, the baseline BCVA and CST did not exactly match. Therefore, we adopted the mean change in BCVA and CST as the primary outcome. Figures 3 and 4 showed the results of the meta-analysis of the effects of intravitreal aflibercept injection on BCVA improvement. The aflibercept treatment group had significantly better BCVA improvement than the focal/grid laser photocoagulation treatment group (MD=13.30; 95%CI: 13.01~13.58; P<0.001) or other treatments (ranibizumab, focal/grid laser photocoagulation, PRP, et al.) group (MD=1.10; 95%CI: 1.05~1.16; P<0.001).

3.4.2 Central subfield thickness

The effects of IAI in CST are shown in Figures 5 and 6. The aflibercept treatment group had higher CST reduction than the focal/grid laser photocoagulation treatment group (MD=-33.76; 95%CI: -45.53 ~ -21.99; P<0.001) or other treatments (ranibizumab, focal/grid laser photocoagulation, et al.) group (MD=-33.76; 95%CI: -45.53 ~ -21.99; P<0.001).

3.4.3 Adverse events

Of the 16 RCTs involved, the overall incidence rates of ocular and non-ocular adverse events were similar across the treatment groups (Figure 7). Regarding the frequency or pattern of serious ocular adverse events, there was no significant difference between the aflibercept group and other treatments (ranibizumab, focal/grid laser photocoagulation, et al.) group (RD=-0.02; 95%CI: -0.06 ~0.01; P=0.41).

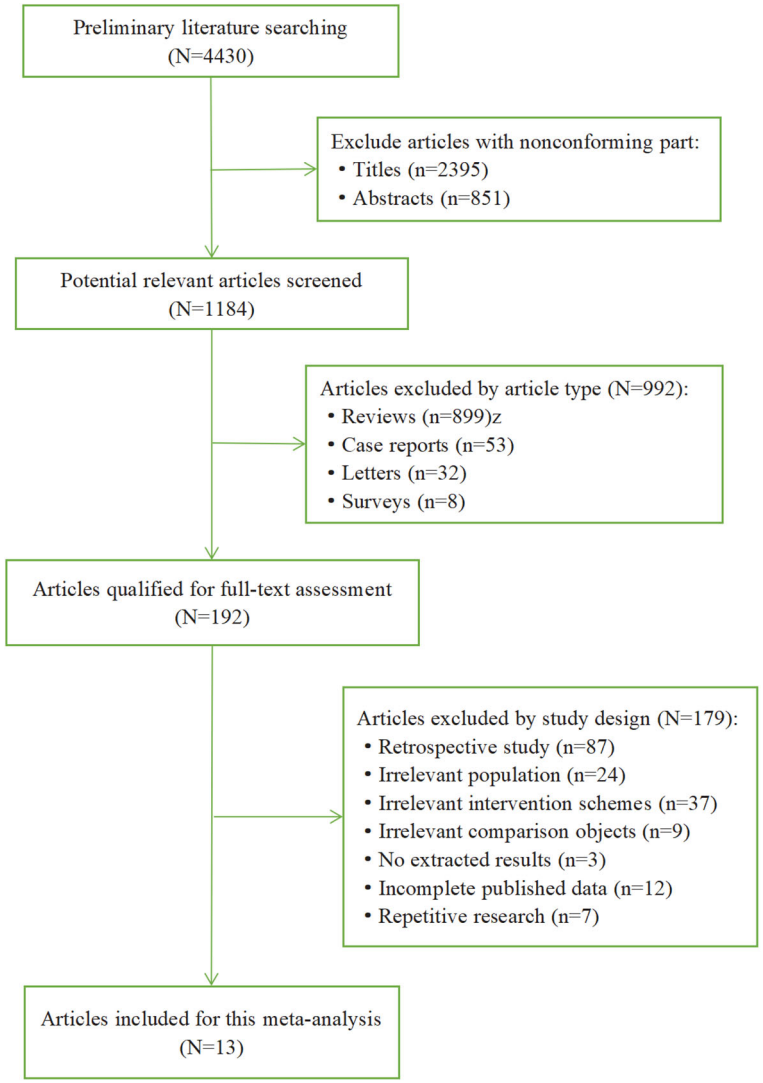


FIGURE 1
Flow chart of literature searching.

4 Discussion

In early 1976, the Diabetic Retinopathy Study group adopted PRP as the gold standard for the treatment of high-risk PDR eyes (25, 26). Since the ETDRS was first published in 1985, focal/grid laser photocoagulation has become the gold standard for the treatment of DME (27). Research on the Diabetic Retinopathy Clinical Research Network (DRCRnet) has confirmed that anti-VEGF drugs are not only effective alternative for PRP in patients with PDR but also as the first-line treatment for DME (28). It is very important for ophthalmologists and policymakers to compare the relative efficacy of DME or PDR treatment with the most reliable method.

Optical coherence tomography (OCT) is a noninvasive and easy-to-perform imaging tool that provides reliable and high-resolution imaging for the observation of retinal anatomy and quantification of the CST (29–31). With the help of OCT and the emergence of anti-VEGF drugs for patients with DR, clinical data suggested that anti-VEGF therapy can reduce macular edema and exudation, thus improving VA, reducing CST, and preventing further vision decline. Our findings are similar to those of previous studies from the viewpoint that anti-VEGF therapy is effective in patients with DME (32, 33). The therapeutic effects of these drugs are obviously superior to those of focal/grid laser photocoagulation separately.

To date, there are few meta-analyses comparing the expected clinical effects of aflibercept with other treatments (ranibizumab, focal/grid laser photocoagulation, PRP, pars plana vitrectomy, et al.) in the management of DME and PDR, especially the research on PDR.Based on this situation, we conducted a meta-analysis to compare the therapeutic effects of drug A with other treatment schemes. We evaluated16 RCTs published after 2015 in this meta-analysis, including 7125 patients who followed up 12 or 24 months. As reported in previous studies, among patients with visual impairment caused by DME, anti-VEGF monotherapy and combined focal/grid laser photocoagulation therapy provided

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TABLE 1 Study characteristics of the included 16 RCTs.

	Study	Design	Disease	Numbers of participants	Gender (F/M)	Interventions details	Follow-up (months)
1	Brown VISTA 2015 (12)	VISTA	DME	461	45.5%/54.5%	Aflibercept 2.0mg Laser photocoagulation	24
2	Brown VIVID 2015 (12)	VIVID	DME	404	38.2%/61.8%	Aflibercept 2.0mg Laser photocoagulation	24
3	DRCR.net 2015 (13)	Protocol T	CI-DME	660	47%/53%	Aflibercept 2.0mg Ranibizumab 0.3mg	12
4	Wells 2016 (14)	Protocol T	CI-DME	660	47%/53%	Aflibercept 2.0mg Ranibizumab 0.3mg	24
5	Sivaprasad 2017 (15)	CLARITY	PDR	232	33.2%/66.8%	Aflibercept 2.0mg PRP	24
6	Fouda 2017 (16)	RCT	DME	42	NR-no rated	Aflibercept 2.0mg Ranibizumab 0.5mg	24
7	Baker 2019 (17)	RCT	CI-DME	702	38%/62%	Aflibercept 2.0mg Laser photocoagulation	24
8	Ozsaygili 2019 (18)	RCT	DME	62	43.5%/56.5%	Aflibercept 2.0mg Dexamethasone 0.7mg	12
9	Chen 2020 (19)	VIVID-East	DME	381	49.7%/50.3%	Aflibercept 2.0mg Laser photocoagulation	12
10	Chatzirallis 2020 (20)	RCT	DME	112	45.5%/54.5%	Aflibercept 2.0mg Ranibizumab 0.5mg	12
11	Antoszyk 2020 (21)	RCT	PDR	205	44%/56%	Aflibercept 2.0mg Vitrectomy with PRP	24
12	Beaulieu 2021 (22)	Protocol V	CI-DME	387	37%/63%	Aflibercept 2.0mg Laser photocoagulation	24
13	Brown KESTREL 2022 (23)	KESTREL	DME	566	37.3%/62.7%	Aflibercept 2.0mg Brolucizumab 6.0 mg	12
14	Brown KITE 2022 (23)	KITE	DME	360	34.7%/65.3%	Aflibercept 2.0mg Brolucizumab 6.0 mg	12
15	Wykof YOSEMITE 2022 (24)	YOSEMITE	DME	940	40.2%/59.8%	Aflibercept 2.0mg Faricimab 6.0 mg	12
16	Wykof RHINE 2022 (24)	RHINE	DME	951	39.1%/60.9%	Aflibercept 2.0mg Faricimab 6.0 mg	12

RCT, randomized controlled trial; F, female; M, male; DME, diabetic macular edema; CI-DME, center-involved diabetic macular edema; PDR, proliferative diabetic retinopathy; PRP, panretinal photocoagulation.

better VA gain than focal/grid laser photocoagulation therapy separately (34). Although the short-term benefit of focal/grid laser photocoagulation combined with anti-VEGF therapy for DME patients was tiny in the DRCR.net Protocol I study, more than one-third of DME patients receiving anti-VEGF therapy delayed focal/grid laser photocoagulation therapy (35, 36). PRP has been the standard of care in the treatment of PDR for decades according to the DRS and the ETDRS (26). Both anti-VEGF therapy and focal/grid laser photocoagulation and PRP achieved remarkable anatomical and functional improvements during early treatment of DME and PDR respectively (37, 38). However, according to our results, for DME with long-term observation (a minimum of 12 months and up to 24 months), IAI had significantly better BCVA improvement than the focal/grid laser photocoagulation treatment

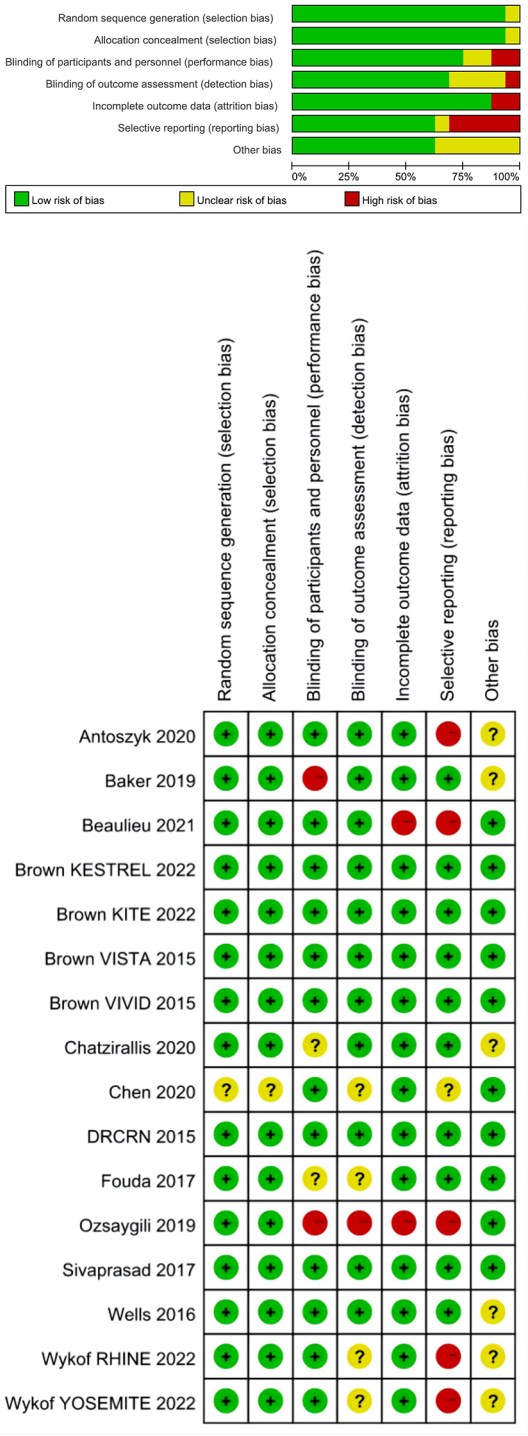


FIGURE 2
Risk of bias graph and summary for each included study.

(MD=13.30; 95%CI: 13.01~13.58; P<0.001) or other treatments (ranibizumab, focal/grid laser photocoagulation, PRP, et al.) (MD=1.10; 95%CI: 1.05~1.16; P<0.001). The visual improvements with IAI were primarily driven by patients with with poor baseline BCVA. In addition, IAI had significantly higher CST reduction than the focal/grid laser photocoagulation treatment (MD=-33.76; 95% CI: -45.53 ~ -21.99; *P*<0.001) or other treatments (ranibizumab, focal/grid laser photocoagulation, et al.) (MD=-33.76; 95%CI: -45.53 ~ -21.99; *P*<0.001). The increased response of patients with intractable DME or PDR may reflect the special pharmacological characteristics of aflibercept. This may be due to the fact that only aflibercept can inhibit both VEGF and placental growth factor (PGF), which are key factors leading to the pathogenesis of DME or PDR (39). More importantly, aflibercept has a faster association

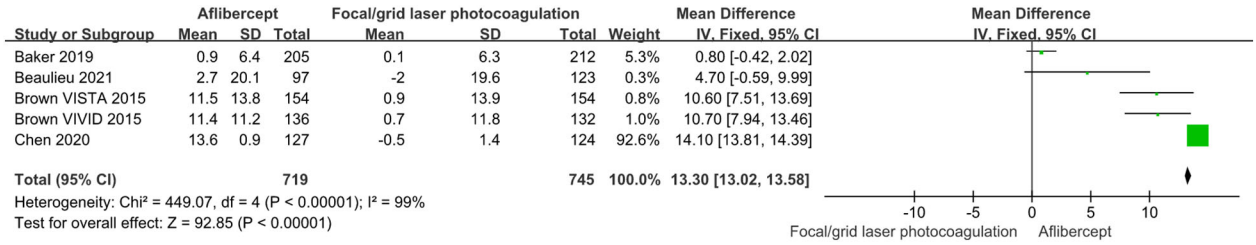


FIGURE 3
The mean changes from baseline in BCVA in the aflibercept group and focal/grid laser photocoagulation treatment group.

rate and a higher binding affinity for VEGF-A, VEGF-B, PlGF-1 and PlGF-2, resulting in accelerating a doubling of response rate (40).

The ability to achieve significant visual improvement with less frequent intravitreal injections and visits will be a valuable strategy for managing DME. In the VISTA and VIVID study, it was reported that the less frequent intravitreal injections schemes of aflibercept 2mg every 8 weeks (2q8) and 2mg every 4 weeks (2q4) can achieve similar effects in visual and anatomical results (12). In clinical practice, IAI 2q8 is also a good choice for working-age patients who have to miss work because of frequent visits. However, further studies are needed to determine the frequency of administration in patients with PDR.

Although anti-VEGF therapy can improve the visual and anatomical functions of patients with DR, focal/grid laser photocoagulation or PRP may still play an important role as an adjuvant therapy (41–43). PRP treatment was not a "one and done" procedure, and the addition of anti-VEGF drugs prevent DR progression and provide a "window period" for PRP. On the other hand, PRP can improve retinal oxygenation and decrease the drive for VEGF production by the retina, thus reducing the number of injections required and the burden of treatment. The combination of PRP and anti-VEGF therapy is acceptable in the real world. What's more, according to the research of Protocol W, although there was no short-term vision benefit, early treatment with aflibercept can positively restore the anatomical structure of NPDR and reduced the risk of PDR or CI-DME with vision loss development in eyes with moderate to severe NPDR (44). Aflibercept may play a certain advantage in the management of DR with different severity.

Refractory vitreous hemorrhage and tractional retinal detachment may occur when PDR develops uncontrollably into advanced pathologies (45–47). For patients with recalcitrant DME, pars plana vitrectomy (PPV) can improve ocular anatomy (48, 49). Under these circumstances, PPV still plays a key role in the treatment of DR. Some studies have shown that anti-VEGF treatment before PPV can reduce intraoperative and postoperative hemorrhage and improve postoperative VA (50). On the positive side, in some cases requiring PPV, the combination of anti-VEGF therapy has a better curative effect. However, whether PPV has a wider effect than continuous anti-VEGF treatment has not yet been confirmed in RCTs.

The main unchangeable determinant of the development of diabetic retinopathy is the duration of diabetes (51). According to

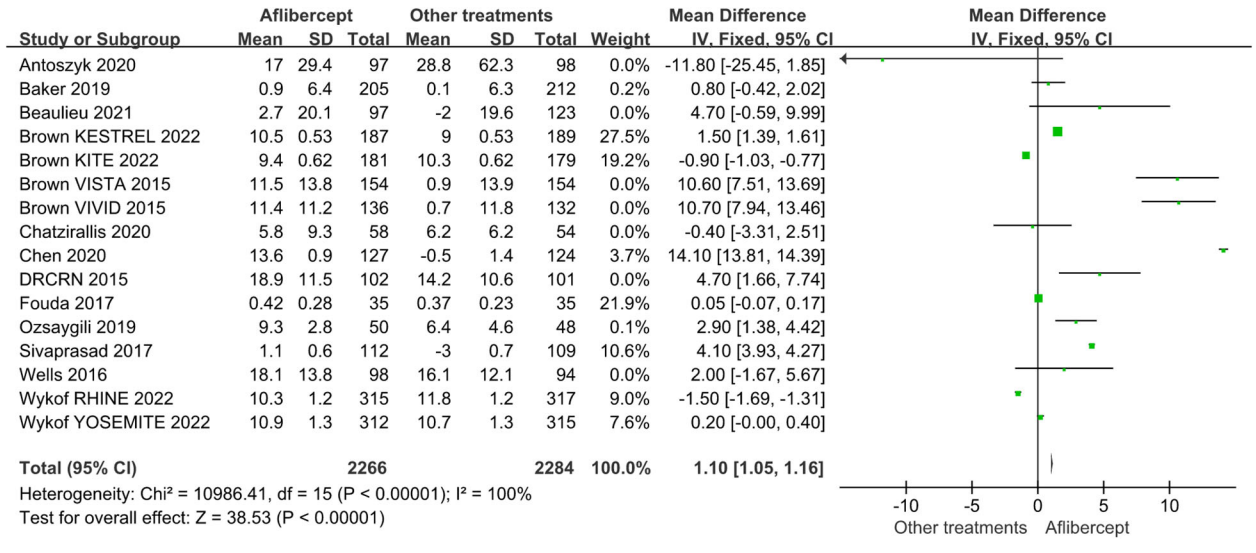


FIGURE 4
The mean changes from baseline in BCVA in the aflibercept group and other treatments group.

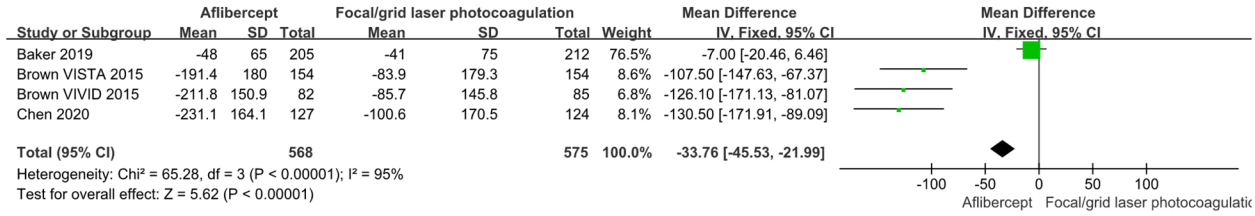


FIGURE 5
The mean changes from baseline in CST in the aflibercept group and focal/grid laser photocoagulation treatment group.

the standards for medical care for diabetes published by the American Diabetes Association in 2021, patients with type 1 diabetes should have a comprehensive ophthalmological examination within 5 years after diagnosis, and patients with type 2 diabetes should have their first fundus examination as soon as possible after diagnosis (52). A large-scale real-world research conducted by Chawla et al. found that the duration of diabetes was a strong predictor of the occurrence and development of DR. When the duration of type 2 diabetes reaches 9.4 ± 6.0 (mean ± SD) years, patients may have retinopathy. Therefore, we should always emphasize the early diagnosis and treatment of diabetic retinopathy in diabetic patients (53). It is worth noting that most people with diabetes in the RCTs we included are middle-aged and elderly people or working people. This kind of people are weak in physical examination consciousness or busy with work, and patients may delay the diagnosis of diabetes for various reasons, and their body are in a state of persistent hyperglycemia without knowing it. As a results, many patients with DR started their disease managements very late, or the medical treatment processes were irregular. Therefore, the scientific and standardized management of national health examination and disease prevention and control can not be ignored.

Studies have shown that the duration of action of anti-VEGF drugs varies among individual patients (32, 49). Moreover, even with appropriate treatment, repeated injections can increase the risk of infection, endophthalmitis, ocular inflammation, stroke, or myocardial infarction. The overall incidence of ocular and non-ocular adverse events in each treatment group was similar, and there was no significant difference between the aflibercept group and the other treatments group. It is a public knowledge that patients with diabetes have a high risk of cardiovascular comorbidities. In addition to diabetes, they are vulnerable to systemic complications. One study suggested that the increase in potential cerebrovascular accidents two years after treatment may be related to pro-epidermal growth factor therapy (54). Anti-VEGF therapy should be used cautiously in patients with myocardial infarction and stroke. Therefore, drug selection, injection frequency and interval, and necessary treatments should be adjusted according to the patient's individual function and anatomical structure.

There are several limitations in this research. First, there may be deviations in the data collection, and only a small number of RCTs were included. In addition, with the rapid development of multi-mode imaging technology, methods for evaluating the prognosis of DR are more diversified. For example, with the addition of swept-source OCT, quantitative evaluation indices are more extensive, which will also affect our results. In future work, we will incorporate more indicators to quantitatively evaluate nonproliferative diabetic retinopathy and PDR. Additional OCTA parameters will be included in subsequent meta-analyses to improve the accuracy

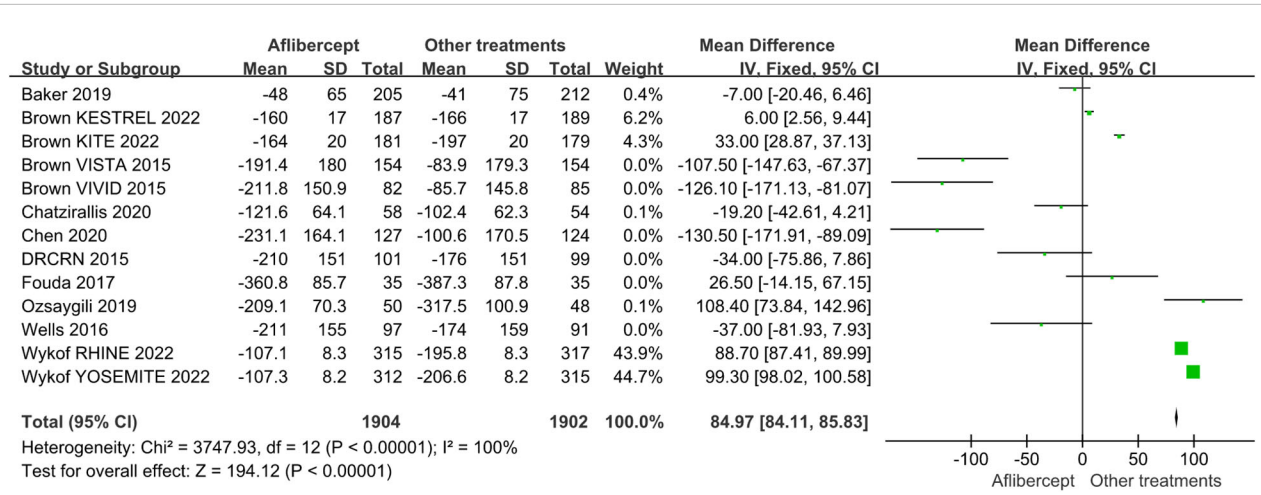


FIGURE 6
The mean changes from baseline in CST in the aflibercept group and other treatments group.

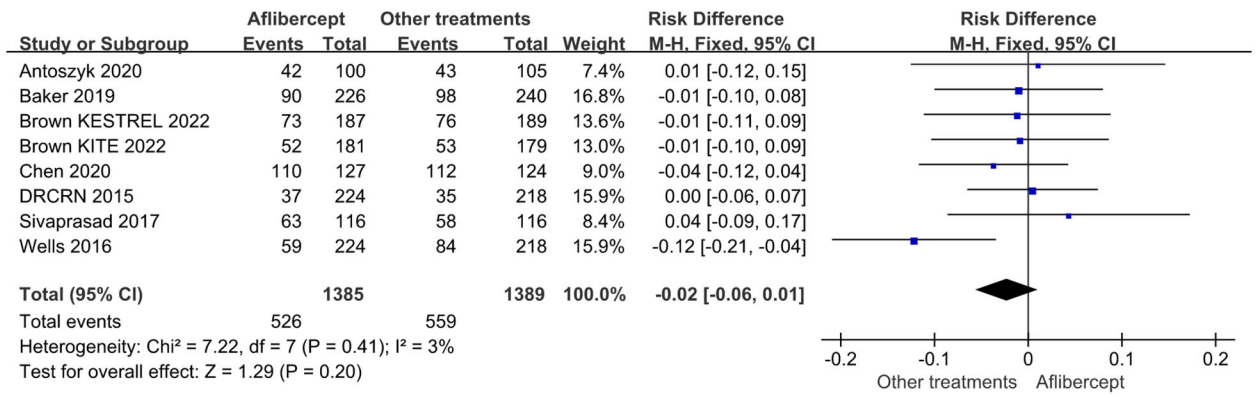


FIGURE 7
The adverse events in the aflibercept group and other treatments group.

and robustness of the above conclusions and to provide better clinical guidance.

5 Conclusions

This meta-analysis showed that the advantages of IAI are obvious in the management of DME and PDR with poor baseline vision for long-term observation (a minimum of 12 months and up to 24 months). Applied IAI separately trended to be more effective than PRP separately with VA improvement for long-term observation. More parameters should be required to assess functional and anatomic outcomes.

Author contributions

TL, XX, and CL designed the study and conducted data extraction and quality evaluation. XX and ZZ drafted and revised the manuscript. ZZ and MF conducted software analysis and data verification. WW and XX sorted data. XY generated the graphics and tables. YS searched literature. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer MC declared a shared affiliation with the authors to the handling editor at the time of review.

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Association of epilepsy, anti-epileptic drugs (AEDs), and type 2 diabetes mellitus (T2DM): a population-based cohort retrospective study, impact of AEDs on T2DM-related molecular pathway, and via peroxisome proliferator-activated receptor γ transactivation

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Introduction: A potential association between epilepsy and subsequent type 2 diabetes mellitus (T2DM) has emerged in recent studies. However, the association between epilepsy, anti-epileptic drugs (AEDs), and the risk of T2DM development remains controversial. We aimed to conduct a nationwide, population-based, retrospective, cohort study to evaluate this relationship.

Methods: We extracted data from the Taiwan Longitudinal Generation Tracking Database of patients with new-onset epilepsy and compared it with that of a comparison cohort of patients without epilepsy. A Cox proportional hazards regression model was used to analyze the difference in the risk of developing T2DM between the two cohorts. Next-generation RNA sequencing was used to characterize T2DM-related molecularchanges induced by AEDs and the T2DM-associated pathways they alter. The potential of AEDs to induce peroxisome proliferator-activated receptor γ (PPARγ) transactivation was also evaluated.

Results: After adjusting for comorbidities and confounding factors, the case group (N = 14,089) had a higher risk for T2DM than the control group (N = 14,089) [adjusted hazards ratio (aHR), 1.27]. Patients with epilepsy not treated with AEDs exhibited a significantly higher risk of T2DM (aHR, 1.70) than non-epileptic controls. In those treated with AEDs, the risk of developing T2DM was significantly lower than in those not treated (all aHR ≤ 0.60). However, an increase in the defined daily dose of phenytoin (PHE), but not of valproate (VPA), increased the risk of T2DM development (aHR, 2.28). Functional enrichment analysis of differentially expressed genes showed that compared to PHE, VPA induced multiple beneficial genes associated with glucose homeostasis. Among AEDs, VPA induced the specific transactivation of PPARγ.

Discussion: Our study shows epilepsy increases the risk of T2DM development, however, some AEDs such as VPA might yield a protective effect against it. Thus, screening blood glucose levels in patients with epilepsy is required to explore the specific role and impact of AEDs in the development of T2DM. Future in depth research on the possibility to repurpose VPA for the treatment of T2DM, will offer valuable insight regarding the relationship between epilepsy and T2DM.

KEYWORDS

epilepsy, anti-epileptic drugs, type 2 diabetes mellitus, next-generation RNA sequencing, peroxisome proliferator-activated receptor γ

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder with various causes, characterized by elevated blood glucose levels both in fasting and postprandial states. This condition leads to multiple complications due to deficient insulin secretion or responsiveness, hindering the metabolism of carbohydrates (1). According to the International Diabetes Federation Diabetes Atlas 9th Edition 2021 (http://www.idf.org/), the number of individuals affected by DM worldwide has risen to 537 million, with three out of four adults living in low- and middle-income countries suffering from this condition. Furthermore, almost 12% of global health expenditure is devoted to DM treatment (1). The prevalence of DM is increasing globally and is projected to rise to 643 million by 2030. DM can be classified into three main types based on the underlying causes: [1] type 1 DM (T1DM), resulting from a deficiency in insulin secretion; [2] type 2 DM (T2DM), which accounts for 90–95% of the DM population, caused by insulin resistance and often an inadequate compensatory insulin secretory response; and [3] gestational DM, a type of DM characterized by high blood glucose during pregnancy with an increased risk of T2DM in the newborn (1). Long-term hyperglycemia is associated with complications such as coronary artery disease (CAD), cerebrovascular disease (CVD), renal failure, blindness, limb amputation, neurological defects, and premature death (1). The primary DM treatment is oral hypoglycemic drugs and insulin injections (1, 2). Additionally, T2DM treatment, and to a lesser extent T1DM, involves lifestyle changes. The oral antihyperglycemic agents used include sulfonylureas (which increase insulin secretion), biguanides (insulin sensitizers, e.g., metformin), α-glucosidase inhibitors (which slow the digestion of starch in the small intestine), meglitinides (which increase insulin secretion), dipeptidyl peptidase-4 inhibitors (DPP-4 inhibitors) (which increase insulin secretion), thiazolidinediones (TZDs, agonists of PPARγ), and sodium-glucose cotransporter (SGLT)-2 inhibitors (2).

Epilepsy is a neurological disorder characterized by recurrent seizures resulting from various factors, such as neurobiological, cognitive, psychological, and social factors (3, 4). The prevalence of epilepsy is 5-10 cases per 1000 persons in most countries (3). Recent studies have evaluated the association between epilepsy and comorbidities like DM, celiac disease, thyroid disease, sclerosis,

and systemic lupus erythematosus (5–7). Patients with epilepsy have higher mortality rates than the general population, owing primarily to pathological causes such as CVD, stroke, and dementia, all of which are also associated with T2DM (8, 9).

Abnormalities in blood glucose levels can trigger focal motor seizures by disrupting the balance between neuronal inhibition and excitation (10). T2DM is associated with an increased risk of neurological disorders (11). Although limited, the available data suggest an association between T2DM and epilepsy development. A population-based study revealed that severe hypoglycemia is linked to an increased risk of epilepsy in T2DM patients. Additionally, T2DM independently increases the risk of epilepsy development (11). Another review supports the association between T2DM and epilepsy (12). A study showed that the incidence of epilepsy in T2DM patients was 1.5 times higher than that in matched controls (11). Epilepsy often co-occurs with obesity in both children and adults. Insulin resistance and pancreatic islet β-cell dysfunction are two primary mechanisms through which obesity leads to T2DM (13). Adiponectin deficiency is a common feature of these pathologies. Adiponectin stimulates fatty acid (FA) oxidation and glucose uptake in skeletal muscles (14). These factors may contribute to the development of T2DM in people with epilepsy or vice versa.

Anti-epileptic drugs (AEDs) are the most commonly used therapy for managing epilepsy, providing lifelong patient treatment. AEDs can effectively control symptoms in about 75% of patients (15), but they can also have significant metabolic side effects, such as insulin resistance (16). Phenytoin (PHE) is a widely used AED that has been associated with elevated fasting plasma glucose levels and increased insulin resistance in a study of 98 patients undergoing AED monotherapy (17). PHE has also been found to alter insulin secretion and increase blood glucose levels in animal models, potentially contributing to insulin resistance (18, 19). Insulin resistance is linked to higher serum leptin levels, which can induce weight gain (20). In contrast, patients receiving valproate (VPA) treatment for epilepsy may experience endocrine changes that lead to weight gain, but VPA has been shown to stimulate pancreatic β-cells and increase the expression of insulin, leptin, and adiponectin (20). Whether the underlying biological mechanisms of epilepsy and T2DM occur sequentially or concurrently with AED treatment remains to be determined.

Li et al. identified several pathways significantly relevant to T2DM, according to their report (21). They used a text mining tool to extract genes associated with T2DM from a literature database and evaluated the relevant pathways using Fisher's exact test based on the cumulative hypergeometric distribution. A total of 6,804 genes and 880 canonical pathways associated with T2DM were confirmed based on gene numbers downloaded from the Molecular Signatures Database (MSigDB), which contains an extensive collection of annotated functional gene sets (22). The PolySearch text mining system was used to identify candidate genes associated with T2DM, which can analyze multiple information sources such as PubMed, OMIM, DrugBank, and Swiss-Prot to produce a list of relevant concepts. The top 10 statistically relevant pathways related to T2DM, ranked by P-value, were attributed to five classes of signaling pathways, including adipocytokine, inflammatory, peroxisome proliferators activated receptor (PPAR), insulin, and T2DM pathway (21).

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily that consist of three subtypes: PPARα (*NR1C1*), PPARβ/δ (*NR1C2*), and PPARγ (*NR1C3*). These receptors are activated by lipid-derived substrates and play distinct roles in energy metabolism (23). PPARα, the first subtype identified, is mainly expressed in the liver, heart, and brown adipose tissue, where it mediates FA oxidation and is the target of hypolipidemic fibrates. PPARβ/δ shares similar functions with PPARα and is ubiquitously expressed, playing a crucial role in FA oxidation in key metabolic tissues such as skeletal muscle, liver, and heart (23). PPARγ is the most highly expressed subtype in white/brown adipose, endothelial, and muscle tissues, and is a master regulator of adipogenesis, modulating processes such as FA transport, uptake by cells, intracellular binding and activation, lipid catabolism, and FA storage. It also significantly modulates glucose homeostasis and insulin sensitivity (23, 24). Upon activation, PPARs form a heterodimer with the retinoid X receptor (RXR) and bind to a specific DNA cis-acting element called a peroxisome proliferator response element (PPRE), thereby initiating gene transcription (25). Prostaglandin J2 metabolites, oxidized low-density lipoprotein particles, and synthetic TZD compounds such as rosiglitazone and pioglitazone are PPARγ activators (24). These drugs have a high affinity for PPARγ, which is an attractive pharmacological target for treating T2DM since it simultaneously modulates several of the pathways disrupted in T2DM and metabolic syndrome by enhancing the expression of genes involved in glucose and lipid metabolism and increasing the expression of glucose transporter type 4 (GLUT4), an insulin-stimulated glucose transport system expressed in muscle cells and the expression of GLUT4 is highly regulated by PPARγ (2, 26, 27). In mice, muscle-specific deletion of PPARγ resulted in severe insulin resistance with milder defects in adipose tissue and liver, altered adipokine expression, increased adiposity, hyperinsulinemia, glucose intolerance, and hypertriglyceridemia (28). Given that skeletal muscle is a major site of fuel oxidation, the loss of muscle PPARγ could affect and impede the utilization of FA and glucose by skeletal muscle, contributing to the development of insulin resistance and T2DM.

The link between epilepsy and T2DM remains unclear, lacking adequate epidemiological evidence. Our goal was to use the National Health Institute (NHI) database to investigate the correlation between epilepsy, AED type, and the incidence of newly diagnosed T2DM in epileptic patients. We also examined the potential underlying biological mechanisms driving the impact of PHE and VPA on T2DM risk. Additional research is needed to clarify the association between epilepsy and T2DM and whether AEDs are involved in the pathophysiological changes that lead to T2DM. Next-generation sequencing (NGS) can help to identify molecular pathways related to each condition and provide insight into comorbidities by assessing molecular interaction networks. Our novel approach integrated clinical database information with data on high-level molecular changes consistently associated with known comorbid diseases.

Our study found that VPA activates PPARγ and increases glucose content in treated muscle cells, which play a significant

role in fuel oxidation. Additionally, it enhances the expression of insulin receptors (IRs) and GLUT4. These results suggest that VPA enhances glucose uptake by upregulating GLUT4 expression and may be used as an insulin-sensitizing agent.

Materials and methods

Data sources

The Taiwan government built a database with high coverage, the National Health Insurance Research Database (NHIRD), which included nearly 99% of Taiwan population. The claims data was obtained from Taiwan National Health Research Institute (NHRI), with the authorization from the NHI Administration, Ministry of Health and Welfare (MHW). This study used the Longitudinal Generation Tracking Database 2005 (LGTD 2005), a subsection of the NHIRD. The LGTD includes the mentioned claims data from 1996 to 2017 belonging to 2,000,000 patients randomly selected from the NHIRD, data available for public access. This database includes information on ambulatory care, inpatient care, dental services, prescription drugs, medical institutions, and physician information. To protect the privacy of the patients and to secure their confidentiality, the identification number of each patient was encrypted, precluding the possibility of the ethical violation of the data. The diagnoses in Taiwan NHI are coded according to the International Classification of Diseases, Ninth & Tenth Revision, Clinical Modification (ICD-9-CM & ICD-10-CM). The Research Ethics Committee of China Medical University and Hospital in Taiwan approved the study [CRREC-109-169 (CR-1)].

Study population

In this study, we included two cohorts of patients: epileptic and non-epileptic (comparison cohort). The epileptic cohort included patients with epilepsy (ICD-9-CM code: 345 or ICD-10-CM code: G40) newly diagnosed from 2000 to 2016. The date when epilepsy was diagnosed was defined as the index date. The non-epileptic comparison cohort included randomly selected patients without epilepsy registered in the LGTD in 2005. It was matched with the epileptic cohort at a 1:1 ratio based on age (per 5 years), sex, and index year. Patients in both cohorts were less than 20 years old, and those with T2DM (ICD-9-CM codes: 250. × 0 and 250. × 2 or ICD-10-CM code: E11) diagnosed before the index date, or with incomplete age or sex information were excluded. The follow-up of this study continued up until the patients withdrawn the insurance, T2DM was diagnosed or until December 31, 2017. The T2DM group included epileptic patients who had ≥ 1 admission or ≥ 2 ambulatory care visits for T2DM within a 365-day period between 2000 and 2017. The patients diagnosed with T1DM (ICD-9-CM: 250. × 1 or 250. × 3 or ICD-10-CM code: E10) between 2000 to 2017 were also excluded.

Outcome measures, comorbidities, and medications

Comorbidities were defined as comorbidities preceding the index date and included hypertension, stroke, hyperlipidemia, atrial fibrillation (AF), chronic obstructive pulmonary disease (COPD), coronary artery disease (CAD), congestive heart failure (CHF), alcohol-related illness, asthma, obesity, and cancer. We analyzed the impact of the following associated medication used in epileptic patients: steroids, statins, thiazide diuretics, and beta-blockers. We extracted data on AEDs, defined as drugs with ATC classification codes N03A and N05BA09. They were classified into the following therapeutical classes: barbiturates and derivatives [including phenobarbital (PB) and primidone], hydantoin derivatives [including phenytoin (PHE)], benzodiazepines [including clonazepam (CLZ), clorazepate, and diazepam], carboxamide derivatives [including carbamazepine (CBZ) and oxcarbazepine], FA derivatives [including valproate (VPA)], and others [gabapentin (GBP), lamotrigine, levetiracetam, pregabalin, topiramate, and zonisamide]. All these therapeutical agents are available in Taiwan.

