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Micronutrients and metabolic diseases

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Micronutrients and metabolic diseases

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Editorial: Micronutrients and metabolic diseases

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micronutrient, vitamin, mineral, iron, phytochemical, polyphenol

Editorial on the Research Topic
Micronutrients and metabolic diseases

Micronutrients, encompassing minerals, vitamins, and phytochemicals, actively involve in diverse metabolic processes, and play critical roles in the maintenance of the normal function of various systems within the body, including cardiovascular, digestive, immune, erythropoiesis, and bone health. A disruption or imbalanced intake of micronutrients will exert adverse impact on human health and potentially contributing to the development of metabolic diseases including but not limited to cardiovascular diseases (CVD), type 2 diabetes, and neurodegenerative diseases (1).

Dietary patterns such as Mediterranean diet and Dietary Approach to Stop Hypertension (DASH), characterized by their richness in antioxidant minerals, phytochemicals, vitamins, and unsaturated fatty acids that may improve cardiometabolic health, have been recommended as preventive or treatment approaches for metabolic diseases (2, 3), including cardiovascular disease and type 2 diabetes (4–6). Supplementation of certain micronutrient in at-risk populations has been proven to be a highly cost-effective intervention for improving metabolic diseases (1). However, the role of many micronutrients in metabolic diseases and the underlying regulatory mechanisms remains unclear. The underlying regulatory mechanisms governing the role of many micronutrients in metabolic diseases remain unclear, emphasizing the importance of continued investigation on the relationship in this field.

Research Topic "*Micronutrients and metabolic diseases*" comprises 22 articles covering the epidemiological and mechanistic studies investigating micronutrient metabolism and their influence on human health. Several articles in this Research Topic systematically evaluated the associations of micronutrients and cardiometabolic risk factors with risk of metabolic diseases. Zhang Y. et al. summarized the relationship among metal ion concentrations and multiple metabolic diseases based on recent studies using ionomic or multi-elemental profiling of different biological samples. Vahid et al. evaluated the association between micronutrient intake and obese/overweight in a case-control population consisting of 1,605 participants. Another prospective cross-sectional study reported that dietary magnesium and potassium intake were associated with lower body fat in 155 Chinese participants with impaired glucose tolerance (Chu et al.). Wan et al. investigated the associations between blood toxic and essential metals with blood lipids, and observed blood lead and blood magnesium concentrations were associated with the dyslipidemia in 998 participants living in Southern China. In a study involving 3,858 Chinese with papillary thyroid microcarcinoma, higher iodine intake was reported to be a risk factor for

nodal metastasis (Zhao et al.). Additionally, a case-control study with 1,012 participants found that dietary intakes of calcium, magnesium, iron, zinc, and copper were inversely associated with the odds of glioma (Zhang W. et al.). These findings suggest that mineral status could serve as valuable indicators for the early detection and prognosis of some metabolic diseases, emphasizing the significance of maintaining adequate and balanced intake of certain micronutrient for overall human health.

For the role of a specific mineral, four articles discussed the role of iron in metabolic diseases. Sun et al. investigated the predictive value of iron-regulatory hormone hepcidin for iron-deficiency anemia risk during pregnancy in a prospective study of 353 Chinese women. Qiu et al. explored the causal association between systemic iron status and 24 specific mental disorders, revealing a detrimental effect of higher body iron stores on depression and psychogenic disorder. A mechanistic study by Bengson et al. dissected iron homeostasis in neural cells, and revealed that ferritinophagy could be implicated in the pathogenesis of neurodegenerative diseases. In the review article by Bao et al., the mechanistic connection between abnormal iron metabolism and osteoporosis induced by diabetes mellitus was discussed.

Vitamin D deficiency or insufficiency has been identified as risk factors for cardiometabolic diseases (7). In this Research Topic, three original studies investigated the associations of vitamin D with cardiometabolic risk factors and related diseases. In a prospective cohort study involving 1,926 individuals, blood vitamin D concentration displayed a non-linear association with risk of type 2 diabetes during a mean follow-up of 3 years (Hu et al.). A cross-sectional study conducted by Shan et al. assessed the association of vitamin D with metabolic syndrome and related risk factors in 1,505 female Chinese (Hu et al.). Chen et al. performed a mechanistic study to evaluate whether vitamin D deficiency during pregnancy could alter the metabolism of glucose and lipids in offspring. Vitamin D deficiency during pregnancy generated adverse effects on the metabolism of glucose and lipids in offspring. Notably, these adverse effects cannot be rescued via vitamin D supplementation after weaning, suggesting that maternal vitamin D deficiency may elevate the risk of metabolic disease for offspring in adulthood (Chen et al.).

Four articles in this Research Topic explored the cardiovascular benefits of phytochemicals. Guo et al. comprehensively reviewed the antioxidant, anti-inflammatory, anti-hypertensive, and lipid-lowering effects of tea polyphenols, as well as the underlying molecular mechanisms. In a meta-analysis of 39 randomized controlled trials by Micek et al., the impact of polyphenols from 100% fruit juices on cardiometabolic risk factors was assessed. They found that anthocyanins were shown to decrease total cholesterol and low-density lipoprotein cholesterol in a dose-response manner (Micek et al.). Another cross-sectional analysis of 12,424 adults from the United States National Health and Nutrition Examination Survey reported that serum carotenoid concentrations were inversely associated with the odds of cardiovascular diseases, especially heart attack and stroke (Wang et al.). Moreover, Zhu et al. conducted a functional study to improve the delivery efficiency of phytochemicals nuciferine and epigallocatechin-3-gallate via loading them into microgel. Oral administration of microgel containing these phytochemicals reduced serum lipids of rats receiving high-fat diet, likely by modulating key genes involved in lipid metabolism and improving the diversity of gut microbiota (Zhu et al.).

Despite of abovementioned works, articles in this Research Topic also explored the associations of vitamin B1 (Wen et al.), niacin (Paolini et al.), zinc (Mitchell et al.), and calcium (Chiu et al.) with cardiometabolic diseases. These articles provided novel epidemiological evidence on the impact of micronutrients on health outcomes and offer mechanistic insights into micronutrient metabolism. The collective findings hold promise for guiding the development of healthier dietary patterns and more effective strategies for the prevention of cardiometabolic diseases.

Author contributions

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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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Preparation of microgel co-loaded with nuciferine and epigallocatechin-3-gallate for the regulation of lipid metabolism