Chemicals, cell cultures, and cytotoxicity assessment

One AED was selected from each group. All chemicals were purchased at the highest-purity grade available and dissolved appropriately. The HepG2, human and mouse rhabdomyosarcoma (RD and C2C12, respectively) cell lines were maintained in Minimum Essential Medium alpha (αMEM: HepG2) and Dulbecco's modified Eagle's medium (DMEM: RD, C2C12), respectively. All cultured cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. Cell viability was measured as previously described by using a modified acid phosphatase (ACP) assay with 4-nitrophenyl phosphate disodium salt as a substrate (29).

mRNA sequencing by using Illumina HiSeq

RD cells were treated with PHE and VPA to study their effects on T2DM pathway. After a 72-hour exposure to drug treatment, gene expression was evaluated in triplicate. Total RNA of each sample was extracted using TRIzol reagent (Invitrogen)/RNeasy Mini Kit (Qiagen), according to manufacturer's instruction. Total RNA of each sample was characterized quantitively and qualitatively using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.), and 1% agarose gel electrophoresis. Next, 1 μg total RNA with RNA integrity number above 6.5 was used for the library preparation. NGS libraries were prepared according to manufacturer's protocol. Poly(A) mRNA isolation was performed using poly(A) mRNA Magnetic Isolation Module or rRNA removal Kit. Next, mRNA fragmentation and priming were performed using

First-strand Synthesis Reaction Buffer and Random Primers. First-strand cDNA was synthesized using ProtoScript® II Reverse Transcriptase (New England Biolabs, Ipswich, MA), and second-strand cDNA was synthesized using Second-strand Synthesis Enzyme Mix (Thermo Fisher Scientific Inc.). The double-stranded cDNA purified using beads was treated with End-Prep Enzyme Mix (New England Biolabs, Ipswich, MA) to repair both ends, and a dA-tailing was added to one reaction, followed by a T-A ligation to add adaptors to both the ends. The size of adaptor-ligated DNA was selected using beads, and fragments of approximately 420 bp (with the approximate insert size of 300 bp) were recovered. Each sample was then amplified using PCR for 13 cycles and P5 and P7 primers, with both primers carrying sequences that can anneal with the flow cell to perform bridge PCR and P7 primer additionally carrying a six-base index allowing for multiplexing. The PCR products were cleaned using beads, validated using Qsep100 (BiOptic, Taiwan), and quantified using Qubit3.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument (Illumina, San Diego, CA, USA), according to manufacturer's instructions. Sequencing was performed using a 2 × 150 bp paired-end (PE) configuration; image analysis and base calling were conducted using HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. The sequences were processed and analyzed using GENEWIZ (Azenta Life Sciences, Inc. USA).

Data analysis

For quality control, to obtain high-quality clean data and to remove technical sequences, including adaptors, PCR primers, or fragments, and sequences with quality of bases lower than 20, the data in FASTQ format was filtered using Cutadapt (V1.9.1). Furthermore, reference genome sequences for mapping and gene model annotation files of relative species were downloaded from the genome websites such as UCSC, NCBI, and ENSEMBL. Hisat2 (v2.0.1) was used to index reference genome sequences. Finally, clean data were aligned to the reference genome by using software Hisat2 (v2.0.1). For expression analysis, first, transcripts in the FASTA format were converted from known gff annotation file and indexed properly. Next, based on a reference gene file, HTSeq (v0.6.1) was used to estimate gene and isoform expression levels from PE clean data. For differential expression analysis, we used DESeq2 Bioconductor package, a model based on the negative binomial distribution, and estimated the dispersion and logarithmic fold changes for data-driven prior distributions; an adjusted P (adjP) value < 0.05 was set to detect differentially expressed genes (DEGs). GOSeq (v1.34.1) was used to identify Gene Ontology (GO) terms annotating a list of enriched genes with a significant adjP of < 0.05, and top GO terms were used to plot directed acyclic graph. We used Kyoto Encyclopedia of Genes and Genomes (KEGG) for the systematic analysis of gene functions. This is a collection of databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances (30) (http://en.wikipedia.org/wiki/KEGG). We used in-house scripts to enrich significant DEGs in the KEGG pathways.

Plasmid construction, transfection, and reporter assay

To study the activation of PPARγ in a luciferase reporter assay, PPARγ expression vectors were amplified from full-length human PPARγ (MGC: 5041, IMAGE:3447380; pCMV-SPORT6; Open Biosystems Inc.) by using PCR and the forward and reverse primers 5′-ACG TTG GTA CAG CTG AAT CCA GAG TCC GCT GA-3′ and 5′-TCT AGA CTA GTA CAA GTC CTT GTA GAT CTC CTG CAG GAG C-3′, incorporating *Kpn*I and *Xba*I sites at the 5′ and 3′ ends, respectively. The PPARγ fragment was then ligated to the pcDNA3 *Kpn*I/*Xba*I site, forming a full-length construct (pcDNA3-PPARγ). To study the activation of the full-length PPARγ receptor, a 3 × repeat of the PPRE 5′-GGA CCA GGA CAA AGG TCA CGT TC-3′ was cloned into a pGL3-basic vector (Promega, Inc.) (31, 32). HepG2 cells were simultaneously co-transfected with the PPARγ reporter system and a β-galactosidase plasmid in each well. Transfections were allowed to proceed for 6 h before drug exposure. After 20 h of drug treatment, the reporter assay was performed as previously described (29).

Small interfering RNAs and siRNA transfection, T0070907 treatment, RNA isolation, qRT-PCR analysis, western blot analysis, and glucose content assessment assay

Small interfering control RNAs (siControl) and siRNA validated for PPARγ were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific Inc.) and applied to RD cells by using Lipofectamine® RNAiMAX reagent (Thermo Fisher Scientific Inc.) for 48 h. The culture was further incubated with or without VPA for 24 h. The PPARγ antagonist, T0070907 (T007), was added 20 minutes (min) before the 24-hour exposure to the VPA treatment.

After 24-hour treatment with AEDs, total RNA was extracted from C2C12 and RD by using a Direct-zol™ RNA MiniPrep kit (ZYMO Research, Irvine, CA, USA), according to manufacturer's protocol. It was further subjected to the synthesis of the first-strand cDNA by using a MultiScribe™ reverse transcriptase kit (Thermo Fisher Scientific Inc.). Genes related to glucose homeostasis and PPARγ target genes were analyzed using qRT-PCR and the Luminaris Color HiGreen qPCR master mix (Thermo Fisher Scientific Inc.) in a real-time PCR system under standard procedures. Each pair of specific primers used for real-time PCR analysis were as follows: *mGLUT4* Forward, 5'- GGA AGG AAA AGG GCT ATG CTG-3', *mGLUT4* Reverse, 5'- TGA GGA ACC GTC CAA GAA TGA-3'; *hGLUT4* Forward, 5'- CAT TCC TTG GTT CAT CGT G-3', *hGLUT4* Reverse, 5'- ATA GCC TCC GCA ACA TAC-3'; *hIR* Forward, 5'- TTT TCG TCC CCA GGC CAT C-3', *hIR* Reverse, 5'- GTC ACA TTC CCA ACA TCG CC-3'; *hPPARγ* Forward, 5'- AAA GAA GCC GAC ACT AAA CC-3', *hPPARγ* Reverse, 5- CTT CCA TTA CGG AGA GAT CC-3'; *β-actin* Forward, 5′-CCT GGC ACC CAG CAC AAT-3′, *β-actin* Reverse, 5′-GCC GAT CCA CAC GGA GTA CT-3′. Cells were treated with AEDs for 24 h, and the expression level of the above-mentioned proteins was measured using Western blot as previously described (29). Glucose content was measured using a commercial colorimetric kit available from BioVision (BioVision, Inc.).

Molecular docking

Molecular docking techniques are used to predict and investigate the steric and electrostatic complementarity between the PPARγ ligand-binding domain (LBD) and putative ligands. Currently, it is a frequently used tool in drug discovery by predicting the intermolecular framework formed between a protein and a ligand responsible for the modulation of the protein (33). In this study, PPARγ structure was extracted from Protein Data Bank (PDB; 6ILQ) and used as a template to dock VPA on PPARγ LBD by using Discovery Studio 4.5. All crystallized H_2O molecules were removed from the protein and the substrate, and hydrogen was added into the CDOCKER module. CDOCKER is a CHARMm-based docking method that has been used to generate highly accurate docked poses. The ligands were conceded to tilt around the rigid receptor (34).

Statistical analysis

To compare the differences between the two cohorts, we used two-sample t-test, when considering age as continuous variable and chi-square test when considering sex, medication, and comorbidities as category variable. The incidence density rate of T2DM was calculated for both cohorts and estimated by dividing the number of events (representing occurrence of T2DM) by follow-up time (per 1000 person-years). The Kaplan-Meier method was used to measure cumulative incidence curves for each cohort and log rank test was applied to assess the difference between two survival curves. We estimated hazard ratios (HRs) and 95% confidence intervals (CIs) for risk of development of T2DM in epileptic and non-epileptic cohorts by using crude and adjusted Cox proportional hazard models. Besides, we evaluated the effect and estimated the risk of T2DM determined by cumulative doses of PHE and VPA, taking into consideration the usage of steroids, thiazide diuretics, and statins. We estimated the annual mean defined daily dose (DDD) of these drugs and further partitioned into three levels. All statistical analyses were performed using SAS statistical software, version 9.4 (SAS Institute Inc., Cary, NC). The figure representing the cumulative incidence curve was plotted by R software. P values < 0.05 were set as having statistical significance.

For the experimental part, the data is presented as mean ± standard error (SE). All experiments were performed at least in triplicate and their mean was used for further analysis by SPSS for Windows, version 20.0 (IBM SPSS, Armonk, NY, USA). One-way analysis of variance (ANOVA) was performed and the significant differences were evaluated by *post hoc* LSD. Results were considered statistically significant at $P < 0.05$.

Results

Baseline characteristics: demographic and association findings

In this study, we totally enrolled 28,178 patients (Table 1), 14,089 patients with epilepsy and 14,089 patients without. In the study population, 61.1% were male. The mean ages of the epileptic cohort and non-epileptic cohort were 49.5 ± 18.9 and 49.0 ± 19.0 years, respectively. In the epileptic cohort, we found a significantly higher proportion of all comorbidities and medications usage than in the non-epileptic cohort. Figure 1 showed that epileptic patients had significantly higher T2DM cumulative incidence than the non-epileptic cohort (log-rank test $P < 0.001$).

Throughout the study, there were 2,246 and 1,933 patients newly diagnosed with T2DM in the epileptic group and non-epileptic group, respectively (Table 2). The incidence of T2DM was 18.6 per 1,000 person-years in the epileptic group and 14.1 per 1,000 person-years in the non-epileptic group. The epileptic patients were 1.31 times more likely to develop T2DM than non-epileptic patients (HR = 1.31, 95% CI = 1.23-1.39). After adjusting for age, sex, comorbidities, and medication, there was still a 1.27-fold increase in the risk of T2DM for the epileptic vs. control group (aHR = 1.27, 95% CI = 1.19-1.36). The sex-specific analysis of the relative risk of T2DM between the epileptic and non-epileptic cohort demonstrated the risk was significantly higher in both women and men with epilepsy than those without. The age-specific analysis indicated the relative risk of T2DM was higher for all age groups in the epileptic cohort than in the non-epileptic cohort. When we assessed the influence of medication on the T2DM risk, the epileptic patients had a significantly increased risk of T2DM when compared with individuals without epilepsy, for both users and non-users of steroids, statins, diuretics, and beta-blockers.

When considering the patients without epilepsy as reference, epileptic patients without AEDs treatment (aHR = 1.70, 95% CI = 1.54-1.88), with hydantoin derivatives-based monotherapy (aHR = 1.26, 95% CI = 1.16-1.37) and overall epileptic patients receiving treatment with AEDs (aHR = 1.19, 95% CI = 1.11-1.27) had higher risk of T2DM (Table 3). Compared to epileptic patients without AEDs treatment, epileptic patients receiving treatment with AEDs had lower risk of T2DM.

Table 4 presents the dose-response relationship between PHE and VPA use and T2DM risk in patients with epilepsy, compared to those without. There was a significantly higher risk of T2DM for patients receiving PHE when using > 150 DDD (aHR = 2.28, 95% CI = 1.99-2.61) than control cohort. However, in epileptic patients treated with VPA, the risk of T2DM decreased dose-dependently with the increase of DDD, although this trend did not reach statistically significance.

RNA-seq and differentially expressed genes analysis

The effects of PHE and VPA on the expression of genes associated with T2DM-related pathway were investigated by using control and PHE- and VPA-treated RD cells to construct cDNA libraries for sequencing on an Illumina NovaSeq instrument; three biological replicates were used, and a total of 2.083×10^8 150 paired-end raw sequencing reads were obtained for all samples. The raw reads were filtered by removing low-

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TABLE 1 Demographic characteristics, comorbidities, and medications in patient with and without epilepsy.

Variable	Epilepsy		P value
	No	Yes	
	N =14089	N =14089	
Sex	N (%)	N (%)	0.99
Female	5485 (38.9)	5485 (38.9)	
Male	8604 (61.1)	8604 (61.1)	
Age, mean (SD)[#]	49.0 (19.0)	49.5 (18.9)	0.02
Stratify age			0.03
≤ 49	7945 (56.4)	7790 (55.3)	
50-65	3043 (21.6)	3009 (21.4)	
≥ 65	3101 (22.0)	3290 (23.4)	
Comorbidity			
Hypertension	3162 (22.4)	5117 (36.3)	< 0.001
Hyperlipidemia	1800 (12.8)	2239 (15.9)	< 0.001
Atrial fibrillation	149 (1.06)	511 (3.63)	< 0.001
Stroke	317 (2.25)	2948 (20.9)	< 0.001
Chronic obstructive pulmonary disease (COPD)	1418 (10.1)	2486 (17.6)	< 0.001
Coronary artery disease (CAD)	1593 (11.3)	2460 (17.5)	< 0.001
Congestive heart failure (CHF)	386 (2.74)	775 (5.50)	< 0.001
Alcohol-related illness	249 (1.77)	1187 (8.43)	< 0.001
Asthma	952 (6.76)	1337 (9.49)	< 0.001
Obesity	78 (0.55)	137 (0.97)	< 0.001
Cancer	303 (2.15)	678 (4.81)	< 0.001
Medication			
Steroids	7236 (51.4)	7771 (55.2)	< 0.001
Statins	962 (6.83)	1348 (9.57)	< 0.001
Diuretics	2683 (19.0)	4728 (33.6)	< 0.001
Beta-blockers	3337 (23.7)	6021 (42.7)	< 0.001

[#]Chi-Square test.

quality reads and reads containing N and adaptor sequences, leaving 2.079×10^8 clean reads for downstream bioinformatic analysis. The clean reads were then mapped onto the human GRCh37 genome by using Hisat2 (v2.0.1): 95.28–96.19% were aligned and about 94.42–94.57% were uniquely mapped. The level of gene expression was measured using the read density: the higher the read density, the higher is the level of gene expression. Gene expression was determined by using a formula, which calculates fragments per kilo bases per million reads (FPKM) based on read counts from HT-seq (v0.6.1). There were 57,905 transcripts expressed in the RNA-Seq data. An absolute fold change of ≥ 2 and an adjusted P-value of < 0.05 were used to detect DEGs by using DESeq2 (v1.6.3) package. The heatmap distinguished different transcription profiles between the 3 groups (Figure 2).

Functional analysis of genes regulated by PHE and VPA treatment

Gene ontology (GO) enrichment analysis was performed to further explore the biological impact of the DEGs induced by PHE and VPA treatments. Several ontologies of GO analysis, such as molecular function, cellular component, and biological process, were obtained. The top biological process terms of the GO analysis associated with up- or down-regulated genes following PHE and VPA treatment, are shown in Figure 3A. The figure shows that treatment-induced changes in gene expression were thoroughly different from the perspective of biological functions. The KEGG analysis revealed PHE- and VPA-induced up- and down-regulated DEGs related to T2DM pathway, which are shown in Figure 3B. When compared with PHE, VPA increased the gene

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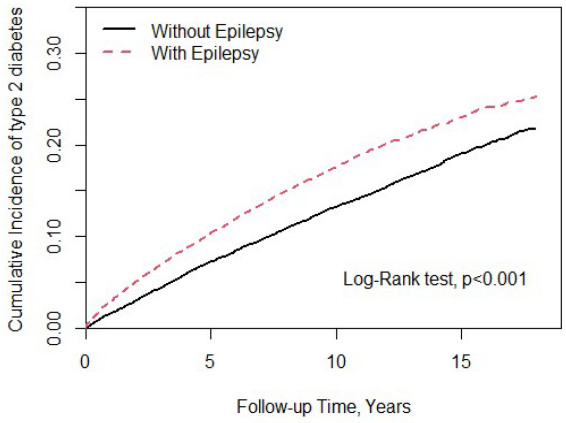


FIGURE 1
Cumulative incidence of type 2 diabetes mellitus (T2DM) compared between with and without epilepsy using the Kaplan-Meier method.

TABLE 2 Comparison of incidence and hazard ratio (HR) of type 2 diabetes mellitus (T2DM) stratified by sex, age, and comorbidities between with and without epilepsy.

	Epilepsy							
	No			Yes				
Variable	Event	PY	Rate#	Event	PY	Rate#	Crude HR (95% CI)	Adjusted HR† (95% CI)
All	1933	136976	14.1	2246	120807	18.6	1.31 (1.23, 1.39)***	1.27 (1.19, 1.36)***
Sex								
Female	736	53627	13.7	953	47699	20.0	1.45 (1.31, 1.59)***	1.43 (1.30, 1.59)***
Male	1197	83349	14.4	1293	73108	17.7	1.22 (1.13, 1.32)***	1.18 (1.08, 1.28)***
Stratify age								
≤ 49	727	90665	8.02	932	82998	11.2	1.41 (1.28, 1.55)***	1.28 (1.16, 1.42)***
50-64	615	25512	24.1	666	21071	31.6	1.31 (1.17, 1.46)***	1.18 (1.04, 1.32)**
≥ 65	591	20799	28.4	648	16738	38.7	1.32 (1.18, 1.48)***	1.25 (1.11, 1.41)***
Comorbidity‡								
No	976	103274	9.45	763	71290	10.7	1.13 (1.03, 1.24)*	1.31 (1.19, 1.45)***
Yes	957	33702	28.4	1483	49517	30.0	1.05 (0.97, 1.14)	1.14 (1.04, 1.25)**
Medication								
Steroids								
No	1064	871444	13.1	1149	71750	16.0	1.23 (1.13, 1.33)***	1.23 (1.12, 1.34)***
Yes	869	55532	15.7	1097	49057	22.4	1.40 (1.28, 1.53)***	1.32 (1.20, 1.45)***
Diuretics								
No	1407	120674	11.7	1417	95957	14.8	1.27 (1.18, 1.37)***	1.32 (1.22, 1.42)***
Yes	526	16302	32.3	829	24851	33.4	1.02 (0.92, 1.14)	1.09 (0.97, 1.23)
Statins								
No	1734	132114	13.1	2006	114613	17.5	1.33 (1.25, 1.42)***	1.31 (1.22, 1.40)***
Yes	199	4862	40.9	240	6194	38.8	0.94 (0.78, 1.14)	0.93 (0.76, 1.15)

(Continued)

TABLE 2 Continued

	Epilepsy							
	No			Yes				
Variable	Event	PY	Rate#	Event	PY	Rate#	Crude HR (95% CI)	Adjusted HR† (95% CI)
Beta-blockers								
No	1326	115246	11.5	1206	84284	14.3	1.25 (1.15, 1.35)***	1.32 (1.21, 1.43)***
Yes	607	21730	27.9	1040	36524	28.5	1.02 (0.92, 1.12)	1.12 (1.00, 1.24)*

Rate#, incidence rate, per 1,000 person-years; Crude HR, crude hazard ratio.
Adjusted HR†: multivariable analysis including age, sex, and comorbidities of hypertension, hyperlipidemia, stroke, COPD, CAD, CHF, alcohol-related illness, asthma, obesity, cancer, impaired glucose tolerance, and gestational diabetes, and medication of steroids, diuretics, statins and beta-blockers.
Comorbidity‡: Patients with any one of the comorbidities, hypertension, hyperlipidemia, stroke, COPD, CAD, CHF, alcohol-related illness, asthma, obesity, cancer, impaired glucose tolerance, and gestational diabetes were classified as the comorbidity group.
*P < 0.05, **P < 0.01, ***P < 0.001.

expression of INSR, VDCC, GK, PYK, PKCζ, PKCε/δ, IRS, and GLUT4 which are beneficial for maintaining glucose homeostasis, while PHE increased only the expression of PI3K in T2DM pathway.

The 3 RNA-Seq datasets were analyzed two by two (control vs PHE; VPA vs PHE). We identified the genes associated with the top 10 pathways involved in T2DM, by applying adjP-criterion, as previously published (21); these comparisons are listed in Table 5. Compared with the control group, PHE-treated group had a substantially lower level of expression of genes beneficial in glucose homeostasis (59.2% vs. 40.8%, CON vs. PHE); the level of expression of genes beneficial in glucose homeostasis, was substantially higher in VPA- than in PHE-treated group (70% vs. 30%, VPA vs. PHE). Genes associated with 5 T2DM-related pathways, according to Li et al. (21) and those significantly different between CON vs PHE and PHE vs VPA are shown in Figure 4.

Evaluation of AEDs effect on T2DM-related gene expression and the involvement of PPARγ

The risk of developing T2DM was lower in patients receiving VPA than in controls. Thus, we intended to perform an in-depth study for verifying the underlying mechanism of action by which AEDs interfere with the risk of developing T2DM. We selected one drug from each category of AEDs to evaluate their effects on cultured cells. Drug concentrations were selected according to the target serum concentrations from trough to peak doses recommended in the

TABLE 3 Incidence, crude, and adjusted hazard ratio (HR) of type 2 diabetes mellitus (T2DM) compared among epilepsy patients with and without anti-epileptic drugs (AEDs) treatment compared to non-epilepsy controls.

Variables					Crude HR (95% CI)	Adjusted HR† (95% CI)	Adjusted HR† (95% CI)
	N	Event	PY	Rate#			
Non-epilepsy controls	14089	1933	136976	14.1	1 (Reference)	1 (Reference)	
Epilepsy without AEDs treatment	2399	528	16765	31.5	2.12 (1.93, 2.34)***	1.70 (1.54, 1.88)***	1 (Reference)
Only barbiturate and derivatives	826	122	10793	11.3	0.83 (0.69, 1.00)*	1.01 (0.84, 1.22)	0.60 (0.49, 0.73)***
Only hydantoin derivatives	4961	809	47558	17.0	1.21 (1.12, 1.32)***	1.26 (1.16, 1.37)***	0.74 (0.66, 0.83)***
Only benzodiazepam derivatives	3052	485	28635	16.9	1.20 (1.08, 1.32)***	1.13 (1.02, 1.25)*	0.67 (0.59, 0.76)***
Only carboxamide derivatives	823	120	8011	15.0	1.07 (0.89, 1.28)	1.23 (1.02, 1.48)*	0.72 (0.59, 0.87)***
Only fatty acid derivatives	910	113	5989	18.9	1.29 (1.06, 1.56)**	1.13 (0.93, 1.37)	0.66 (0.54, 0.81)***
Only others§	584	69	3056	22.6	1.50 (1.18, 1.91)***	1.24 (0.97, 1.59)	0.73 (0.57, 0.94)***
Overall epilepsy with AEDs treatment	11156	1718	104042	16.5	1.17 (1.10, 1.25)***	1.19 (1.11, 1.27)***	

Rate#, incidence rate, per 1,000 person-years; Crude HR, crude hazard ratio.
Adjusted HR†: multivariable analysis including age, sex, and comorbidities of hypertension, hyperlipidemia, stroke, COPD, CAD, CHF, alcohol-related illness, asthma, obesity, cancer, impaired glucose tolerance, and gestational diabetes, and medication of steroids, diuretics, statins and beta-blockers.
§Other AEDs including gabapentin, lamotrigine, levetiracetam, pregabalin, topiramate, and zonisamide.
*P < 0.05, **P < 0.01, ***P < 0.001.

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TABLE 4 Hazard ratio (HR) and 95% confidence intervals (CIs) for type 2 diabetes mellitus (T2DM) associated with average defined daily doses (DDD) of phenytoin (PHE) and valproate (VPA).

	Crude HR	(95% CI)	Adjusted HR†	(95% CI)
Non-epilepsy controls	1.00	(reference)	1.00	(reference)
Epilepsy with PHE				
≤ 30 DDD	0.96	(0.86, 1.07)	1.04	(0.93, 1.16)
31-150 DDD	1.01	(0.88, 1.16)	1.08	(0.94, 1.24)
> 150 DDD	2.19	(1.92, 2.49)***	2.28	(1.99, 2.61)***
P for trend		< 0.001		< 0.001
Epilepsy with VPA				
≤ 40 DDD	0.88	(0.68, 1.13)	1.11	(0.86, 1.44)
41-160 DDD	0.80	(0.55, 1.17)	0.95	(0.65, 1.39)
> 160 DDD	0.71	(0.50, 1.01)	0.83	(0.59, 1.19)
P for trend		0.02		0.47

Crude HR, crude hazard ratio.
Adjusted HR†: multivariable analysis including age, sex, and comorbidities of hypertension, hyperlipidemia, stroke, COPD, CAD, CHF, alcohol-related illness, asthma, obesity, cancer, impaired glucose tolerance, and gestational diabetes, and medication of steroids, diuretics, statins and beta-blockers.
***P < 0.001.

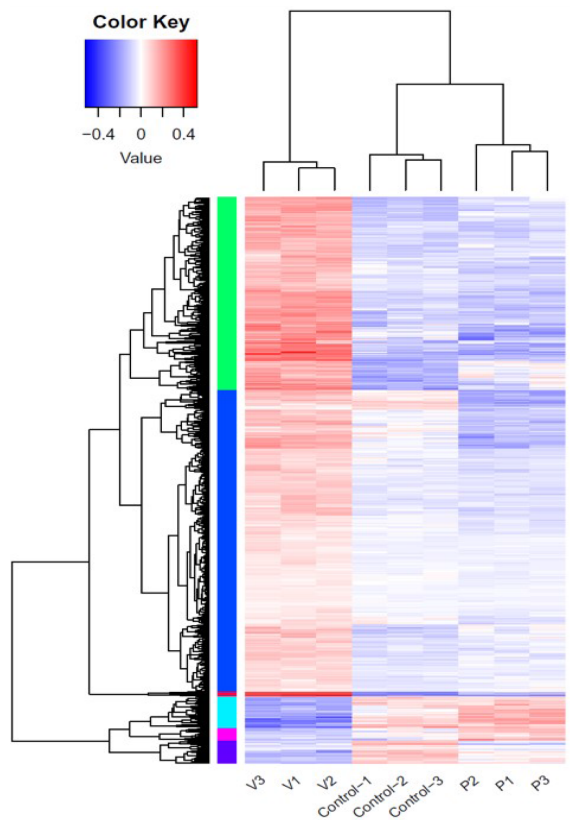


FIGURE 2
Differential gene expression in control, phenytoin (P)-, and valproate (V)-treated cells. Heatmap of all differentially expressed genes in the control, phenytoin (PHE)-, and valproate (VPA)-treated samples.

Handbook of Pharmacotherapy (35). Since hepatic toxicity of AEDs is well documented (36), we conducted a cell viability assay in 2 rhabdomyosarcoma cell lines: human RD and mouse C2C12 cells. An ACP assay was used to assess cytotoxicity following the exposure of these two cell lines to AEDs for 24 h. Cytotoxicity was not significantly different between the AEDs regardless of the concentration (Figure 5).

Since AEDs may affect the outcome of T2DM development, we evaluated the effects of AEDs on the expression of GLUT4; as the skeletal muscle is the major site for fuel oxidation (37, 38). In C2C12 cells, VPA significantly induced the expression of GLUT4 up to 3.2- and 8.9-fold after treatment with 346.7 and 693.4 μM VPA, respectively (Figure 6A). However, other AEDs did not induce GLUT4 mRNA expression. Treatment of RD cells with 346.7 and 693.4 μM VPA induced GLUT4 mRNA expression up to 3.2- and 5.7-fold, respectively (Figure 6B). Increased GLUT4 protein expression was observed in cells treated with VPA, but not in those treated with CBZ and PHE (Figure 6C). The interaction between insulin and its target cells leading to the manifestation of insulin-induced effects, relies on IR. Its activation triggers different cellular processes such as glucose uptake and protein synthesis, through an intracellular signaling network (13). We found that treatment with 346.7 and 693.4 μM VPA induced IR mRNA expression up to 2.4- and 3.9-fold, respectively (Figure 6D); this effect was also confirmed by assessment of protein expression (Figure 6E). However, no significant change in PPARγ mRNA expression was noted after treatment with any AED (Figure 6F). Furthermore, we determined the intracellular glucose content in VPA-, CBZ-, and PHE-treated cells by using glucose colorimetric assay; we found that the intracellular content of glucose was higher in VPA-treated cells than in CBZ- and PHE-treated cells. For cells

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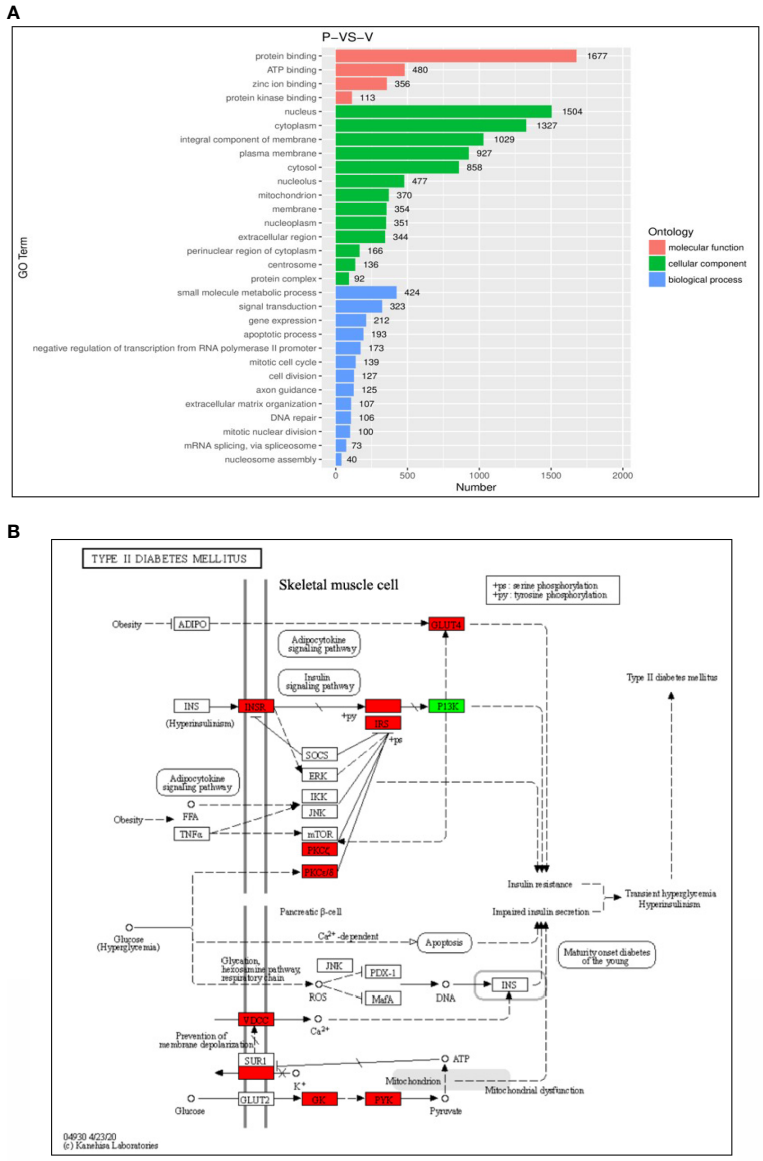


FIGURE 3
Functional enrichment analysis of differential gene expressions (DEGs). **(A)** Top representatives of gene ontology biological process terms and **(B)** KEGG analysis of T2DM-related DEGs obtained using the samples treated with phenytoin (P) and valproate (V). The red box indicates gene expression: VPA > PHE; the green box indicates gene expression: PHE > VPA, $P < 0.05$ in **(B)**.

treated with peak concentrations of CBZ and PHE, intracellular glucose was reduced compared with cells treated with trough concentrations (Figure 7). These results suggest that, compared with other AEDs, VPA can induce the expression of GLUT4 and IR, and thus enhance glucose uptake and increase intracellular glucose concentration.

VPA specifically transactivates PPARγ-mediated PPRE promoter activity and mRNA expression of GLUT4

The ability of AEDs to transactivate PPARγ in a cell-based system was assessed using luciferase assay. All AEDs were tested using trough and peak concentrations. A reporter construct containing 3 × PPRE upstream of a luciferase reporter was transfected into HepG2 cells, which were then treated with AEDs or troglitazone (TGZ, used as a positive control). PPARγ expression was the highest following TGZ treatment (25.4-fold; Figure 8A). Even with the present of PPARγ, PHE, CBZ, CLZ, and GBP did not transactivate the PPRE promoter. Only VPA significantly transactivated the PPRE promoter, especially in the presence of PPARγ (12.3- and 27.7-fold increases with 346.7 and 693.4 μM VPA, respectively; Figure 8B). The specificity of VPA's effect was confirmed in the PPARγ reporter assay by blocking the response to VPA with a PPARγ-LBD antagonist, T007. The presence of T007 inhibited the VPA effect in the PPRE reporter assay, in a concentration-dependent manner (Figure 8B) and reduced mRNA expression of GLUT4 (Figure 8C).

TABLE 5 Summary of genes significantly different between the 2 groups (CON vs. PHE, left part; VPA vs. PHE, right part).

	Gene numbers significantly different from CON and PHE groups (P < 0.05)	Gene expression (gene numbers that beneficial for blood glucose homeostasis) CON vs. PHE		Gene numbers significantly different from VPA and PHE groups (P < 0.05)	Gene expression (gene numbers that beneficial for blood glucose homeostasis) VPA vs. PHE	
KEGG adipocytokine signaling pathway	18	10	8	12	9	3
KEGG T2DM	6	4	2	17	14	3
BIOCARTA PPARA pathway	17	11	6	16	11	5
Reactome regulation of lipid metabolism by PPARα	23	15	8	15	8	7
KEGG PPAR signaling pathway	12	5	7	20	15	5

After we confirmed VPA can specifically induce GLUT4 expression via PPARγ transactivation, we used 3 kinds of PPARγ siRNA (siPPARγ-430, -830, and -1234) to determine whether VPA can also transactivate PPARα or PPARβ/δ. We found that these siPPARγ specifically acted against PPARγ, but not PPARα/β/δ (Figure 9A). We selected siPPARγ-830 to investigate the inhibitory effect on the expression of PPARγ and GLUT4 (Figures 9B, C). The mRNA expression of PPARγ and GLUT4 was reduced by siPPARγ-830 even in the presence of VPA. These results showed that VPA can specifically induce GLUT4 expression by transactivation of PPARγ signaling pathway.

Molecular docking

Molecular docking was performed to simulate the interactions between PPARγ and its ligand. The ligand of PPARγ was virtually docked to the LBD of PPARγ (PDB entry: 6ILQ) by using the docking program CDOCKER. The docking analysis revealed that the interaction between VPA and PPARγ has an energy score of 32.667. The binding model indicates VPA binds to LBD at residues ARG288, ILE341, GLU343, MET329, and LEU333 (Figure 10). Thus, we showed that VPA acts as a partial agonist of PPARγ, providing insight for designing novel PPARγ modulators.

Discussion

To our knowledge, this is the first population-based study reporting a correlation between epilepsy, AEDs use, and the risk of T2DM, in Asian populations. The risk of T2DM is 1.27 times higher in epileptic patients than in the general population after adjusting for confounding factors and comorbidities (P < 0.001). Furthermore, for patients receiving hydantoin derivatives-based treatment, the risk of T2DM had a 1.26-fold increase when compared with non-epileptic controls. However, even without AEDs treatment, epileptic patients had a higher risk of T2DM than the control cohort (aHR, 1.70; 95% CI, 1.54-1.88, P < 0.001). Furthermore, the risk of T2DM decreased in epileptic patients treated with AEDs when compared to those not treated. Our dose response sub-analysis demonstrated that PHE increased, while VPA decreased the risk of T2DM, in a dose-dependent manner. We also analyzed the T2DM-related pathways interfered by PHE and VPA and the treatment-induced biological disturbances that contribute to T2DM development. In addition, we demonstrated that among AEDs, VPA may act as an effective PPARγ ligand increasing glucose uptake and thus, may be beneficial for epileptic patients by reducing the risk of T2DM development.