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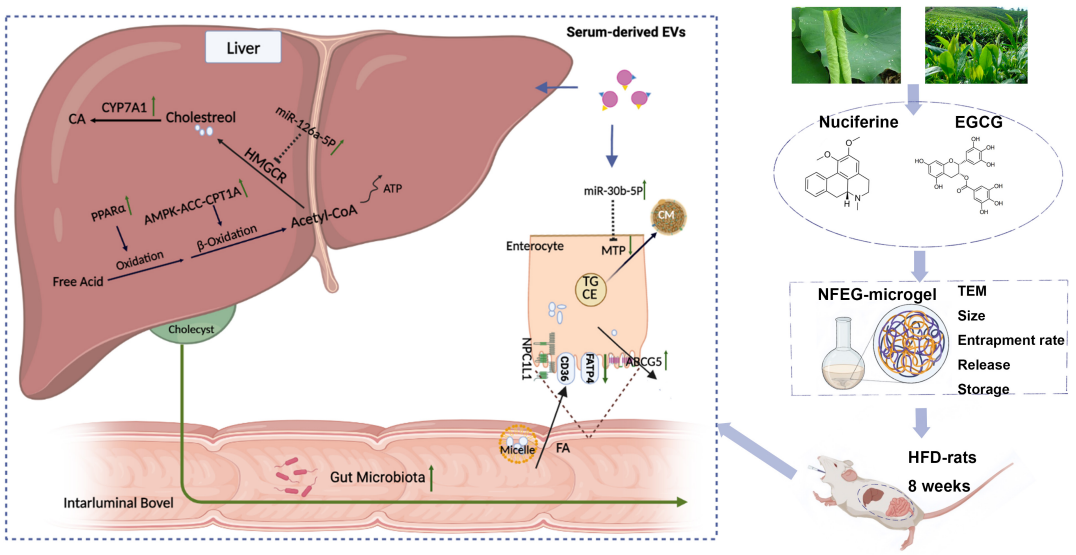
This study aims to enhance the stability and bioavailability of nuciferine (NF) and epigallocatechin-3-gallate (EGCG) by loading NF into liposomes and then incorporating the liposomes and EGCG into porous microgels (NFEG-microgel) prepared with chitosan and proanthocyanidin. Analysis of particle size (0.5–3.0 μm), electron microscopy, rheology, stability, and simulated gastrointestinal release confirmed that the prepared microgels had high encapsulation rate and good stability and release characteristics. Intervention experiments were performed by orally administering NFEG-microgel to high-fat diet rats to evaluate its efficacy and regulatory mechanism for blood lipid metabolism. NFEG-microgel intervention significantly reduced the body weight and serum lipid level, and the mechanism was related to the expression regulation of key genes involved in lipid metabolism and miRNAs (miR-126a-5p and miR-30b-5p) in serum extracellular vesicles. In addition, NFEG-microgel improved the diversity of gut microbiota by enriching short-chain fatty acids (SCFA)-producing bacteria and reducing harmful bacteria, suggesting that it can ameliorate lipid metabolism by regulating the intestinal flora community in rats.

KEYWORDS

nuciferine, epigallocatechin-3-gallate, microgel, miRNAs, lipid metabolism genes, gut microbiota

Introduction

Nuciferine (NF, PubChem CID: 10146) is an aromatic ring-containing alkaloid extracted from *Nelumbo nucifera* leaves and has a wide variety of pharmacological activities, such as lipid lowering (1), anti-inflammatory, antioxidant, anti-liver injury, and anticancer (2). NF also inhibits the accumulation of lipid in 3T3-L1 preadipocytes by regulating the expression of lipid gene (3) and improves the lipid



GRAPHICAL ABSTRACT

NFEG-microgel was prepared and intervention experiments were performed by orally administering NFEG-microgel to high-fat diet rats to evaluate its efficacy and regulatory mechanism for blood lipid metabolism. The amelioration of lipid metabolism was relative to regulating the expression of key genes which interacted with miR-126a-5p and miR-30b-5p in serum extracellular vesicles and the intestinal flora community in rats.

profile in mouse diabetic model by activating peroxisome proliferator-activated receptors-α (PPAR-α)/peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) (4). In addition, NF treatment changes the composition of the intestinal microbiota of rats fed with high-fat diet (HFD) (5).

Epigallocatechin-3-gallate (EGCG), the major polyphenolic catechin of green tea, is the most abundant antioxidant catechin accounting for about 50∼80% of total catechins content. Although the biological effects of EGCG have not been completely discovered, it can enhance lipid metabolism, has anti-inflammatory and antioxidant activities, and can reduce tumor incidence. Increasing evidence indicates that EGCG has a wide range of biological activities, including activating AMP-activated protein kinase (AMPK), promoting lipid metabolism, and improving insulin resistance, which has a multipronged preventive and therapeutic effect on non-alcoholic fatty liver disease (NAFLD) (6). In addition, EGCG can improve different types of liver injury (7).

Nuciferine has extremely low water solubility and dispersion, and its oral bioavailability is only 3.9% (8). Its stability, bioavailability, and absorptivity can further decline due to gastrointestinal conditions, which is similar to the trend of the stability and bioavailability of EGCG. In humans, the maximum plasma concentration of EGCG is only 0.15 μM after consuming two cups of green tea (4). The double-layer encapsulation of liposome and microgel is adopted to improve the stability and bioavailability of NF and EGCG. In this process, NF and EGCG are encapsulated simultaneously to play a synergistic role at different points in the metabolic cycle. Microgels (d. 1∼350 μm) are swellable polymer networks with hydrophilic functionalities that allow them to entrap large amounts of water without collapse and are usually prepared by polymer (polysaccharides and/or proteins) cross-linking through ionic, physical, or covalent interactions (9). Microgels have been widely used in the encapsulation of various unstable substances, such as astaxanthin (10) and polyphenols (11), to improve their stability and bioavailability and control their release. *Myrica rubra* leaf proanthocyanidin extract (MLPE) and chitosan are abundant natural materials widely used in the food industry because of their safety and good functional properties. Chitosan coating can increase the stability of liposomes in various biological fluids including simulated gastric fluid (SGF) and simulated small intestinal fluid (SIF), the adhesion of mucosa, and the solubility of drugs (12).

Lipid metabolism is a process of lipid synthesis and degradation in cells, including lipid decomposition, storage for energy and synthesis of structural and functional lipids. Its main function is to transport lipids to the peripheral tissues for use or to transport lipids back to the liver for recycling and removal. And their disorders will lead to the occurrence of many diseases that seriously endangers people's life and health. According to the functional division, lipid metabolism is roughly composed of three parts: exogenous lipid absorption, endogenous lipid synthesis, and adverse cholesterol transport (13). Lipid metabolism disorders lead to the increase of total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C), and the decrease of high-density lipoprotein cholesterol (HDL-C) which can induce a variety of diseases such as non-alcoholic fatty liver disease and varying evolutions. The liver is a vital organ of the

regulation of lipid steady state. Excessive accumulate of lipid in the liver and overloaded liver cause subsequent series of inflammation, insulin resistance, oxidative stress, mitochondrial dysfunction, and intestinal flora disturbance, in the occurrence and development of NAFLD, resulting in multiple attacks on the liver (14). And the published literatures show that changes in the expression or activity of miR-34a, miR-33, miR-122, and miR-21 are the key mechanisms responsible for the development of NAFLD and its progression toward a serious status (15). Animal studies elucidated that the intestinal microbiota, which is consistently rich in proteobacteria, may have a causal role in NAFLD (16).

In our study, NFEG-microgel was prepared, characterized, and used to prevent and inhibit dyslipidemia or NAFLD. Its regulating mechanism for lipid metabolism disorder was also investigated and clarified.

Materials and methods

Materials and instruments

Nuciferine was bought from MedChemExpress Co., Ltd. (Shanghai, China, purity > 98%). Standard EGCG was obtained from Beijing Solarbio Technology Co., Ltd. (Beijing, China, purity > 98%). Chitosan of medium molecular weight was purchased from Sigma–Aldrich (St. Louis, MO, USA, purity > 75%). Lipo3000 transfection reagent was acquired from Thermo Fisher Co., Ltd. TRIzol® Plus RNA Purification Kit and Transcriptor First Strand cDNA Synthesis Kit were bought from Thermo Fisher (Waltham, MA, USA). Dual-luciferase reporter assay system was purchased from Promega Co., Ltd. (Madison, WI, USA).