T2DM is associated with an increased risk of several neurological disorders, such as dementia, Parkinson's disease, and multiple sclerosis (11). Data regarding the comorbid association of T2DM and epilepsy exist, however they are sparse. Thus, a population-based study estimating the risk of epilepsy in patients with T2DM, revealed severe hypoglycemia as well as T2DM increased the risk of epilepsy (11). Moreover, a review confirmed the association between T2DM and epilepsy (12). Additionally, epilepsy is often comorbid with obesity (39, 40). These patients are less physically active compared to the general population and thus, they have increased BMI, decreased aerobic strength, poor self-respect, and high levels of anxiety and depression (41). In addition, 61% of the T2DM cases are attributable to obesity, and the risk of T2DM doubles with a weight gain of 5-8 kg (42). In our study, after adjusting comorbidities such as obesity, the correlation between epilepsy and the risk of T2DM still remained significant, suggesting epilepsy may independently increase the risk of T2DM development. Several studies have pointed to the fact that there is an association between epilepsy and T1DM. A meta-analysis evaluated the association between T1DM and epilepsy using data from various sources, including Pubmed, ISI Web of Knowledge, Embase, and Cochrane Library. Of the 13 studies identified, three cohort studies met the inclusion criteria (43). The results showed that T1DM is associated with an increased risk of epilepsy, and this association is also observed in patients younger than 18 years old. The findings indicate that individuals with T1DM have a significantly higher risk of developing epilepsy compared to those without the condition. A review examines the potential relationship

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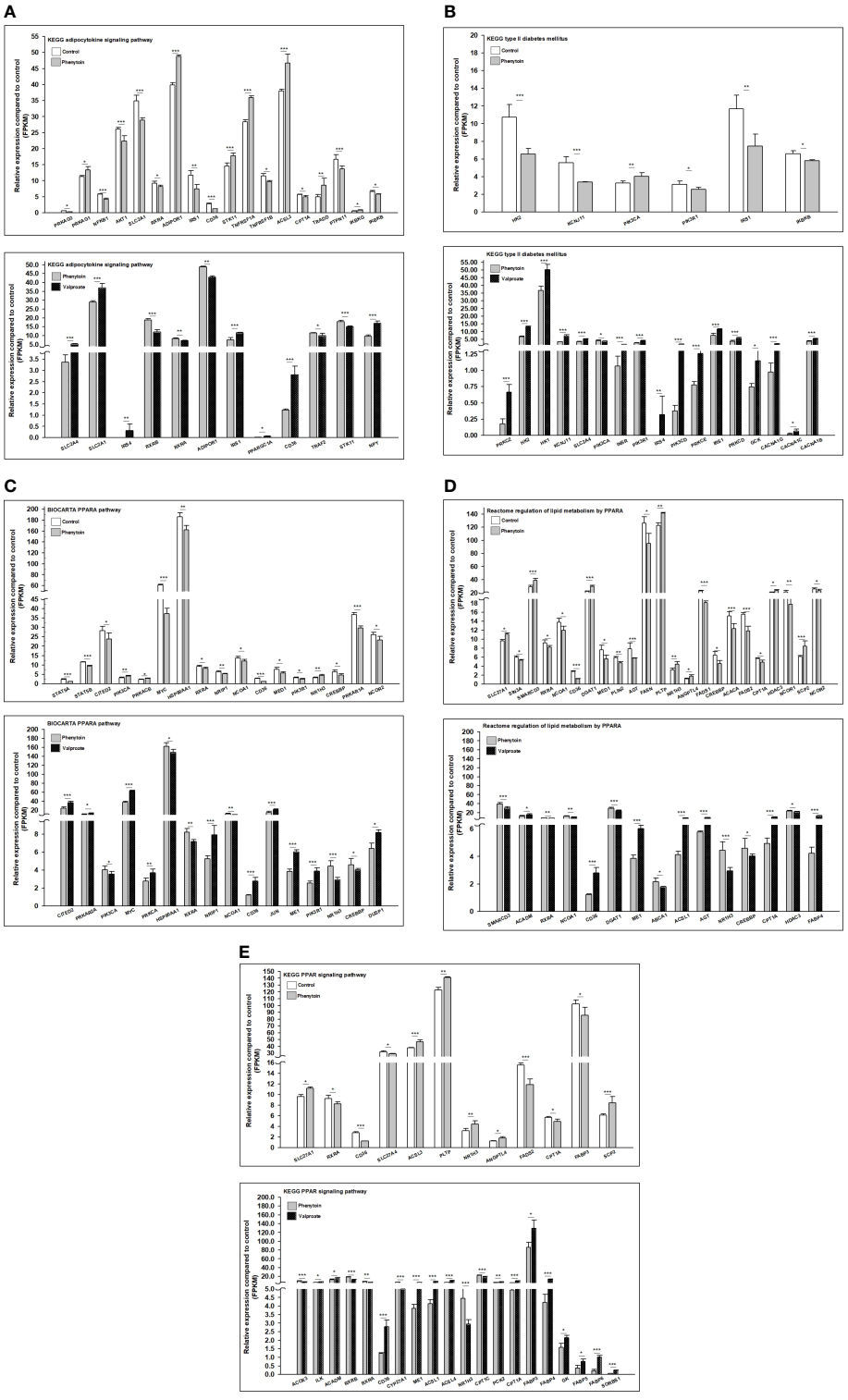


FIGURE 4
Gene family expression (presented by FPKM) of T2DM-related pathways from RD cells treated with DMSO, PHE, and VPA. Cells were treated with DMSO, PHE (79 μM), and VPA (693.4 μM) for 72 h; mRNA was extracted; and RNA-Seq and DEG analyses were performed. **(A)** KEGG adipocytokine signaling pathway; **(B)** KEGG T2DM pathway; **(C)** BIOCARTA PPARA pathway; **(D)** Reactome regulation of lipid metabolism by PPARα; **(E)** KEGG PPAR signaling pathway. *P < 0.05, **P < 0.01, ***P < 0.001.

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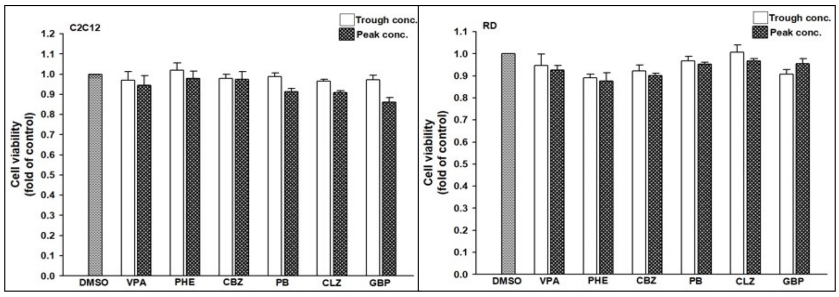


FIGURE 5
Viability of C2C12 and RD cells after exposure to anti-epileptic drugs (AEDs). (Left) C2C12 and (Right) RD cells were exposed to valproate (VPA, trough: 346.7 μM, peak: 693.4 μM), phenytoin (PHE, trough: 40 μM, peak: 79 μM), carbamazepine (CBZ, trough: 17 μM, peak: 51 μM), phenobarbital (PB, trough: 43 μM, peak: 172 μM), clonazepam (CLZ, trough: 0.06 μM, peak: 0.22 μM), and gabapentin (GBP, trough: 12 μM, peak: 117 μM) for 24 h. Cell viability was monitored using cellular acid phosphatase activity by using 4-nitrophenyl phosphate disodium salt as a substrate. The data are shown as the mean ± SE (error bars; n = 3).

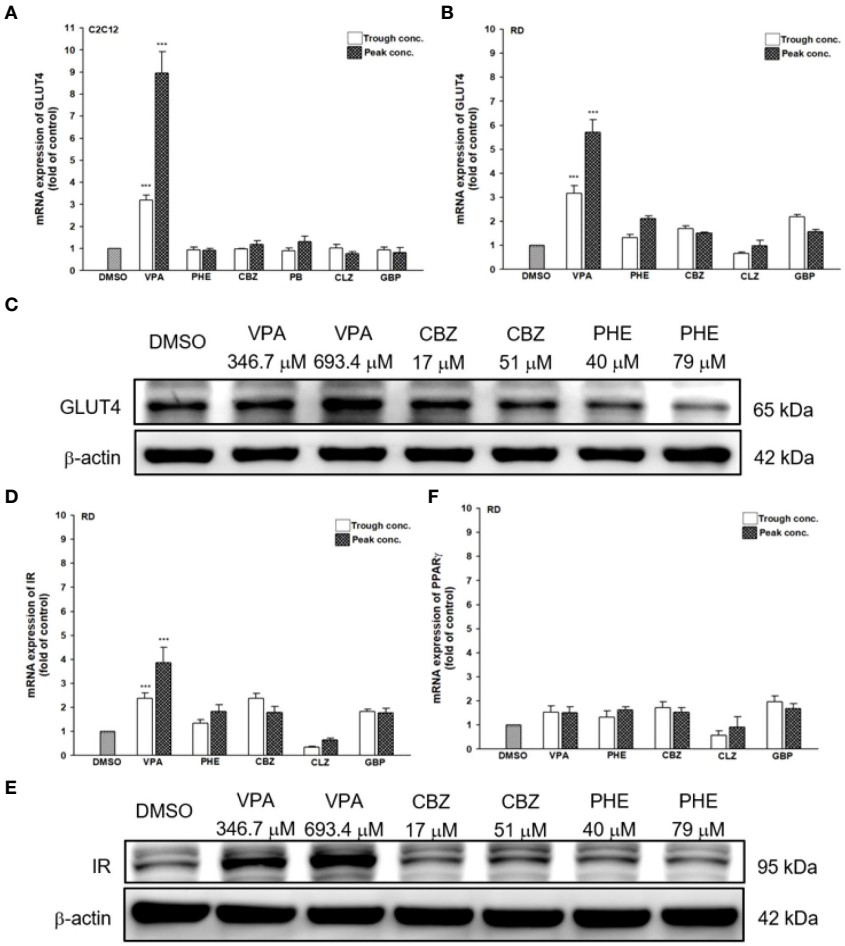


FIGURE 6
Gene and protein expression of T2DM-related genes after treatment with AEDs. **(A)** C2C12 and **(B, D, F)** RD cells were treated for 24 h with AEDs (each with trough and peak concentrations); after treatments, total RNA was extracted, and the expression levels of GLUT4, IR, and PPARγ and β-actin as a control were analyzed using qRT-PCR. Values were normalized to the expression of β-actin, with the levels of DMSO-treated cells set at 1. Results are expressed as means ± SE (n = 3). ***P < 0.001 compared with control cells as indicated. **(C, E)** RD cells were treated for 24 h with VPA, CBZ, and PHE. Whole-cell extract was harvested, and the expression levels of **(C)** GLUT4 and **(E)** IR and the internal control (β-actin) were analyzed using western blot analysis. A representative blot is shown.

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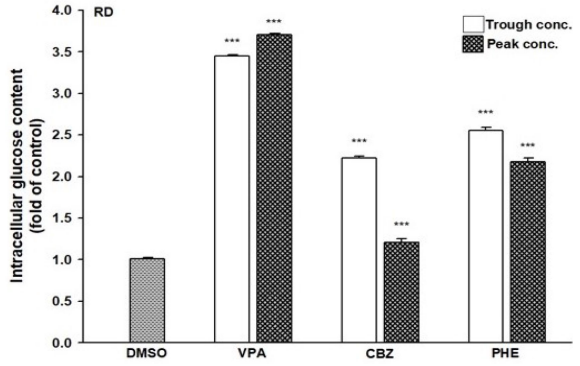


FIGURE 7
Intracellular glucose content in AEDs-treated cells. RD cells were treated with VPA, CBZ, and PHE for 24 h; cell lysates were extracted; and glucose contents were analyzed using a glucose colorimetric assay kit, according to manufacturer's instructions. Values were normalized to individual protein contents, with the levels of DMSO-treated cells set at 1. Results are expressed as means ± SE (n = 3). ***P < 0.001 compared with control cells as indicated.

between epilepsy and T1DM and investigates the underlying mechanisms. The presence of anti-glutamic acid decarboxylase antibodies (GAD-Abs), which are associated with T1DM and various neurological disorders, including epilepsy, may play a role in this link (44). Additionally, metabolic conditions such as hypoglycemia and hyperglycemia, which are common in diabetic patients, may also be implicated. However, the exact pathogenetic mechanisms behind this association remain unclear.

In epileptic patients, T2DM might develop because of mitochondrial dysfunction, adiponectin deficiency, or obesity, these three mechanisms might sequentially or concurrently induce the biological drivers of T2DM in epileptic patients (45). Drug-induced diabetes is a clinical condition reported by physicians, although the underlying mechanisms of the diabetogenic effects are unknown: alteration of insulin secretion and sensitivity, direct cytotoxic effects on pancreatic cells, and increase in glucose production may be involved (16). Furthermore, drug-induced diabetes is becoming a global issue because its potential to induce severe CVD complications. A study in Taiwan using the NHI reference database revealed that 71% of epileptic patients used AED monotherapy (46). Approximately one third of epileptics take two or more AEDs. CBZ, PHE, and VPA are the most frequently used drugs in monotherapy, and a combination of CBZ and VPA is used in polytherapy (46). PHE induces hyperglycemia particularly at toxic concentrations, secondary to the inhibition of insulin release and a subsequent post-binding defect in insulin action (16). Patients with PHE-induced hyperglycemia have an increased insulin requirement which suggests the occurrence of insulin resistance. However, the reduction of PHE dose resulted in improvement of hyperglycemia. *In vitro* studies assessing the effect of PHE on both insulin receptor binding and post-binding function in a primary culture system of adipocytes showed a 57% reduction in maximum [^{14}C]3-0-

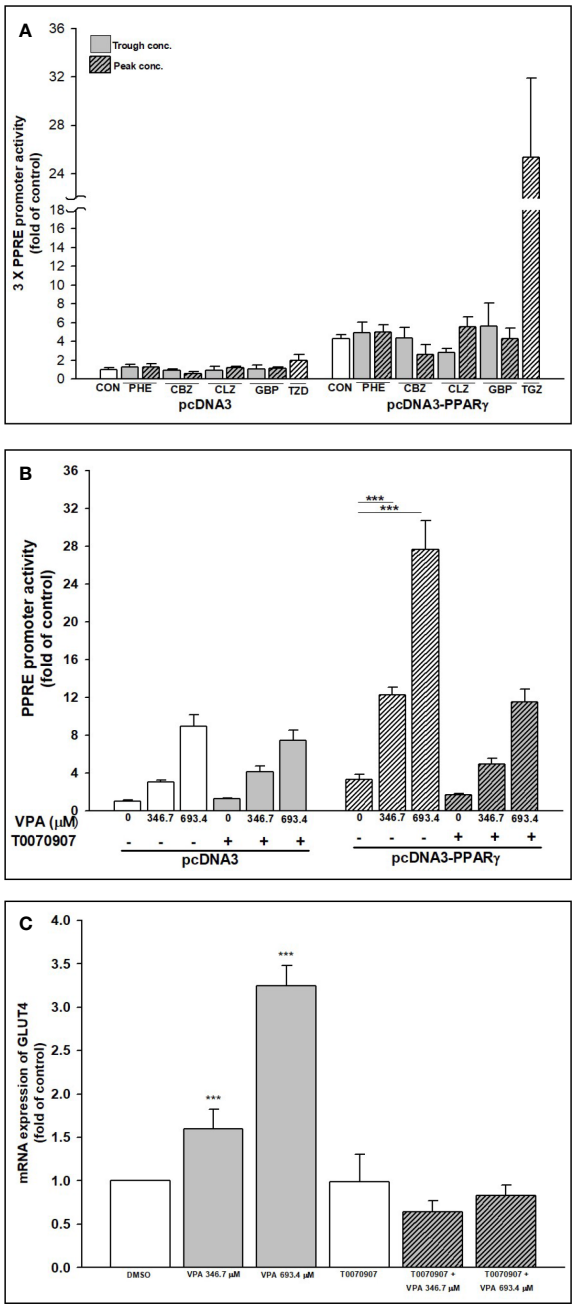


FIGURE 8
Transactivation of PPARγ–PPRE promoter activity and GLUT4 expression after AEDs treatment. Transient transcriptional assays of 3 × PPRE reporter activity were performed in HepG2 cells to determine the effects of **(A)** AEDs- and **(B)** VPA-, VPA + T0070907-mediated activation of PPARγ. HepG2 cells were co-transfected with a control vector (pcDNA3) or a PPARγ expression plasmid (pcDNA3-PPARγ) and a 3 × PPRE reporter plasmid. Transfected cells were subsequently exposed to AEDs for 24 h. Results are expressed as means ± SE (n = 4), with the levels of DMSO-treated cells set at 1. ***P < 0.001 compared with control cells as indicated. **(C)** RD cells were treated with VPA alone or in combination with T0070907; after treatments, total RNA was extracted, and the expression levels of GLUT4 and β-actin were analyzed using qRT-PCR. Values were normalized to the expression of β-actin, with the levels of DMSO-treated cells set at 1. Results are expressed as means ± SE (n = 3). ***P < 0.001 compared with control cells as indicated.

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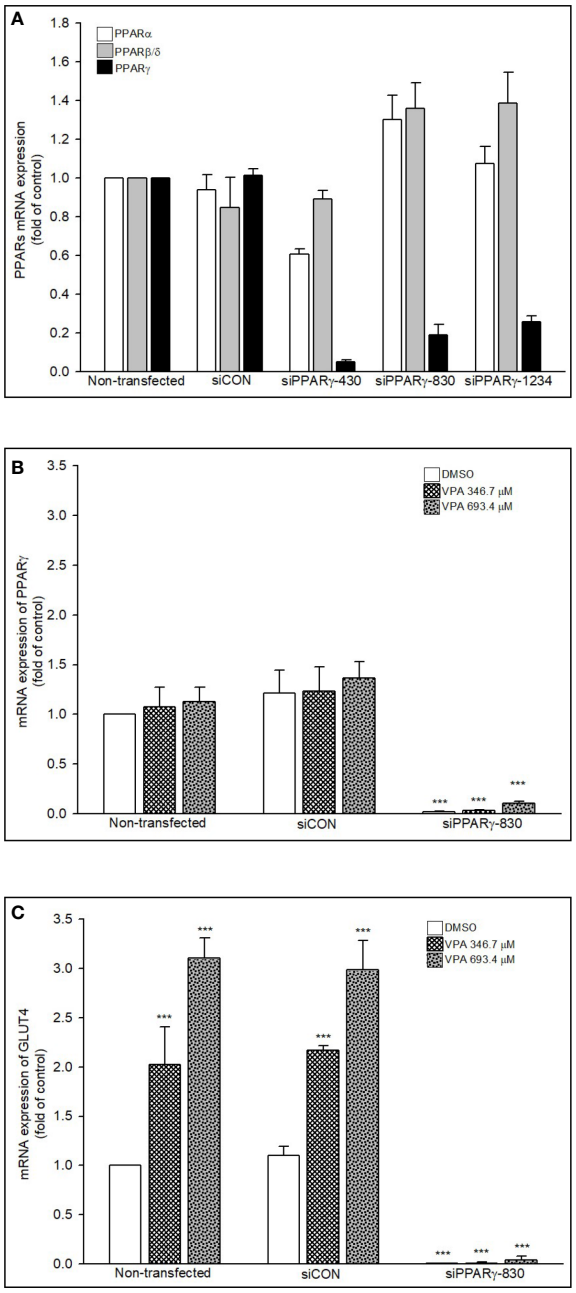


FIGURE 9
Knockdown of PPARs and mRNA expression of PPARs and GLUT4. **(A, B)** RD cells were transfected with 30 nM siControl or siPPARs for 48 h or treated with **(C)** VPA for additional 24 h. Total RNA was extracted, and the expression levels of PPARs, GLUT4, and β-actin were analyzed using qRT-PCR. Values were normalized to the expression of β-actin, with the levels of DMSO-treated cells set at 1. Results are expressed as means ± SE (n = 3). ***P < 0.001 compared with control cells as indicated.

methylglucose transport in the presence of PHE and no effect on maximum insulin binding. Therefore, PHE administration can result in insulin resistance by induction of a post-binding defect in insulin action. VPA treatment may increase the risk of insulin resistance and consequently of T2DM (47–49). However, the studies investigating these effects have small dimensions. Further efforts are needed to explore the relationship between AEDs use and occurrence of diabetes. Our cohort study demonstrated epilepsy increased the risk of T2DM development. However, in patients receiving AEDs treatment, this risk decreased. Moreover, increasing PHE dosage increased the risk of T2DM, while increasing the DDD of VPA decreased the risk. Thus, clinicians should be aware that both medication and the disease itself may alter glycemic status. Patients receiving drugs known to alter blood glucose levels should be identified and closely monitored.

We further investigated the mechanisms underlying these effects determined by PHE and VPA. Skeletal muscle is the primary target of various factors, for increasing insulin-stimulated glucose uptake, utilization, and disposal (14). RD cells preferentially use glycolytic over oxidative metabolism (50). They exhibit a low but significant insulin-stimulated glucose uptake, in the range of 20–25%. Thus, we chose RD cells to determine the mechanisms of the diabetogenic effect of PHE/VPA treatment. After drug treatment, gene expression was evaluated in triplicate. We used high throughput RNA sequencing (RNA-seq) for its unbiased ability to detect expressed genes with greater sensitivity and accuracy than gene expression microarrays. With appropriate bioinformatics tools, regulatory events affecting genes and associated pathways can be identified more efficiently than in single-gene assays. These tools were applied to identify genes and pathways modified by PHE or VPA in RD cells, when compared to the corresponding not treated controls. To understand the effect of PHE and VPA on the gene expression of the whole muscle genome, RNA-seq was performed on RD cells cultured for 72 hrs in triplicate. After read alignment and gene expression quantification, differential expression analysis of genes and pathways was undertaken. After analysis, the possible candidate genes with significant response ($P < 0.05$) and those corelated with T2DM (21) were selected and compared between the PHE and VPA groups. We observed that, control, PHE, and VPA treated samples clustered into distinct groups. Statistical analysis showed that PHE and VPA treatment induced differential expression of genes in RD cells, with FDR ≤ 0.05, the top differentially expressed genes ranked by significance are shown in heatmap form. We identified genes that had significantly different levels of expression among treated group. These gene sets included KEGG adipocytokine signaling pathway, KEGG T2DM, BIOCARTA PPARA pathway, reactome regulation of lipid metabolism by PPARα, and KEGG PPAR signaling pathway. These findings show an important effect of

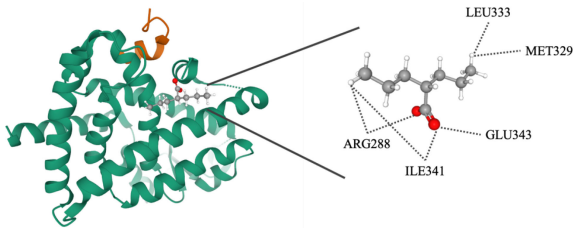


FIGURE 10
Molecular docking analysis of valproate on PPARγ. Superimposition of docked compounds in the PPARγ-binding pocket of the 3D structure.

these AEDs on RD cells. The number of genes beneficial for glucose homeostasis were substantially higher in control vs. PHE, and VPA vs. PHE (59.2% vs. 40.8%, 70% vs. 30%, respectively). VPA increased the expression of GLUT4, IR, and thus intracellular glucose content. We hypothesized that VPA might alter the levels of expression of these genes by PPARγ transactivation and VPA induced GLUT4 expression by PPARγ transactivation.

PPARγ, the molecular target of the TZDs, modulates multiple pathways involved in insulin sensitivity. It represents a promising target for drugs against pathologies such as T2DM and metabolic syndrome (2). Both TZDs, rosiglitazone and pioglitazone, are still available in many countries for the T2DM control (51), although the European Medicines Agency and U.S. Food and Drug Administration issued various warnings on their CVD safety issues. In patients with T2DM, when compared with placebo or active controls, rosiglitazone increases significantly the risk of heart failure, and to some extent that of myocardial infarction, but not the risk of stroke, and it does not influence the cardiovascular mortality and all-cause mortality (52). Compared to rosiglitazone, pioglitazone exerts beneficial effects on the plasma lipid profile, leading to a lower risk of acute myocardial infarction, stroke, or heart failure (51). However, the clinical use of pioglitazone is also limited by the occurrence of several adverse events, including body-weight gain, fluid retention, and possibly bladder cancer (53). Thus, it needs to develop safer PPARγ to reduce the abovementioned adverse reactions.

Skeletal muscle is the main tissue responsible of glucose uptake, and various factors can stimulate this process and increase glucose utilization (37). In contrast, muscle contraction increases insulin-mediated glucose disposal. GLUT4 levels in skeletal muscle are adjusted by transcriptional regulation, and overexpression of GLUT4 in skeletal muscle increases insulin- and contraction-stimulated glucose uptake and metabolism (54). Impaired insulin stimulation of glucose uptake in adipose tissue and skeletal muscle is one of the earliest defects detected in insulin-resistant states (55). In these states, the level of expression of GLUT4 is substantially lower in adipose tissue, as reported by preclinical and clinical studies, however it is not modified in muscle tissue, which indicates a tissue-specific nature of GLUT4 gene regulation in pathological and physiological states (55). GLUT4 density in muscle fibers from DM patients was 9% lower than in those from weight-matched non-diabetic obese subjects and 18% lower than in those from the lean control group (56). Thus, decrease of GLUT4 expression leads to insulin resistance and consequently to T2DM. Increased muscle GLUT4 expression improves insulin action and glucose disposal and enhances muscle glycogen storage (38). The regulation of GLUT4 gene possesses interest from a clinical point of view, because insulin-mediated glucose homeostasis depends on GLUT4 protein levels in muscles (56). PPARγ binds to cis elements on the GLUT4 promoter and keeps it in a repressed state; TZDs binding to PPARγ causes the detachment of corepressors and the attachment of coactivators, which subsequently leads to increased GLUT4 expression and enhanced insulin responsiveness (37). There are reports of VPA reducing the blood glucose level and fat deposition in adipose tissue and liver of mice and rats (57, 58). In addition, VPA treatment determines lower fasting plasma glucose levels (59), and can be safely prescribed, not causing insulin resistance and its associated complications (60). VPA-treated patients presented lower blood glucose levels compared with controls (59, 61–63). However, VPA induced weight gain in several clinical studies (64–66), although the exact mechanisms remain unknown. Recently, the possibility of repurposing VPA for the treatment of different diseases was investigated (67–69). Here, we demonstrate for the first time that VPA may be repurposed for the treatment of T2DM as it specifically transactivates PPARγ. Furthermore, we demonstrated VPA as a PPARγ agonist may reduce the risk of TD2M in epileptic patients. Further research is needed to confirm these results.

The PPARγ protein comprises an N-terminal regulatory domain, a central DNA-binding domain, and a C-terminal LBD (amino acids 204-477). The LBD consists of 13 α-helices and a small four-stranded β-sheet. Helix H12 of the ligand-dependent activation domain (AF-2), is essential for ligand binding and PPAR activation (2). The large size and the flexibility of the binding pocket allow PPARγ to interact with structurally distinct ligands. The binding pose indicates VPA interacts with the LBD at 5 residues. Thus, we showed that VPA could act as a new partial PPARγ agonist, providing insight for the development of novel PPARγ modulators.

Our study had several limitations. First, the database we used may have misclassified epilepsy and T2DM, limiting the reliability and validity of the study. However, in Taiwan, universal health insurance is distributed, and as a result, specialists enact a peer review system, to reduce the possibilities of bias. Second, some factors associated with T2DM are unmeasured or poorly captured in claims data (e.g. smoking habits, alcohol intake, body mass index, lifestyle and dietary habits, and family history of T2DM, are not available in NHIRD database). However, they are unlikely to be associated with the choice of epilepsy treatment. Therefore, the magnitude of their impact is negligeable and they would likely not influence our findings. Third, this claims database was initially created for charging purposes, and as some information was anonymized, we could not get any individual information directly from the patients. Fourth, exposure, outcome, or covariate misclassification cannot be excluded, as this claims database does not include clinical laboratory data such as blood glucose and HbA1c levels, and thus, we could not assess the severity of T2DM. Exposure was evaluated based on medication dispensing. In this study, both epilepsy and T2DM were accurately diagnosed and coded by specialists according to standard symptomatic criteria, research facility information, and imaging findings. To minimize outcome misclassification, we used validated, age-specific definitions of T2DM with high positive predictive values in health care databases. Additionally, this study reduced the confounding effect of medication by adjusting for comorbidities. However, more information should be obtained from other databases to conduct a comprehensive prospective study or randomized controlled trial to further investigate the relationship between epilepsy, AEDs, and T2DM. Most important, evidence derived from a retrospective cohort study is typically lower in statistical quality than that derived from clinical trials, because of numerous sources of inherent bias, including the classification bias. However, the NHI

program has a high coverage rate, and medical reimbursement specialists and peer reviewers scrutinize all insurance claims, ensuring that the diagnoses and coding of diseases are highly reliable. The nondifferential misclassification allows that our results are not invalidated by the classification bias.

This study had several strengths. First, we used a nationwide, population-based cohort of patients with anonymized data to minimize selection bias. Additionally, we evaluated the effect of epilepsy and AEDs on the risk of development of T2DM over a prolonged follow-up period. Moreover, by adjusting for age and sex in a 1:1 ratio, we accounted for confounders that may affect the occurrence of T2DM. Finally, although detection bias may have occurred if patients had more hospital visits than the control population by increasing the possibility of detecting T2DM, the risk of T2DM was still increased 1.27-fold in patients with ≥ 2 hospital visits per year. Overall, this is the first study to investigate the epilepsy-associated risk for developing T2DM and evaluate the effects of AEDs on T2DM-related pathway gene expression and on PPARγ transactivation.

Conclusion

On the basis of our cohort study and NGS database research, we found that epilepsy is associated with an increased risk of developing T2DM. Treatment with AEDs decreased this risk when compared to no treatment. However, increased DDD of PHE, but not of VPA, increased risk of T2DM when compared to non-diabetic population. These findings highlight that the initial choice of one AED could impact the incidence of T2DM. Patients and clinicians concerned about the potential metabolic adverse effects of the AEDs treatment should consider initiating therapy with VPA, which possesses the lowest risk of T2DM. We suggest a novel paradigm to increase GLUT4 expression in skeletal muscle cells, by using an AED used in clinical practice that also has regulatory effects on PPARγ. We believe that further unveiling the mechanisms that regulate GLUT4 gene expression in diabetes will hopefully result in finding effective ways to improve overall insulin sensitivity in epileptic populations. We demonstrated for the first time that PHE and VPA administration have different effects on the level of expression of genes related to T2DM-associated pathway. We demonstrated that VPA could induce expression of GLUT4 via the specific transactivation of PPARγ, and thus increase glucose uptake in muscle cells. Furthermore, it also increases the expression of IR. Future preclinical studies will clarify whether VPA can mitigate the risk of T2DM. In this context, repurposing VPA provides a time- and cost-effective alternative for preventing the occurrence of T2DM in patients with epilepsy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Research Ethics Committee of China Medical University and Hospital in Taiwan. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization, NT, TYW, YJF, and YPL. Methodology, NT, TYW, YJF, and YPL. Software, CLL, FYC, CCNW, CYH, and FJT. Validation, TYW, and YPL. Formal analysis, CLL, FYC, CCNW, and CYH. Investigation, NT, TYW, YJF, and YPL. Resources, CLL, CCNW, CYH, FJT, and YPL. Data curation, CLL, FYC, CCNW, and YPL. Writing-original draft preparation, all authors. Writing-review and editing, all authors. Visualization, all authors. Supervision, NT, TYW, YJF, and YPL. Project administration, FJT, and YPL. Funding acquisition, NT, TYW, YJF, and YPL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

αMEM	Minimum Essential Medium alpha
ACP	Acid phosphatase
adjP	Adjusted P
AEDs	Anti-epileptic drugs
AF	Atrial fibrillation
aHR	Adjusted hazards ratio
ANOVA	One-way analysis of variance
BMI	Body mass index
CAD	Coronary artery disease
CBZ	Carbamazepine
CHF	Congestive heart failure
CIs	Confidence intervals
CLZ	Clonazepam
COPD	Chronic obstructive pulmonary disease
CVD	Cerebrovascular disease
DDD	Defined daily dose
DEGs	Differentially expressed genes
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DPP-4	Dipeptidyl peptidase-4
FA	Fatty acid
GBP	Gabapentin
GK	Glycerol kinase
GLUT4	Glucose transporter type 4
GO	Gene Ontology
HCS	HiSeq Control Software
HRs	Hazard ratios
ICD-9-CM & ICD-10-CM	International Classification of Diseases Ninth & Tenth Revision Clinical Modification
IR	Insulin receptor
IRS	Insulin receptor substrate
KEGG	Kyoto Encyclopedia of Genes and Genomes
LBD	Ligand-binding domain
LDL	Low-density lipoprotein
LGTD	Longitudinal Generation Tracking Database
MHW	Ministry of Health and Welfare
MSigDB	Molecular Signatures Database
NGS	Next-generation sequencing
NHI	National Health Institute
NHIRD	National Health Insurance Research Database
NR	Nuclear receptor
PB	Phenobarbital
PDB	Protein Data Bank
PE	Paired-end
PHE	Phenytoin
PI3K	Phosphoinositide 3-kinases
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
PYK	Pyruvate kinase
RXR	Retinoid X receptor
SE	Standard error
SGLT	Sodium-glucose cotransporter
siRNAs	Small interfering RNAs
T007	T0070907
T2DM	Type 2 diabetes mellitus
TZDs	Thiazolidinediones
VDCC	Voltage-gated calcium channels
VPA	Valproate

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Performance of artificial intelligence in diabetic retinopathy screening: a systematic review and meta-analysis of prospective studies

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Aims: To systematically evaluate the diagnostic value of an artificial intelligence (AI) algorithm model for various types of diabetic retinopathy (DR) in prospective studies over the previous five years, and to explore the factors affecting its diagnostic effectiveness.

Materials and methods: A search was conducted in Cochrane Library, Embase, Web of Science, PubMed, and IEEE databases to collect prospective studies on AI models for the diagnosis of DR from January 2017 to December 2022. We used QUADAS-2 to evaluate the risk of bias in the included studies. Meta-analysis was performed using MetaDiSc and STATA 14.0 software to calculate the combined sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio of various types of DR. Diagnostic odds ratios, summary receiver operating characteristic (SROC) plots, coupled forest plots, and subgroup analysis were performed according to the DR categories, patient source, region of study, and quality of literature, image, and algorithm.

Results: Finally, 21 studies were included. Meta-analysis showed that the pooled sensitivity, specificity, pooled positive likelihood ratio, pooled negative likelihood ratio, area under the curve, Cochrane Q index, and pooled diagnostic odds ratio of AI model for the diagnosis of DR were 0.880 (0.875-0.884), 0.912 (0.99-0.913), 13.021 (10.738-15.789), 0.083 (0.061-0.112), 0.9798, 0.9388, and 206.80 (124.82-342.63), respectively. The DR categories, patient source, region of study, sample size, quality of literature, image, and algorithm may affect the diagnostic efficiency of AI for DR.

Conclusion: AI model has a clear diagnostic value for DR, but it is influenced by many factors that deserve further study.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42023389687.

KEYWORDS

artificial intelligence, diabetic retinopathy, meta-analysis, diagnostic accuracy, prospective study

1 Introduction

Diabetic retinopathy (DR) is one of the leading causes of blindness among middle-aged and older people worldwide (1, 2). According to international standards, DR is mainly divided into nonproliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) based on the condition of the fundus. As a common complication of diabetes, it is estimated that up to 30% of diabetic patients will eventually develop various types of DR (3), and 10% of diabetic patients are at risk of blindness (4).

Fundus color photography plays a key role in the screening of DR, which has traditionally relied on the clinical experience of ophthalmologists or retinal specialists who comprehensively evaluate the patient's condition through routine ophthalmology examination, fundus scope, optical coherence tomography (OCT), and other methods. However, as DR is an insidious disease, most patients are not consciously aware of the existence of the disease, especially those living in areas with underdeveloped medical facilities, making it challenging for ophthalmologists to make an accurate and timely diagnosis of the patient's condition from fundus color photography. In addition, ophthalmologists often adopt different intervention methods for DR patients with different disease states. Therefore, in order to delay the occurrence and development of DR, reduce the blinding rate, and improve the quality of life of patients, it is necessary to accurately distinguish the various types of DR at an early stage of the disease.

As deep learning technology has advanced in recent years, the application of artificial intelligence (AI) in the medical field is receiving increasing attention. It involves the analysis and evaluation of image-related data through the establishment of relevant databases and application models, and information processing (5). In the field of ophthalmology, AI is widely used in DR, age-related macular degeneration (AMD), glaucoma, cataract, and other diseases (6), and fully autonomous diagnostic systems have already been developed (7). As a potential method to assist clinical ophthalmologists in the diagnosis and treatment of DR, one of the obvious advantages of AI is its high diagnostic accuracy. Studies have shown that the accuracy of DR diagnosis can reach up to 90% by using a deep learning mode (8–10), and 80% or above with a machine learning model (11–13). Therefore, AI can effectively relieve the pressure on ophthalmologists by conducting mass image screening and improve their efficiency of ophthalmologists in the diagnosis and treatment of related diseases and complications, thus solving the problem of insufficient medical resources and promoting the comprehensive development of blindness prevention and treatment strategies. At present, diagnostic meta-analyses on the accuracy of DR detection by AI have mainly focused on a specific algorithm (14, 15). Additionally, most studies are based on the mining of publicly available datasets, which lack verification in the real world. Although these datasets are not updated in time, they are repeatedly cited in many meta-analyses. Moreover, in previous meta-analyses, most of the included studies were retrospective studies, which may cause bias in the real world setting. The present meta-analysis will be systematically investigated the performance and application status of AI in diagnosing DR based on fundus color photographs in the real world in the last five years. Furthermore, the factors that might affect the diagnostic effect of AI through subgroup analysis will be explored. Our results can further validate the role of AI in clinical decision making.

2 Materials and methods

2.1 Search strategy

Two reviewers (KL and SM) searched relevant prospective studies in the Cochrane Library, PubMed, Embase, Web of Science, and The Institute of Electrical and Electronics Engineers (IEEE) databases over the last 5 years. Each of the other reviewers re-evaluated whether the search strategy was appropriate and whether the included literature was consistent with the research purpose. The terms of our search were as follows: ("Diabetic Retinopathy" OR "Diabetic Retinopathies" OR "Retinopathies, Diabetic" OR "Retinopathy, Diabetic", then combined these items using AND with "Artificial intelligence" OR "machine learning" OR "deep learning" OR "neural network") AND ("diagnosis" OR "screen" OR "classification" OR "discriminate") AND ("performance" OR "sensitivity" OR "specificity" OR "accuracy" OR "area under the curve" OR "auc"). The meta-analysis was conducted following the PRISMA (16) (Supplementary Table S4).

2.2 Study selection and eligibility criteria

Criteria for inclusion in the meta-analysis were: (1) the study was a diagnostic study; (2) the subjects were type 2 or type 1 diabetic patients with DR; (3) the diagnostic measure was AI technology, and DR was diagnosed from fundus color images; (4) the study was complete with available data on sensitivity (SE), specificity (SP), number of patients and controls, and other outcome indicators; (5) prospective research in the last 5 years. Exclusion criteria: (1) Diagnostic measures do not belong to AI technology and no AI-related algorithm was used; (2) studies with duplicate data and incomplete original data; (3) studies with incomplete or inaccessible outcome indicators. ZW and ZL selected the studies independently according to the inclusion and exclusion criteria above. If there was a difference of opinion among the reviewers, a joint consultation was held with a third reviewer (XZ) before making a decision. Here are populations, interventions, comparators, outcomes, and study designs (PICOS) in our study. Population of our research comes from type 2 or type 1 diabetic patients with DR; interventions: patients with DR are diagnosed by AI technology.; comparators: patients with DR are diagnosed by clinical doctors;.study design: the study was a diagnostic study.

2.3 Data extraction

After obtaining the full articles, two reviewers (ZW and ZL) independently summarized the features of the included studies and extracted outcome indicators related to the diagnostic efficacy of AI

from each study. We addressed divergence between the two reviewers' data extraction by discussion and consultation with a third investigator (KL). Reviewers directly extracted SE, SP, and number of DR patients and total participants from the included studies. These indicators were used to calculate the outcome variables for the diagnostic meta-analysis, namely true-positives (TP), false-positives (FP), false-negatives (FN), and true-negatives (TN), which were then entered into contingency tables, followed by subsequent meta-analysis. If a study contained different types of DR or different algorithms, and there were multiple contingency tables, we assumed that they were independent of each other.

2.4 Quality assessment

To assess the quality of the included Studies, two investigators (SM and XZ) used Quality Assessment of the Diagnostic Accuracy Studies 2 (QUADAS-2) (17) and RevMan 5.3. QUADAS-2 scale includes four bias risk assessment parts, namely patient selection, index test, reference standard, and flow and timing. Each part has two or three questions. If all the answers were "Yes", that part was considered as low risk. Additionally, patient selection, index test, and reference standard were also evaluated in terms of the clinical applicability. If the answers to these assessment parts were "low risk", it indicates that the included studies are less biased.

2.5 Data synthesis and analysis

We used MetaDiSc software (version 1.4) for the outcome variables (TP, FP, FN, TN). Summary receiver operating characteristic (SROC) plots and coupled forest plots were used to visualize the merger results. The I^2 test and Cochrane-Q test were used to evaluate heterogeneity caused by possible non-threshold effects in this meta-analysis. If I^2>50%, it was considered as significant heterogeneity. Subsequently, bivariate random effects model was used to calculate the pooled sensitivity, specificity, area under the curve (AUC), diagnostic odds ratio (DOR), positive and negative likelihood ratios (LR+ and LR-, respectively), among which area under the SROC curve indicates the diagnostic value of AI for DR. In order to explore how categories of DR, source of patients, sample size, country, quality of included studies and images, and different algorithms can influence the merged results, we performed subgroup analysis according to the above factors.