The following instruments were used in this study: Zetasizer Nano ZS90 (Malvern Instruments Co., Ltd., Malvern, UK), MIKRO 22R centrifuge (Kirchlengern, NRW, Germany), OLYMPUS BX60 fluorescent microscope camera (OLYMPUS Corporation, Tokyo, Japan), transmission electron microscope (TEM) (Hitachi, Tokyo, Japan), and CFX384 multiplex real-time fluorescence quantitative PCR instrument (Bio-Rad, Hercules, CA, USA).

Manufacturing of nuciferine-loaded liposomes

Nuciferine-loaded liposomes (NF-liposomes) were manufactured by solvent evaporation and ultrasonic homogenization following a previous method (17) with some modifications. In brief, 1 g of soybean phospholipids (Shanghai Taiwei Pharmaceutical Co., Ltd., Shanghai, China) and 0.2 g of cholesterol (Shanghai Titan Chemical Co., Ltd., Shanghai, China) were dissolved in trichloromethane and stirred with magnetic force. After complete distribution, 0.2 g of NF dissolved in methanol was added and the mixture was stirred evenly. The organic solvent was evaporated by rotation (30 rpm) at 30°C to form a light-yellow film, which was then dispersed with 0.05 M phosphate buffered saline (PBS). Ultrasonic crushing was performed in an ice bath for 6 min to obtain a translucent liposome solution. Finally, particle size, zeta potential, and polydispersity index (PDI) were analyzed by Zetasizer Nano ZS90. Encapsulation rate was determined by high-performance liquid chromatography (HPLC).

Preparation of chitosan-procyanidin microgels loaded with nuciferine and epigallocatechin-3-gallate

Chitosan–procyanidin microgels were prepared following the methods of Zhang et al. (18) and Zou et al. (19) with some modifications. Chitosan was dissolved in 1% acetic acid solution to a concentration of 6 mg/ml. The liposome solution loaded with NF was mixed with chitosan solution, and magnetic stirring was performed for 1 h. MLPE with purity of 70% and average polymerization degree of 6.7 was extracted in our lab. The two concentrations of MLPE (2 and 6 mg/ml) were added to the above solution (EGCG was dissolved in advance so that the final concentration was 2 mg/ml), and magnetic stirring was performed for at least 3 h to obtain NF-EGCG double-encapsulated microgel (NFEG-microgel). After overnight refrigeration, the encapsulation rate and stability of the two concentrations of MLPE microgels were compared. The results showed that the encapsulation rate was high and stable upon the addition of 2 mg/ml MLPE in the microgel. Thus, subsequent microgels were prepared with this concentration.

Characterization

Encapsulation efficiency

After the preparation of microgels, encapsulation efficiency was determined by ultrafiltration centrifugation. In brief, 0.5 ml of NFEG-microgel solution was centrifuged in an ultrafiltration centrifuge tube at 4,000 g for 30 min. The liquid supernatant of the outer tube, which contained unencapsulated NF and EGCG, was obtained. The content of NF and EGCG was determined by HPLC, and the encapsulation efficiency of NFEG-microgel was calculated according to the following formula:

$$\text{Encapsulation efficiency} = \frac{W_1 - W_2}{W_1} \times 100\%,$$

where W1 is the total NF or EGCG weight of NFEG-microgel, and W2 is the weight of NF or EGCG in the supernatant.

Morphological observation

The sample was first dispersed and dropped onto a carbon coppered grid, and the morphological characteristics

of the microgel prepared with chitosan and MLPE were photographed by TEM.

Average particle size, polydispersity index, and zeta potential

Particle size was measured by LS13 320 Laser particle size analyzer (Beckman coulter, Brea, CA, USA), and zeta potential, and PDI were analyzed by Zetasizer Nano ZS90 after the sample was dispersed.

Rheological property analysis

The rheological properties of samples were determined using Discovery Hybrid Rheometer (TA Instruments, Wilmington, DE, USA) (20). The measured shear rate (τ) increased from 0.01 to 500 s^{-1} and was used to evaluate the viscosity and shear stress of the microgel.

Simulated gastrointestinal digestion of NFEG–microgel *in vitro*

In brief, 5 ml of microgel was first digested in 5 ml of SGF with pH of 2.0 for 2 h, followed by the addition of 10 ml of SIF and incubation for 6 h. The pH was adjusted to 7.0 with 0.1 M $NaHCO_3$ solution following a previous method with some modifications (11). Incubation was carried out in a shaker under 120 rpm/min and 37°C. The samples were extracted at 0.5, 1, 2, 4, 6, and 8 h, and the same volume of release media was added. SGF composed of 0.9 g of NaCl and 0.14 g of pepsin (1:3,000, Beijing Solarbio Technology Co., Ltd., Beijing, China) was dissolved in 100 ml of 0.1 mol/L HCl aqueous solution at a final pH of 2.0. The simulated SIF was composed of 0.225 g of trypsin (1:250, Hefei Bomei Biotechnology Co., Ltd., Hefei, China) and 1.125 g of pig bile salt (China National Institute for Food and Drug Control, Beijing, China).

Storage stability

Nuciferine solution dissolved in 0.5% sodium carboxymethyl cellulose, EGCG solution, and NFEG-microgel were stored at 4°C for 12 weeks at their corresponding concentrations. Their storage stability was evaluated by their apparent appearance and encapsulation rate (21).

Animal sample collection and processing

Thirty healthy male Wistar rats (180 ± 20 g) with special pathogen free were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China) with animal license number SCXK (Shanghai, China) 2017-0005. The rats were raised in standard conditions (temperature 25 ± 1°C, relative humidity 40–60%, and 12 h light/dark cycles) and had free access to food and water. Their dietary consumption was recoded daily. All animal procedures were permitted by the Laboratory Animal Ethics Committee (2022-005) of China Jiliang University (Hangzhou, China). After a week of acclimatization, the rats were randomly divided into the following six groups with five rats each: blank control (NC), HFD, NF-loaded microgel treatment with high-fat diet (HFD + NF), EGCG-loaded microgel treatment with HFD (HFD + EGCG), NF and EGCG co-loaded microgel treatment with HFD (HFD + NFEG), and empty microgel treatment with HFD (HFD + MG). The NC group received commercial normal diet, and the remaining groups received HFD purchased from Fanbo Animal Feed Biotechnology Co., Ltd. (Shanghai, China) and consisted of 10% lard, 10% yolk powder, 6% casein, 3% maltose, 2% cholesterol, 1.2% premix, and 0.1% cholate. The rats in HFD + NF group intragastrically received NF at 20 mg/kg/day, those in HFD + EGCG group intragastrically received EGCG at 20 mg/kg/day (22), and those in HFD + NFEG group intragastrically received the same NF (20 mg/kg/day) and EGCG (20 mg/kg/day) for 8 weeks (23), The volume of the treatments was 10 ml/kg of rat body weight. The rats in NC and HFD + MG groups intragastrically received the same volume of water and empty microgel, respectively, for 8 weeks. Food intake was recorded daily, and body weight was monitored weekly. Blood samples were collected from the tail every 2 weeks and then centrifugated at 1,500 × g for 15 min at 4°C to separate the serum. The biochemical indexes of the serum were detected using the corresponding kits. Triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL-C), and low-density lipoprotein (LDL-C) kits were purchased from Thermo Fisher Co., Ltd. (Waltham, MA, USA). At the end of the experiment, the rats were sacrificed. Blood, liver, small intestine, and cecum contents were obtained and stored at −80°C. The liver was sectioned, preserved in formalin fixative, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E).

Real-time quantitative PCR analysis

Total RNA of liver and small intestine tissues was extracted using TRIzol® Plus RNA Purification Kit following the manufacturer's protocol. Single-stranded cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit. Real-time quantitative PCR (RT-PCR) was conducted to determine the expression of PPARα, cholesterol 7 alpha-hydroxylase A1 (CYP7A1), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), adenosine monophosphate activated protein kinase (AMPK), ACC in the liver, carnitine palmitoyl transferase 1A (CPT1A), and ATP-binding cassette G5 (ABCG5), fatty acid translocase CD36 (CD36), Niemann-Pick C1-like 1 (NPC1L1), fatty acid transport protein 4 (FATP4), and microsomal triacylglycerol transfer protein (MTP) in the small intestine mRNA. Quantitative PCR primers were designed using Primer Premier 6.0 and Beacon Designer 7.8 and synthesized by Shenggong Bioengineering Co., Ltd. (Shanghai, China). RT-PCR was performed with PowerUp™ SYBRTM Green Master Mix (Applied Biosystems, Foster City, CA, USA) using CFX384

Real-Time Fluorescence Quantitative PCR System (Bio-Rad, Hercules, CA, USA). The primer sequences are shown in Table 1. The reaction conditions were as follows: 95°C for 1 min and 40 cycles of 95°C for 15 s and 63°C for 25 s to collect fluorescence. The expression of genes was normalized in reference to the housekeeping gene GAPDH, and the relative gene expression in the six groups was statistically analyzed using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Radio immunoprecipitation assay lysis. buffer including protease inhibitor cocktail (Thermo Fisher, Waltham, MA, USA) was used for the extraction of the total proteins of liver and small intestine, and the protein concentration was determined using BCA quantitative kit (Beyotime, Shanghai, China). The total extracted proteins were separated by SDS-PAGE for 2 h and then transferred to a PVDF membrane for 2 h. The membrane was incubated with T-TBS (containing 5% BSA) for 1 h and subsequently with the following primary antibodies: rabbit anti-CYP7A1 (1:1,000, Biorbyt orb539102, Cambridge, UK), anti-PPARα (1:1,000, Abcam ab126285, Cambridge, UK), anti-ABCG5 (1:1,000, Proteintech 27722-1-AP, Chicago, IL, USA), anti-NPC1L1 (1:500, Thermo Fisher PA5-72938, Waltham, MA, USA), anti-CPT1(1:500, Abcam ab128568, Cambridge, UK), and anti-GAPDH (1:10,000, Abcam ab181602, Cambridge, UK) as internal control at 4°C overnight. After being washed with T-TBS, the membranes were incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (1:5,000, Thermo Pierce, Waltham, MA, USA) and goat anti-rabbit IgG secondary antibody (1:5,000, Thermo Pierce, Waltham, MA, USA) at room temperature for 1 h. Protein expression was visualized on X-ray films using SuperSignal® West Dura Extended Duration Substrate (Thermo Pierce, Waltham, MA, USA). Image J 1.8.0 was used to analyze the optical density values of bands, and each test was repeated three times.

Isolation and characterization of serum extracellular vehicles

The cell fragments in the serum were removed by centrifugation at 10,000 rpm for 30 min, and the supernatant was transferred to Beckman L-100XP Ultracentrifuge (Brea, CA, USA) at 100,000 × g for 75 min and then discarded. The precipitate was washed and resuspended with PBS for another centrifugation at 100,000 × g for 75 min (24). After centrifugation, the supernatant was discarded, and the precipitate was resuspended with 200 μl of PBS to obtain serum extracellular vehicles (EVs). Quantification was performed using BCA kits (Thermo Fisher Co., Ltd., Waltham, MA, USA). In characterizing EVs, three or more proteins must be reported in at least a semi-quantitative manner, single vesicles must be examined, and the size distribution of EVs must be measured (25). EVs were characterized by TEM HT7700, and the average EV particle size was analyzed by Flow NanoAnalyzer N30E (Xiamen Fuliu Biological Technology Co., Ltd., Xiamen, China). EV-labeled proteins tetraspanins (CD9 and CD63) and endosome or membrane-binding proteins (TSG101) were quantitatively detected by Western blot.

QRT-PCR assay for miRNAs in serum extracellular vehicles

For the selection of miRNAs closely related to the mRNAs of lipid metabolism, the species was first selected as rats on miRDB.[1] Several mRNAs related to lipid metabolism were then imported. One example is CYP7A1, that is, all miRNAs related to CYP7A1 gene were analyzed. For reliable results, all the miRNAs related to CYP7A1 gene obtained from the last website were inputted in another web site TargetScanMouse.[2] Hundreds of related genes were obtained for each miRNA. CYP7A1 was searched in this list. If the results overlap, then this miRNA will be selected as an alternative. Finally, miR-21-5p, miR-30b-5p, miR-33-5p, miR-27a-3p, and miR-126a-5p were identified as possibly interacting with target genes PPARα, MTP, CYP7A1, ABCG5, and HMGCR, respectively (14).

First, serum EV miRNA was extracted as follows. EVs were added with 300 μl of binding buffer, shaken evenly, and centrifuged at 12,000 *g* for 10 min. The supernatant was transferred to a spin cartridge, and the precipitate was retained and added with anhydrous ethanol to obtain a final ethanol concentration of 70%. The mixture was transferred to the second spin cartridge and centrifuged at 12,000 *g* for 1 min. The waste was discarded, and the sample was centrifuged again at 12,000 *g* for another 1 min after 500 μl of wash buffer was added to the spin cartridge. The waste liquid was discarded, and the above steps were repeated. Idling centrifugation (12,000 g, 5 min) was performed to dry the adsorption column, and centrifugation was conducted at 12,000 *g* for 2 min. Finally, the spin cartridge was added with 50 μl of RNase-free ddH_2O and then stored at −80°C after being placed at room temperature for 2 min (26). qRT-PCR was performed in line with the above steps. The reverse-transcription primer sequences are shown in Table 2, and the qRT-PCR primers are displayed in Table 3.

Double luciferase assay

293T cells in logarithmic growth phase were seeded in 24-well plates and transfected with Lipofectamine™ 3000 Transfection Reagent at approximately 60% confluence.

1 http://www.mirdb.org

2 https://www.targetscan.org/vert_80/

TABLE 1 Real-time PCR primers in rat liver and small intestine.

Gene	GenBank accession	Reverse transcription primer sequences (5′–3′)	Size (bp)
Rat GAPDH	NM_017008.4	GAAGGTCGGTGTGAACGGATTTG	127
		CATGTAGACCATGTAGTTGAGGTCA	
Rat PPARα	NM_013196.2	GGAGGCAGAGGTCCGATT	131
		TCAGCAAGGTAACCTGGTCATTCAA	
Rat CPT1A	NM_031559.2	GCACATTAGACCGTGAGGAACT	138
		CCTTGATATGTTGGATGGTGTCTGT	
Rat ACC	NM_022193.1	GAGGTTGGCTATCCAGTGATGA	102
		CTGTCTGAAGAGGTTAGGGAAGT	
Rat CYP7A1	NM_012942.2	CAAGACGCACCTCGCTATTCTCT	113
		CTTCAGAGGCTGCTTTCATTGCT	
Rat HMGCR	NM_013134.2	CCTGCGTGTCCCTGGTCCTA	125
		CCTTTGGGTTACTGGGTTTGGT	
Rat AMPK	XM_008763901.1	GATTTGCCCAGTTACCTCTTTCC	156
		CACTGCGAGCTGGTCTTGA	
Rat CD36	AF072411.1	CGGTTGGAGACCTACTCATTGA	147
		CCACTTCCTCTGGGTTTTGC	
Rat FATP4	NM_001100706.1	CCTCTACCACTCAGCAGGAAA	156
		CGGCAAAGCTCACCAATGTAC	
Rat ABCG5	NM_053754.2	CTTCTGTGCCAAATAACCCAATG	135
		GGATGACAAGAGTCGGGATGAA	
Rat NPC1L1	NM_001002025.1	GCTGCTGTTTCTGACCCTGTTT	141
		CCCACTTCAAGGTATCGGTTCAG	
Rat MTP	NM_001033694.1	GTTCTCCCAGTACCCGTTCTTGGT	100
		CCTCCCTGTGGATAGCCTTTCAT	

TABLE 2 Reverse transcription primer sequences of miRNAs.