We used the midas package in STATA14.0 to conduct a sensitivity analysis of the included studies to explore the source of heterogeneity. Furthermore, the incidence rate of DR (30%) was taken as the prior probability, and the posterior probability was calculated according to the summarized LR+ and LR-. The results were visualized in STATA14.0 and displayed with fagan plots. We have assess publication bias by plotting Deek's funnel plot. The funnel plot is asymmetric when significant publication bias is present. All statistical results were considered significant if the two-tailed p value<0.05.

3 Results

3.1 Selection and characteristics of the eligible studies

A flowchart of the literature search and study selection process is presented in Figure 1. Firstly, relevant studies were retrieved successively from the relevant databases according to the retrieval strategy, which yielded 2748 studies in total. Thereafter, duplicate studies, meta-analyses, reviews, conference files, studies whose full text could not be obtained, and studies whose title and abstract were inconsistent with the research content were eliminated. After the preliminary screening, we obtained 72 original studies. Next, we excluded studies that were not of interest, studies that were not prospective or cross-sectional, or had incomplete data for meta-analysis. Finally, 21 studies were used for quantitative synthesis of the meta-analysis (18–38). Table 1 summarizes the outcome variables included in the study. The population included in the study was selected from the real world from cross-sectional or prospective studies, thus avoiding bias due to case-control studies. Among them, seven, 17, five, and four studies evaluated any DR, referable DR (RDR), more-than-mild DR (mtmDR), and vision-threatening DR (VTDR), respectively. In addition, 19 studies included patients from the clinic, seven from the general community, and seven from the ordinary population. We explored the algorithm used by each study for diagnosing DR, image quality, region where the study was conducted, and sample size (Table 1). Table 2 summarized additional data about the patients, such as sex, age, type of diabetes, diabetes duration, co-morbidities and soon. The study was registered in the PROSPERO (CRD42023389687).

3.2 Quality assessment

Figures 2, 3 show the summary chart and bar chart, respectively, for quality evaluation of the included studies, and Supplementary Table S1 shows the process of quality evaluation using QUADAS-2. We found that seven studies answered no in patient selection, all studies performed well in the index test, and nine studies did not provide clear information for evaluating the reference standard. The included studies performed poorly in evaluating the flow and timing of patient selection; additionally, when evaluating patient selection, index test, and reference standard, all studies showed low risk with regard to clinical applicability concerns, indicating the high credibility of this meta-analysis.

3.3 Threshold analysis and heterogeneity test

The data were imported into MetaDiSc software (version 1.4) for analysis. It was found that the spearman correlation coefficient between the sensitivity logarithm and (1-specificity) logarithm was 0.001 (p=0.996>0.05), which was not significant. Therefore, there

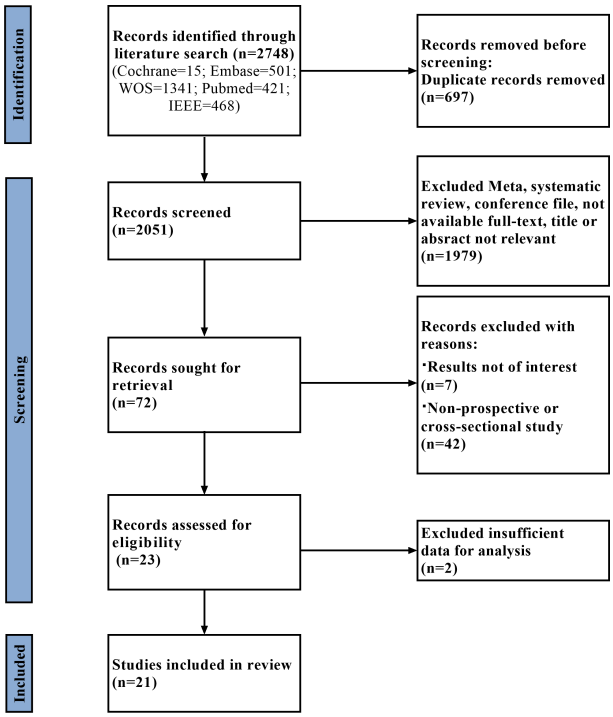


FIGURE 1
Flow diagram of literature selection.

was no threshold effect (Supplementary Table S2) in this study. We then combined the DOR of all studies, and the Cochrane-Q test showed Cochrane-Q=1437.57, P=0.000<0.01, indicating that heterogeneity was caused by the non-threshold effect in this study. Moreover, the SE, SP, LR+, LR-, and DOR were all greater than 50% (Table 3). The above results show that heterogeneity existed between the studies, and may be related to the population, age, algorithm, and literature quality. Consequently, a random effects model (REM, DerSimonian-Laird method) was adopted to synthesize the above five indicators.

3.4 Synthesis of results

MetaDiSc software was used to analyze the included data. The pooled SE was 0.880 (0.875-0.884), pooled SP was 0.912 (0.99-0.913), pooled LR+ was 13.021 (10.738-15.789), pooled LR- was 0.083 (0.061-0.112), combined AUC=0.9798, Q index =0.9388, and pooled DOR was 206.80 (124.82-342.63). Corresponding (SROC) plots and coupled forest plots are shown in Figure 4; Table 2, respectively. For further analyzing the diagnostic efficacy of AI in diagnosing any DR, 0.3 was set as the pretest probability. On drawing fagan nomogram (Figure 5), it was found that the positive post-test probability was 93% and negative post-test probability was 3%. Next, we grouped all included studies in accordance with categories of DR (any DR/RDR/mtmDR/VTDR), patient source (clinical-based/community-based/population-based), country (non-Asia/Asia), sample size (<5000 eyes/>5000 eyes), quality of literature (low quality/medium quality/high quality), image pixels (<1000*1000/>1000*1000), algorithm (convolutional neural network/machine learning/neural network/others [deep learning, regression tree algorithm]), and performed subgroup analysis. The results are shown in Table 4, and the SROC plot of each subgroup is shown in Supplementary Figures S1-S7.

3.5 Meta regression and sensitivity analysis

To explore the source of heterogeneity, we performed meta regression according to the conditions of subgroup analysis using MetaDiSc software. We found that the p value of the algorithm term was 0.033<0.05, indicating that when different AI models are uses to diagnose DR, the algorithms used by the different models may be the source of heterogeneity. The results are shown in Table 5. STATA 14.0 was selected for conducting the sensitivity analysis. It can be clearly seen in Supplementary Figure S9 that there were three original studies with strong sensitivity and the results did not differ significantly. The deleted results are shown in Supplementary Figure S8 and Supplementary Table S3. Other original studies did not demonstrate obvious sensitivity. Overall, the results of our study were stable.

3.6 Publication bias

STATA 14.0 was used to first conduct a publication bias test for all data included in the study, followed by a publication bias test for different categories of DR. The results are shown in Supplementary

TABLE 1 Summary of the data obtained from the included studies.

Author	Year	country	reference standard	Categories of DR	TP[a]	FP[b]	FN[c]	TN[d]	Sample size (eyes)	Source of patients	Quality of literature	pixels	algorithm
Baget-Bernaldiz et al. (18)	2021	Spain	four expert retina ophthalmologists	any DR	2310	11	49	11816	14186	Health Care Area	low	Not clear	convolutional neuronal network
				RDR	1453	10	50	12673	14186	Health Care Area	low	Not clear	convolutional neuronal network
Bode, B.W et al. (19)	2019	US	Wisconsin Reading Center	mtmDR	296	209	14	1182	1701	primary care and eye care facilities	high	Not clear	machine learning
				vtDR	60	178	3	1436	1677	primary care and eye care facilities	high	Not clear	machine learning
Do Rio et al. (20)	2022	UK	retinal specialist	any DR	556	2358	216	14076	17206	community	medium	Not clear	deep neural network
Gulshan et al. (21)	2019	India	retinal specialist	mtmDR	615	95	77	1118	1905	eye care centers	high	Not clear	neural network
				VTDR	418	141	11	1376	1946	eye care centers	high	Not clear	neural network
Li et al. (22)	2022	China	retinal specialist	RDR	147	44	9	947	1147	Shanghai General Hospital	medium	800*800	deep learning
Ming et al. (23)	2021	China	Two licensed ophthalmologists	RDR	19	5	5	292	321	community	low	Not clear	convolutional neural network
				any DR	30	6	6	279	321	community	low	Not clear	convolutional neural network
Natarajan et al. (24)	2019	India	a vitreoretinal resident and a vitreoretinal surgeon	RDR	23	60	0	311	394	Population-based	high	Not clear	neural network
				any DR	36	28	6	324	394	Population-based	high	Not clear	neural network
Noriega et al. (25)	2021	Mexico	3 retina specialists	RDR	48	5	2	45	100	Mexican ophthalmic hospital	low	224*224	deep learning
Pawar et al. (26)	2021	India	four ophthalmologists	RDR	47	14	0	150	211	rural community eye clinic	low	Not clear	deep learning
Rego et al. (27)	2021	Portugal	3 ophthalmologists	RDR	38	7	9	241	295	primary care offices	low	Not clear	convolutional neural network
Rogers et al. (28)	2020	Finland	a board of experts.	RDR	486	944	109	4213	5752	clinical-based	medium	4288*2848	deep learning
				VTDR	52	700	8	4992	5752	clinical-based	medium	4288*2848	deep learning
Sandhu et al. (29)	2020	US	clinical ophthalmologists	any DR	75	1	0	35	111	a single academic medical center	high	1024*1024	machine learning
				mtmDR	75	1	0	35	111	a single academic medical center	high	1024*1024	machine learning

(Continued)

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TABLE 1 Continued

Author	Year	country	reference standard	Categories of DR	TP[a]	FP[b]	FN[c]	TN[d]	Sample size (eyes)	Source of patients	Quality of literature	pixels	algorithm
Scheetz et al. (30)	2021	Australia	two retinal specialists	RDR	31	21	1	150	203	clinical-based	low	Not clear	deep learning
Sosale et al. (31)	2020	India	vitreoretinal specialist	RDR	121	23	1	152	297	population-based	low	750*1334	convolutional neural networks
				any DR	105	8	16	168	297	population-based	low	750*1334	convolutional neural networks
Sosale et al. (32)	2019	India	retina specialist	RDR	231	49	17	603	900	population-based	low	512*512	Convolutional Neural Networks
				any DR	210	29	42	619	900	population-based	low	512*512	Convolutional Neural Networks
Tang et al. (33)	2021	China	retina specialist	RDR	178	11	10	214	413	clinical-based	low	2600*2048	convolutional neural networks
Ting et al. (34)	2017	China	a retinal specialist(>10 years' experience)	RDR	976	2929	102	31941	35948	community	high	Not clear	convolutional neural networks
				VTDR	514	3154	0	32280	35948	community	high	Not clear	convolutional neural networks
				mtmDR	298	4026	22	31602	35948	community	high	Not clear	convolutional neural networks
Wang et al. (35)	2021	China	three trained graders	RDR	192	291	6	2115	2604	Clinica-based	medium	299*299	convolutional neural networks
Wongchaisuwat et al. (36)	2021	Thailand	a retinal expert	RDR	18	77	3	884	982	Clinical-based	medium	Not clear	convolutional neural networks
			a retinal expert	RDR	21	104	2	547	674	Clinical-based	medium	Not clear	convolutional neural networks
Yao et al. (37)	2022	China	two senior ophthalmologists	mtmDR	42	26	9	45	121	Clinical-based	low	Not clear	regression tree algorithm
Zhang et al. (38)	2020	China	a panel of three experts	RDR	8265	2306	1657	28437	40665	diabetes centers	low	800*800	neural network

[a]TP, true positive;
[b]FP, false positive;
[c]FN, false negative;
[d]TN, true negative; DR, diabetic retinopathy; RDR, referable DR; mtmDR, more-than-mild DR; VTDR, vision-threatening DR.

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TABLE 2 Summary of additional characteristics of the included studies.

Author	Year	Categories of DR	Sex (male %)	Age	Type of diabetes	Random blood sugar	Hemoglobin A1c	Diabetes duration	Co-morbidities		
									BP[a]	Obesity[b]	BMI[c] (kg/m2)
Baget-Bernaldiz et al. (18)	2021	any DR	54.6	63.7 years (mean)	type 2 diabetes	NM[d]	NM	NM	NM	NM	NM
		RDR	54.6	63.7 years (mean)	NM	NM	NM	NM	NM	NM	NM
Bode, B.W et al. (19)	2019	mtmDR	50.1	<65 years, 75.1% >=65 years, 24.9%	type 2 diabetes	NM	>=9%	NM	NM	NM	NM
		vtDR	50.1	<65 years, 75.1% >=65 years, 24.9%	type 2 diabetes	NM	>=9%	>=5 years	NM	NM	NM
Do Rio et al. (20)	2022	any DR	47.9	57.7 years (mean)	type 2 diabetes	>=8.9%	NM	NM	NM	NM	NM
Gulshan et al. (21)	2019	mtmDR	58.1	56.6 years (mean)	NM	NM	NM	NM	NM	NM	NM
		VTDR	58.1	56.6 years (mean)	NM	NM	NM	NM	NM	NM	NM
Li et al. (22)	2022	RDR	68.4	50 yeas (mean)	NM	NM	8.67%	9.79 years (median)	NM	overweight	25.67
Ming et al. (23)	2021	RDR	45.7	69.3 years (mean)	NM	NM	NM	11.2 years (mean)	NM	NM	NM
		any DR	45.7	69.3 years (mean)	type 2 diabetes	NM	NM	11.2 years (mean)	NM	NM	NM
Natarajan et al. (24)	2019	RDR	48.4	51.3 years (mean)	NM	NM	NM	NM	NM	NM	NM
		any DR	48.4	51.3 years (mean)	NM	NM	NM	NM	NM	NM	NM
Noriega et al. (25)	2021	RDR	NM	NM	NM	NM	NM	NM	NM	NM	NM
Pawar et al. (26)	2021	RDR	51.45	52.84 years (mean)	NM	NM	NM	NM	NM	NM	NM
Rego et al. (27)	2021	RDR	NM	NM	NM	NM	NM	NM	NM	NM	NM
Rogers et al. (28)	2020	RDR	34.2	60 years (mean)	NM	NM	NM	NM	NM	NM	NM
		VTDR	34.2	60 years (mean)	NM	NM	NM	NM	NM	NM	NM
Sandhu et al. (29)	2020	any DR	52	20-82 years	NM	NM	NM	NM	NM	NM	NM
		mtmDR	52	20-82 years	NM	NM	NM	NM	NM	NM	NM
Scheetz et al. (30)	2021	RDR	50	56 years (median)	type 1 diabetes (34%) type 2 diabetes (65%)	NM	NM	13 years (median)	NM	NM	NM
Sosale et al. (31)	2020	RDR	58	55 years (mean)	type 1, 2 diabetes	NM	8%	11 years (mean)	NM	overweight	27
		any DR	58	55 years (mean)	type 1, 2 diabetes	NM	8%	11 years (mean)	NM	overweight	27
Sosale et al. (32)	2019	RDR	NM	NM	NM	NM	NM	NM	NM	NM	NM

(Continued)

TABLE 2 Continued

Author	Year	Categories of DR	Sex (male %)	Age	Type of diabetes	Random blood sugar	Hemoglobin A1c	Diabetes duration	Co-morbidities		
									BP[a]	Obesity[b]	BMI[c] (kg/m2)
		any DR	NM	NM	NM	NM	NM	NM	NM	NM	NM
Tang et al. (33)	2021	RDR	52.5	60.81 years (mean)	type 1 diabetes (6%) type 2 diabetes (94%)	NM	NM	11.84 years	NM	NM	NM
Ting et al. (34)	2017	RDR	51.02	60.16 years (mean)	NM	NM	7.54%	3.7 years (median)	132/73	overweight	27.22
		VTDR	51.02	60.16 years (mean)	NM	NM	7.54%	3.7 years (median)	132/73	overweight	27.22
		mtmDR	51.02	60.16 years (mean)	NM	NM	7.54%	3.7 years (median)	132/73	overweight	27.22
Wang et al. (35)	2021	RDR	58.5%	59.1 years (mean)	NM	Fast blood glucose: 5.60 ± 1.17mmol/L	NM	6.44 years (mean)	129/75	no obesity	24
Wongchaisuwat et al. (36)	2021	RDR	NM	NM	NM	NM	NM	NM	NM	NM	NM
		RDR	NM	NM	NM	NM	NM	NM	NM	NM	NM
Yao et al. (37)	2022	mtmDR	61.16%	56.64 years (mean)	NM	NM	8.73%	9 years (mean)	NM	NM	NM
Zhang et al. (38)	2020	RDR	58.25%	18-29 years,3.4% 30-39 years,9.3% 40-49 years,21.3% 50-59 years,35.0% 60-69 years,25.3% >=70 years,5.7%	NM	NM	<6.5%,9.3% (6.5-6.9)%,7.5% (7.0-7.9)%,15.3% (8.0-8.9)%,14% (9.0-9.9)%,12.2% >=10%,27.9%	<5 years,42.2% 5-10 years,17.8% 10-15 years,13.3% 15-20 years,6% >=20 years,4%	NM	NM	NM

[a]BP, blood pressure,(mmHg, systolic blood pressure/diastolic blood pressure);
[b]obesity, according to the BMI index conversion (BMI=18.5-25kg/m^2, no obesity; BMI=25-30kg/m^2, overweight; BMI=30-35kg/m^2, mild obesity; BMI=35-4040kg/m^2, moderate obesity; BMI>40kg/m^2, severe obesity);
[c]BMI, Body Mass Index, weight/height^2;
[d]NM, Not Mentioned.

Figure S10. For all the included studies, p value was 0.84>0.05, which means that there was no publication bias in this study, based on the symmetry of the funnel plot.

4 Discussion

In recent years, several studies have investigated the use of AI for the assessment of incidence and diagnostic accuracy of DR. Meanwhile, the application of AI in the medical field is being continuously developed and subdivided (39). However, due to the different image quality of fundus color photos, algorithms used by AI models, and performance of cameras used in different studies, the AI diagnostic effect varies significantly among different studies (40, 41). Our study is the first meta-analysis to include all prospective studies and use multiple algorithms to evaluate the diagnostic efficacy in DR, rather than only deep learning or machine learning. Firstly, relevant studies were retrieved from medical databases according to the retrieval strategy, and the retrieved studies was screened according to the existing guidelines for diagnostic reviews (42) to ensure the rigor of the study. In the final meta-analysis, we included a total of 21 original studies, involving 129,759 eyes. All studies were conducted in the real world, avoiding the bias caused by retrospective studies.

There was no threshold effect in this analysis, but the heterogeneity among studies was high. Therefore, the random effects model was used to combine all indicators. DOR and AUC were the main indicators to judge the relationship between the diagnostic results and DR. The pooled DOR=206.80 and AUC=0.9798 for all included studies indicated that AI had a high diagnostic performance for DR. In order to make the study results more clinically relevant, we drew fagan plots and concluded that if AI showed a positive result based on fundus color photography, the probability of the patient having DR was 93%. If the AI diagnosis was negative, the patient had a 3% chance of DR.

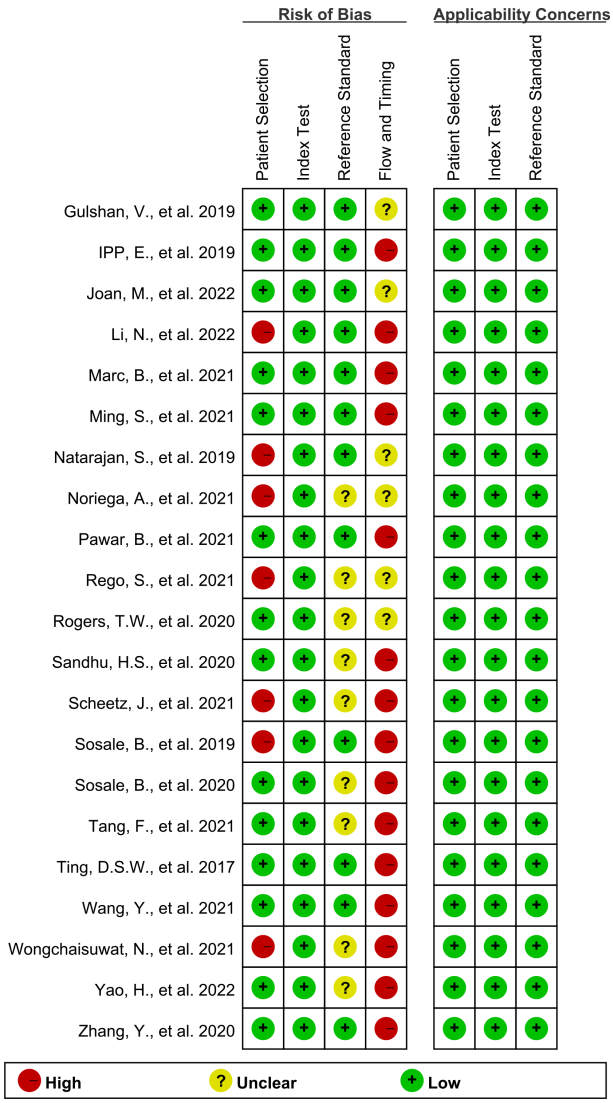


FIGURE 2
QUADAS-2 summary plot of bias risk assessment.

To explore the source of heterogeneity, we conducted meta regression, and found that the differences in AI algorithms may be the source of heterogeneity. To further explore the factors influencing the AI diagnosis of DR, we performed a subgroup analysis. We found that studies with patients from clinics, hospitals, or medical research centers had higher diagnostic efficacy than those with patients from other sources, and this may be because patients from hospitals or medical research centers are more representative, and retinopathy can be diagnosed more accurately by clinicians with a lower error rate; besides studies from non-Asian countries had higher diagnostic efficacy than studies from Asian countries, and we believed that this is because non-Asian countries have carried out artificial intelligence algorithm diagnosis DR for a long time, trained the algorithm more times, had a large data set,

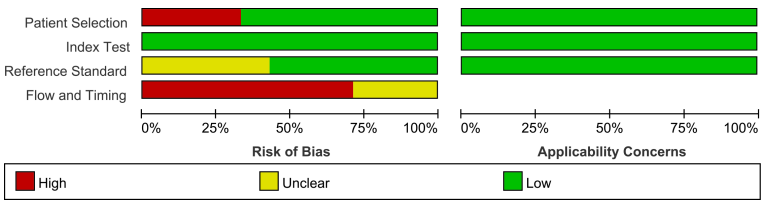


FIGURE 3
QUADAS-2 bar plot of bias risk assessment.

TABLE 3 The combined predictive value of all included studies.

Index	Merge value	95% Cis	I^2 (%)	P value
Se[a]	0.880	0.875-0.884	97.3	0.00
Sp[b]	0.912	0.911-0.913	99.5	0.00
DOR[c]	206.80	124.82-342.63	97.8	0.00
LR+[d]	13.021	10.738-15.789	99.0	0.00
LR-[e]	0.083	0.061-0.112	96.5	0.00

[a]Se, sensitivity;
[b]Sp, specificity;
[c]DOR, diagnostic odds ratio;
[d]LR+, positive likelihood ratio;
[e]LR-, negative likelihood ratio.

included more cases, and had relatively high data quality. Moreover, we have found that the greater the number of eyes included in the study, the higher the diagnostic efficacy. We hypothesized that the more cases included, the more times the algorithm would be trained, so the more accurate the diagnosis would be. When the image pixel was taken as the standard to judge the image quality, it was found that the higher the image quality, the higher the DOR value of the diagnostic result, which is similar to the findings of Yip et al. (43). When the algorithm was taken as a subgroup for analysis, the diagnostic effectiveness of the convolutional neural network (CNN) algorithm was significantly higher than that of other algorithms. CNN is the most widely used in the field of medical imaging, which approximates the work efficiency and reliability of experienced clinicians (44). We also found that when the included studies were of high quality, the heterogeneity was significantly lower than when studies were of lower quality. The high diagnostic performance of the above results maybe the result of the large number and high representativeness of the included studies. This may be because high-quality research used more training of AI models, and clinical trials are better arranged, scientific, and include more representative cases, so the results are more reliable.

Several studies have found that for screening, risk stratification, management, and prognosis of DR, the effect of AI cannot be ignored. Firstly, an AI-based automated system can improve the efficiency and coverage of DR diagnosis and treatment, since the traditional DR diagnosis and treatment process only relies on a

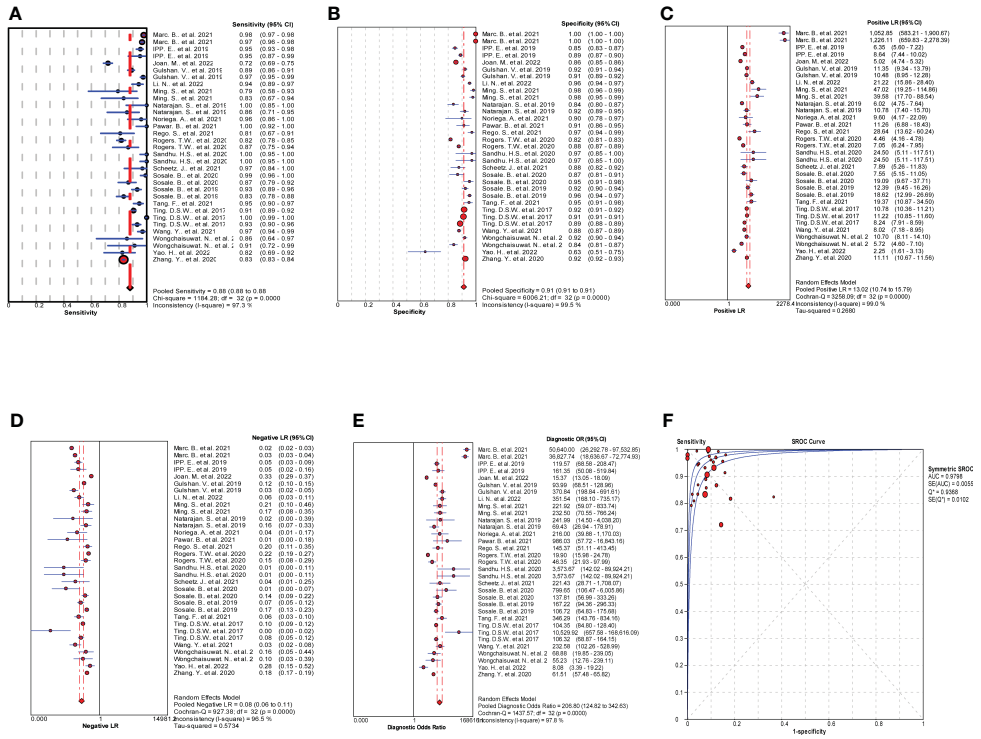


FIGURE 4
Results of meta-analysis and forest plots of all the included studies. **(A)** Forest plot of pooled Se. **(B)** Forest plot of pooled Sp. **(C)** Forest plot of pooled positive likelihood ratio (LR+). **(D)** Forest plot of pooled negative likelihood ratio (LR-). **(E)** Forest plot of pooled diagnostic odds ratio (DOR). **(F)** Summary receiver operating characteristic (SROC) plots.

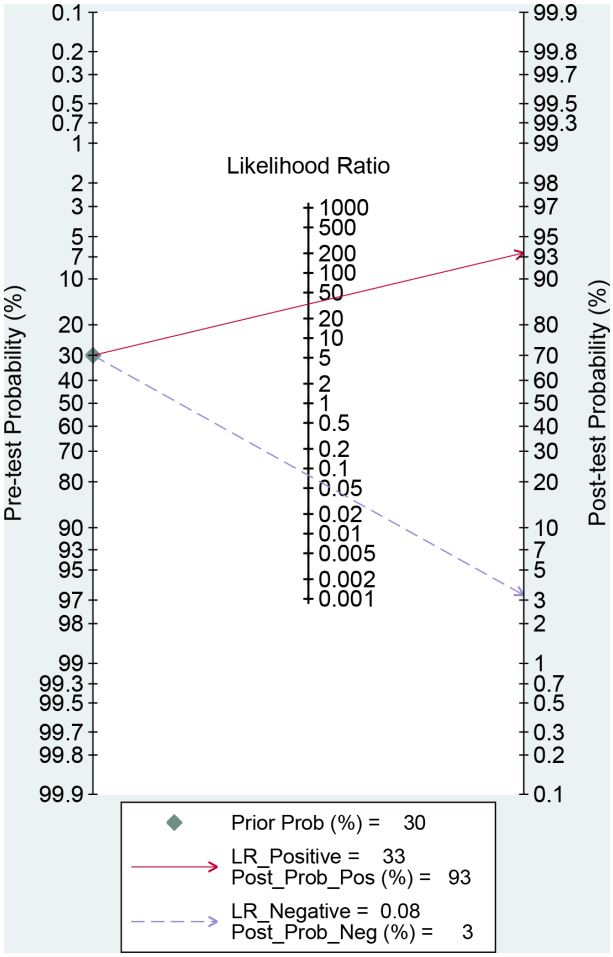


FIGURE 5
Fagan nomogram of artificial intelligence (AI) for the diagnosis of any diabetic retinopathy (DR).

pattern of manual identification, which is easily affected by the experience, skills, and other factors related to the ophthalmologist or relevant technical personnel; therefore, the efficiency of DR screening is relatively low (45). Secondly, DR Patients in remote areas can miss the opportunity to undergo timely treatment due to the lack of skilled ophthalmologists (46). The application of AI in telemedicine can solve this problem (41). Thirdly, it can help clinicians to develop appropriate treatment strategies based on the individual disease of the patients. Clinically, proliferative diabetic retinopathy (PDR) patients are usually treated with laser, intravitreous injection of anti-vascular endothelial growth factor (VEGF), or corticosteroid drugs (47). If the above treatment is given at an early stage to patients with non-proliferative diabetic retinopathy (NPDR) due to an incorrect diagnosis, it will not only waste medical resources, but may also cause serious complications (48). Moreover, studies have shown that AI-based DR screening is more cost-effective than manual grading, and may help in providing cost-effective, convenient, and effective medical services (49).

As a novel diagnostic tool, there are still many problems with AI: (1) Although AI is getting better at diagnosing eye diseases, in our study, the false negative rate (FNR) was 12% and false positive rate FPR was 8.8%, which cannot be ignored. Further exploration of imaging features, increasing the sample size of the training set and test set, or further improving the performance of the algorithm are all feasible methods to solve these problems (43). (2) At present, the models established by various AI algorithms are still considered "black box". This model lacks "explanatory ability" for the diagnosed diseases, that is, it cannot provide the reasons for the diagnostic results to clinicians (50, 51). (3) Since most current studies have detected DR through fundus imaging, the results may not be applicable to other eye diseases and imaging methods.

The ophthalmologist will play an important role in judging the clinical value of emerging AI technologies, in addition to a guiding role in integrating complementary imaging information with clinical data to provide more complete diagnostic information (52). Even if AI can diagnose DR independently, the ophthalmologist will eventually have to issue a report and take legal risks (53); therefore, legislation is required to clarify the respective scope of responsibility between doctors and companies providing AI services, which may also promote the popularization of AI diagnostic services. What is satisfactory is that currently both doctors and patients have a positive attitude towards the diagnostic efficacy of AI (54, 55), which may lay a foundation for their subsequent cooperation.

TABLE 4 Results of subgroup analysis.

Subgroup	Number of study	[pooled Se (95%CI)/I^2]	[pooled Sp (95%CI)/I^2]	AUC	[DOR (95%CI)/I^2]	[RDOR[a] (95%CI)/P value]
Categories of DR						0.68 (0.23-2.02)/0.4747
any DR	7	0.91 (0.900-0.919)/98.7	0.917 (0.914-0.920)/99.8	0.9846	400.16 (23.709-6754.0)/99.2	
RDR	17	0.861 (0.855-0.867)/96.0	0.924 (0.923-0.926)/99.4	0.9780	205.59 (110.14-383.76)/97.1	
mtmDR	5	0.916 (0.900-0.930)/87.5	0.886 (0.883-0.890)/94.1	0.9417	74.829 (33.069-169.32)/89.0	
VTDR	4	0.979 (0.969-0.987)/92.6	0.906 (0.903-0.908)/95.5	0.9446	299.16 (48.362-1850.6)/92.4	
Source of patient						0.69 (0.22-2.13)/0.5044
clinical-based	19	0.879 (0.873-0.883)/97.8	0.935 (0.933-0.937)/99.6	0.9804	246.49 (108.49-560.05)/98.3	
community-based	7	0.874 (0.861-0.886)/98.0	0.899 (0.897-0.900)/99.0	0.9778	171.47 (53.469-549.90)/98.0	
population-based	7	0.913 (0.893-0.929)/88.4	0.898 (0.889-0.906)/91.0	0.9738	144.06 (96.850-214.29)/31.9	
Country						0.33 (0.06-1.80)/0.1906
non-Asia	12	0.922 (0.915-0.929)/98.1	0.920 (0.917-0.922)/99.8	0.9854	399.01 (78.607-2025.3)/99.1	
Asia	21	0.862 (0.856-0.868)/95.5	0.909 (0.908-0.911)/96.4	0.9721	135.23 (96.122-190.26)/88.7	
Sample size						2.58 (0.46;14.61)/0.2697
<5000 eyes	18	0.913 (0.897-0.927)/82.8	0.915 (0.907-0.922)/91.5	0.9744	151.75 (87.790-262.30)/73.7	
>5000 eyes	15	0.877 (0.872-0.882)/98.7	0.912 (0.911-0.913)/99.8	0.9828	253.11 (121.86-525.71)/99.0	
Quality of included studies						0.63 (0.22-1.87)/0.3925
low	15	0.875 (0.870-0.880)/98.1	0.957 (0.955-0.958)/99.5	0.9815	356.42 (96.015-1323.0)/98.4	
medium	7	0.807 (0.788-0.824)/94.6	0.860 (0.856-0.864)/97.4	0.9487	60.018 (28.850-124.86)/94.9	
high	11	0.935 (0.927-0.943)/93.2	0.904 (0.902-0.905)/96.2	0.9850	157.27 (100.48-246.13)/78.7	
Pixels of image						0.83 (0.33-2.10)/0.6860
<1000*1000	6	0.840 (0.833-0.847)/93.8	0.923 (0.920-0.926)/94.2	0.9740	151.90 (78.281-294.76)/89.6	
>1000*1000	7	0.883 (0.864-0.901)/93.5	0.853 (0.847-0.860)/95.4	0.9703	207.04 (55.647-770.29)/93.0	
Algorithm						0.42 (0.23-0.77)/0.0072
CNN[b]	16	0.952 (0.947-0.957)/94.0	0.922 (0.920-0.923)/99.7	0.9862	402.96 (136.89-1184.6)/97.6	
ML[c]	4	0.967 (0.948-0.981)/74.4	0.874 (0.861-0.885)/84.7	0.7278	289.92 (82.140-1023.3)/62.8	

(Continued)

TABLE 4 Continued

Subgroup	Number of study	[pooled Se (95%CI)/I^2]	[pooled Sp (95%CI)/I^2]	AUC	[DOR (95%CI)/I^2]	[RDOR[a] (95%CI)/P value]
NN[d]	5	0.842 (0.835-0.849)/96.3	0.923 (0.920-0.926)/88.9	0.9685	111.69 (60.069-207.67)/89.7	
others[e]	8	0.799 (0.780-0.818)/92.8	0.857 (0.853-0.861)/96.7	0.9366	50.616 (25.408-100.83)/92.9	

[a]RDOR, relative diagnostic odds ratio;
[b]CNN, convolutional neural network;
[c]ML, machine learning;
[d]NN, neural network;
[e]others, deep learning, regression tree algorithm.

This study has the following limitations: (1) the collected DR data lacked proliferative diabetic subtype or further classification of DR, which may affect the evaluation of the diagnostic value; (2) some studies lacked four-grid table data or contained a small number of samples; therefore, the diagnostic value of the representative algorithm may not be truly reflected; (3) In the meta regression, we did not further analyze patient information, such as age, sex, and duration of the disease, which may be a source of heterogeneity and need further study; (4) only English studies were included, which may cause a bias due to the lack of literature in other languages; (5) The gold standard is the decision made by an ophthalmologist or retinologist based on the fundus color image, which means that AI may not perform well on images that an ophthalmologist cannot recognize; (6) Most of the AI algorithm models used in studies are self-developed or debugged models. Since the researchers did not clarify the pre-training degree and learning amount of each model, we could not include these factors in the analysis; (7) there are problems with direct comparison of diagnostic accuracy. As can be seen from the high diagnostic accuracy of VTDR, the diagnostic accuracy differs between mild retinopathy and severe retinopathy. Therefore, the overall accuracy will change depending on the composition ratio of the disease stage of the image for accuracy verification. For example, if many of the accuracy verification images are of mild retinopathy, it is difficult to recognize the lesions, so false negatives increase and accuracy is predicted to decrease.

In conclusion, this meta-analysis suggests that AI-based fundus color imaging has a high predictive ability for DR. The diagnosis rate is much higher than the manual, method, which can contribute to the clinical development of the follow-up strategy or diagnosis and treatment plan and has a high practical application value. However, AI still has a certain rate of missed diagnosis and misdiagnosis, and is easily affected by the patient source, number and representativeness of sample, algorithm of the AI model, quality of images, use of cameras, and type of algorithm. Correspondingly, the performance of AI for diagnosis of DR can be further improved by obtaining more detailed patient data, collecting a large number of samples from multi-centers, deep mining of image features, optimizing AI algorithm architecture, and using high-resolution cameras for images. If the diagnosis and treatment strategies formulated by ophthalmologists are combined with AI, the work efficiency can be greatly improved and the

TABLE 5 Meta regression of included studies.

Var	Coeff.	Std. Err.	p value	RDOR	[95%CI]
Cte.	9.872	2.0250	0.0001	—	—
S	0.300	0.2752	0.2861	—	—
categories[a]	-0.382	0.5265	0.4747	0.68	(0.23;2.02)
source[b]	-0.370	0.5465	0.5044	0.69	(0.22;2.13)
country[c]	-1.106	0.8214	0.1906	0.33	(0.06;1.80)
quality[d]	-0.456	0.5233	0.3925	0.63	(0.22;1.87)
pixels[e]	-0.183	0.4477	0.6860	0.83	(0.33;2.10)
algorithm[f]	-0.861	0.2933	0.0072	0.42	(0.23;0.77)
sample[g]	0.949	0.8396	0.2697	2.58	(0.46;14.61)

Tau-squared estimate = 3.0652 (Convergence is achieved after 7 iterations).
[a]categories, categories of diabetic retinopathy (any DR/RDR/mtmDR/VTDR).
[b]source, source of patient (clinical-based/community-based/population-based).
[c]country, region where the study was conducted (non-Asia/Asia).
[d]quality, quality of included studies (low/medium/high).
[e]pixels, pixels of fundus color photograph (<1000*1000/>1000*1000).
[f]algorithm, algorithm of applied artificial intelligence model (convolutional neural network/machine learning/neural network/others [deep learning, regression tree algorithm]).
[g]sample, sample size of included studies (<5000 eyes/>5000 eyes).
"—", null value.

utilization rate of medical resources can be increased, in addition to providing a more scientific and efficient way for early screening, diagnosis, and treatment of DR.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

KL and SM were responsible for searching the studies in the databases. ZW and ZL designed the entire research idea, selected the appropriate studies after careful searching, and extracted the data from each study. SM and XZ completed the quality assessment. Final meta-analysis was completed by ZW. XZ and YD conceived the study and wrote the first draft. The four authors jointly revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1197783/full#supplementary-material

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Development of predictive nomograms clinical use to quantify the risk of diabetic foot in patients with type 2 diabetes mellitus

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Objective: The aim of the study was to explore the risk factors for diabetic foot disease in patients with type 2 diabetes mellitus and to establish and verify the nomogram model of DF risk in patients with T2DM.