Gene	GenBank accession	Reverse transcription primer sequences (5′–3′)
rno-mir-27a-3p	MIMAT0000799	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCGGAA
rno-mir-33-5p	MIMAT0000812	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCAAT
rno-mir-126a-5p	MIMAT0000831	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGCGTA
rno-miR21-5p	MIMAT0000790	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA
rno-miR30b-5p	MIMAT0000806	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCTGA

Before transfection, the medium was replaced with a fresh one. MiRNA-30b-5p mimics and miRNA-126a-5p mimics were prepared at 20 pmol/well, and recombinant plasmid pmirGLO-MTP-WT or pmirGLO-MTP-MUT and pmirGLO-HMGCR-WT or pmirGLO-HMGCR-MUT were added at 500 ng/well. The 24-well plates with fresh medium were placed in an incubator at 37°C with 5% CO_2 for 8 h. After 48 h, each well was washed with PBS twice and added with 250 μl of 1 × PLB lysate before the cells were lysed at room temperature. Approximately 100 μl of LAR II was absorbed in a black 96-well plate, followed by the addition of 20 μl of lysis solution. After the reading of mixture was checked, Stop & Glo substrate (100 μl) was added within 10 s and the new reading was recorded again.

TABLE 3 Real-time PCR primers of miRNAs.

Gene	Forward primer and universal primer (5′–3′)
rno-mir-33-5p-F	CGCGGTGCATTGTAGTTGC
rno-mir-27a-3p-F	GCGCGTTCACAGTGGCTAAG
rno-mir-126a-5p-F	GCGCGCATTATTACTTTTGGTACG
rno-miR21-5p-F	GCGCGTAGCTTATCAGACTGA
rno-miR30b-5p-F	GCGCGTGTAAACATCCTACAC
Universal reverse primer (micro-R)	AGTGCAGGGTCCGAGGTATT

Gut microbiota composition analysis

Microbial DNA was extracted from rat cecal content samples with the Fast DNA SPIN kit (MPBIO, CA, USA) in

accordance with the instructions. The V4–V5 region of the bacteria 16S ribosomal RNA gene was amplified by PCR under specific conditions (95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min) and using primers 515 F (5′-barcode-GTGCCAGCMGCCGCGG-3′) and 907 R (5′-CCGTCAATTCMTTTRAGTTT-3′); the barcode of which has an eight-base sequence unique to each sample. Amplicons were extracted from 2% agarose gels, purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor^{TM}-ST (Promega, Madison, WI, USA). The Purified PCR products were quantified by Qubit®3.0 (Life Invitrogen). The pooled DNA product was used to construct Illumina Pair-End library following Illumina's genomic DNA library preparation steps. The amplicon library was then paired-end sequenced (2 × 250) on an Illumina Novaseq platform (Mingke Biotechnology Co., Ltd., Hangzhou, China). The image data files obtained by high-throughput sequencing were converted into sequencing reads, which were then stored in FASTQ files including their sequence information and sequencing quality information. For reliable results in subsequent analysis, the raw data were first filtered through Trimmomatic v0.33, CutAdapt 1.9.1 and custom Perl scripts were then used to identify and remove primer sequences, and high-quality reads without primer sequences were finally generated. USEARCH (version 10[3]) was employed to assign sequences with ≥ 97% similarity to the same OTUs. Tukey's method and R software were used for the comparison of alpha diversity index between samples (P = 0.05). Beta diversity analysis and mapping were performed on QIIME (27) to reveal differences and similarities among the samples as measured by principal coordinate analysis (PCoA) with R software. LEfSe method was applied to compare the differential abundances of bacteria among groups at family and genus levels (28). Only those taxa with a log LDA score > 4 were considered.

Statistical analysis

Data were shown as mean ± standard deviation (SD). Statistical differences were analyzed by one-way ANOVA with Tukey's multiple comparison test using SPSS 18.0 ($P < 0.05$ indicated significant differences).

Results

Characterization of NFEG-microgel

The encapsulation rates of NF and EGCG by NFEG-microgel were 90 and 86%, respectively, and that of NF by liposomes was 93.5%. The average particle size, PDI, and zeta potential of NF-liposome were 104.8 nm, 0.226, and +59.8 mV, respectively, and those of NFEG-microgel were 2595 nm, 0.532, and −41.6 mV, respectively. Absolute zeta potentials greater than 30 were considered as stable. The microstructural morphologies of NFEG-microgel were observed by TEM as shown in **Figure 1A**. All the microgels had regular spherical or subspherical shape with neat edges. The average particle size of NFEG-microgel was 0.5∼3.0 μm (**Figure 1B**), which was in line with the above results. According to the rheological data, the shear viscosity of NFEG-microgel decreased with the increase in shear rate. The mathematical power-law function model was fitted to the obtained data using the cf tool in MATLAB R2020a to determine the consistency index k and fluidity index n. The fitting coefficient R^2 was 0.99, indicating that NFEG-microgel conforms to the power-law fluid, that is, it is a non-Newtonian fluid. The k value was 0.899, and the n value was 0.265. If $n < 1$ in power-law fluid, then NFEG-microgel is a fake plastic fluid (**Figure 1D**).

Release

The NF in NFEG-microgel had a good release ability under SGF and SIF conditions with release rates of 70.6% at 0.5 h in SGF and 85.5% at 4 h in SIF. A high release rate was maintained up to 8 h. However, the release characteristics of EGCG in NFEG-microgel differed from those of NF. EGCG was released slowly in SGF and reached about 48% release rate at 2 h. Meanwhile, NFEG-microgel entered the digestive SIF, promoting the sustained release of EGCG in the microgel. The release rate reached 71.5% at 4 h and remained high up to 8 h (**Figure 1C**). The microgels protected EGCG and ensure its slow and sustained release because chitosan coating can increase the adhesion of mucosa.

Storage stability

The storage stability of NFEG-microgel was evaluated by its apparent appearance upon storage at 4°C for 12 weeks. **Figures 1E,F** show that even after being stored for 12 weeks, the freshly prepared NFEG-microgel still showed good stability in terms of color, uniformity, and encapsulation rate and maintained 76 and 68% encapsulation rates for NF and EGCG, respectively. Meanwhile, NF solution of the same concentration dissolved in 0.5% sodium carboxymethyl cellulose exhibited precipitation at the 1st week, indicating the conventional suspension dosage form of NF had disadvantages such as uneven dispersion and instability. The same concentration of EGCG solution exhibited discoloration at the 4th week, indicating that the stability of conventional solution was poor (**Figure 1E**). In conclusion, NFEG-microgel increased the water solubility, dispersion, and stability of NF and enabled its sustained and stable release. Moreover, NFEG-microgel protected the storage stability of EGCG, which is easily oxidized and discolored when placed in a complex external environment. NFEG-microgel also

3 http://drive5.com/uparse/

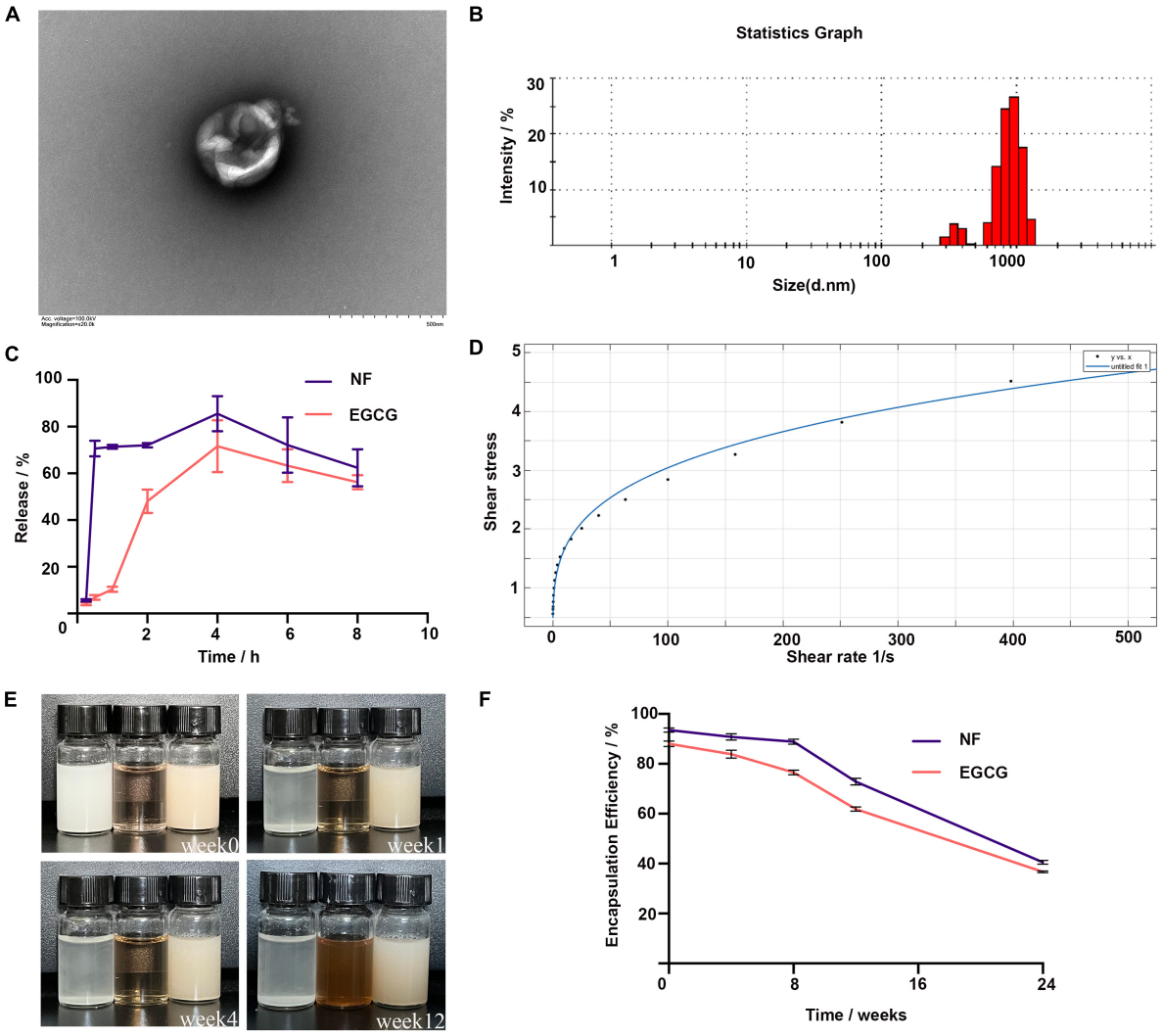


FIGURE 1
Characterization of NFEG-microgel. **(A)** NFEG-microgel observed by TEM, **(B)** average particle size of NFEG-microgel, **(C)** simulated NFEG-microgel digestion in SGF and SIF, **(D)** rheological fitting curve, **(E)** storage of NFEG-microgel at 4°C for 12 weeks (from left to right, NF solution, EGCG aqueous solution, and NFEG were shown), and **(F)** encapsulation rates during storage for 24 weeks.

inhibited the direct digestion and destruction of EGCG in the intestine due to the adhesion of chitosan to intestinal mucosa.

NFEG-microgel significantly reduced the body weight and ameliorated lipid metabolism disorder in high-fat diet rats

The liver of the rat in each group was photographed (Supplementary Figure 1). In terms of appearance, the liver of rat in the HFD + NF, HFD + EGCG, HFD + NFEG, HFD + MG, and HFD groups was larger and showed different degrees of yellowing compared with that in the normal group. The liver color of the HFD + NFEG group was the closest to that in the normal group. The body weights of different groups showed an increasing trend. At the end of HFD feeding for 8 weeks, the body weight of rats was significantly higher in the HFD group than that in the NC group but lower than that in the HFD + NF, HFD + EGCG, HFD + NFEG, and HFD + MG groups. The HFD + NFEG group showed the most remarkable reduction in body weight (Figure 2A). The total 8-week feed consumption in kilograms (kg) for the NC, HFD + NF, HFD + EGCG, HFD + NFEG, HFD + MG, and HFD groups was 5.67, 5.31, 5.04, 5.61, 5.51, and 5.69 kg, respectively. According to these data, the normal diet consumption per day was consistent with the rising trend of body weight gain in the NC group. A slight decrease in HFD consumption per day was observed in the other groups

from the 5th week to the last week. The trends of water intake per day were in accordance with the diet consumption, and no difference in weekly water consumption was found among the groups.

As shown in Figure 2B, the NC, HFD + NF, HFD + EGCG, HFD + NFEG, HFD + MG groups had significantly lower serum TC, TG, and LDL-C concentrations ($P < 0.05$) and significantly higher HDL-C content ($P < 0.05$) than the HFD group, except for the HFD + MG group whose HDL-C content showed no significant difference from that in the HFD group. The difference between HFD + NFEG and HFD groups was the most significant ($P < 0.01$). In particular, the HFD + NFEG group had significantly lower TC and LDL-C contents than the HFD + NF group and significantly different TG and HDL-C contents compared with those in the HFD + NF and HFD + EGCG groups ($P < 0.05$).

Rat liver H&E staining results showed that the hepatocytes filled with large lipid composition had balloon-like changes, and the hepatocyte nuclei were constricted and deformed in the HFD group. Meanwhile, the amounts of lipid droplets and the degree of liver fat cavitation in the HFD + NF, HFD + EGCG, and HFD + NFEG groups were reduced compared with those in the HFD group, indicating that HFD-induced hepatic fat accumulation could be reduced by NF and EGCG (Figures 2C–H). These results demonstrated that NFEG-microgel has a better effect on improving the lipid profile, hepatic steatosis, and liver injury than HFD or feeding with single package NF or EGCG-microgel.