Methods: The clinical data of 705 patients with type 2 diabetes who were hospitalized in our hospital from January 2015 to December 2022 were analyzed retrospectively. According to random sampling, the patients were divided into two groups: the training set (DF = 84; simple T2DM = 410) and the verification set (DF = 41; simple T2DM = 170). Univariate and multivariate logistic regression analysis was used to screen the independent risk factors for DF in patients with T2DM in the training set. According to the independent risk factors, the nomogram risk prediction model is established and verified.

Results: Logistic regression analysis showed age (OR = 1.093, 95% CI 1.062–1.124, P <0.001), smoking history (OR = 3.309, 95% CI 1.849–5.924, P <0.001), glycosylated hemoglobin (OR = 1.328, 95% CI 1.173–1.502, P <0.001), leukocyte (OR = 1.203, 95% CI 1.076–1.345, and LDL-C (OR = 2.002, 95% CI 1.463–2.740), P <0.001) was independent risk factors for T2DM complicated with DF. The area of the nomogram model based on the above indexes under the ROC curve of the training set and the verification set is 0.827 and 0.808, respectively; the correction curve shows that the model has good accuracy; and the DCA results show that when the risk threshold is between 0.10–0.85 (training set) and 0.10–0.75 (verification set), the clinical practical value of the model is higher.

Conclusion: The nomogram model constructed in this study is of high value in predicting the risk of DF in patients with T2DM and is of reference value for clinicians to identify people at high risk of DF and provide them with early diagnosis and individual prevention.

KEYWORDS

diabetic foot, nomogram model, prediction, risk factors, type 2 diabetes mellitus

Introduction

Over the past decades, the rapidly increasing prevalence and incidence of diabetes mellitus have become major public health issues worldwide. In 2015, it was estimated that around 415 million people are living with diabetes worldwide, according to the International Diabetes Federation, and this number continues to grow and has been projected to grow up to 642 million by 2040 at the same time, with a growth rate of 55% over the next 20 years (1). Epidemiological studies have shown that the incidence of diabetic foot (DF) (2) in diabetic patients ranges from 15% to 25% in their lifetime, and it is the leading cause of non-traumatic amputation (3). DF is a serious complication of diabetes, which is closely related to peripheral vascular, neuropathy, and increased mechanical pressure of the foot (4). If the patients with DF are not treated in time, the disease will progress, resulting in lower limb ulcers, gangrene, and osteomyelitis, which will lead to more serious adverse outcomes such as amputation or even death. The quality of life of type 2 diabetic patients with diabetic foot was significantly decreased, and their socio-economic burden was significantly increased (5). However, the early clinical manifestations of DF patients are not obvious, and there are some difficulties in diagnosis. In view of the possible serious harm of diabetic foot, we urgently need to put forward an effective evaluation method to predict the occurrence of DF in the early stages (6). Previous studies (7) have mainly focused on the risk factors for DF, but there is no intuitive and simple method to more accurately assess the risk of diabetic foot in patients with type 2 diabetes. As a forecasting tool, the nomogram model can show the corresponding relationship between a specific disease and risk factors through an intuitive graphical scoring system and accurately predict the risk of disease occurrence (8). Based on the above advantages, the purpose of this study is to build a nomogram model to predict the risk of DF in patients with T2DM, to guide clinicians in identifying people at high risk of DF, and to provide them with early diagnosis and individualized prevention.

Materials and methods

Study design and participants

To solve this clinical problem, we followed the methods of the article we published earlier (9) and improved them. We designed and implemented a retrospective study, and 705 patients with T2DM who were hospitalized in Wuhan Fourth Hospital and Zhongnan Hospital from January 2015 to December 2022 were included in this study at all. Some of the patient data came from the data collected in our previous study (9), but most of the population data for patients with diabetic foot and type 2 diabetes mellitus were derived from the collected data. There were 461 males with an average age of 55.70 ± 11.83 years and 244 females with an average age of 64.52 ± 11.39 years. With reference to the method of Jiang et al. (10), according to the principle of random sampling, the cases were divided into two groups: the training set (n = 494, including 84 T2DM patients with DF and 410 simple T2DM patients) and the verification set (n = 211, including 41 T2DM patients with DF and 170 simple T2DM patients). The ratio between them was 7:3. Criteria for selecting the participants were as follows: Inclusion criteria: (1) All patients met the diagnostic criteria for type 2 diabetes published by the 1999 WHO diagnostic criteria; (2) the age of patients was over 18 years; and (3) all patients gave informed consent to this study. Exclusion criteria: (1) Type 1 DM patients or secondary DM patients; (2) patients with severe hepatic and renal dysfunction; (3) diabetic patients during pregnancy and lactation; (4) patients with malignant tumors or acquired immune dysfunction; and (5) patients with a severe lack of case data. This study is in line with the Helsinki Declaration and has been approved by the Ethics Committee of Wuhan Fourth Hospital (KY2023-032-01).

Data collection

A clinical investigation case report form (CRF) we designed was used to collect the clinical data of the patients from the Hospital Information System (HIS) system of the hospital. We collected demographic data of the patients including, gender, age, BMI, diabetes course, smoking history, etc.; results of the first biochemical examination of fasting venous blood after admission, including fasting blood glucose (FBG), glycated hemoglobin (HbA1c), white blood cell (WBC), albumin (ALB), triglyceride (TG), blood uric acid (BUA), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and fibrinogen (FIB). Results of an ultrasonographic examination of atherosclerosis or plaque in the lower extremities of patients. We set up an epidata database to collect the required survey data according to the CRF, and following data input, a parallel check of all data by two people was performed to control the quality of the data.

Statistical analysis

Continuous variables with a normal distribution and non-normal distribution were expressed as "mean ± standard deviation (x ± s)" and "the median (lower quartile, upper quartile) [M (P25p75)]", respectively. The T-test was used for continuous variables following a normal distribution; otherwise, the Wilcoxon rank-sum test was used for continuous variables without a normal distribution. Categorical variables were expressed as percentage component ratios, and the Chi-square test was used for comparison between groups. Two-sided tests were used for all statistical data, and p-values <0.05 were considered statistically significant. Confidence intervals (CI) were all set at 95%. All statistical analysis was carried out using SPSS 26.0 software.

Establishment and verification of nomogram model

Firstly, the risk factors of T2DM complicated with DF were screened by univariate logistic regression analysis, and then the

selected risk factors were introduced into multivariate logistic regression analysis to determine the independent risk factors of DF in patients with T2DM. R4.2 software was used to establish a nomogram prediction model based on all independent risk factors for DF in patients with T2DM. The accuracy of the model was tested by using a calibration curve containing 2,000 bootstrap samples, and the nomogram prediction model was validated internally and externally. The area under the curve (AUC) of the receiver operating characteristic curve was used to estimate the performance of a nomogram model. Furthermore, decision curve analysis (DCA) quantifies the net benefit to patients with T2DM at different threshold probabilities, and the clinical application value of the nomogram was determined. All the statistical analyses were performed with R4.2 and the corresponding ROCR, rms, and ggDCA packages.

Results

Baseline clinical characteristics of participants

A total of 705 patients with T2DM were included in this study, with an average age of 57.72 ± 12.00 years old. Among them, there were 494 patients with T2DM in the training set, with an average age of 57.17 ± 11.62 years, of whom 66.19% were male, and 211 patients with T2DM in the verification set, with an average age of 59.01 ± 12.76 years, of whom males account for 63.51%. In the statistical analysis, there was a significant difference in serum BUA level between T2DM patients in the training set and verification set ($p < 0.05$); there was no significant difference in other clinical indexes between the two groups ($p > 0.05$), as shown in Table 1.

Univariate and multivariate logistic regression analysis

The variables with statistically significant differences in training sets were introduced into the univariate logistic regression analysis. The results showed that age, course of diabetes, history of smoking, lower extremity atherosclerosis, or plaque, HbA1c, WBC, and LDL-C were the risk factors of DF in patients with T2DM ($p < 0.05$), while BUA was the protective factor of DF in patients withT2DM ($p < 0.05$). LDL-C were the risk factors for DF in T2DM patients ($p < 0.05$), while BUA was a protective factor for DF in T2DM patients ($p < 0.05$). With DF in T2DM patients as the dependent variable, the above six risk factors (age, course of diabetes, history of smoking, lower extremity atherosclerosis or plaque, HbA1C, WBC, and LDL-C) were put into multiple factors logistic regression analysis. The inclusion and exclusion criteria for independent variables were 0.05. Forward stepwise regression was used to select the variables. The results showed that only five factors, including age (OR = 1.093, 95% CI 1.062–1.124, P <0.001), history of smoking (OR = 3.309, 95% CI 1.849–5.924, P <0.001), HbA1c (OR = 1.328, 95% CI 1.173–1.502, P <0.001), WBC (OR = 1.203, 95% CI 1.076–1.345, P = 0.001), and LDL-C (OR = 2.002, 95% CI 1.463–2.740, P <0.001), became independent risk factors for DF in patients with T2DM, as shown in Table 2.

Establishment of a nomogram prediction model

Based on the results of the multi-factor logistic regression analysis, five separate influence factors of DF in T2DM patients

TABLE 1 Differences in characteristics between the training set group and the verification set group.

Variables	Verification set (n = 211)	Training set (n = 494)	X^2/Z/T-value	P-value
Gender (male/female)	134/77 (63.51%)	327/167 (66.19%)	0.472	0.492
Age (years)	59.01 ± 12.76	57.17 ± 11.62	1.872	0.062
BMI (kg/m^2)	24.96 (22.46, 27.10)	24.78 (22.49, 27.06)	−0.156	0.876
Course of T2DM (years)	8.00 (3.00, 10.00)	7.00 (3.00, 10.00)	−1.172	0.241
History of smoking	65/146 (30.81%)	174/320 (35.22%)	1.287	0.257
atherosclerosis	85/125 (40.28%)	203/291 (41.09%)	0.023	0.879
HbA1c (%)	8.60 (7.10, 10.60)	8.25 (6.90, 10.00)	−1.673	0.094
FBG (mmol/L)	9.18 (6.94, 12.63)	9.30 (7.01, 12.30)	−0.093	0.926
WBC (10^9/L)	5.86 (4.59, 7.33)	6.35 (4.72, 7.97)	−1.468	0.142
ALB (g/L)	34.88 ± 5.48	35.79 ± 5.29	−2.080	0.038
BUA (mmol/L)	333.60 (256.80, 406.70)	332.40 (276.38, 398.95)	−0.641	0.521
TG (mmol/L)	1.44 (1.03, 2.46)	1.14 (0.88, 1.37)	−7.882	<0.001
HDL-C(mmol/L)	0.98 (0.84, 1.17)	0.98 (0.83, 1.17)	−0.247	0.784
LDL-C (mmol/L)	2.65 (2.06, 3.20)	2.65 (2.03, 3.24)	−0.148	0.883
FIB (mg/dl)	400.28 (354.56, 443.47)	394.37 (358.99, 443.19)	−0.029	0.977

TABLE 2 Independent risk factors of DF in patients with T2DM.

Variables	β	SE	Wald	P	OR (95% CI)
Age	0.089	0.014	38.198	<0.001	1.093 (1.062, 1.124)
History of smoking	1.197	0.297	16.224	<0.001	3.309 (1.849, 5.924)
HbA1c	0.283	0.063	20.262	<0.001	1.328 (1.173, 1.502)
WBC	0.185	0.057	10.593	0.001	1.203 (1.076, 1.345)
LDL-C	0.694	0.16	18.806	<0.001	2.002 (1.463, 2.740)

(age, smoking history, HbA1C, WBC, and LDL-C) were introduced into R4.2 software to establish a nomogram model for predicting DF risk in T2DM patients, as shown in Figure 1A. According to variables such as age, smoking history, HbA1c, WBC, and LDL-C, we can get different scores on the top score line (0–100 points); the total score is obtained by adding all the variable scores, and then the corresponding probability of DF in patients with T2DM can be found on the risk line; the higher the total score, patients with T2DM have a higher risk of developing DF. Here is an example to better understand the nomogram model: if the patient with T2DM is 70 years old and complicated by a smoking history, an HbA1c of 9.2%, a WBC of 5.21 * 10^9/L, and an LDL-C of 2.72 mmol/L, the probability of DF is estimated to be 42% (Figure 1B).

Verification of nomogram prediction model

The receiver operating characteristic (ROC) curve was used to evaluate the performance of the model in predicting the risk of DF in T2DM patients. The area under the curve (AUC) of the ROC was 0.827 in the training set (Figure 2A) and 0.808 in the verification set (Figure 2B), which indicated the model had good performance. A total of 2,000 bootstrapping samples were generated to replace the original samples, and the whole modeling process was repeated to obtain calibrated curves to verify the accuracy of the nomogram model. The calibration curve of the nomogram after correction illustrated that the model has good consistency between the predicted and observed values of the training set and the verification set, and the accuracy is high (Figures 3A, B). In addition, the DCA curve showed that when the risk threshold was between 0.10–0.85 (training set) and 0.10–0.75 (verification set) (Figures 4A, B), patients got higher clinical net benefits, indicating that the model has a wide range of safe application thresholds and high clinical practical value.

Discussion

With the rapid growth of diabetes worldwide and the trend of aging, diabetes and its complications are becoming the focus of global public health challenges (11). In the previous literature (12), 40% to 60% of non-traumatic amputations were caused by DF, and the hospital mortality rate of DF patients can be as high as 12% (13). In clinical practice, some patients are insensitive to pain because of neuropathy, and the decrease in arterial blood flow may make local skin redness and other inflammatory phenomena not obvious, which makes it difficult to make an early diagnosis of DF and delay the time of treatment. Once the patients are complicated with infection and necrosis, the disease progresses rapidly, and clinical treatment is extremely difficult, the risk of amputation and death of the patients will be greatly increased (6). Based on the above, it is particularly important to recognize and deal with DF early and promptly. We urgently need to put forward an evaluation method that can effectively predict the occurrence of DF.

According to the statistical results in the previous research, factors such as advanced age, long course of disease, poor control of blood glucose, neuropathy and vascular disease, infection, and so on will increase the risk of DF in patients with T2DM, even the risk of amputation and death (14); however, the relevant assessment tools that can effectively forecast the risk of DF in patients with T2DM have been rarely studied previously (15, 16). Therefore, it makes sense to produce a systematic, simple, and efficient clinical tool for predicting the risk of DF in patients with T2DM. The nomogram model can synthesize multiple risk factors and predict the probability of final events graphically, and it has been widely used in various fields (17). However, there is no nomogram model to predict the risk of DF in patients with T2DM. Therefore, according to the above research, we incorporate several related potential risk factors, explore the independent risk factors for DF in patients with T2DM, and construct a nomogram model to predict the risk of DF in patients with T2DM.

Our study showed that age, smoking history, HbA1c, WBC, and LDL-C were independent risk factors for the occurrence of DF, which was consistent with the results reported in previous literature (7). Age, as an independent risk factor for the occurrence of DF, has a significantly increased risk in elderly patients, which may be related to diabetes-related complications with age, such as peripheral neuropathy and vascular disease, chronic renal insufficiency, and so on (18). Previous studies have confirmed that smoking not only increases the risk of DF (19) but also has a great risk of recurrence (20). Similar results have been obtained in this study. Smoking significantly increases the risk of DF, which may be related to the death of nerve and vascular-related cells induced by oxidative stress, the decrease of tissue blood flow and oxygen supply, and the increase in chronic inflammation (21). As we all know, long-term exposure to hyperglycemia will damage vascular endothelial cells and basement membranes, hinder the

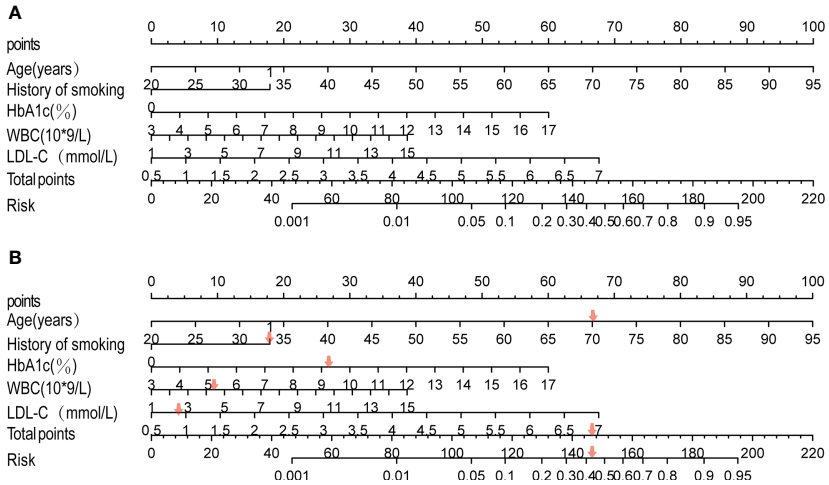


FIGURE 1
Development of the risk nomogram **(A)** and the dynamic nomogram for an example **(B)**. The nomogram to predict the risk of diabetic foot in patients with T2DM was developed with predictors including age, history of smoking, HbA1C, WBC, and LDL-C. Smoking history, 1; no smoking history, 0.

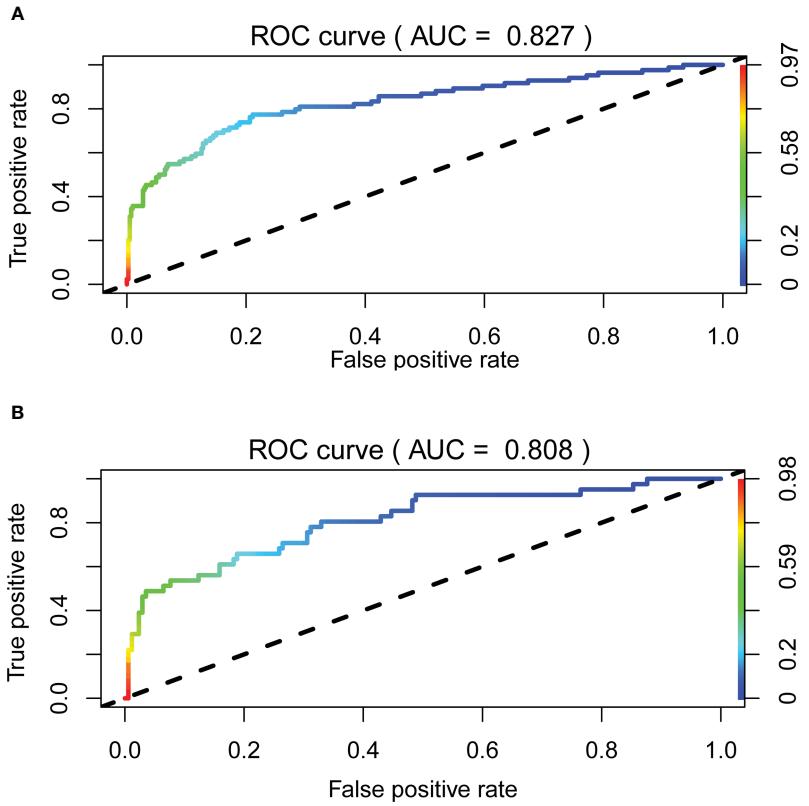


FIGURE 2
The accuracy of the nomogram for predicting DF using the ROC curve, the area under the ROC curve AUC represents the ability of the model to distinguish between DF and simple type 2 diabetes. When AUC = 0.5 said that the probability of the model distinguishing diabetic foot patients was 50%, it indicated that the probability of the model distinguishing diabetic foot patients was 100%. The closer the AUC was to 1, the stronger the distinguishing ability of the model was. The area under the ROC curve of the training set and verification set of the model (AUC) was 0.827 **(A)** and 0.808 **(B)**, respectively. It showed that the model has good distinguishing ability.

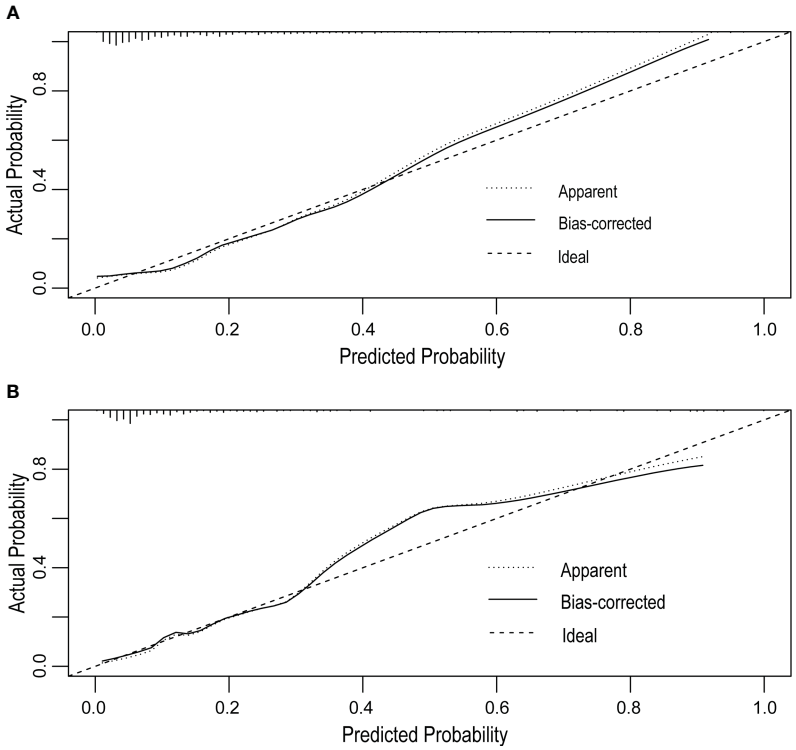


FIGURE 3
Calibration curves of the DF risk in nomogram prediction. The thick dotted line represents an ideal prediction, and the thin dotted line represents the predictive ability of the nomogram. The closer the thick dotted line fit is to the thin dotted line, the better the predictive accuracy of the nomogram is. The calibration curves of the training set **(A)** and the verification set **(B)** have good agreement between the prediction probability and the actual probability, and the average absolute errors are all less than 0.05.

blood supply of tissue microcirculation, and thus accelerate the occurrence and development of atherosclerosis and damage to the peripheral nerves of the foot under a hypercoagulable state, resulting in local ischemia of the foot (22). In this study, HbA1c was used to reflect the control level of blood glucose in patients within 2–3 months (23), which proved that hyperglycemia plays an important role in the risk of DF in patients with T2DM. Therefore, maintaining a stable blood glucose level in patients with T2DM is of great significance to prevent the occurrence of DF (24). In addition, as a sensitive indicator of acute inflammation, the level of WBC can reflect the occurrence and outcome of inflammation. The level of WBC in patients with DF is higher than that in patients with simple T2DM and is positively correlated with the degree of infection (25, 26). Therefore, WBC can be used as one of the indexes to predict the risk of DF in patients with T2DM. We also found that the level of LDL-C is an independent risk factor for DF in patients with T2DM, which may be due to the excessive production of LDL-C and the deposition of cholesterol on the walls of peripheral arteries to form atherosclerosis (27). As a result, the blood supply to the lower extremities and feet is insufficient, and the patient is in a state of ischemia and hypoxia for a long time, resulting in tissue damage and even necrosis.

Based on the results of univariate and multivariate logistic regression analysis in the training set, we successfully constructed a nomogram model to predict the risk of DF in T2DM patients and validated the model internally and externally by using the verification set data. The correction curve was accomplished by generating 2,000 bootstrapping samples to replace the original samples and repeating the entire modeling process. The results showed that the area under the curve (AUC) of ROC in the training set was 0.827 and the area under the curve (AUC) of ROC in the verification set was 0.808, which indicated that the model has a good degree of differentiation. Then the correction curve results showed that the prediction probability of the model in the training set and verification set was like that of the actual DF occurrence, which indicated that the prediction model was accurate. In addition, the DCA curve indicated that when the risk threshold was between 10% and 85%. The clinical net income of the patients was higher, indicating that the safe application threshold of the model was wide, the clinical practical value was high, and the data in the verification set have achieved similar results.

Previously, we successfully established a nomogram prediction model to predict the risk of amputation in patients with diabetic feet and applied it to clinical practice with remarkable results (9). But it is

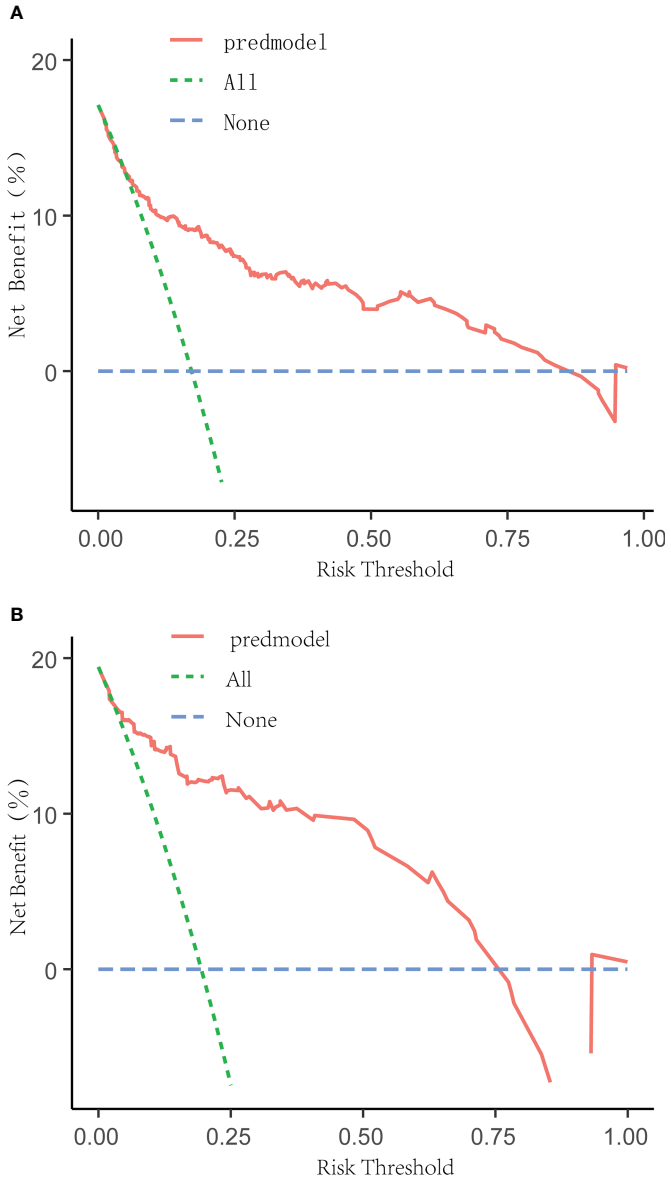


FIGURE 4
Decision curve analysis for the DF risk nomogram. The y-axis measured the net benefit. The green dotted line represented the assumption that all patients had DF. The blue dotted line represented the assumption that all patients had no DF. The solid red line represented the risk nomogram. The threshold ranges of the nomogram model in the training set **(A)** and verification set **(B)** are 0.10 to 0.85 and 0.10 to 0.75, respectively, indicating that the model has a wide range of safety and high clinical practical value.

well known that when diabetic patients progress to diabetic foot, their prognosis is often poor, and many patients are at risk of amputation or even death. Therefore, it is particularly important to identify diabetic foot patients early in their lives. However, the models for predicting diabetic foot are rare. Based on previous work (9), we established and validated the nomogram model for predicting diabetic foot in patients with type 2 diabetes. The model showed good results. This is of great significance for the early assessment of the occurrence of DF. This model provides a very effective means for the risk factor analysis and early diagnosis of diabetic foot.

As far as we know, this is one of only a few limited nomogram models with high accuracy to visually predict the risk of DF in patients with T2DM based on clinical indicators. However, there are still several limitations to our current study. First, the sample size is limited; second, insufficient diabetic foot risk factors were included in the model; although this nomogram has achieved satisfactory accuracy, there is still a lot of room for further prospective multi-center validation to confirm and improve the reliability of this nomogram and its clinical application; third, this study was designed retrospectively; due to limited data availability, not all clinical indexes were included in the study, and further improvement is needed to explore a more comprehensive and accurate reinforcement model. In conclusion, our study further confirmed that age, smoking history, HbA1c, WBC, and LDL-C

were independent risk factors affecting the occurrence of DF. Based on the above indicators, we constructed a nomogram model to predict the risk of DF in patients with T2DM. This model can effectively quantitatively evaluate the risk of DF in patients with T2DM, provides a simple and intuitive tool for clinical workers to identify people at high risk of DF, and has guiding significance for early diagnosis and individual prevention of DF patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

BP and RM contributed equally to the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The circadian rhythm: an influential soundtrack in the diabetes story

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Type 2 Diabetes Mellitus (T2DM) has been the main category of metabolic diseases in recent years due to changes in lifestyle and environmental conditions such as diet and physical activity. On the other hand, the circadian rhythm is one of the most significant biological pathways in humans and other mammals, which is affected by light, sleep, and human activity. However, this cycle is controlled via complicated cellular pathways with feedback loops. It is widely known that changes in the circadian rhythm can alter some metabolic pathways of body cells and could affect the treatment process, particularly for metabolic diseases like T2DM. The aim of this study is to explore the importance of the circadian rhythm in the occurrence of T2DM via reviewing the metabolic pathways involved, their relationship with the circadian rhythm from two perspectives, lifestyle and molecular pathways, and their effect on T2DM pathophysiology. These impacts have been demonstrated in a variety of studies and led to the development of approaches such as time-restricted feeding, chronotherapy (time-specific therapies), and circadian molecule stabilizers.

KEYWORDS

diabetes mellitus, circadian rhythm, chronotherapy, metabolic syndrome, sleep disorder, lifestyle

1 Introduction

According to the World Health Organization (WHO), diabetes mellitus (DM) is a metabolic disorder characterized by high level of blood glucose (1) that could damage most of the vital organs including the cardiovascular system (2), eyes (3), kidneys (4), and nervous system (5, 6). More than 90% of all instances of diabetes mellitus are type 2 diabetes mellitus (T2DM) (7), which is characterized by a combination of two different factors: inability of the pancreatic β-cells in producing sufficient amounts of insulin and lack of insulin adsorption by its targeted cells (8). Type 2 Diabetes Mellitus patients are normally obese people with accumulation of body fat, especially in the abdominal area (9). The worldwide changes in the lifestyle patterns including consumption of high-calorie foods (10), and the aging population (11) are the primary causes of the T2DM epidemic.

Fine-tuning the molecular pathways involved in the synthesis and release of insulin and their response in tissues is required to ensure that insulin would meet the body's metabolic demands (12). Therefore, the smallest metabolic imbalance in any of the relevant pathways could lead to the pathogenesis of T2DM (13). For instance, Adipokine dysregulation and increased free fatty acid release from adipose tissue are two of the inflammatory events that contribute to the development of insulin resistance in the whole body (14).

According to the epidemiological statistics, the predicted rates of T2DM are alarmingly high. The International Diabetes Federation estimates that by 2045, there will be 700 million people worldwide with diabetes (7). In 2019, 4.2 million deaths occurred among people with diabetes between the ages of 20-79 years old. Moreover, in the same year, more than $720 billion was paid for the treatments related to the diabetes, showing its extreme economic burden. The true burden of disease is likely underreported as one in three people with diabetes is underdiagnosed. Most patients with diabetes are middle-aged (40-59 years old) (15) and more than 80% of them live in low- to middle-income countries, which presents additional treatment challenges. Cardiovascular disease (CVD) is the main cause of mortality in T2DM patients compared to those without diabetes, while it could also lead to other vascular-related outcomes and stroke (2). Besides, diabetes can affect other organs such as pancreas (both β and α cells) (16), liver (17), muscle (18), kidneys (19), brain (20), gut (21), and adipose tissue (22). Furthermore, changes in gut microbiota (23), immunological diseases (24), and inflammation (25) are increasingly being recognized as important pathophysiological variables in the onset of metabolic syndrome (26).

Type 2 diabetes can result from the interaction of numerous inherited and environmental risk factors (27, 28). One of the interesting factors that has been proven to have a role in prevalence of T2DM is genetic and environmental changes in circadian rhythm. For example, T2DM is significantly more common in those who work in shifts, such as night and evening shifts (29). It has been demonstrated that days and/or weeks- long exposure to acute circadian disruption leads to changes in glucose metabolism as well (30).

2 Pathogenesis of T2DM

Pathogenesis of T2DM is fueled by inducing insulin resistance in skeletal muscle, liver, and fat so that insulin signaling is disturbed (31). Insulin resistance leads to a significant reduction in the glucose clearance, especially through the skeletal muscles that are the primary organ responsible for the postprandial glucose reduction (32). Muscle insulin resistance is due to a combination of factors, including but not limited to 1) insufficient insulin-mediated transport of glucose transporter type 4 (GLUT4) to the plasma membrane; 2) reduced glycogen accumulation; 3) decreased oxidation of glucose; and 4) altered the activity of mitochondria (32, 33). Fasting hepatic glucose production is elevated in an insulin resistance caused by impaired regulation of gluconeogenesis. In a hepatic insulin resistance state, livers produce more glucose after a meal since their ability to metabolize glucose is impaired (34). Finally, reducing the insulin-mediated glucose transport, lipid absorption, lipolysis, and inflammation in adipose tissue can lead to an increase in the plasma free fatty acids (FFAs) (35, 36).

Cellular insulin resistance is resulted from the accumulation of abnormal lipids in insulin-sensitive tissues like liver, muscles, and adipose tissue (36). Indeed, activation of insulin signaling cascade in muscle and liver cells is inhibited by the accumulation and trafficking of lipid messengers such as ceramides and diacylglycerols that are elevated in obesity (37). Besides, cumulation of intracellular diacylglycerols and ceramides could induce the inactivation of insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) and create insulin resistance (38, 39). Protein kinase C and other atypical serine/threonine kinases trigger this pathway (40). Poor adipocyte metabolism has been linked to both obesity and T2DM, leading to an increase in lipolysis and FFA levels and an elevation in the expression level of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) from activated adipose tissue macrophages (41–43). As a result, abnormal metabolism in adipose tissue of patients with T2DM is attributed to the excessive fat storage in target tissues via mediating alterations in the insulin signaling cascade in the skeletal muscle and liver.

Hyperglycemia cannot occur without the presence of both insulin resistance and pancreatic islet failure, both of which are characteristic of T2DM (44). Patients with T2DM who suffer from islet failure have impaired glucose-stimulated insulin production and uncontrolled glucagon release. Reduced β-cell populations and impaired β-cell secretory capacity contribute to the impaired glucose-stimulated insulin secretion (45). Increased apoptosis of β-cells along with the development of dedifferentiated α-cells have been linked to the β-cell mass reduction observed in type 2 diabetes (45). Moreover, exposure to islet amyloid peptide oligomers (IAPP), hyperlipidemia, and hyperglycemia is associated with the activation of intracellular oxidative and/or endoplasmic reticulum stress (46, 47). There is also limited evidence that changes in β-cells number and activity is the underlying reason of the defected glucagon suppression observed in diabetes; however, these possibilities need further investigation (48, 49).

2.1 Glucose homeostasis mechanism

Signals from organs such as pancreas, brain, gut, and liver exert considerable control on blood glucose homeostasis. The rise in blood glucose levels following a meal is the primary stimulus for insulin release from β-cells (50–52). The glucose transporter 2 (GLUT2) facilitative glucose transporter, found on the surface of β-cells, is responsible for glucose uptake from the circulation (53). During glycolysis, as glucose enters the cells and its level raises, the ATP/ADP ratio and ATP production increase (54). The increased ATP leads to the inhibition of ATP-sensitive K^+ channels (K_{ATP}), channels that are responsible for the constant resting potential in the absence of stimuli via pumping K^+ ions (55, 56). This leads to the reduced outward K^+ current, followed by membrane depolarization, and thus, opening the voltage-gated Ca^+ channels (VDCC) (57, 58). Insulin is secreted when granules containing the hormone fuse with the cell membrane, a process triggered by the increased intracellular calcium concentration.

The action of insulin on cells is mediated by its attachment to the extracellular subunits of insulin receptors on the cell surface; that leads to the phosphorylation of this receptor, the tyrosine receptor kinases (RTKs), followed by the activation of downstream pathways mediating the cellular effects (59, 60).

Another hormone emitted by the pancreas is called glucagon, which is released by alpha cells in the pancreatic islets when blood glucose levels drop. It counteracts with the insulin role in glucose regulation via increasing the production and secretion of glucose. Ketogenesis and lipolysis in the liver are both boosted by glucagon (51). The liver senses glucose levels in the portal vein and sends a signal to the arcuate nucleus (ARC) via vagal afferents, leading to smaller meals and increased postprandial satiety (61, 62). In addition to activating the G-protein-coupled receptors (GPCR) pathways in the ARC, glucagon has also the ability to pass through the blood-brain barrier (BBB) in animal models which suggests that it may directly act on the central nervous system to control hunger (63, 64).

Amylin, which is released together with insulin by pancreatic cells, inhibits the activity of orexigenic neurons in the anterior cingulate cortex to reduce hunger (65). Amylin inhibits gastric secretions and postprandial glucagon release through stimulating the region postrema in the medulla of the brainstem. Somatostatin, the hormone secreted from the pancreatic cells, has roles in regulation of appetite, digestion, and glucose metabolism via exocrine, endocrine, and brain pathways (66–68). It could suppress the expression of different hormones, from glucagon and insulin to gastrin, prolactin, secretin, and thyroid stimulating hormone (69), digestive enzymes, stomach acid, and bile produced by the digestive system (70–73).

The adrenal glands produce corticosteroids, which regulate many bodily functions. The release of this class of hormones follows a 24-hour cycle, controlled by the circadian clock. They can be broken down into two primary groups: mineralocorticoids and glucocorticoids (74). Glucocorticoids have anti-inflammatory effects in addition to regulating carbohydrate, protein, and lipid metabolism, whereas mineralocorticoids such as aldosterone manage the balances of fluid and electrolyte through modifying the renal tubules activity (74). Cortisol, the primary endogenous glucocorticoid in human physiology that is related to the stress, stimulates gluconeogenesis to raise blood glucose levels under both stress and hypoglycemia (75, 76).

Postprandially, cells in the digestive tract secrete a class of peptide hormones called incretins, which play a role in controlling both blood glucose and nutrient absorption. Gastric inhibitory peptide (GIP) and Glucagon-like peptide-1 (GLP-1) are two primary incretins that lower blood glucose via increasing the insulin release from pancreatic cells (77, 78). In addition to controlling the adsorption rate of nutrients into the body, incretins reduce the speed of stomach emptying. Dipeptidyl peptidase-4 (DPP-4) could inhibit the GLP-1 and GIP activity, so the inhibitors of DPP-4 along with several types of GLP-1 and GIP analogues have been applied in clinical therapy of T2DM; however, more research is needed to clarify the therapeutic use of these medications for T2DM (79) (Figure 1).

2.2 Circadian rhythm of glucose homeostasis: Diabetic vs healthy

It has been known for nearly half a century that normal human subjects exhibit a diurnal cycle in glucose tolerance. The rise in plasma glucose concentrations is much lower in the evening than in the morning after ingesting glucose orally or receiving an intravenous infusion of glucose. Mice and rats, like humans, have been shown to have diurnal rhythms (80). They have a lower glucose tolerance during the day (resting phase) than at night since they are nocturnal (active at night). The reasons behind impaired glucose tolerance include insulin resistance, high hepatic glucose production (HGP) from the liver, and impaired β-cell function. Research shows that insulin sensitivity and β-cell responsiveness to glucose are both lower in healthy adults after dinner than before breakfast. In people with normal glucose levels, the function of HGP in the diurnal regulation of glucose homeostasis is unclear. Researchers have found conflicting results regarding the diurnal variation in HGP: some have found a decrease in HGP associated with sleep and an increase in HGP at dawn, while others have found no diurnal rhythm in 24-hour fasting HGP or a decrease in HGP before breakfast compared to the lunch and dinner. One possible explanation for the divergent findings is that the participants in these trials had diverse eating habits on the day of testing. Patients with T2DM commonly experience the dawn phenomenon (hyperglycemia in the morning), which may be explained by the diurnal cycle of HGP. The increased HGP in the morning before breakfast has been linked to both a circadian modulation of HGP, as demonstrated by rodent research, and a prolonged overnight fast, which causes a rush of counterregulatory hormones (such as cortisol, growth hormones, and norepinephrine) (81–83).