NFEG-microgel significantly regulated the mRNAs in liver and small intestine

Key genes associated with lipid metabolism in the liver and small intestine were detected, and differences were observed among the groups. HFD + NFEG had significant effects on liver PPARα, AMPK, ACC, CYP7A1, CPT1A, and HMGCR ($P < 0.01$) compared with HFD. The expression of AMPK, CPT1A ($P < 0.01$), and CYP7A1 ($P < 0.05$) genes in the HFD + NFEG group were significantly higher than those in the HFD + NF and HFD + EGCG groups (Figure 3A). HFD + NFEG downregulated the expression of NPC1L1, MTP, CD36, and FATP4 and significantly repressed the downregulation of ABCG5 in the small intestine of HFD rats ($P < 0.01$). The HFD + NFEG group had significantly higher expression of ABCG5 gene ($P < 0.01$) and significantly lower expression of MTP genes ($P < 0.05$) compared with the HFD + NF and HFD + EGCG groups. FATP4 expression in the HFD + NFEG group was significantly decreased compared with that in the HFD + NF group ($P < 0.01$). CD36 and FATP4 expression showed no significant differences between the HFD + EGCG and HFD + NFEG groups, indicating that EGCG mainly inhibited lipid absorption after its release in the intestine. In addition, the expression of NPC1L1 and CD36 in the HFD + MG group was as low as that in the HFD + NFEG group (Figure 3B), indicating that the blank microgel had a similar effect on lipid absorption.

NFEG-microgel significantly regulated the protein expression of genes in liver and small intestine

Western blot was used to detect liver PPARα, CYP7A1, HMGCR, and CPT1A and small intestine NPC1L1, MTP, and ABCG5. As shown in Figure 4, differences in the expression of these proteins were observed among the groups. The protein expression levels of PPARα, CYP7A1, and CPT1A were significantly higher in the HFD + NF, HFD + EGCG, and HFD + NFEG groups than in the HFD group ($P < 0.01$) and were higher in the HFD + NFEG group than in the HFD + NF and HFD + EGCG groups ($P < 0.05$). The protein expression level of HMGCR was significantly higher in the HFD and HFD + MG groups than other groups ($P < 0.05$) where that in the HFD + NFEG group was the lower than others ($P < 0.05$). The protein expression levels of NPC1L1 and MTP significantly decreased in the HFD + NF, HFD + EGCG, and HFD + NFEG groups compared with those in the HFD group ($P < 0.01$) and were lower in the HFD + NFEG group than in the NC, HFD + NF and HFD + EGCG groups ($P < 0.05$). The protein expression levels of ABCG5 were significantly higher in the HFD + NF, HFD + EGCG, and HFD + NFEG groups than in the HFD group ($P < 0.01$) and were higher in the HFD + NFEG group than in the HFD + NF and HFD + EGCG groups ($P < 0.01$). Meanwhile, the protein expression levels of PPARα, CPT1A, HMGCR, NPC1L1, and ABCG5 in the HFD + MG group showed no significant differences from those in the HFD group ($P > 0.05$), except for NPC1L1 and MTP ($P < 0.05$).

Identification of extracellular vehicles in the serum

As shown in the TEM observation in Figure 5A, the serum EVs had spherical structures and saucer shape. Their particle size range within 50–140 nm (Figure 5B), and the average particle sizes of the NC, HFD + NF, HFD + EGCG, HFD + NFEG, HFD + MG, and HFD groups were 86.92, 83.71, 88.08, 96.01, 92.45, and 84.69 nm, respectively. For EV characterization, three marker proteins were reported in at least a semi-quantitative manner. Western blot analysis showed that the expression levels of the surface proteins of EVs, CD9, CD63, and TSG101 were high in the EVs (Figure 5C).

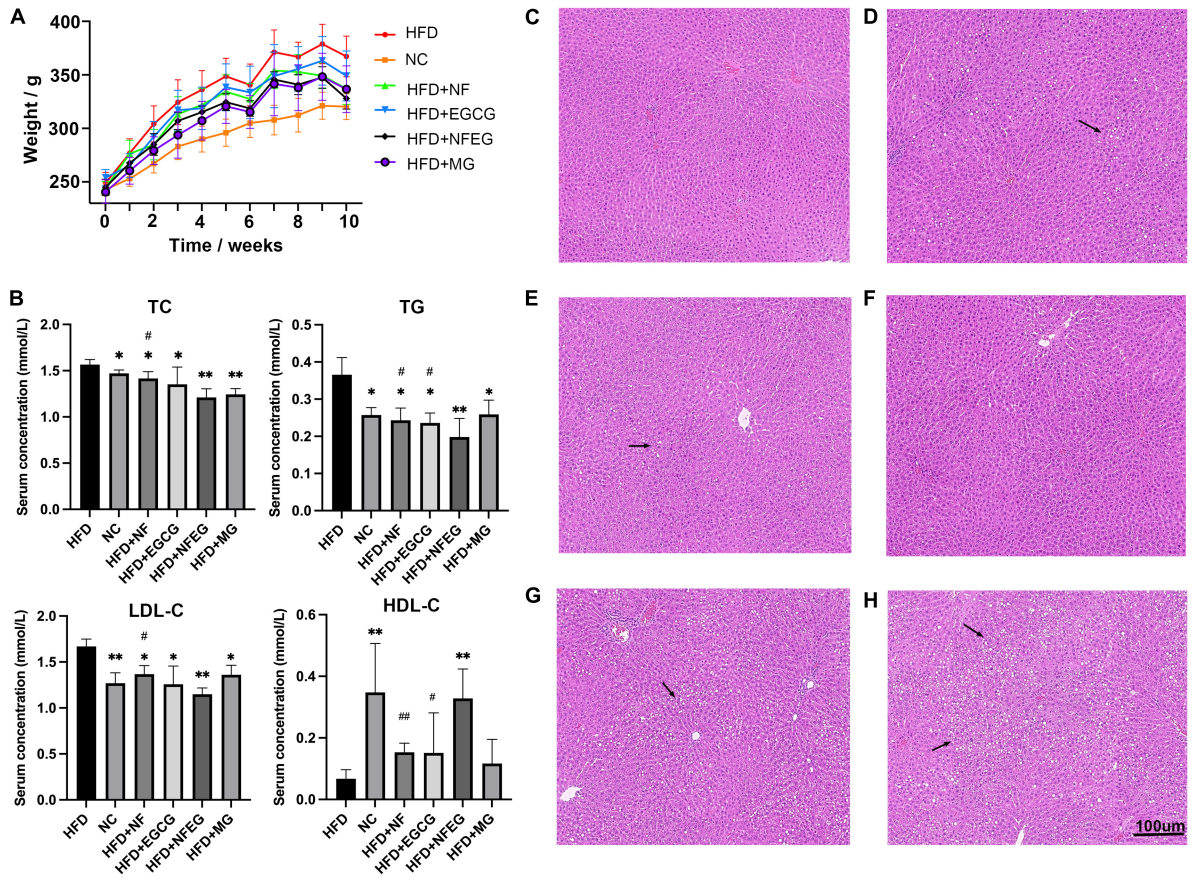


FIGURE 2
Body weight, serum biochemical indexes and H&E staining in different treatment groups: **(A)** body weight of rats, **(B)** serum TC, TG, LDL-C, and HDL-C concentrations, liver H&E staining **(C)** NC group, **(D)** HFD + NF group, **(E)** HFD + EGCG group, **(F)** HFD + NFEG group, **(G)** HFD + MG group, and **(H)** HFD group. (*$P < 0.05$) and (**$P < 0.01$) vs. HFD group; ($^{\#}P < 0.05$) and ($^{\#\#}P < 0.01$) vs. HFD + NFEG group.