Patients with T2DM frequently have fasting/morning hyperglycemia, which can be partially attributed to an increase in the overnight production of endogenous glucose. Endless laboratory

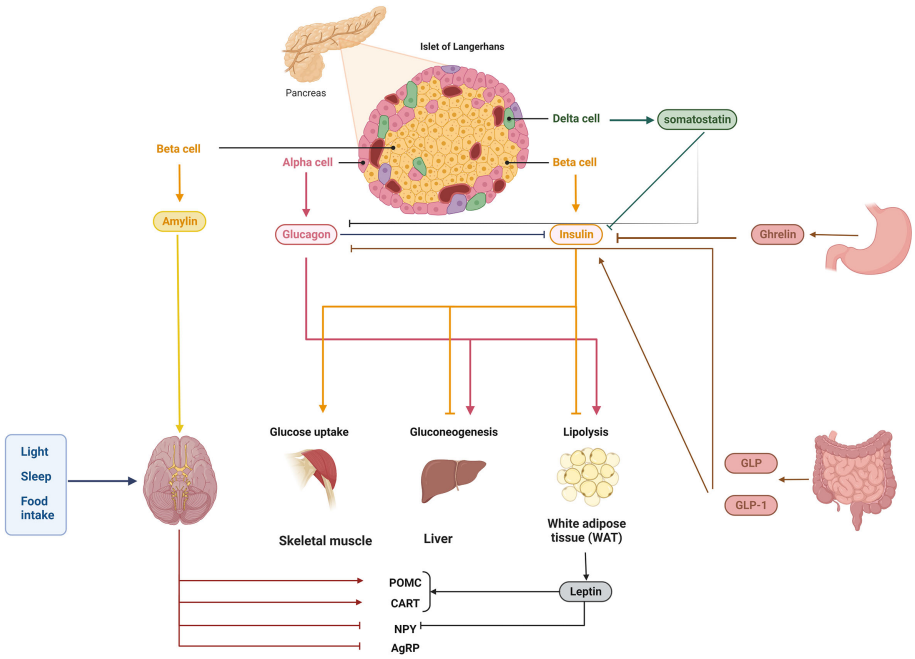


FIGURE 1
Homeostasis of blood glucose is controlled by a complex network of interactions. Multiple signals from the pancreas and further away from the brain, liver, muscle, intestine, stomach, and adipose tissue regulate blood glucose homeostasis. Glucagon, insulin (together with amylin), pancreatic polypeptide, and somatostatin are produced by cells in the pancreatic islets. Islet cells emit insulin in response to high glucose, such as from food intake; this insulin is detected by numerous peripheral tissues, which then synthesize or induce several molecules/pathways, such as lipogenesis and gluconeogenesis, and block others, such as glycogenolysis. Importantly, insulin secretion can be regulated both locally and at a distance, for example, by hormones and incretins in the colon and the stomach. Blood glucose homeostasis can be regulated by endogenous hormones like glucocorticoids, which also display rhythmicity, and by exogenous variables like light exposure and food consumption, which modulate circadian rhythms.

work has been prompted by the observation of the daily fluctuations of glucose regulation in an effort to pinpoint and characterize the underlying endogenous circadian rhythms of glucose regulation. With the need to better understand diabetes and its effects, more studies in differential glycemic control over the course of the day in people with and without diabetes is needed (84).

3 The circadian rhythm: an overview

Without any environmental cues, circadian rhythms are generated by an internal molecular clock that runs for about 24 hours. A "master" clock present in the suprachiasmatic nucleus (SCN) of the hypothalamus coordinates the molecular clocks in several peripheral tissues into a unified, hierarchical system. About 20,000 neurons make up the SCN to create a highly integrated circadian network (85). Light signals from the retina are processed by the master clock, which then relays the obtained information to the peripheral clocks through the endocrine and systemic cues (86, 87). The molecular architecture and the ability to generate sustained circadian rhythms are shared by the master and peripheral clocks situated in SCN neurons and across peripheral tissues, with the degree of intercellular connectivity between these clocks being the primary distinction.

The systemic changes (like body temperature), metabolic changes (for example by changes in exercise and food intake), changes in sleep pattern (e.g. shift work or jetlag) and the circulating hormones are the regulators of the phase of the SCN clock, while the phase of peripheral clocks can be adjusted independent of the SCN clock (85, 86, 88). This is related to the high degree of intercellular coupling of the SCN neurons, which create a neuronal network resistant to the phase perturbations of the internal cues. This networking logic makes sure that the master clock always stays on time by keeping its intrinsic 24-hour timing in sync with the solar cycle, while the peripheral clocks adjust to show the local metabolic status of the tissues in which they function (Figure 2) (85).

Pancreatic islet cell subtypes, skeletal myocytes, adipocytes, and hepatocytes are all examples of metabolically important peripheral tissues/cells that have self-sustaining and cell-autonomous circadian oscillators (89–93). Without consistent stimulation from the SCN, these cell-autonomous oscillators can keep cellular processes rhythmic over the course of a 24-hour day (94). However, the SCN clock appears to integrate a complicated multi-level hierarchical oscillator network by coordinating and synchronizing tissue circadian oscillators in response to neuronal, humoral, and behavioral inputs (95, 96).

It was previously believed that the SCN only used a single biological mechanism for the regulation of the phase entrainment and circadian physiology of the peripheral tissue oscillators; however, recent research suggests otherwise (97). The autonomic nervous system, specifically the parasympathetic and sympathetic

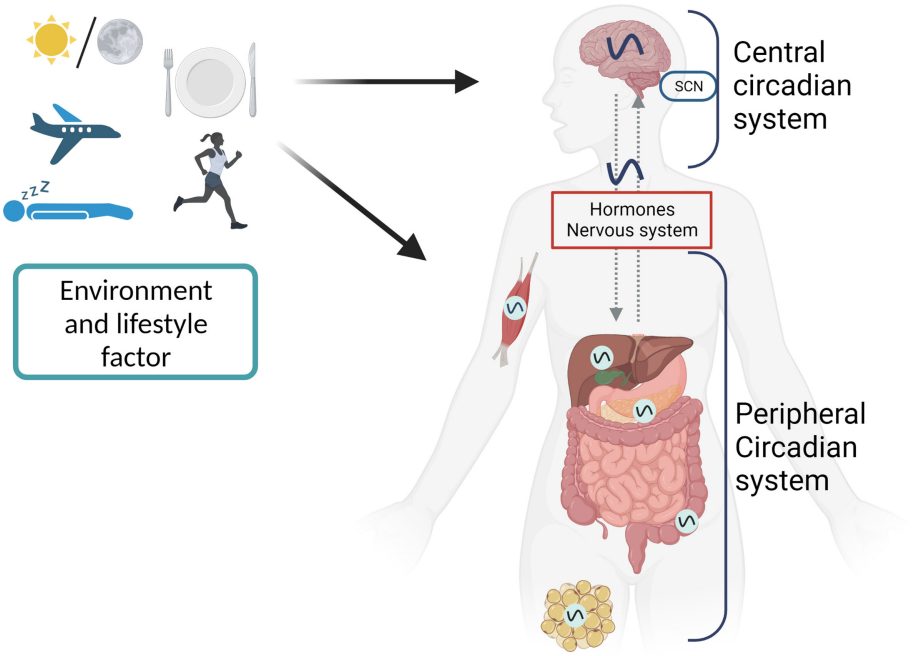


FIGURE 2
The first level of circadian disruption occurs when environmental cycles and/or behavioral cycles are out of sync with the SCN. Second, internal misalignment between the central clock and the peripheral clocks, generated by, for example, misaligned meals, can create circadian disruption at the organismal level. Another manifestation of this is an improper phase relationship between the peripheral clocks of various organs. Desynchronization between cells inside tissue can alter circadian rhythms on a cellular level.

axons, aids in SCN-dependent entrainment of circadian rhythms. Autonomic regulation of the hepatic circadian clock, which is responsible for the circadian rhythms in the production of hepatic glucose and ambient glycemia, is mediated by the sleep-wake circadian network (98, 99). The SCN plays a pivotal role in cell types that are vulnerable to daily dietary challenges by indirectly managing the entrainment of peripheral oscillators via modification of the fasting/feeding cycles. It is suspected that a combination of food-related metabolites and gut-related hormonal factors mediates the circadian entrainment of peripheral oscillators in response to meals, although the specific processes involved remain unknown (100). The SCN also controls circadian cycles in temperature and hormones (e.g., glucocorticoids) (101). In mammals it manages peripheral oscillator entrainment and uses redundant communication channels to fine-tune the temporal regulation of a wide variety of circadian rhythms.

3.1 Molecular pathway of the circadian rhythm

The cell-autonomous molecular clock of the mammals consists of two transcription/translation feedback loops (TTFL) that interact with each other and generate strong 24-hour rhythms for gene expression. The core of TTFL is composed of four integral clock proteins: two activators (CLOCK and BMAL1) and two repressors (Period circadian protein homolog;PER1 and PER2) (102, 103). Their location and stability are regulated by kinases and phosphatases (kinases: CKI, phosphatases: PP1, PP5). CLOCK: BMAL1 is a type of transcription factor with basic helix-loop-helix-PAS (PER-ARNT-SIM) structure that stimulates the expression of the *Per* and *Cry* (Cryptochrome) repressor genes and other genes responsible for the clock-controlled output (104). The heterodimer formed in the cytoplasm between PER and CRY proteins is transported to the nucleus, so that it could inhibit further transcriptional activity via interacting by CLOCK: BMAL1. After about 24 hours, PER and CRY proteins are destructed through a ubiquitin-dependent processes, releasing the regulation of CLOCK: BMAL1. Phosphatases (PP1 and PP5) are used for preventing and regulating the activity of casein kinases (CKI and CKI, respectively), which is a critical factor for controlling the clock intrinsic period (Figure 3) (105, 106).

Sleep phase issues are shared by mice and humans when either the intrinsic duration of the clock is reduced (S662G) or a loss-of-function mutation occurs in CKI (T44A). By altering the location and stability of PER, pharmacological inhibition of casein kinases was shown to have a significant role in period length determination. Transcriptional activators (retinoid-related orphan receptors RORa, b, and c) and repressors (REV-ERBa and REV-ERBb) are involved in generation of the second TTFL (107, 108). ROR/REV-TTFL-induced delay in the expression of *Cry1* is critical for the appropriate circadian timing; however, rhythmic changes in the abundance of BMAL1 are not necessary for driving the core loop of TTFL. Gene expression is appropriately timed for local physiology thanks to the cooperation and interlocking of feedback loops that induce phase delays in the output of the circadian transcription and make a robustness against the environmental disturbance and probable noises (109).

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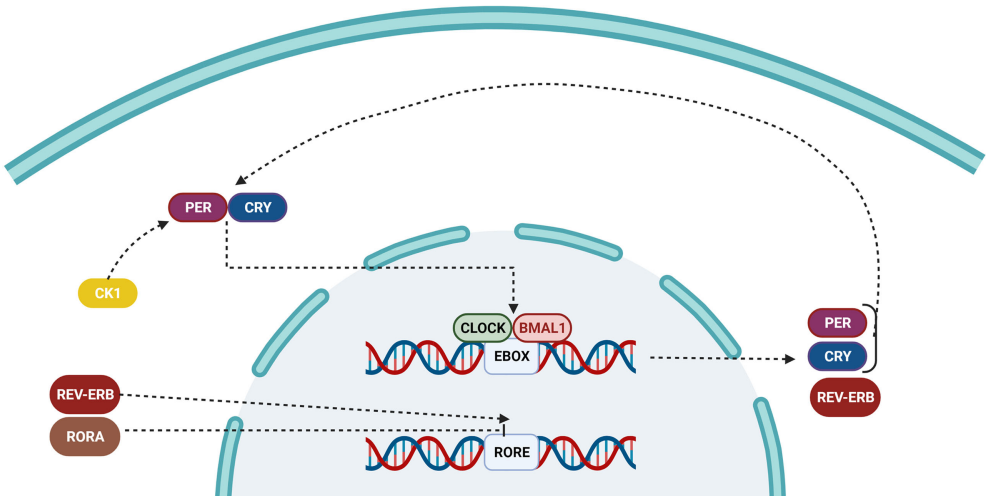


FIGURE 3
Pathway of the circadian clock inside a cell. Over time, the accumulation of PER and CRY proteins binds to CLOCK/BMAL1, and switches it from an active to an inactive state, preventing it from repressing the transcription of downstream genes. The ROR/REV-ERB complex controls the primary feedback loop by influencing RORE.

4 Diabetes and the circadian rhythm: a new frontier in diabetes research

4.1 Genetic signatures in the human genome

The metabolic risk of T2DM can be increased or decreased by specific single nucleotide polymorphisms (SNPs) in humans. Lipid metabolism can also cause SNPs in circadian genes (such as *PER3*), and on the other hand, the presence of SNPs in circadian genes (such as *CLOCK* and *CRY1* in intestinal cholesterol absorption) can play a role in fat metabolism and increase the chance of T2DM (110–114). Risk factors for developing metabolic syndrome (such as hypertension) are also associated to a SNP presented in the structure of neuronal PAS domain protein 2 (NPAS2), a paralog of CLOCK with the ability of binding to the BMAL1 (115). *Bmal1* SNPs are associated with high blood pressure in addition to T2DM, hyperglycemia, and gestational diabetes (116, 117). Patients with *Cry2* and *Per2* SNPs are prone to the glucose tolerance disorder, and individuals with *Per2* SNPs, who overeat and experience stress-related eating, tend to acquire more weight (115, 118, 119).

These SNPs could also affect the expression level of several other genes. For example, the sleep/wake cycles are aided by the secretion of more melatonin in the evening and less during the day. Elevated fasting glucose levels could impair cellular functions and increase the risk of T2DM in the presence of two SNPs in the melatonin receptor 1B gene (*Mtnr1b*) (120, 121). Melatonin secretion patterns could be affected by these SNPs and lead to an increased risk in diabetes. Accordingly, it seems that the risk of metabolic syndrome, obesity, and T2DM could be significantly influenced by SNPs in the molecular circadian clock and the genes regulated by those SNPs (122).

In a 2021 study, researchers showed a genetic network between the circadian cycle and T2DM (123). In this study, integrating gene expression patterns with the biomolecular networks of the genome-scale in diabetes samples allowed for a meta-analysis of data pertaining to genes involved in T2DM and different types of cancers (including bladder, breast, pancreatic, liver, colon, and rectum cancers). They found that both of these disorders shared a common deregulation of a number of genes. For example, although the expression level of *Arntl2* and *Agrp* were increased in T2DM and cancer samples, *Usp2*, *Ezh2*, *Igf1*, *Klf10*, and *Ntrk3* were downregulated (123). Phenotypic changes in T2DM appear to be linked with the malignant transformation, which may partially be related to the alterations in mRNA. Using these genes for survival analysis, they discovered associations between only *Arntl2*, *Usp2*, and *Igf1*. Survival was poor in BLCA and BRCA cancer samples where *Arntl2* was upregulated, while downregulation of *Usp2* and *Igf1* had a negative effect (123).

4.2 Peripheral circadian rhythm: a central role in diabetes

4.2.1 Pancreatic circadian rhythm

It has long been understood that individuals' ability to maintain their blood glucose levels stable throughout the day is varied with the time. Indeed, improved glucose tolerance is related to higher effects of glucose and β-cell targeting secretagogues on insulin secretion, which is first observed at the start of the active circadian cycle (124, 125). Classic experiments using a 72h glucose clamp methodology corroborated these first data, showing that humans exhibit strong circadian rhythms of insulin secretion independent of feeding and glycemia (29). Moreover, the cell-autonomous circadian clock appears to oversee the diurnal rhythms in the glucose-induced insulin secretion. These diurnal rhythms are maintained in the isolated pancreatic islets and pure β-cell populations in human, animal, or *in vitro* models (29, 126).

Besides, circadian fluctuations have also been seen in the glucagon production *in vivo* and *in vitro* (islet cultures and isolated α-cells). However, our knowledge of the circadian regulation of the α-cell glucagon response is considerably limited (127).

Many research groups have established the effects and roles of the autonomous circadian clock (e.g., Per1 and Per2) in pancreatic islets (127–130). The results of these studies demonstrated that clock gene expression in islet cells exhibits robust independent circadian rhythms with a clear 24h period, phase, and amplitude of circadian oscillations. Moreover, they showed that SCN entrains the circadian clocks phases of the islets via modulating the feeding behavior. It was also seen that shifts in photoperiod and/or obesogenic meals can jumble the islet circadian clock (131). Besides, the pure populations of α- and β-cells exhibit robust cell-autonomous circadian oscillators, with discrete phases in transcriptional and functional oscillations (89, 132).

Similar to other metabolically active cells such as skeletal muscle and liver, the transcriptional profile of islet cells is subject to circadian oscillations regulated by the cellular clocks. Around one-third of the transcripts in mouse islets show circadian cycles, which is consistent with previous findings. The biological pathways involved in the secretion and exocytosis of insulin (including SNARE interactions, vesicular transport, and exocytosis), mitochondrial function, processing and transporting of protein in endoplasmic reticulum, and cell turnover regulation, are enriched in circadian-expressed transcripts in islet cells (such as DNA damage and repair and DNA replication) (133, 134). Recent research has shown that each of the distinct islet cell subtypes have their own circadian transcripts, with expression peak during the feeding or fasting (activating or inactivating) of the circadian cycle, which corresponds to the glucagon and insulin release peak (130, 135).

4.2.2 Liver circadian rhythms

Liver plays a critical role in orchestrating the glucose homeostasis's circadian regulation throughout the day. It is the diurnal change in the amount of hepatic glucose synthesis that regulates the circadian variations in blood glucose under fasting conditions (136). Because of this, circadian rhythms in the concentrations of blood glucose are lost if the circadian clock of SCN (or liver-specific) is disrupted (137). The liver also acts in the circadian modulation of postprandial glucose metabolism since the circadian changes in the amounts of glucose production by the liver could affect the diurnal cycles of meal tolerance and insulin sensitivity. In addition, both animal and human models have shown that the rates of gluconeogenesis and glycogenolysis, as well as glycogen storage strongly fluctuate during the day (29, 138).

Hepatocytes exhibit specific diurnal oscillations in gene expression and morphological properties such as volume and size of cells (139–141). Hepatocytes display patterns of circadian expression for a number of metabolic genes, including *Glut2*, *Gck*, *Pepck*, and *Gcgr*. These genes are critical for regulating hepatic glucose production, and lipid oxidation in hepatic cells, respectively. Both nuclear and cytoplasmic proteome of hepatocytes undergoes significant diurnal variation associated with rhythm in cellular metabolism, proliferation, DNA repair, ribosome synthesis, and intracellular protein trafficking. Multiple diurnal rhythms in liver function, as well as the corresponding of diurnal oscillations in the transcriptome and proteome of the liver, appear to be primarily regulated by fasting-feeding cycles. Consequently, while assessing the mechanisms regulating daily circadian rhythm in the liver, it is important to distinguish between clock-dependent and clock-independent factors (such as feeding-driven factors) (142). Besides it as important to add that feeding may be a stronger cue for hepatic circadian rhythm than light-dark cycle as feeding restricted to resting phase inverts metabolic rhythm of liver (143, 144).

Scientists have uncovered some of the processes and targeted molecules related to the clock-controlled transcriptional regulation of hepatocytes by utilizing chromatin immunoprecipitation (ChIP-Seq) and deep sequencing. For instance, Rey and coworkers mapped almost 2,000 BMAL1 binding sites across the entire genome in the liver (145). Additional evidence have shown that hepatic clocks are critical mediators of glucose homeostasis since most of the binding sites were located/mapped in promoter areas of genes related to the metabolism of hepatic glucose (like *Glut2* and *G6pc*) and lipid (such as *Agpat6* and *Dgat2*). This finding is supported by the *in vivo* test observation that showed mice with a liver-specific deletion of *Bmal1* exhibit systemic glycemia and glucose intolerance due to the absence of rhythmic regulation of glucose metabolism related genes (e.g *Glut2*) (146, 147). Newer studies built on these observations, demonstrating that hepatic *Bmal1* is essential for controlling hepatic oxidative capacity through orchestrating intracellular mitochondrial dynamics such as mytophagy and fission (146, 148).

Through the activation of different transcriptional and translational regulators, hepatic clocks coordinate the cellular metabolism regulation via an "indirect" method. By performing a ChIP-Seq analysis on binding sites of BMAL1 of the liver, they were able to show that "transcriptional control" was the most significantly enriched annotated functional cluster, included about 82 DNA transcription factors and 18 liver-expressed nuclear receptors. In lipid and cholesterol metabolism, hepatic clocks activate Kruppel-like 10 (*Klf10*) and the nuclear receptor *Ppar* (peroxisome proliferator-activated receptor) (149–151). The fact that both *Klf10* and *Ppar* regulate Bmal1 promoter activity demonstrates the intricate relationship between the circadian clock and metabolism of liver (149, 150). Finally, research have shown that proteins involved in the circadian clock (such as CRY 1 and 2, and PER2) attune the regulation of circadian over hepatic glucose metabolism via modulating the signaling of cAMP and the post-translational function of nuclear receptor. Diabetic mice with overexpression of *Cry1* in the liver had reduced gluconeogenesis and blood glucose levels. Researchers convincingly showed that *Cry1* expression during the circadian clock inhibits the key enzymes of gluconeogenic activation through binding and blocking the Gs subunit of hepatic glucagon receptor (152, 153). Growing documents indicate that hepatic clocks play a critical role in the regulation of glucose production and uptake and insulin sensitivity in T2DM.

4.2.3 Muscle circadian rhythms

It was revealed by *in vivo* and *in vitro* studies skeletal myocytes contain functional, self-sustaining, and independently oscillating circadian rhythm. It is important to note that circadian fluctuations occur for almost every physiological factor involved in glucose homeostasis in skeletal muscle (92, 154, 155). Changes in protein and lipid metabolism, as well as glucose oxidation and uptake, deposition of glycogen, the oxidative capability of mitochondria, and expression and translocation of the GLUT4, occur in skeletal muscle on a daily basis (156–158). Consistent with its basic function, the circadian system increases metabolic flexibility, as evidenced by the fact that the maximum physiological activity of skeletal muscle in uptake and oxidation of glucose is coincided with the commencement of circadian cycles in both human and animal (mouse) tests. In accordance with the transcriptional control by the endogenous circadian clock mechanism, circadian rhythms in skeletal muscle activities (such as glucose absorption) are maintained in isolated muscle cells (158). In addition, T2DM is linked to alterations in the circadian modulation of glucose uptake and oxidation in muscle, suggesting that the disruption of the circadian clock is a factor that could affect the pathogenesis of insulin resistance in skeletal muscle of patients with diabetes (159, 160).

The strongest evidence that confirmed the critical role of biological clock in regulating the function of skeletal muscle is provided by the studies done on *Bmal1* knockout mice models. The loss of *Bmal1* is unique among clock genes because it eliminates circadian rhythms in behavior, physiology, and molecular processes. Notably, muscle-specific transgenic rescue can correct the profound decrease in the mass, function, and mitochondrial density of skeletal muscle resulted from a systemic loss of *Bmal1* (161). *Bmal1* knockout mice models have been established to clarify the function of this gene in the regulation of glucose metabolism in skeletal muscle. It is interesting to note that Bmal1 knockouts generally show a significant reduction in glucose and insulin tolerance, resulting from reduced insulin-stimulated glucose uptake, which is a major factor in T2DM (162, 163).

Disturbing the glucose uptake related to the *Bmal1* deletion could also reduce the expression level of TBC1D1, a protein essential for GLUT4 translocation from the cytoplasm to the plasma membrane (164, 165). Notably, reduction in the amounts of glucose transport documented in patients with T2DM also affects skeletal muscle. Deletion of *Bmal1* in muscle could change the cellular metabolism toward increasing the lipid usage and storage, and reduce the expression and activity of essential metabolic enzymes that are responsible in the breakdown and oxidation of glucose (164). Again, this is consistent with the phenotypic alterations in T2DM patients' skeletal muscles. Extensive experimental data suggest that impaired insulin-mediated glucose transport, absorption, storage, and oxidation in skeletal muscle contribute to the onset and progression of T2DM (166).

4.2.4 Adipocyte circadian rhythm

In addition to act as an energy source, white adipose tissue (WAT) has metabolic and endocrine functions including blood glucose level regulation. The body utilizes the triglycerides (TGs) stored in WAT for energy through a process called lipolysis, in which the TGs are broken down into FFAs and glycerol (167). Lipolysis and lipogenesis, as two main paths of fat metabolism in the body, play a very important role in glucose homeostasis, since the excess fat are accumulated in different tissues especially liver and muscles, and cause insulin resistance and interfere with glucose homeostasis (168). Human plasma-free fatty acid concentrations have been shown to vary throughout the day, and this diurnal variation has been believed to be caused by the cyclic changes of the insulin signaling cascade activation in adipose tissue (169).

The circadian clock has been demonstrated to govern adipose tissue functions such as lipolysis, adipogenesis, and metabolic inflammation in mammals because of the dramatic daily variations in energy needs (170, 171). Obesity is reliably induced in Clock and/or Bmal1 mutant mice, likely as a result of elevated levels of plasma FFAs and triglycerides. Studies in rodents and humans have also uncovered the importance of circadian expression for many of the genes that are critical for the appropriate regulation of metabolism of adipocyte in different adipose tissue depots, suggesting that circadian clocks play a role in regulating adipocyte metabolism (91, 172). As an illustration, one study demonstrated that the heterodimer of BMAL1 and CLOCK had a transcriptional regulation on the activity of two important lipolysis pacemaker enzymes that lead to the clock-regulated release of FFA and glycerol (173). Adipocytes had a reduced ability to produce polyunsaturated fatty acids in Bmal1-deficient animals. This could be due to decreased expression of Elovl6 and Scd1, two critical genes involved in the elongation and desaturation of FFA (173, 174).

Adipose tissue is known as the energy stores as well as one of the largest endocrine organs due to its seemingly endless capacity to expand and produce a wide range of hormones and cytokines. Leptin, adiponectin, resistin, visfatin, omentin, and inflammatory cytokines (such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1)) are just some of the known hormones that are released by adipocytes in both obesity and diabetes (175, 176). Macrophages found in adipose tissue are one of the best-known contributors in the insulin resistance induced by obesity. Adipose tissue macrophages (ATMs) have been demonstrated to convert from anti-inflammatory M2 phase to pro-inflammatory M1 type in diabetes conditions, and release the pro-inflammatory cytokines such as TNF-α, IL-6) and IL-12 (43, 177, 178). Although the precise role of the circadian clock in regulating inflammation in adipose tissue remains unclear, there is mounting evidence that suggests disruptions in these pathways can trigger inflammatory reactions (179). For instance, the expression level of TNF-α and macrophage-1 antigen (MAC1) in WAT was increased in obese mice with a disrupted circadian clock (177, 178, 180). Macrophages with a disturbed circadian clock (*Per2* mutant) showed higher M1 macrophage polarization in WAT and exhibited heightened pro-inflammatory activation in response to LPS. Downregulation of PPAR, an important clock-regulated transcription factor used for the polarization of M2 macrophage, in macrophages replicated the same results in obese mice with *Per1/2*-disrupted myeloid cells (41). Overwhelming evidence suggests that the circadian clock could

regulate the biology of adipose tissue from multiple aspects (such as lipogenesis, lipolysis, insulin sensitivity, inflammatory pathways, etc.) so that the disruption of this clock could lead to an increase in the adiposity, insulin resistance, and susceptibility to the development of T2DM (176, 181).

4.3 Environmental and epidemiological factors

4.3.1 Light and circadian rhythm

Constant light exposure decreased the domain (the difference between the highest and lowest points) of the SCN rhythmicity in mice, as measured by electrophysiological monitoring. As a result, food consumption rose while energy expenditure fell (87, 182). In a study in mouse models, the effect of the light cycle on fat and glucose metabolism was investigated. Results demonstrated the effect of light on obesity and development of diabetes. Body weight gain, adipocyte area, glucose intolerance, and insulin resistance were all augmented in low-fat diet (LFD) and high-fat diet (HFD) mice exposed to the circadian-disrupting (CD) cycle under higher intensity (HI) but not lower intensities (LI) (183). In addition, LFD-HI therapy was associated with an increase in blood and hepatic triglyceride levels independent of the light cycle. Another benefit of CD cycle was reflected in the improvement of glucose and lipid metabolism in HFD-LI. Moreover, the deleterious effects of CD cycle on the metabolism were mitigated under the LI condition, particularly in HFD mice (183) (Figure 4).

4.3.2 Food intake and circadian rhythm

The hypothalamus, adipose, and hepatic clocks are all affected by a high-fat diet in animal models. Circadian rhythms are also affected by the timing of meals. The postprandial glucose response to a meal may be affected by the time of day, which would have a profound impact on T2DM (184). Earlier mealtime consumption has been linked to lower levels of postprandial glucose in both observational and experimental investigations. Increasing the protein and fat content of evening meals has been found to be a straightforward method for lowering postprandial glucose levels (184). Foods with a low glycemic index (GI) have a negative impact on the glycemic response if they were taken in the morning than later (185). The glycaemic response can be lowered by carefully timing the consumption of fat and protein with carbohydrate items like bread and rice. There is interesting evidence that changing dietary habits such as time and number of meals, the interval between meals, and the amount of each meal can influence postmeal glucose levels and metabolism. Instead of solely considering the dietary content of a meal, people with diabetes can benefit from considering these recommendations to improve their glycaemic management (185).

The majority of food consumed by mice maintained on a 12h light/12h dark cycle (12:12h LD) is consumed during the dark phase. However, in a study that was conducted to determine the effects of the time of feeding on weight gain, it was concluded that mice that were only fed during the light phase acquired more weight compared to those that were only fed during the dark phase (186). In another experiment, the effect of time-restricted feeding (TRF)

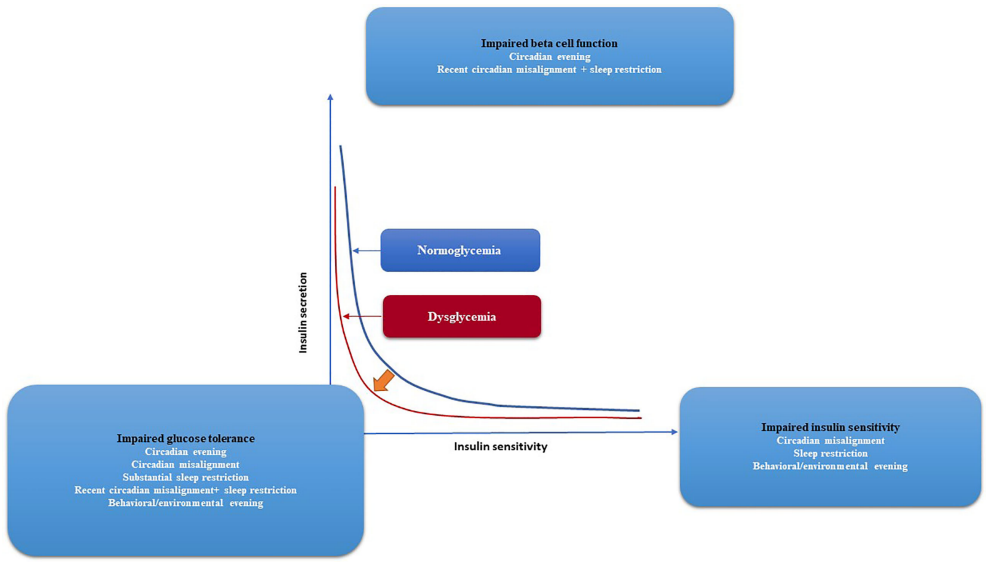


FIGURE 4
Glycemic control and the risk of developing diabetes, together with the effects of circadian and behavioral factors are depicted graphically. The two hyperbolae for normoglycaemia (blue line) and dysglycaemia (red line) illustrate the correlation between insulin secretion and insulin sensitivity (red line). Lower insulin sensitivity requires more insulin secretion for normal blood glocuse levels. Impaired glucose tolerance arises if insulin secretion is unable to compensate for diminished insulin sensitivity, as shown by a leftward shift of the curve. Circadian disarray, sleep restriction, and, to a lesser extent, behavioral/environmental lateness all have negative effects on insulin sensitivity, according to experiments. Both recent circadian misalignment and nocturnal awakening have negative effects on beta cell function. Evidence suggests that a number of factors—including behavioral/environmental evening, circadian misalignment, significant sleep restriction, recent circadian misalignment with sleep restriction, and severe sleep restriction—combine to reduce glucose tolerance. The progression from normoglycemia to dysglycemia and eventually diabetes may be influenced by circadian and behavioral factors that affect glycaemic regulation.

on preventing obesity and metabolic syndrome was investigated in the liver of *Rev-erb* α/β and *Bmal1* knockout mice and all organs of Cry1;Cry2 knockout mice (187). When these mice were given free access to food, they quickly became obese and displayed metabolic abnormalities that were determined by their genetic makeup. However, when they were fed with the same diet but with TRF, they were in a healthy weight range with no metabolic disorders. It was discovered through transcriptome and metabolome analysis that TRF lowered hepatic fat buildup and improved cellular resistance to metabolic stress (187).

Same as what was seen in animal studies, the timing of meals has been proven to affect the risk of diabetes development in humans. A study in 2018 has shown that there is a direct link between eating dinner late and gene MTNR1B (melatonin receptor 1B), which affects glucose metabolism, increased body fat and weight gain (188). Previously, the relationship of this gene with type 2 diabetes has been proven (189). Since exogenous melatonin has also been demonstrated to cause decreased glucose tolerance, the suggested role of melatonin in this pathway is in line with that discovery (190). Time-restricted eating (TRE), eating within a specific daily window, has been shown to improve the metabolic indices such as atherogenic lipids, weight, visceral fat, and blood pressure in people with metabolic syndrome. In 2019, a randomized controlled clinical trial was conducted to evaluate the association of 9-hour TRF with diabetes risk in men. In this study, 15 men were randomly divided into two early (TRFe) or delayed (TRFd) groups, and during and before (to achieve baseline glucose) the study, the volunteers' blood glucose and insulin were measured. The level of blood lipids and steroid hormones were measured in all volunteers. The results showed that TRF can improve glycemic responses regardless of whether it is early or delayed (191). The benefits of TRE were tested and reported by a small sample size (n=15–20) and a larger clinical study (192, 193).

4.3.3 Work shift and circadian rhythm

Shift work, and circadian misalignment more generally, are associated with the increased risk of metabolic syndrome, obesity, and T2DM. A real-world investigation comparing day-shift and night-shift workers supports the hypothesis that circadian misalignment is a risk factor for metabolic disorders (134). Results showed that compared to day-shift workers, night-shift workers had higher levels of glucose, insulin, and triacylglycerol after eating a meal. A meta-analysis of 12 observational studies reported that shift work is associated with an increased risk of T2DM (9%), compared to those who have never worked shifts (194). It's important to note that workers whose shifts alternate throughout the week are at a greater risk than those whose schedules remain stable. This is probably because both food intake and light exposure are an inappropriate time when the body is not prepared for them. Also, similar to the human results, rats subjected by a simulated shift-work procedure (using rotating running wheels) became obese and experienced a flattening of their glucose rhythms. Accelerated cell dysfunction and loss along with impaired GSIS were observed in rats whose 12:12h LD cycles were disrupted by continuous light exposure (131, 195). Research on *Bmal1* knockout mice has revealed the critical function of *Bmal1* in mitigating oxidative stress and adjusting to circadian rhythm disturbances. Therefore, mice with a dysfunctional version of this gene are more likely to develop diabetes (196).

The timing of food consumption, light exposure, and nutrient content have all been recognized as important factors used for regulating the metabolic clock in both rodents and humans. These results suggest that time-specific therapy (chronotherapy) and other strategies that target the circadian system, like the analogs of synthetic circadian protein, could be useful for the management of metabolic syndrome and T2DM.

5 Chronotherapy; new weapon in a war against diabetes

Chronotherapy consists of maintaining an optimized circadian rhythm and regulating the timing of drugs to achieve more efficacy and fewer side effects (197). An example of medication timing is prescribing statins to be used at night. The reason is that statins target 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase. The activity of this enzyme is highest at night and during sleep; therefore, targeting it at night with statins can be more effective (198).

A dopamine agonist, bromocriptine, is an adjunctive therapy for T2DM. Following a 24h cycle, hypothalamic dopaminergic activity drives hepatic gluconeogenesis and adipocyte lipolysis (199). When taken within 2h of awakening, bromocriptine is expected to inhibit this drive, helping to prevent hyperglycemia and dyslipidemia (199). Despite FDA approval for the treatment of T2DM and evidence of the drug's effectiveness in lowering blood glucose levels, bromocriptine is rarely used in actual clinical practice (199). In a study in 2021, results showed that in addition to greater effectiveness, the reduction of complications, especially cardiovascular complications, can be seen in the treatment with bromocriptine based on the circadian rhythm; however, with all this evidence, further preclinical and clinical studies are still needed to identify the exact relationship between circadian rhythm and bromocriptine (200, 201).

Despite laboratory evidence that showed the time-dependent effects of metformin on blood glucose and its interaction with the circadian rhythm molecular components, no clinical trials have looked at the optimal time of day to take this drug (202, 203). Scientists found that the time of day significantly affected the initial drop in blood glucose and the levels of lactate in the blood of healthy mice after metformin treatment (204). They further showed that circadian time has significant effects on the activation of AMP-activated protein kinase (AMPK) in the liver via affecting the kinetics of metformin. Loss of *Bmal1* expression in the liver modifies the AMPK induction and blood glucose response to metformin, while can't completely eliminate the diurnal variations. Combined, these findings highlight the complexity with which circadian rhythms influence physiologic responses to metformin (204). In addition to the effect of the circadian cycle on metformin, it has also had very significant effects on modifying the

circadian cycle of different tissues (such as liver and muscles) in diabetes (205, 206).

The endocrine cells of pineal glands secrete a hormone called melatonin, which was the first circadian-based medication to be studied for its potential to treat diabetes (207, 208). Since its production and secretion are governed by direct neural inputs from the SCN, it has been argued that the rhythmic secretion of melatonin is a hormonal response to the central circadian clock (209). Indeed, the SCN and peripheral circadian oscillators are directly affected by exogenous melatonin treatment via the ubiquitously expressed melatonin receptors. Thus, it has been proven that supplementing with melatonin at the proper time improves the global circadian rhythms in both mice and humans (209, 210). By improving the insulin signaling cascade of the cells, melatonin treatment in animals has been found to reduce obesity and attenuate insulin resistance of the liver and skeletal muscle. In isolated T2DM islets, it was able to enhance the survival of β-cells and improve the insulin secretion (stimulated by glucose); therefore, reduce the oxidative and endoplasmic reticulum stress (211–213). Although many studies have pointed out the potential therapeutic effects of melatonin in the control and treatment of diabetes, there is still a need to conduct targeted clinical studies for determining the effectiveness of this drug in T2DM treatment (214–217). In another study, the effectiveness of melatonin and metformin at the same time in the treatment of diabetes was discussed in rat that had become diabetic due to circadian rhythm disorder and obesity (218). As a stand-alone therapy, melatonin improved the activity of circadian rhythms, suppressed the induction of β-cell failure, and enhanced the tolerance of glucose. On the other hand, metformin improved the sensitivity of insulin and glucose tolerance without affecting circadian rhythms; however, it was taken to help with sleep issues (218). Importantly, melatonin and metformin demonstrated synergistic activities to ameliorate obesity, insulin sensitivity, circadian activity, and suppression of islet cell failure in CDO rats, and so slowing the onset of metabolic dysfunction. Based on the results of this research, it seems reasonable to consider treating metabolic and circadian dysfunctions together as a means of preventing and treating obesity and T2DM (218).

More research on small-molecule pharmacological enhancers of the circadian system has been conducted in recent years to improve the clock-regulated physiological outputs. Subsequently, several pharmacological agents have been created with established efficacy in modifying metabolic function in pre-clinical investigations, all of which focus on the core circadian oscillator (219, 220). Initially, thiazolidinedione, a medication used to treat diabetes, was identified as the first synthetic agonist for ROR (Retinoic Acid-Related Orphan Receptors) and the only medication available for the treatment of insulin resistance (221, 222). Nobiletin, another ROR inhibitor and a naturally occurring flavonoid has been discovered to increase clock amplitude through activation of the ROR nuclear receptor (223). Specifically, administration of Nobiletin for an extended periods of time improved the glycemia, glucose tolerance, insulin resistance, and ectopic fat formation in two different mouse models of obesity and T2DM (224, 225). Rev-ERB agonist, a recently found pharmacological regulator of the circadian system was shown to have the capability of modifying the gene expression of circadian metabolic and ameliorate glycemia, lipidemia, and ectopic fat deposition in mice (226). One of these compounds is berberine, which intervenes in the circadian cycle through three pathways; 1) reducing the activity of the luciferase reporters Nlrp3 and Bmal1, 2) increasing the repressor activity of REV-ERB, and 3) reducing the expression levels of REV-ERB target genes (227). In another clinical trial about the effect of berberine in the treatment of diabetes, the results provided evidence that berberine is a safe and effective therapeutic agent for diabetes, particularly when used in combination with other medications. This may be useful in the future for directing the clinical application of berberine and the development of drugs to treat type 2 diabetes and dyslipidemia (228).

Cell-based phenotypic screening for drugs that modulated circadian rhythmicity led to the discovery of the first CRY agonist (KL001) (229, 230). The mechanism of action of KL001 involves blocking the interaction of the ubiquitin ligases that bind to the main binding pocket of CRY and tag them for destruction. In order to extend the repressive phase of the clock, CRY's lifetime can be extended by binding tiny molecules in the binding pocket (229). Improvements in glucose tolerance in the db/db mice model of diabetes have been observed *in vivo* with compounds that were discovered through *in silico* drug design research began with KL001 (231).

6 In-silico approach: systematic study in biology and pharmacology

Understanding diseases, determining the relationship between various cellular pathways, and selecting an appropriate treatment all benefit greatly from the use of various computational and simulation technologies (such as artificial intelligence, molecular docking, etc.). Systems biology refers to the discipline that uses computational tools and models of complex biological systems to systematically investigate biological phenomena (232, 233).

These computational analyses and sophisticated models have also found application in the field of pharmacology. Therapeutic efficacy and side effect predictions (including organ toxicity and genetic toxicity) and medication repositioning could be improved with the help of these computational investigations. System pharmacology is the name given to this branch of pharmacology (234, 235).

6.1 Drug combination prediction

Because of their potential to boost efficacy or lessen unwanted side effects, combination treatments are increasingly being used in the treatment of a wide range of disorders. In addition, optimizing the inhibition of different cell pathways can enhance the process of treating a disease when several pathways contribute to its incidence or treatment. Given the large number of available medications as well as intricate physiological pathways, the use of computational methods to predict the ideal therapeutic combination is critical and can save time and money on various research (236–238).

For example, in 2021, Teng and et.al, used *In silico* method for predicting effective drug combinations for T2DM treatment. They compiled data on genes linked to T2DM and built a disease module for the condition. They then identified promising medications by assessing their spatial closeness to the disease module. Gene Set Enrichment Analysis (GSEA) was then used to narrow down the list of possible medications based on the drug-induced gene expression profiles. This network-based approach had also been used in predicting potential drug combinations for type 2 diabetes (237).

Considering the relationship between circadian cycle and type 2 diabetes and the specificity of different cellular pathways, the use of these computational methods can speed up the use of combination therapy based on circadian cycle in the treatment of diabetes.

6.2 Drug repositioning

To date, more than 5,000 drugs have been approved by the US Food and Drug Administration in various therapeutic indications, and many drugs are in different phases of clinical studies. This volume of drugs and available data on their effects on cells and different cellular pathways gives us the possibility to find new targets for drugs that have previously been approved and used for other targets without the need to design new drugs; This reuse of drugs is called drug repositioning (239). The use of drug repositioning can significantly reduce the cost and time of new treatments. A very indicative example of the application of drug repositioning was seen in the treatment of Covid-19. In addition to many computational and clinical studies, many drugs mentioned in computational studies could show appropriate effects in clinical studies. They were used as a treatment option in the treatment protocol for Covid-19 (240, 241).

Considering that no known drugs has yet been introduced as an effective intervention in the circadian cycle and approved in clinical trials, implementing drug repositioning to suggest effective medicinal compounds on different targets in the circadian cycle can be very practical and bring forth the treatment based on the circadian rhythm.

7 Conclusion

Various studies have shown the effect of circadian rhythm in the creation, progression, and treatment of diabetes, especially T2DM. These studies, which were mostly laboratory- and animal-based, have provided suitable evidence for designing and conducting further wider studies, especially at the clinical level. Moreover, chronotherapy has been proposed as one of the most trending areas of pharmacology, which is very important in making personalized medicine a reality in terms of more effectiveness and less side effects. Also, with a better understanding of chronotherapy, we can suggest combination treatments based on the combination of drugs with environmental and lifestyle factors. New laboratory tools and techniques such as organ-on-a-chip, microfluidic-based diagnostic tools, and new generation sequences allow us to conduct more studies on different aspects of the circadian rhythm and its relationship with diabetes. Also, with the progress of computational tools and biological modeling, as well as the huge growth of data science and artificial intelligence, chronotherapy appears to be a very promising approach in the treatment of different types of diseases.

Author contributions

Conceptualization: MM, AH, and PS. Methodology: MM and AA. Investigation: AH, MK, MA-P, and AT. Resources: MM and AZ. Writing—original draft preparation: AH, MK, MA-P, AT, and PP. Writing—review and editing: MM, AZ, AA, and PS. All authors contributed to the article and approved the submitted version.

Conflict of interest

Author AA was employed by Xsphera Biosciences Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Efficacy and safety of high-dose intramuscular vitamin D_2 injection in type 2 diabetes mellitus with distal symmetric polyneuropathy combined with vitamin D insufficiency: study protocol for a multicenter, randomized, double-blinded, and placebo-controlled trial

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Background: Distal symmetric polyneuropathy (DSPN) is the most common chronic complication of type 2 diabetes mellitus (T2DM). DSPN may lead to more serious complications, such as diabetic foot ulcer, amputation, and reduced life expectancy. Observational studies have suggested that vitamin D deficiency may be associated with the development of DSPN in T2DM. However, interventional studies have found that low-dose vitamin D supplementation does not significantly improve neuropathy in DSPN. This study aims to evaluate the efficacy and safety of intramuscular injection of high-dose vitamin D (HDVD) in T2DM with DSPN combined with vitamin D insufficiency.

Methods and analysis: We will conduct a multicenter, randomized, double-blinded, and placebo-controlled trial in four large hospitals. All eligible participants will be randomly assigned to either the vitamin D_2 supplement or placebo control group and injected intramuscularly monthly for 3 months. Additionally, anthropometric measurements and clinical data will be collected at baseline and 3 months. Adverse events will be collected at 1, 2, and 3 months.

The primary outcome measure is the change in the mean Michigan Neuropathy Screening Instrument (MNSI) score at baseline and 3 months post-intervention. We will use the gold-standard liquid chromatography-tandem mass spectrometry method to distinguish between 25(OH)D_2 and 25(OH)D_3 levels. The MNSN score before the intervention will be used as a covariate to compare the changes between both groups before and after the intervention, and the analysis of covariance will be used to analyze the change in the MNSI score after HDVD supplementation.

Discussion: Glycemic control alone does not prevent the progression of DSPN in T2DM. Some studies have suggested that vitamin D may improve DSPN; however, the exact dose, method, and duration of vitamin D supplementation are unknown. Additionally, neuropathy repair requires HDVD supplementation to sustain adequate vitamin D levels. This once-a-month intramuscular method avoids daily medication; therefore, compliance is high. This study will be the first randomized controlled trial in China to analyze the efficacy and safety of HDVD supplementation for patients with T2DM and DSPN and will provide new ideas for pharmacological research and clinical treatment of diabetic neuropathy.

Clinical trial registration: https://www.chictr.org.cn/, identifier ChiCTR2200062266.

KEYWORDS

type 2 diabetes mellitus, distal symmetric polyneuropathy, high-dose vitamin D supplementation, Michigan neuropathy screening instrument, randomized controlled trial

1 Introduction

According to the latest International Diabetes Federation statistics for 2021, the total number of people with diabetes worldwide is 536.6 million—and China accounts for a quarter (1). The prevalence of diabetes among adults in China is 12.8%, higher than the global average of 10.5%. Moreover, the number of people with diabetes in China is approximately 129.8 million, and those with type 2 diabetes mellitus (T2DM) account for > 90%. Therefore, T2DM is a serious public health problem in China (2). Additionally, the chronic complications caused by T2DM are a threat to national health. Notably, the complications involving peripheral nerves—peripheral neuropathy—can affect 75% of people with T2DM. Peripheral neuropathy occurs as early as in the pre-diabetic state of obesity, indicating its close relation to diabetes, obesity, and other metabolic diseases (3, 4).

Distal symmetrical polyneuropathy (DSPN) is the most representative manifestation of nerve damage in T2DM and is more common than other spinal nerves, cranial nerves, and autonomic neuropathy lesions (5). DSPN develops slowly, starting with small nerve fibers at the end of the limb and progressing to larger nerves, including sensory and motor nerves. The symmetrical onset of numbness and sensory abnormalities is the earliest clinical symptom in many patients with DSPNs. Moreover, approximately one-third of patients experience burning, pin and needle sensations, and pain. These painful symptoms are unbearable and lead to insomnia and depression, reducing patients' quality of life (6, 7). Additionally, patients with DSPN are prone to complications, such as diabetic foot ulcers, due to a long-term lack of self-sensory protection, leading to serious complications, such as irreversible amputation and shortened life expectancy. Finally, as the disease progresses, people's ability to work becomes severely affected, causing a serious economic burden and wastage of resources for individuals, families, and society; therefore, DSPN requires more attention (8, 9).

The clinical efficacy of DSPN treatment is poor. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study showed that glycemic control alone in T2DM does not reduce the incidence of DSPN, suggesting that hyperglycemia is not the only factor influencing the development of DSPN. Recently, clinicians have been looking for alternative treatments to improve DSPN (10). The etiology and pathogenesis of DSPN have not been elucidated; however, its development involves or is accelerated by abnormal insulin signaling pathways, microcirculatory disorders, non-enzymatic advanced glycosylation end product pathways, oxidative stress, and other factors leading to mitochondrial dysfunction, DNA damage, and apoptosis (9, 11).

Abbreviations: T2DM, type 2 diabetes mellitus; DSPN, distal symmetric polyneuropathy; MNSI, Michigan Neuropathy Screening Instrument; HDVD, high-dose vitamin D.

Vitamin D is a fat-soluble ring-opening steroid discovered over 100 years ago and includes vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol). Vitamin D_2 is derived from foods, such as mushrooms, offal, egg yolks, and fatty sea fish. Vitamin D_3 is converted from 7-dehydrocholesterol in the skin by ultraviolet light. Vitamins D_2 or D_3 require two hydroxylation processes, mediated by 25-hydroxylase in the liver and 1α-hydroxylase in the kidneys, to become active as 1,25-$(OH)_2D_2$ or 1,25-$(OH)_2D_3$, respectively. At present, activated vitamin D has gone beyond the traditional concept of vitamin and as it can play a hormone-like role, so 1,25-$(OH)_2D$ is also known as D hormone. Therefore, some scholars refer to the unactuated form of vitamin D as a D prohormone. Additionally, activated vitamin D is directly involved in gene regulation *via* the vitamin D receptor (VDR), which has several biological roles. Since vitamin D receptors are found in bones, kidneys, parathyroid glands, small intestine, nerve tissue, and pancreatic β-cell, vitamin D may have other biological roles in addition to its traditional role of regulating calcium, phosphorus, and bone metabolism. These roles include facilitating cell differentiation and proliferation, reducing superoxide, regulating lipid metabolism and inflammatory factors, reducing superoxide, promoting nerve growth factors, regulating immunity, reducing cytokine storms, and reducing COVID-2019 mortality (12–16).

Considering all these functions, vitamin D is important for health; however, its insufficiency is a widespread public health problem. Vitamin D nutritional status is determined by measuring serum 25-(OH)D levels—a sum of 25-$(OH)D_2$ and 25-$(OH)D_3$. The Endocrine Society recommends a 25-(OH)D level of 30 ng/mL (17); a patient is considered to have a vitamin D -insufficient state if this level is not reached (17–19). Studies have shown that vitamin D deficiency occurs in 74.7% of people with T2DM and affects people with prediabetes. Moreover, studies have shown that people with low vitamin D levels and prediabetes have a significantly higher risk of developing diabetes (20, 21).

A study by the National Health and Nutrition Examination Survey showed that the risk of developing DSPN increases by 2.59 times in people with vitamin D deficiency (22). Another study suggested that vitamin D deficiency may be an independent risk factor for DSPN (23). In an observational study, Seham et al. (24) found that vitamin D levels were significantly lower in patients and DSPN than in those with T2DM without DSPN and confirmed that 87.6% of those with DSPN had vitamin D deficiency, and the prevalence of vitamin D deficiency in T2DM without DSPN was only 45%.

However, the mechanisms by which vitamin D deficiency specifically affects DSPN are unknown. Vitamin D may improve glycemic control by promoting β-cell secretion and increasing insulin sensitivity. Additionally, vitamin D may reduce oxidative stress and prevent nerve damage (25). Animal studies have shown that vitamin D increases the synthesis of nerve growth factors in rats (26). Vitamin D is a potent inducer of neurotransmitters and can reduce nerve demyelination and improve axonal regeneration (27, 28).

A meta-analysis showed that vitamin D supplementation effectively reduced neuropathic pain and prevented further nerve damage (29). The association between vitamin D and pain may be due to nociceptive calcitonin gene-related peptide (CGRP)-positive neurons. These neurons have a distinct vitamin D phenotype, and their ligands and receptors are hormonally regulated. A study reported that vitamin D receptor expression in the growth cones and CGRP expression increased rapidly with vitamin D deficiency, indicating a possible connection between vitamin D and pain. Additionally, this study suggested the presence of lower pain tolerance in patients with vitamin D deficiency (30, 31).

Adequate vitamin D levels are not achieved with sunlight and food intake alone; therefore, additional supplements are required. Studies have shown that different doses of vitamin D supplementation may produce different results in DSPN treatment. A clinical study by Karonova et al. (32) showed that high-dose vitamin D (HDVD) supplementation in patients with DSPN and vitamin D deficiency achieved adequate status in all patients; however, only half of the low-dose group achieved adequate status. Moreover, the severity of neuropathy was accompanied by a significant reduction in inflammatory markers in the HDVD group; however, these markers were not altered in the low-dose group. Basit et al. (33) showed a significant decrease in total pain score and a reduction in positive symptoms in diabetic neuropathy patients treated with HDVD. Silva et al. (34) treated patients who had diabetic cardiac autonomic neuropathy with HDVD and showed significant improvement in parameters related to resting heart rate variability without adverse effects. Notably, neuropathy repair and symptom improvement are slow; therefore, we decided to use HDVD supplementation to repair neuropathy.

It is currently believed that vitamin D_2 and D_3 supplementation have the same effect of correcting vitamin D insufficiency, and it is believed that injection is better than oral supplementation (17, 35). Indeed, lower daily doses of vitamin D supplements require longer time to increase vitamin D levels in the body and are less likely to sustain sufficient levels. In addition, daily medication reduced compliance and executive function in patients, thereby reducing the number of people who reach sufficient state of vitamin D. Therefore, some scholars have proposed that the injection of HDVD supplementation as a method of administration with lower frequency and longer interval will be more conducive to obtain desired intervention outcomes (36, 37). Compared to vitamin D_3, vitamin D_2 has a lower binding affinity to vitamin D-binding proteins (VDBP). This means that taking vitamin D_2 instead of vitamin D_3 will result in higher levels of free vitamin D concentration in the blood, which will bind better to VDR (38, 39). Indeed, vitamin D_2 has been used clinically for more than 50 years (39). Therefore, we use vitamin D_2 injection as a strategy to correct vitamin D insufficiency.

The traditional nerve conduction test reflects only the lesions of large nerve fibers but not those of small fibers, such as fine myelinated A δ, unmyelinated C, and autonomic nerve fibers, which mediate nociceptive pain and temperature perception (5). Michigan Neuropathy Screening Instrument (MNSI) comprises a questionnaire and physical examination. The questionnaire contains 15 questions that address DSPN symptoms, with a maximum score of 13 points, and is completed by the patients.

The physical examination is performed by the physician and includes ankle reflexes, vibration perception, foot ulceration, and feet appearance. Physical examination has a maximum score of 8 points for both feet. Multiple studies have demonstrated that MNSI is a reliable and effective tool for assessing DSPN. Additionally, compared with nerve conduction tests, MNSI avoids invasive examinations and the discomfort of electrical stimulation. Moreover, MNSI may avoid missing early small-fiber lesions in DSPN (40–44). Therefore, we will use the MNSI to assess the changes in neuropathy after HDVD supplementation.

The development of DSPN is relatively reversible in the early stage; however, it becomes a refractory nerve injury in the later stage, and the condition is persistent and recurrent. Correcting vitamin D deficiency helps prevent and treat DSPN. However, the specific dosage, long-term treatment effects, and related side effects should be confirmed in high-quality randomized controlled trials. Few clinical trials have investigated the effects of vitamin D supplementation on neuropathy in China's population with DSPN and T2DM. Therefore, this study aims to explore the efficacy of HDVD supplementation in treating DSPN in T2DM, assess its safety, and collect information on adverse reactions.

2 Methods and analysis

2.1 Study design

We will conduct a multicenter, prospective, randomized, double-blinded, and placebo-controlled clinical trial. First, participants will be assigned to the HDVD supplementation or placebo control group in a 1:1 block randomization scheme. Next, intramuscular injections will be administered at baseline and 1 and 2 months later. Moreover, we will collect anthropometric measurement data, fasting blood glucose, β-cell function, hemoglobin A1c, and other biochemical data. Lastly, MNSI will be performed at baseline and 3 months later. This study will follow the Comprehensive Reporting Test Standard Guidelines (45). The study flow chart is shown in Figure 1.

2.1.1 Study setting

We will recruit participants from four large hospitals: Longyan First Affiliated Hospital of Fujian Medical University, Longyan Traditional Chinese Medicine Affiliated Hospital of Fujian University of Traditional Chinese Medicine, Longyan Boai

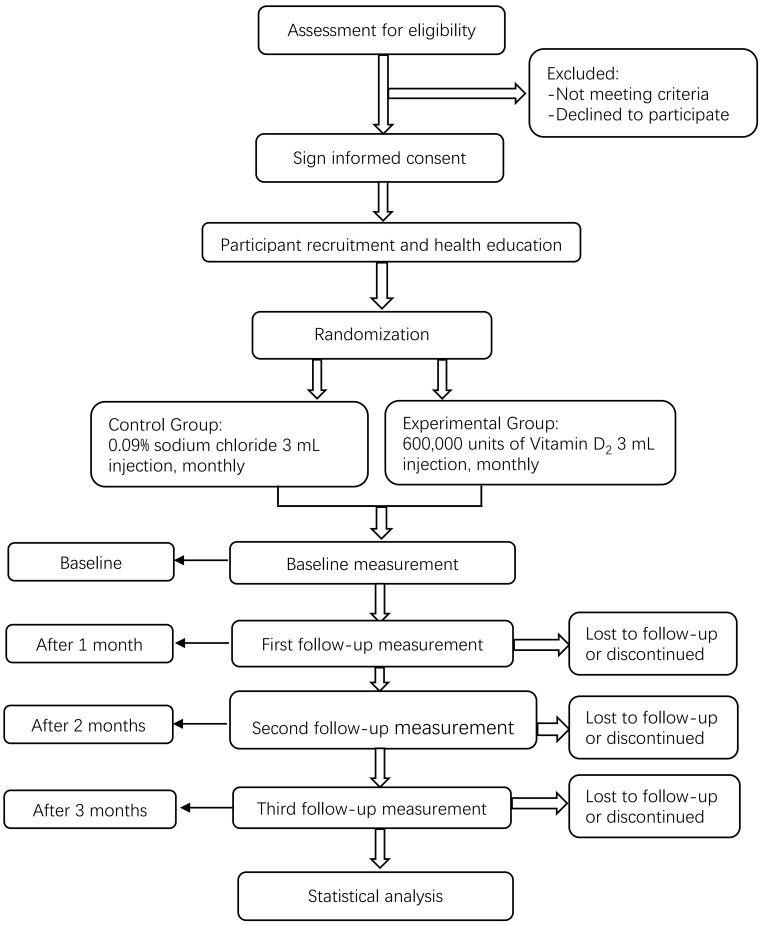


FIGURE 1
Trial flow chart.

TABLE 1 Inclusion criteria and exclusion criteria.

Inclusion criteria	Exclusion criteria
1. All participants live permanently in Longyan City, South China (latitude and longitude 115 ° 50 ′ 56 "-117 °44 ′ 15" E, 24 ° 22 ′ 31 "-26 °2 ′ 35" N).	1. Patients without glucose homeostasis such as diabetic ketoacidosis and hypoglycemia.
2. Aged between 20 and 79 years.	2. Patients with hyperthyroidism, hypothyroidism, or parathyroid disease.
3. 25(OH) vitamin D < 30 ng/mL	3. Severe disability or mental illness and pregnant and lactating women.
4. All participants will sign a written informed consent form.	4. Alcoholic or drug abusers, a vegetarian who never eats fish or meat.
5. The diagnosis of type 2 diabetes mellitus will be confirmed according to the diagnostic criteria of the World Health Organization in 1999.	5. Chronic gastrointestinal diseases such as intestinal tuberculosis, ulcerative colitis, or irritable bowel syndrome.
6. The diagnosis of distal symmetric polyneuropathy will be diagnosed according to the Guidelines for the Prevention and Treatment of Type 2 Diabetes Mellitus in China (2020 Edition). Five neurological examinations will be performed, including ankle reflexes, pinprick pain, vibration, pressure, and temperature sensations. If a patient has one clinical symptom (numbness, pain, and paresthesia) and any of the five neurological examinations are abnormal, the patient will be diagnosed with DSPN. Additionally, patients will be diagnosed with DSPN if two of the five neurological examinations are abnormal, without any clinical symptoms, Figure 2.	6. The participant has recently taken the following medications or supplements: immunosuppressants or glucocorticoids, such as prednisone at doses >10 mg/day; drugs such as phenytoin sodium, phenobarbital, and rifampicin that may affect the catabolism of vitamin D; vitamin D or active vitamin D, such as calcitriol; and drugs that directly affect nerves (such as chemotherapy drugs, vitamin B6 or Neurobion). 7. Patients with large areas of skin pigmentation or skin diseases. Those who engage in long-term outdoor activities or have tropical tourism plans in the next 3 months. Using sunscreen on the face and hands 8. Hyperphosphatemia and renal rickets.
	9. Blood calcium level >11 mg/dL (2.75 mmol/L), history of urinary tract stones, and sarcoidosis.
	10. Acute stress diseases, such as shortness of breath, fever and diarrhea, severe leukopenia, malignant tumor, liver dysfunction (bilirubin> 1.5 times the upper limit of normal value, alanine aminotransferase> 2 times the upper limit of normal value) or organic diseases, such as decreased renal function eGFR*<20 mL/min/1.73 m2.
	11. Neuropathy caused by other causes, such as cervical and lumbar lesions (nerve root compression, spinal stenosis, cervical and lumbar degeneration), cerebral infarction, Guillain–Barre syndrome, and serious arteriovenous vascular lesions (venous embolism, lymphangitis).
	12. Participants considered unsuitable for the study based on the investigator's judgment.

DSPN, Distal symmetric polyneuropathy; eGFR, estimated glomerular filtration rate.

Hospital, and Longyan Shanghang County Hospital. All participants will provide written informed consent to participate in the study.

2.1.2 Participants

The inclusion and exclusion criteria for the target participants are listed in Table 1. All diagnostic criteria will be based on the guidelines and expert consensus of the World Health Organization and China. To recruit as many eligible patients with DSPN as possible, we will distribute recruitment leaflets to outpatients and hospital wards and release recruitment information using the WeChat mobile application (version 8.0.32, Tencent Holdings Limited, Shenzhen, China) and other online forms. Those interested can contact the recruiter directly *via* phone. If a participant experiences a serious adverse event or disease (such as anaphylactic shock or myocardial infarction) during the trial, whether related to the investigational drug or not, the person will be treated immediately and withdrawn from the trial. Moreover, the participants will be allowed to withdraw from the trial at any time.

2.1.3 Sample size estimation

In a previous study of patients with peripheral neuropathy who received vitamin D supplementation for a 1.19-point reduction in MNSI physical examination score with a standard deviation of 1.29 points (46). We assumed a two-sided α error probability of 0.01 and a power of 0.95. We used PASS version 11 (NCSS, LLC. Kaysville, Utah, USA) to estimate the sample size. After estimation, the total sample size was 84 cases, with 42 in each group. Considering that 20% of the participants could be lost to follow-up or refusal of follow-up, we plan to enroll 53 patients per group (total 106 patients).

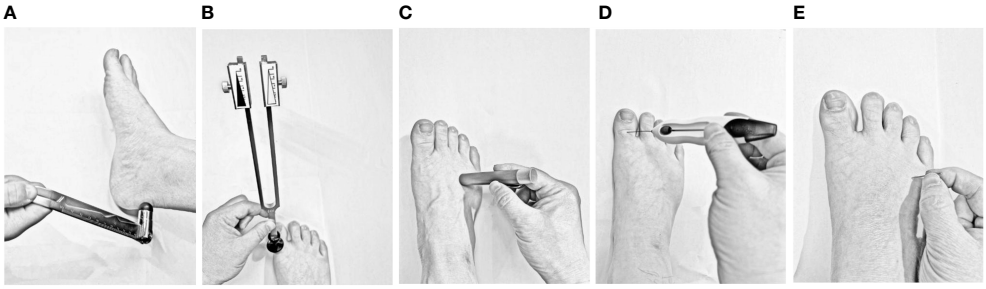


FIGURE 2
Schematic of five neurological examinations used to diagnose distal symmetric polyneuropathy **(A)** Ankle reflexes: The examiner should gently tap the Achilles tendon on one side of the subject's foot using a percussion hammer. The ankle reflex is considered absent if the foot cannot be plantarflexed, weakened if the plantar flexion is not significant, and positive if the bilateral ankle reflexes are weakened or absent. **(B)** Vibration sensing: After striking a 128 Hz tuning fork, it is placed on the dorsal side of the big toe of both feet to observe whether the participants can feel the tuning fork's vibration. Vibrational sensation is positive if the sense of vibration on either side disappears or decreases. **(C)** Temperature sensing: One end of the temperature tester (metal or polyester) is s placed on the skin of the dorsum of the foot (avoiding calluses, ulcers, scars, and necrotic tissue). The result is positive if the participant cannot correctly distinguish the temperature at either end. **(D)** Pressure sensing: A 10 g Semmes–Weinstein monofilament is placed on the dorsal side of the hallux with gradually increasing pressure, repeated four times on each side, eight times in total on both feet, and 1 point is recorded if no pressure is sensed once. The result is considered positive if the total score is $\geq$ 5 points. **(E)** Pinprick pain sensing: Gently prick the skin of the dorsum of the foot with the tip of a pin without causing skin damage. Participants who cannot feel pain or are hyperresponsive to pain are considered positive.

2.2 Randomization, allocation concealment and blinding

All eligible participants will be assigned a unique identification number in the order of enrollment. Randomized assignment of subjects is fundamental to clinical trial design. While performing a simple sequence of random numbers can ensure independence among subjects and avoid potential selection biases, doing so has the potential to lead to an imbalance in the distribution of important covariates across treatment groups. Therefore, we will opt for the block randomization scheme (47). A person independent of the research center will generate a block randomization coding scheme using R software (version 4.1.2, R Foundation for Statistical Computing, Vienna, Austria). Next, all participants will be assigned in a 1:1 ratio to the placebo control or HDVD supplementation groups.

In order to avoid selection bias to the maximum extent possible, we will adopt the method of avoiding leakage of grouping information. The goal is to balance out all known and unknown factors between the two groups, so that differences in outcomes between the groups could ultimately be attributed to the effect of HDVD supplementation. To this end, we are going to use the method of allocation concealment. A staff member who determines the grouping of random sequences will not be involved in the inclusion of subjects. Neither our investigators nor our subjects are aware of the random sequence and the corresponding group during the diagnosis of DSPN, during the eligibility evaluation stage such as reviewing whether patients met the inclusion and exclusion criteria, and during the stage of soliciting patients' willingness to participate in the trial.

Each protocol will be placed in an opaque envelope, marked with the code, and sealed. In order to reduce information bias, including measurement bias and implementation bias, we will double-blind the trial for the investigator and participants. Once the participants are enrolled, a third person, independent of the participant and investigator, will open the envelopes individually, and the executor will perform the corresponding injection per the envelope protocol.

2.3 Intervention

All participants will receive diabetes health education and follow-up. Medical nutrition therapy will be recommended for every patient with diabetes, and they will be advised to avoid overeating and undereating, which causes oxidative stress due to glucose fluctuations, exacerbating peripheral nerve damage. Moreover, self-monitoring of blood glucose will be recommended for every participant. The optimal blood glucose control target will be 4.4 mmol/L < fasting blood glucose < 7.0 mmol/L, 2-h postprandial blood glucose < 10.0 mmol/L, and avoid hypoglycemia. Next, exercise therapy, avoidance of foot injuries, and wearing white socks frequently will be recommended for every patient with diabetes to detect minor local injuries early.

After randomization, all participants will receive an intramuscular injection of the trial drug (vitamin D_2 600,000 units, 3 mL, monthly for 3 months) or placebo (0.09% sodium chloride 0.0027 g, 3 mL, monthly for 3 months) into the gluteus maximus. Vitamin D_2 is produced by Jiangxi Gannan Haixin Pharmaceutical Co. Ltd. (Ganzhou, Jiangxi, China), and sodium chloride by Hubei Xinghua Pharmaceutical Co. Ltd. (Wuhan, Hubei, China). Each injection will be packaged in an opaque sealed package with an instruction leaflet and label. Drug dispensing will be performed by a third person independent of the implementer and participant. It is critical that all participants remain in the follow-up cohort as long as possible, and the efforts of all investigators and related personnel will improve participant

adherence to the intervention and follow-up schedule. If the participant refuses to continue the follow-up, the investigator will determine the reason and encourage the participant to continue the intervention and follow-up.

2.4 Outcome assessment

2.4.1 Primary and secondary outcomes

The primary outcome measure is the change in the mean MNSI score at baseline and 3 months post-intervention, reflecting the peripheral neuropathy status of the participant.

The secondary outcome measures include 1. changes in vitamin D level, including 25(OH) D_2, 25(OH)D_3, and 25(OH)D; 2. changes in fasting blood glucose, 2-h postprandial blood glucose, and hemoglobin A1c; 3. changes in fasting C-peptide and 2-h postprandial C-peptide; 4. changes in homeostasis model 2 for assessing insulin resistance, insulin sensitivity, and β-cell function (HOMA2-IR, HOMA2-%S, and HOMA2-%B, respectively) by HOMA2 software (version 2.2) available from the Oxford Centre for Diabetes, Endocrinology, and Metabolism (48).; 5. changes in systolic blood and diastolic blood pressure; 6. changes in triglyceride, total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol; and 7. changes in the maximum bimanual grip strength and quality of life score.

2.4.2 Demographic and anthropometric data

Baseline data will be collected at enrollment for all participants. The demographics will include name, age, sex, race, residence, occupation, marital status, and education. Anthropometric measurements will include height, weight, body mass index, waist circumference, and hip circumference. Additionally, we will measure vital signs, such as body temperature, heart rate, and blood pressure, simultaneously.

2.4.3 Clinical data collection and follow-up

Venous blood samples will be collected from participants at baseline and designated visit points for biochemical data, such as lipid, glucose, and uric acid. The method of venous blood sampling is as follows. First, the participants will fast for 10–12 h, and a qualified nurse will collect blood samples at 6:30–8:30 am. Next, the blood samples will be tested for 25(OH)D_2, 25(OH)D_3, and 25(OH)D levels using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Fuzhou King Med Clinical Laboratory (Fuzhou, Fujian, China). The executive assistants involved in data collection and evaluation will be blinded to participants' groupings. All clinical data collection processes are presented in Table 2.

2.4.4 Safety assessment and adverse events reporting

We will evaluate liver function, albumin, renal function, calcium, phosphorus, potassium, sodium, chloride, and other biochemical parameters at baseline and post-intervention to evaluate the safety of the HDVD. All adverse events reported during the study will be closely monitored and recorded. In this trial, an adverse event is any unfavorable or unexpected occurrence related to the intervention. The investigator will ask the participants questions regarding adverse reactions. Participants will be called on days 1, 3, and 7 after each injection to determine any adverse events (Table 3). Moreover, participants will be asked to self-report any adverse events at any time. All adverse events will be professionally addressed, closely monitored by a physician, and treated as necessary. Serious adverse events will be reported to the study director—responsible for managing adverse reaction reports—within 1 h. Additionally, related serious adverse events will be reported to the Ethics Committee of Longyan First Affiliated Hospital of Fujian Medical University. The Ethics Committee is independent of the investigators, and the investigators are not members of the Ethics Committee.

2.5 Data management

After enrollment, all participants will be given a unique identification number, which will substitute participants' names in the database to protect their privacy. All data will be entered using the EpiData Software (Version 3.1, Denmark), and two data entry clerks will enter the case report form into the same database and cross-check to ensure the accuracy of the data. Finally, all written materials and encrypted electronic files containing the study's data will be stored and backed by the study director. These documents will be retained for > 3 years after this study.

2.6 Data analysis

Any participant who completes the randomization will be included in the intention-to-treat set for data analysis. In cases when random or unforeseeable missing data occur, the missing data will be processed by multiple imputation method and sensitivity analysis will be carried out (49). The safety set which contains at least one safety assessment and adverse event report will be used to analyze the safety of HDVD supplementation. Quantile-quantile plot will be used to determine if the data conforms to a normal distribution. For descriptive analysis, the results of continuous variables (age and body mass index, among others) that conform to normal distribution will be expressed as mean ± standard deviation, and those that do not conform to normal distribution will be expressed as median and interquartile range. Categorical variables (sex and smoking status) will be expressed as frequencies and percentages.

Baseline data will be compared between the HDVD supplementation and placebo control groups. In HDVD supplementation or placebo control group, a paired sample t-test will be used for post-intervention versus pre-intervention comparisons. The difference value of MNSI before and after intervention will be performed by independent sample t-test test to compare the difference between the two groups. If the data does not conform to normal distribution, the Mann-Whitney U test will be used for analysis. The chi-square test will be used for categorical variables.

TABLE 2 Participant flow of enrolment, intervention, and outcome measurements.

		PS	M0	M1	M2	M3
Enrolment	Recruitment	√				
	Eligibility Screening	√				
	Signed written informed consent	√				
	Allocation		√			
Intervention	Health education, medical nutrition therapy, exercise therapy, self-monitoring of blood glucose, and disease follow-up		√	√	√	√
	High-dose vitamin D_2 /placebo injection		√	√	√	
Outcomes	Demographic characteristics and basic information (sex, age, marital status, place of residence, and education level)		√			
	Personal life status (smoking status, drinking status, beverage intake status, sunshine reception, living alone, and menstrual status for females)		√			
	Duration of diabetes, strict diet control, and moderate aerobic exercise for >150 min per week		√			
	Number of self-monitoring of blood glucose per week and occurrence of hypoglycemia		√			
	Comorbidities (hypertension, hyperuricemia, obesity, osteoporosis, and nonalcoholic fatty liver disease)		√			
	Other diabetic complications (diabetic retinopathy, diabetic nephropathy, diabetic foot ulcer, coronary atherosclerotic heart disease, cerebrovascular disease, and peripheral vascular disease)		√			
	Anthropometric measurements (height, weight, body mass index, waist, and hip circumference) and blood pressure (systolic and diastolic blood pressure)		√			√
	Metabolic parameters (FBG, 2hBG, HbA1C, FCP, 2hCP, triglycerides, total cholesterol, HDL-C, LDL-C, uric acid, HOMA2-IR/%S/%B)		√			√
	Intima-media thickness of carotid and dorsalis pedis artery by ultrasound, fundus examination, bone mineral density		√			√
	25-(OH) Vitamin D_2, 25-(OH) Vitamin D_3, and 25-(OH) Vitamin D tested by LC-MS/MS method		√			√
	Maximum bimanual grip strength measured by a dynamometer		√			√
	Short Form -12 Quality of Life Questionnaire		√			√
	MNSI score		√			√

PS, prior to study; M0, baseline assessment; M1, 1 month from baseline; M2, 2 months from baseline; M3, 3 months from baseline; HOMA2-IR/%S/%B; homeostasis model assessment index 2 for assessing insulin resistance/insulin sensitivity/function of islet β-cells; LC-MS/MS, liquid chromatography-tandem mass spectrometry; *MNSI, Michigan Neuropathy Screening Instrument; FBG, fasting blood glucose; HbA1c, hemoglobin A1c; 2hBG, 2-h postprandial blood glucose; FCP, fasting C-peptide; 2hCP, 2-h postprandial C-peptide; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Furthermore, we will use the MNSI score before intervention as a covariate, and the F value will be calculated using analysis of covariance (ANCOVA) to analyze the change in the MNSI score after HDVD supplementation. Finally, if the number of adverse events occurring during the study is sufficient, the chi-square test or Fisher's exact probability method will be used to compare differences in adverse events between both groups.

All data will be analyzed using the R software (version 4.1.2; R Foundation for Statistical Computing, Vienna, Austria). Additionally, all statistical analyses will be performed using a two-sided test, considering $P < 0.05$ statistically significant.

2.7 Data monitoring

This study will be supervised by the Ethics Committee of the Longyan First Affiliated Hospital of Fujian Medical University, whose members have no conflicts of interest with this study. The study director will have access to all results and decide when to terminate the study based on its progress. Additionally, the study director will grant other members of the study's team the right to disseminate the trial results by publishing papers.

2.8 Special instructions

During the study, participants will be told to avoid high-fat and high-carbohydrate diets inappropriate for T2DM patients. Moreover, participants will be informed to avoid excessive sun exposure and consumption of salmon, mushrooms, and other foods that may influence vitamin D levels. Furthermore, participants will be asked to spend 20 min daily in the sun outdoors as usual. Next, participants will be notified 3 days before the next injection and follow-up to avoid being unable to participate in the injection in time due to a busy schedule or forgetting the time. Moreover, if the participants cannot visit the hospital on time on weekdays, executive assistants will use overtime to inject and follow them up as necessary on a case-by-case basis. Finally, we will provide a 24-h

TABLE 3 Safety assessment and adverse event follow-up of participant.

	M0	M1	M2	M3
Liver function: alanine aminotransferase, aspartate aminotransferase, total bilirubin, albumin, globulin	√			√
Renal function: creatinine, blood urea nitrogen, and eGFR calculated by the CKD-EPI equation	√			√
Creatine kinase (CK), creatine kinase myocardial band (CK-MB), lactate dehydrogenase (LDH), alkaline phosphatase (ALP)	√			√
Electrolytes: calcium (Ca), phosphorus(P), potassium(K), magnesium (Mg), sodium (Na), chloride (Cl)	√			√
Closely follow up on the physical health after injection, and receive consultation and feedback at any time within 24 h	√	√	√	√
Did the participant experience any adverse reactions after the injection (whether or not determined to be related to this injection)	√	√	√	√
Digestive adverse reactions: decreased appetite, constipation or diarrhea, abdominal pain, nausea, or vomiting	√	√	√	√
Skin adverse reactions: pruritus, allergic dermatitis, rash, swelling, and pain at the injection site	√	√	√	√
Nervous system adverse reactions: metallic sensation in the mouth, persistent headache, convulsions, and insomnia	√	√	√	√
Urinary adverse reactions: cloudy urine, nocturnal polyuria, and hematuria	√	√	√	√
Skeletal system adverse reactions: bone pain and myalgia	√	√	√	√
Respiratory and circulatory system: shortness of breath, palpitations, hypertension, shock, hypotension, and chest pain	√	√	√	√
Hematologic and endocrine adverse reactions: rapid weight loss or gain, fever, and fear of cold	√	√	√	√
Other adverse reactions: fatigue, asthenia, dry mouth, and fall	√	√	√	√
Record treatment for adverse reactions (only for participants with adverse events)	√	√	√	√

M0, baseline assessment; M1, 1 month from baseline; M2, 2 months from baseline; M3, 3 months from baseline; eGFR, estimated glomerular filtration rate; CKD-EPI, the Chronic Kidney Disease Epidemiology Collaboration equation.

telephone consultation service and other measures to ensure the best attendance and avoid losses to follow-up.

3 Discussion

DSPN can induce diabetic foot ulcer and amputation, increasing the social burden and consuming medical resources. The United States spends > $10 billion yearly to treat DSPN. Early identification and treatment of DSPN can save at least 80% of medical costs (50–52). Therefore, preventing the further development of DSPN has attracted our attention.

Vitamin D deficiency is a growing global health problem, even in countries like the United Arab Emirates, which receives plenty of sunshine yearly (18). This implies that the vitamin D produced by the sun's ultraviolet rays is insufficient. A recent meta-analysis showed that vitamin D deficiency in T2DM increased the risk of DSPN by 1.22 times (53). This evidence highlights an early treatment window for patients with T2DM and the possibility that vitamin D supplementation may prevent the development of DSPN. A previous retrospective study found that vitamin D levels in patients with T2DM and DSPN were one-third lower than those without DSPN, suggesting that vitamin D may affect DSPN progression (18). Furthermore, clinical studies have shown that HDVD supplementation can significantly reduce the pain score of DSPN, reduce demyelination, improve axonal regeneration, and even improve the quality of life of patients with DSPN (33, 34, 54).

Notably, patients with vitamin D deficiency may not rely solely on increased sun exposure and dietary supplements; therefore, additional vitamin D supplements are needed. Supplementation is achieved mainly by oral administration and intramuscular injection of vitamin D pharmaceutical preparations. However, recovery from neuropathy is slow, and most patients have difficulty maintaining a daily low dose of vitamin D supplements. Moreover, it also takes longer to achieve vitamin D sufficiency with low-dose supplementation.

Since low-dose vitamin D supplementation cannot quickly correct this deficiency, some scientists have begun to try HDVD supplementation programs. A clinical study conducted by Masood et al. (55) with a single dose of 600000 units of vitamin D showed that it took only 2 months for 70% of people with vitamin D deficiency to achieve an adequate status, indicating that high-dose vitamin D supplementation can correct vitamin D deficiency quickly. This means the high-dose regimen could reduce the time traditionally required to reach the target. Additionally, a previous study by Xu et al. (56) demonstrated that a single injection of 600000 units of vitamin D increased 25(OH)D level by 10.3 ng/mL over 12 weeks, indicating that HDVD injection therapy is effective and durable in raising vitamin D concentrations. A clinical study by Diamond et al. (35) showed that a regimen of 600000 units of vitamin D injected intramuscularly once yearly increased participants' vitamin D levels by an average of 128%, and all participants' vitamin D levels were normalized. Furthermore, a bioavailability study by Cipriani et al. (57) revealed that vitamin D levels peaked on day 120 after a single intramuscular dose of 600000 units of vitamin D, and the conversion to 1,25 $(OH)_2D$ was more stable and less volatile after intramuscular administration than after oral administration. Overall, the studies described above suggest that intramuscular HDVD is a more effective method of vitamin D supplementation than daily oral low-dose vitamin D

supplementation. Moreover, the HDVD regimen has better compliance owing to fewer supplements, no need to take medication daily, and less forgetting or omission. However, few studies have investigated whether an HDVD can improve peripheral neuropathy. Our study aims to determine the efficacy of HDVD in T2DM patients with DSPN.

To make our study feasible, our study plan will exclude patients with severe renal insufficiency. Because 1α-hydroxylase is tightly regulated in the body, safe dose range for vitamin D supplementation is wide and there is no excess of 1,25-$(OH)_2D$ that is formed in the body. Previous studies have shown that poisoning is only possible when the 25-hydroxyvitamin D levels is greater than 224 ng/mL (560nmol/L) (17, 58, 59). A prospective randomized controlled study by Mehta et al. showed significant improvement in neuropathy in patients with DSPN who received HDVD supplementation for 6 months. The study included patients with chronic kidney disease (CKD) stages 1-4 and excluded patients with CKD stage 5 (eGFR <15 mL/min/1.73 m^2). The authors showed that 25-hydroxyvitamin D levels were significantly increased 6 months after the HDVD supplementation (before: 18.91 ± 5.3 ng/mL vs. after: 53.71 ± 7.8 ng/mL, $p<0.05$); however, serum creatinine level was not increased (before: 1.99 ± 0.9 mg/dL vs. after: 1.82 ± 1.8 mg/dL,$p>0.05$). This study provides strong evidence that HDVD supplementation does not worsen renal function in DSPN patients with an eGFR greater than 15 mL/min/1.73 m^2 (60). It is well known that kidney function gradually declines with age; however, a clinical study of monthly intramuscular injection of HDVD in very elderly people with an average age of 89.6 years has attracted our attention. The results of this study showed no further decline in renal function after intervention (creatinine level, before: 97.38 ± 18.08 mg/dL vs. after: 101.12 ± 20.39 umol/L, $p>0.05$), and there was no significant change in blood calcium levels (before: 2.30 ± 0.14 mg/dL vs. after:2.34 ± 0.11 mmol/L, $p>0.05$) (61). Our study will also collect data on changes in renal function, blood calcium after HDVD.

Many researchers have explored whether increasing the dose of vitamin D supplementation will cause side effects or adverse reactions. In a study by Binkley et al. (37) administering 50000 units of vitamin D monthly to people aged > 65 years did not cause any toxicity or side effects. Another clinical study by Bian et al. (61) in people aged > 80 years showed that supplementation with 600000 units of vitamin D monthly for more than half a year might be safe. However, the sample size used in these studies was small. Therefore, we will use a monthly regimen of high-dose intramuscular vitamin D injections closely observed by the implementer to achieve better compliance and an earlier time to target. Additionally, our study will address the safety of monthly HDVD supplementation in individuals with insufficient vitamin D levels.

Although vitamins D_2 and D_3 have the same efficacy in exerting physiological effects, previous studies have suggested that vitamin D_2 supplementation may affect vitamin D_3 levels (56). This may be related to increased CYP24A1 enzyme activity in the liver, promoting the conversion of 25$(OH)D_3$ to 24,25-$(OH)_2D_3$, thereby inactivating it (57, 62). Additionally, 25$(OH)D_2$ and 25 $(OH)D_3$ compete for the binding to VDBP (63, 64). Notably, traditional immunoassays for vitamin D do not distinguish between 25$(OH)D_2$ and 25$(OH)D_3$, which may underestimate 25 $(OH)D_2$ levels. Testing for vitamin D_2 can rule out the effects of sunlight (65). Therefore, we will use the gold standard LC-MS/MS method to distinguish between 25$(OH)D_2$ and 25 $(OH)D_3$ levels.

The strength of this study design is that it will determine whether an improvement in neuropathy accompanies an increase in vitamin D concentration due to HDVD supplementation. Notably, DSPN is associated with muscle atrophy; thus, decreasing the quality of life. Although previous studies suggested that vitamin D supplementation may improve muscle strength (52, 54, 66), our study will explore the effects of HDVD from multiple dimensions, such as quality of life and muscle strength. We will use the Short Form-12 (SF-12) questionnaire, comprising eight domains, to assess the quality of life. The SF-12 has satisfactory validity and reliability, showing good internal consistency in the psychological (Cronbach's α=0.83) and physical component summaries (Cronbach's α=0.81) (67). Moreover, we will measure the maximum grip strength of both hands before and 3 months post-intervention to assess changes in muscle strength.

This study design has some limitations. First, the trial will be conducted only in China's population with T2DM and DSPN. Therefore, the results may not be directly applicable to other ethnic groups. Second, the latitudes of southern China will be selected for this study; therefore, the results may not be directly applicable to other latitudes. Nevertheless, the study can provide certain evidence that may require further verification in other regional or international multicenter studies. Third, owing to the difficulty in recruiting patients with DSPN, it will be difficult to maintain a balance between the groups. However, we will further correct factors, such as age, sex, and body mass index, through stratified analysis and by applying the analysis of covariance statistical strategy as far as possible.

In conclusion, this study will provide clinical evidence demonstrating the efficacy and safety of post-treatment HDVD supplementation for DSPN. Additionally, if HDVD supplementation can improve neuropathy, this study will provide new directions for future pharmacological research and clinical management of diabetic neuropathy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Longyan First Affiliated Hospital of Fujian Medical University (approval number: LYREC2022-013-01). The patients/participants provided their written informed consent to participate in this study.

Author contributions

TC, XX, PW, and MT participated in the study design. XX, PW, and MT supervised the study.TC prepared the first of the manuscript. TC and SL conducted the statistical strategy. LH performed sample size estimation. LH, XL, JC, SW, YZ, YL, LX, YQ, LQ, and YX edited and revised the manuscript. PW reviewed the methodology and the entire contents of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Co-occurrence of hypertension and type 2 diabetes: prevalence and associated factors among Haramaya University employees in Eastern Ethiopia

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Background: Both hypertension (HTN) and diabetes are public health concerns in low- and middle-income countries, particularly in sub-Saharan African countries. The co-occurrence of HTN and diabetes is associated with an increased risk of mortality, morbidity, and reduced productivity in the working force. In Ethiopia, there is limited evidence on the co-occurrence of HTN and type 2 diabetes (T2DM). Therefore, this study was conducted to assess the co-occurrence of HTN and T2DM and their associated factors among Haramaya University employees in Eastern Ethiopia.

Methods: A cross-sectional survey was conducted among 1,200 employees at Haramaya University using a simple random sampling technique from December 2018 to February 2019. Demographic and behavioral factors were collected on a semi-structured questionnaire, followed by measurement of anthropometry and blood pressure. Blood glucose and lipid profile measurements were performed by collecting 6 ml of venous blood samples after 8 h of overnight fasting. Data were entered into EpiData 3.1 version and analyzed using Stata 16 software. Bivariable and multivariable logistic regressions were applied to observe the association between independent variables with co-occurrence of HPN and T2DM using odds ratio, 95% confidence interval (CI), and p-values of ≤ 0.05 were considered statistically significant.

Results: The prevalence of HTN and T2DM was 27.3 and 7.4%, respectively. The co-occurrence of HTN and T2DM was 3.8%. The study found that being older (AOR = 3.97; 95 % CI: 1.80–8.74), khat chewing (AOR = 2.76; 95 % CI: 1.23–6.18), body mass index $\geq$ 25 kg/m^2 (AOR = 5.11; 95 % CI: 2.06–12.66), and sedentary behavior $\geq$8 h per day (AOR = 6.44; 95 % CI: 2.89–14.34) were statistically associated with co-occurrence of HTN and T2DM. On the other hand, consuming fruits and vegetables (AOR = 0.10; 95 % CI: 0.04–0.22) and a higher level of education (AOR = 0.39; 95% CI: 0.17–0.89) were negatively statistically associated with the co-occurrence of HTN and T2DM.

Conclusion: The co-occurrence of HTN and T2DM was prevalent among the study participants. This may create a substantial load on the healthcare system as an end result of increased demand for healthcare services. Therefore,

rigorous efforts are needed to develop strategies for screening employees to tackle the alarming increase in HTN and T2DM in university employees.

KEYWORDS

co-occurrence, hypertension, diabetes mellitus, university employees, Ethiopia

Introduction

Non-communicable diseases (NCDs) are the primary causes of morbidity and mortality globally. It causes 41 million death each year, equivalent to 71% of all deaths globally (1, 2). Almost 75% of all disease deaths and 82% of the 16 million people who died prematurely or before reaching 70 years of age occur in low- and middle-income countries (3) including 39.3% in Ethiopia (4).

Hypertension (HTN) and diabetes (DM) are global public health problems (5). They have been confirmed as two of the major risk factors for cardiocerebrovascular diseases as leading causes of death and disability among adults (6). It has been found that individuals with both HTN and DM have a greater risk of cardiocerebrovascular disease than those with only one condition (7). Hypertension (HTN) and DM are also the challenges of the healthcare system in low- and middle-income countries (8), due to changes in their diet habits and lifestyle and increased sedentary behavior (9, 10). Hypertension (HTN) and DM share common comorbidities and risk factors (11). Complications related to HTN and DM are reduced quality of life and productivity (12), such as the increased risk of stroke (13), cardiovascular diseases, end-stage renal disease (14, 15), retinopathy, depression, impaired health-related quality of life, and increased healthcare costs (16–18).

The coexistence of HTN and type 2 diabetes (T2DM) has been documented in previous studies (19, 20). Epidemiological studies have documented that DM predisposes to HTN (16, 17). For instance, a study in the USA showed that up to 75% of adults with diabetes also have hypertension (21), and in China, ∼15 million people have both HTN and DM (22).

In Ethiopia, about 1.7 million cases and 23,157 deaths were related to DM in 2019 (23). Moreover, evidence shows that the pooled prevalence of HTN was 21.8%, which was the highest prevalence reported in Addis Ababa at 25.4% and the lowest in the Tigray region at 15.4% (24). In Ethiopia, reports indicate that among university employees and civil servants in Addis Ababa, the prevalence of HTN was 13.9 and 27.3%, respectively (25, 26). Similarly, studies showed that DM among university employees and governmental civil servants at Guji Zone was 4.7 and 3.9%, respectively (25, 26). Moreover, studies showed that co-occurrence of HTN and T2DM accounted for ∼40–75% of patients (27).

Although the co-occurrence of HTN and T2DM is believed to be prevalent among university employees in Ethiopia, there is limited evidence. Therefore, this study aimed to assess the co-occurrence of HTN and T2DM among university employees in Eastern Ethiopia.

Materials and methods

Study area

This study was conducted among Haramaya University employees from December 2018 to February 2019. Haramaya University is located ∼510 kilometers east of Addis Ababa, Ethiopia. The university has ∼7,176 employees, 28.1% women and 71.9% men. In terms of job mix, 21.1% of the staff are academic and the other 78.9% are grouped under technical and administrative staff.

Study design and period

An institutional-based cross-sectional study was conducted among 1,200 university employees who were randomly selected. The study participants in the age range of 18 to 65 years and who were working for at least 6 months were included. Self-declared physical disability and pregnant women were excluded from the study. A single population proportion formula was used to calculate the sample size using OpenEpi 3.1. based on a previous study in China (22); double burdens of hypertension and T2DM were estimated as 3.8% with a 95% confidence level (the critical value $Z\alpha/2 = 1.96$), a 2% margin of error, and a 10% non-response rate. Finally, a total of 1,200 study participants were recruited. The sample was drawn using simple random sampling techniques based on the proportion to the size of their respective departments using the payroll roster as the sampling frame.

Data collection and tools (questionnaire)

Data were collected by trained nurses and laboratory technicians using a structured questionnaire adapted from the WHO STEPS, through face-to-face interviews, physical measurements, and biochemical tests. A pre-test was conducted on 5% of the final sample size at another nearby public university. Data collectors were trained on how to conduct interviews, anthropometric measurements, and field data collection procedures before directing them to gather data needed from data providers. Then, study participants were appointed on the next day morning in fasting condition to collect blood samples at the university clinics by trained medical laboratory technologists. The field research supervisors closely supervised the data collection process and checked the completeness and accuracy of the filled data on a daily basis. A field guide and data collection manual were used as a reference during the training. Standard

operating procedures (SOP) were followed starting from the sample collection up to reporting of the result.

Variables and measurements

The dependent variable is the co-occurrence of hypertension and T2DM. Blood pressure was measured using a digital measuring device (Microlife BP A50, Microlife AG, Switzerland) with participants sitting after resting for at least 10 min. Then, three times blood pressure measurements were taken in an interval of at least 5 min between consecutive measurements. In doing so, the mean systolic and diastolic BP from the second and third measurements was analyzed and diagnosed as hypertension when the mean systolic and diastolic blood pressure was greater or equal to 140/90 mm Hg (28). To determine blood glucose level and lipid profile, 6 ml of venous blood samples was collected from the participant's antecubital arm in a seating position after 8 h of overnight fasting. The collected samples were then directed into the sterile vacuum tube (Gel Clot Activator) and placed on the rack for 10–20 min for clotting formation.

The specimen tubes were then centrifuged at 3,000 revolutions per minute to extract the serum from the whole blood; fasting serum triglyceride and blood sugar were analyzed using the standard enzymatic colorimetric method (HUMAN Gesellschaft fur Biochemica und Diagnostica mbH Max-Planck-Ring, Germany), while HDL-c was analyzed using direct homogenous standard enzymatic assay colorimetric test (HUMAN Gesellschaft fur Biochemica und Diagnostica mbH Max-Planck-Ring, Germany) (29). Lipid profile and fasting blood glucose concentrations were reported in mg/dL (30). The prevalence of T2DM was defined as fasting blood glucose of ≥126 mg/dl or a self-reported diagnosis of diabetes (31).

Smoking status was categorized as never-smoking, current smoking (at least one cigarette per week), and former smoking (quit smoking more than 12 months) (32, 33). Current drinkers were those who drank once or more in the prior month of data collection. The habit of using Khat (Catha edulis) was grouped into never, occasional, and habitual (frequent). The total physical activity score was computed as the sum of all metabolic equivalent (MET)-minutes per week for vigorous-intensity physical activity, moderate-intensity physical activity, and walking. The sum of MET-minutes per week was categorized as high (3,000 MET-minutes or above), moderate (between 2,999 and 600 MET-minutes), and low (<600 MET-minutes).

Anthropometric data (weight, height, waist, and hip circumference) were collected according to the WHO STEPS manual (28). A BMI of ≥25.0 was defined as overweight/obese according to the WHO STEPS (31). Waist circumference was measured midway between the costal margin and the iliac crest with a tape line (31).

Statistical analysis

Data were double-entered into EpiData version 3.1 and cleaned, coded, and then transferred to STATA version 16 for analysis. The data were checked for missing values and outliers. The magnitude of hypertension, T2DM, and co-occurrence were summarized using proportion and frequency. A binary logistic regression model was used to determine factors associated with the co-occurrence of hypertension and T2DM. Information criteria (AIC/BIC) were used to select the final optimal model. The multicollinearity was checked using a scatter matrix and VIF. Interaction between variables was also checked, and the effect of omitted variable effect was tested using "ovtest." Model fitness was checked with Hosmer–Lemeshew test, overall model statistics were checked with "fitstat" command, and the *p*-value of the Hosmer–Lemeshew was found to be 0.25. The magnitude of variables with a *p*-value of <0.25 in bivariate analysis results obtained were taken for the multivariable analysis. Associations between outcome variables and covariates were reported with an adjusted odds ratio with a 95% confidence interval, and finally, statistical significance was considered at a *p*-value of <0.05.

Results

Socio-demographic characteristics of the study participants

A total of 1,200 employees were enrolled, with 1,164 providing data, resulting in a 97% response rate. The majority of study participants were men, 598 (51.4%) resulting in a sex ratio of 1:1.06. The mean age of the study participants was 35 (±9.4 SD) years, with a range of 20–60 years. In the majority of the study participants, 755 (64.9%) were non-office workers and 739 (63.5%) attended college and above education level. The mean reported per capita annual income of participants was 1,05,059.1 (±49,960.38 SD) (Table 1). Nearly 37 and 33% of study participants had high total cholesterol and triglyceride level.

Approximately half of the study participants, 571 (49.1%), had low physical activity and, 561 (48.25), were alcohol consumers. Moreover, 396 (34.5) were khat chewers, and 413 (35.5%) were overweight/obese. In addition, 131 (11.3) of the study participants were ever smokers. Table 2 shows details of the anthropometric, biochemical, and lifestyle characteristics of the subjects.

The prevalence of HTN and T2DM

The mean systolic and diastolic blood pressure were 124.4 (±16.6SD) mmHg and 79.5 (±10.5SD) mmHg, respectively. The overall prevalence of HTN was 290 (27.3 %); of which, 128 (28.3 %) were women and 162 (27.1 %) were men. Of these, 246 (21.1 %) were previously unknown that they had hypertension, and only 44 (3.8%) were known hypertensive individuals. The mean and highest values of fasting blood glucose were 87.69 (±29.62SD) mg/dL and 341 g/dL, respectively. This study shows that the overall prevalence of T2DM (≥ 126 mg/dL) after overnight fasting was 86 (7.4 %); of which, 40 (7.1 %) were women and 46 (7.7 %) were men. From these, 2.3% were known T2DM individuals and 5.1% were individuals with undiagnosed T2DM.

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TABLE 1 Socio-demographic characteristics of civil servants of Haramaya University in Eastern Ethiopia, 2019 ($n = 1,164$).

Socio-demographic variables	Category	Frequency (*n*)	Percent (%)
Sex	Male	598	51.4
	Female	566	48.6
Age in years	<45	941	80.8
	≥45	223	19.2
Occupation	Non-office worker	755	64.9
	Office worker	409	35.1
Level of education	Primary school (grade 1–8)	193	16.6
	Secondary school (grade 9–12)	232	19.9
	College and above (grade 12+)	739	63.5
Monthly salary income in ETB	<2,000	367	31.5
	2,000–4,000	328	14.4
	4,001–6,000	168	28.2
	>6,000	301	25.9
Ethnicity	Oromo	509	43.7
	Amhara	549	47.2
	Others[a]	106	9.1
Marital status	Never married	427	36.7
	Married	667	57.3
	Divorced/ widowed	70	6.0
Religion	Orthodox	722	62.0
	Muslim	219	18.8
	Protestant	197	16.9
	Others[b]	26	2.3

[a]Gurage, Tigraway, Harari, and Wolaita.
[b]Catholic, Traditional; ETB, Ethiopia Birr.

TABLE 2 Biochemical, anthropometric, and lifestyle characteristics of the study participants of Haramaya University employees, Eastern Ethiopia, 2019 (1,164).

Variable	Category	Frequency (*n*)	Percent (%)
LDL	Normal (<130 mg/dl)	903	77.6
	High (≥ 130 mg/dl	261	22.4
Total cholesterol	Normal (<200 mg/dl)	736	63.2
	High (≥ 200 mg/dl)	428	36.8
HDL	Desirable (≥ 50 mg/dl)	912	78.4
	Low (< 50 mg/dl)	252	21.6
Triglycerides	Normal (<150 mg/dl)	785	67.4
	High (≥ 150 mg/dl)	379	32.6
Waist circumference	Normal	619	53.2
	High	545	46.81
Body Mass Index	< 25 kg/m^2	751	64.5
	≥ 25 kg/m^2	413	35.5
Khat chewing	Yes	398	34.0
	No	768	66.0
Physical activity level	Low (≤600 MET min/week)	226	19.4
	Moderate (600–2,999 MET min/week)	367	31.5
	High (≥3,000 MET min/week)	571	49.1
Smoking status	Yes	151	11.3
	No	1,033	88.7
Alcohol consumption	Yes	561	48.2
	No	603	51.8
Serving fruits and vegetables	0 servings per day	56	4.8
	At least one-time servings per day	1,108	95.2
Sedentary behavior	<8 h per day	928	79.7
	≥8 h per day	236	20.3

Co-occurrence of HTN and T2DM and associated factors

Of the total study participants, 44 (3.8%) had both HTN and T2DM. Of these, 4.5% who had co-occurrence of HTN and T2DM were men (Figure 1).

In multivariable logistic regression analysis, age, intake of fruits and vegetables, khat chewing, educational status, BMI, and sedentary behavior were significantly associated with the co-occurrence of HTN and T2DM.

Participants aged 45 years and above were almost four times more likely to have the co-occurrence of HTN and T2DM compared to participants aged younger than 45 years. Employees who attended college and above were 61% less likely to have the co-occurrence of HTN and T2DM than those who attended

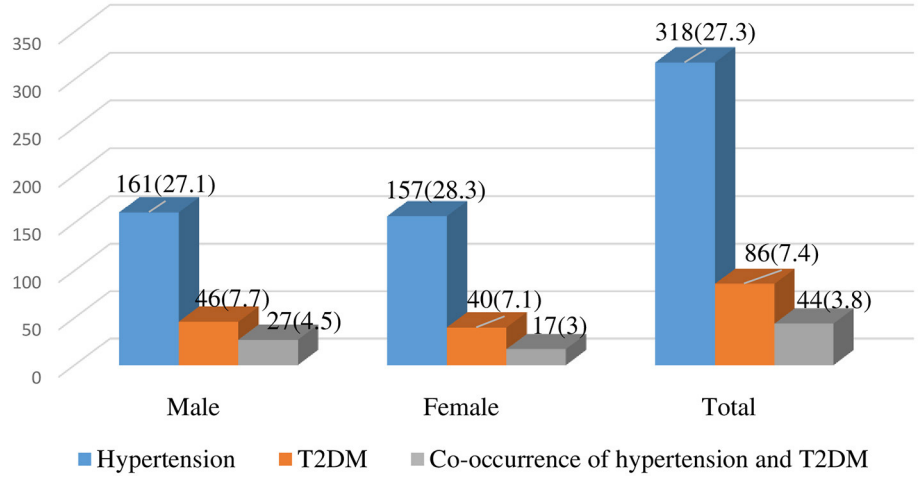


FIGURE 1
The prevalence of HTN, T2DM, and co-occurrence of HTN and T2DM among Haramaya University employees, Eastern Ethiopia, 2019.

less than or equal to secondary school. Khat chewers were nearly three times more likely to have the co-occurrence of HTN and T2DM as compared to non-khat chewers. Moreover, the odds of co-occurrence of HTN and T2DM were five times higher among those whose BMI was >25 kg/m^2 compared with those whose BMI was <25 kg/m^2. Similarly, the odds of co-occurrence of HTN and T2DM among participants having a sedentary lifestyle was approximately 6 times higher than those who do not live a sedentary life. On the other hand, study participants who had servings of fruits and vegetables at least once per day were 90% less likely to have co-occurrence of HTN and T2DM as compared to those who did not consume servings of fruits and vegetables (Table 3).

Discussion

This study found the overall prevalence of HTN, T2DM, and co-occurrence of HTN and T2DM were 27, 8, and 4%, respectively. Co-occurrence of HTN and T2DM was significantly increased with advanced ages, body mass index >25 kg/m^2, khat chewers, those with sedentary behaviors, and a higher level of education, while co-occurrence of HTN and T2DM was significantly lower among individuals those who reported consuming one or more servings of fruits and vegetables per day.

In this study, more than one in four employees had HTN. This finding is consistent with a study conducted in Wollo, North Ethiopia (34); Sidama, South Ethiopia (35); and Debre Birhan, North Ethiopia (36); and Addis Ababa, Ethiopia (25). However, the finding of this study is higher than that of the study conducted in Wolaita, Southern Ethiopia (37); Hawassa, Southern Ethiopia (38); and Togo (39). This may be due to the difference in study participant characteristics and study setting. For instance, our study population is university employees, whereas the others included working adults. In addition, the high prevalence of hypertension in our study could be due to a more sedentary lifestyle, increased pressure from the high workload, and changes in dietary patterns (40). However, the finding of this study is lower than study in Southern (41), northwestern (42) and northern Ethiopia (43), southern Nigeria (44) and Bangladeshi (45). This difference may be due to the sample size and setting of the study. Changing nutritional intake combined with increasingly sedentary lifestyles was reported to increase the emergence of chronic diseases such as hypertension in developing countries (9, 10).

Similarly, ~8% of the study participants were found to have T2DM. This is consistent with the study conducted in northern Ethiopia (43) and Addis Ababa (46), but the percentage of prevalence was higher than the study conducted in southern Nigeria (44) and Taiwan (47). This may be due to a high level of physical inactivity and poor consumption of fruits and vegetables in the study area and an unhealthy diet due to the entry site of a processed and packed diet. However, this finding is lower than the percentage of prevalence found in eastern Sudan (48) and north Sudan (49), Spain (50), and France (51). This may be due to working types and conditions, income, and educational status in urban centers of high-income countries that predispose them to stay home, consume unhealthy diets, and have multiple behavioral risk factors.

This study showed that ~4% of the study participants had both HTN and T2DM. This finding is consistent with studies conducted in Bangladesh (19) and China (22). This finding coincides with the fact that the lifestyle of working adults in Ethiopia has radically changed in the last decade due to the changing working environment, concentration of the middle-aged population, urban dwellers, risk of sedentarism, less physical activities in the workplace, better access to technology, and leisure lifestyle status. However, this finding is lower than the percentage of prevalence found in Ghana (52) and the USA (53). This difference may be due to the study units used (public employees in this study and any adult in the other two studies) and other socio-demographic and dietary patterns of the study population. Accumulating evidence has shown that hypertension affects the majority of individuals with diabetes

TABLE 3 Factors associated with co-occurrence of hypertension and type 2 diabetes among Haramaya University employees, Eastern Ethiopia, 2019 ($n = 1,164$).

Characteristics	Variable	HTN&T2DM co-occurrence		COR (95 % CI)	AOR (95 % CI)	*P*-value
		Yes (*n*/%)	No (*n*/%)			
Sex	Male	27 (4.5)	571 (95.5)	1	1	
	Female	17 (3.0)	549 (97.0)	0.65 (0.35–1.21)	0.52 (0.21–1.27)	0.150
Age group	<45 years	17 (1.8)	924 (98.2)	1	1	
	≥45 years	27 (12.1)	196 (87.9)	7.49 (4.00–14.00)	3.97 (1.80–8.74)	0.001
Level of education	≤ Secondary school	27 (6.4)	398 (93.7)	1	1	
	College and above	17 (2.3)	722 (97.7)	0.35 (0.19–0.65)	0.39 (0.17–0.89)	0.025
Marital status	Never married	5 (1.2)	422 (98.8)	1	1	
	Married	35 (5.3)	632 (94.7)	4.67 (1.82–12.03)	1.44 (0.46–4.53)	0.553
	Divorced/widowed	4 (5.7)	66 (94.3)	5.12 (1.34–19.54)	1.67 (0.30–9.42)	0.560
Ever cigarette smoking	Non-smoker	34 (3.3)	999 (96.7)	1	1	
	Smoker	10 (7.6)	121 (92.4)	2.69 (1.35–5.35)	0.97 (0.39–2.43)	0.950
Serving fruits and vegetables per day	None	20 (29.0)	49 (71.0)	1	1	
	At least once	24 (2.2)	1,071 (97.8)	0.06 (0.03–0.11)	0.10 (0.04–0.22)	0.000
Khat chewing	No	16(2.1)	752 (97.9)	1	1	
	Yes	28(7.2)	368 (92.8)	3.18 (1.73–5.83)	2.76 (1.23–6.18)	0.014
Body Mass Index	< 25 kg/m2	8 (1.1)	743 (98.9)	1	1	
	≥ 25 kg/m2	36 (8.7)	377 (91.3)	8.87 (4.08–19.27)	5.11 (2.06–12.66)	0.000
Sedentary behavior	< 8 h per day	12 (1.3)	916 (98.7)	1	1	
	≥ 8 h per day	32 (13.6)	204 (86.4)	11.97 (6.06–23.65)	6.44 (2.89–14.34)	0.000
Alcohol consumption	No	16 (2.7)	587 (97.3)	1	1	
	Yes	28 (5.0)	533 (95.0)	1.93 (0.03–3.60)	1.30 (0.58–2.88)	0.521
Family history of HPN	No	31 (3.3)	912 (96.7)	1	1	
	Yes	13(5.9)	208 (94.1)	1.84 (0.95–3.58)	1.47 (0.58–3.70)	0.418
Family history of DM	No	35 (3.3)	1,040 (96.7)	1	1	
	Yes	9 (10.1)	80 (89.9)	3.34 (1.55–7.20)	1.47 (0.49–4.44)	0.494

mellitus which dramatically increases the risk of morbidity and mortality from cardiovascular disease (54, 55). The co-existence of HTN and T2DM is a major contributor to the development and progression of microvascular and macrovascular complications, which in turn complicates the treatment strategy and increases healthcare costs.

This study showed that advanced age was significantly associated with the co-occurrence of HTN and T2DM. This finding is consistent with studies conducted in North Wollo Zone, Amhara Region (34); Wolaita Zone, Southern Ethiopia (37); Northwest Ethiopia (42) and Southern Ethiopia (38); Sidama Zone, South Ethiopia (35); and Ghana (56) where older age or advance in age is found to increase the risk of hypertension and/or diabetes. Over-abundant food consumption combined with a more sedentary lifestyle among older people mostly accounts for the increase in chronic non-communicable diseases (57, 58).

This study demonstrates that the co-occurrence of HTN and T2DM was 61% lower among public servants with a college and above education level. Consistent with this finding, studies showed that people who have a low socioeconomic status have a higher risk of non-communicable diseases (NCDs) than more advantaged groups. Social inequalities accounted for more than half of inequalities in major NCDs (59–61). Being educated is more effective in reducing unhealthy diet, substance use, physical inactivity, and better access to healthcare for timely diagnosis and treatment (62).

Study participants who chew khat were approximately three times more likely to have the co-occurrence of HTN and T2DM as compared to non-khat chewers. This finding is consistent with a study conducted in Southern Ethiopia (35) and Southwest Ethiopia (63), which indicates that the risk of either hypertension or diabetes was increased among people who chewed khat. Evidence shows that khat chewing has a significant effect on increasing fasting plasma glucose (64) and mean blood pressure (65).

Similarly, our study showed that employees who had sedentary behavior (≥8 h per day) are more likely to have the co-occurrence of HTN and T2DM than their counterparts. This finding is

consistent with studies conducted in Southern Ethiopia (41) and China (66). Insufficient physical activity is one important cause of most chronic diseases and results in substantial disease in quality of life (67, 68). Physical activity primarily prevents or delays chronic diseases. Preventing sedentary behavior with increased promotion of physical activity needs to be part of any healthcare system. BMI≥25 Kg/m^2 was among the modifiable risk factors found to increase co-occurrence of HTN and T2DM in this study. Similar studies in Ethiopia showed BMI≥25 Kg/m^2 or being overweight or obese (35, 36, 38, 69) increased the risk of HTN or diabetes or both. This finding is also consistent with a study by Tseng where "BMI/obesity is significantly linked to blood pressure/hypertension throughout the range of BMI in diabetic patients in either sex regardless of a previous hypertension history" (70). Dietary mediators seem to play a significant role in the pathogenesis of cardiovascular disease, among different socioeconomic layers (71).

In this study, study participants who had servings of fruits and vegetables at least once per day were less likely to have both HTN and T2DM as compared to their counterparts who do not consume servings of fruits and vegetables. A study conducted in Ghana (56) showed high fruit intake was associated with a lower risk of hypertension. Several studies showed that unhealthy diets and physical inactivity are well-recognized modifiable behavioral risk factors for NCDs (72–74). Studies showed that the majority of cardiovascular diseases can be attributed to major risk factors, such as low intake of fruits and vegetables rather than consuming more foods that are nutritionally poor and consuming energy-dense foods high in sugar and/or saturated fats or excessively salty (22). People with type 2 diabetes are encouraged to optimize dietary patterns to reduce their risk for cardiovascular diseases, such as diabetes and related comorbidities (75). This study revealed very critical evidence on the co-occurrence of HTN and T2DM, which can be very useful for policymakers in low- and middle-income countries for early interventions to limit the pace of such non-communicable diseases.

Limitations and strengths of the study

The limitation of this study is reliance on self-reporting, which contributes to recall and social desirability bias that potentially underestimates behavioral factors. This study may not be generalizable to all employees in Eastern Ethiopia as the study population was drawn only from one institution. However, the strengths of this study include large sample size and a standardized questionnaire based on World Health Organization's (WHO) STEP approach to collect data. Moreover, this study was the first of its kind among university employees in Ethiopia and can be fairly generalized for this category of workers in areas where there are contextual working adults.

Conclusion

Approximately 4% of Haramaya University employees had co-occurrence of HTN and T2DM. Factors associated with the co-occurrence of HTN and T2DM were older age, overweight/obesity, sedentary behavior, higher level of education, consumption of khat, and less intake of fruits and vegetables. These findings call for the health promotion interventional strategies targeting the aforementioned determinants. Increasing awareness of controlled consumption of "khat," lifestyle modifications, and strengthening job periodic screening programs of high-risk populations are recommended.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Health Research Ethics Review Committee (Ref. No. IHRERC/196/2018) of Haramaya University, College of Health and Medical Sciences. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AM conceived the study and drafted the proposal. AT, AM, and LR had substantial contributions to the study design and development of the data collection checklist and drafted, wrote, and corrected the manuscript. All authors read the manuscript and approved it.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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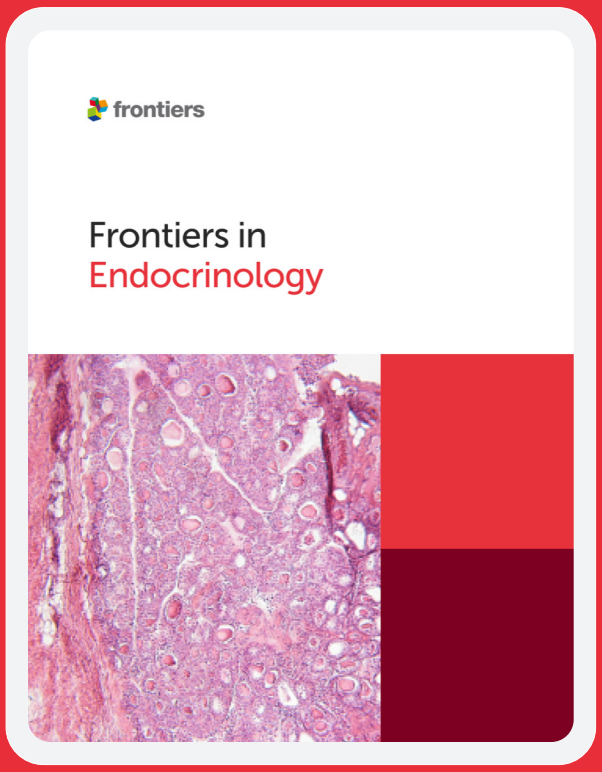
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