NFEG-microgel significantly regulated the level of miRNA in serum extracellular vehicles

Five miRNAs closely associated with lipid metabolism were detected. Compared with those in the HFD and HFD + MG groups, the expression levels of miR-30b-5p and miR-126a-5p in the NC, HFD + NF, HFD + EGCG and HFD + NFEG groups were significantly increased ($P < 0.05$). No significant difference was observed in the expression level of miR-21-5p, miR-33-5p, and miR-27a-3p among the groups. In addition, the expression level of miR-30b-5p in the HFD + NFEG group was higher than that in the NC, HFD + NF, and HFD + EGCG groups ($P < 0.05$), and the expression level of miR-126a-5p showed no significant differences among the NC, HFD + NF, HFD + EGCG, and HFD + NFEG groups (Figure 5D). In accordance with the qRT-PCR results of lipid metabolism genes in the liver and small intestine, miR-126a-5p and miR-30b-5p may interact with their target genes HMGCR and MTP, respectively.

Effects of NFEG-microgel on dual-luciferase assay

qRT-PCR results elucidated that the expression levels of miR-126a-5p and miR-30b-5p in the serum EVs significantly differed among the six treatment groups and showed negative correlation with the expression of HMGCR and MTP. TargetScan and miRDB databases predicted possible interaction sites between miR-126a-5p and miR-30b-5p and their target genes HMGCR and MTP mRNA, respectively. The interactions of miR-30b-5p-MTP and miR-126a-5p-HMGCR were further tested by constructing the reporter plasmids of the potential action points of HMGCR and MTP and conducting dual-luciferase reporter assay.

As shown in Figures 5E,F, the relative fluorescence value of rno-miR-126a-5p + HMGCR-WT co-transfection group were lower ($P < 0.05$) than that of miR-NC + HMGCR-WT co-transfection group. After HMGCR mRNA mutation, no difference in relative fluorescence value was observed between the co-transfections of miR-NC+HMGCR and

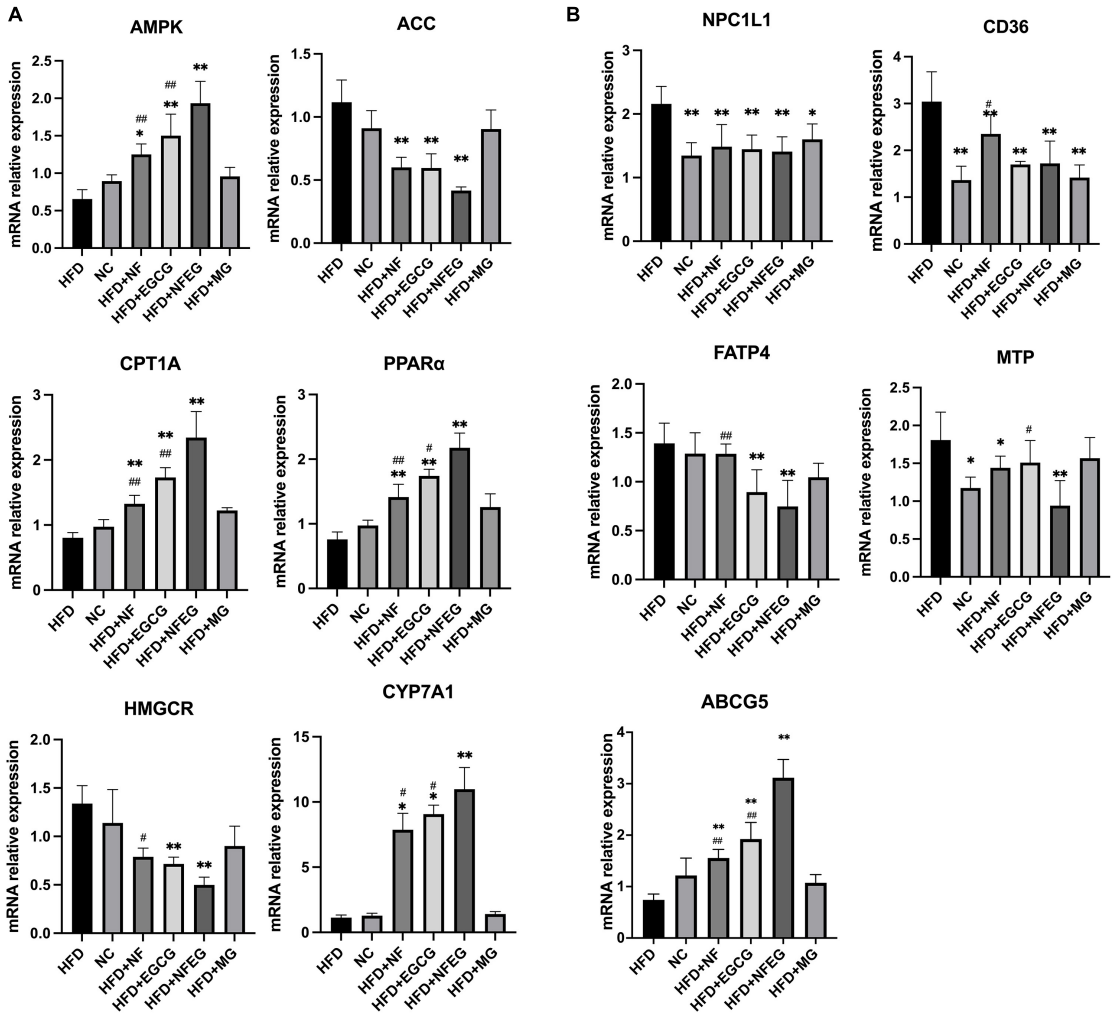


FIGURE 3
mRNA levels in rat liver and small intestine: **(A)** expression of AMPK, ACC, CPT1A, PPARα, HMGCR, and CYP7A1 in rat liver; and **(B)** expression of NPC1L1, CD36, FATP4, MTP, and ABCG5 in rat small intestine. (*$P < 0.05$) and (**$P < 0.01$) vs. HFD group; ($^{\#}P < 0.05$) and ($^{\#\#}P < 0.01$) vs. HFD + NFEG group.

rno-miR-126a-5p + HMGCR groups ($P > 0.05$; Figure 5E). Dual-luciferase reporter assay results of miR-30b-5p and MTP showed similar results (Figure 5F). The above results implied the existence of interaction sites for miR-126a-5p and miR-30b-5p and their respective target mRNAs, HMGCR and MTP. These sites may be the key to relieve NAFLD by post-transcription mechanism between miRNAs and mRNAs.

NFEG-microgel altered the composition and diversity of gut microbiota in high-fat diet rats

The gut microbiota may be an underlying target to treat obesity and related metabolic diseases. Bacterial 16S rRNA in stool was conducted to examine whether NFEG-microgel can alter the composition of the gut microbiota. Analysis of α-diversity index (Chao1 index, Shannon index, and Simpson index) reflecting gut microbiota showed that α-diversity increased with the increase in Chao1 index and Shannon index and the decrease in Simpson index (Figure 6A). Significant differences were observed among the HFD + NFEG and HFD, HFD + MG, and HFD + NF groups. Bray–Curtis PCoA analysis of OTU abundance of each rat revealed that compared with that in the NC group, the intestinal microbiota in the HFD group showed significant structural changes along the first principal component (PC1) and the second principal component (PC2). Compared with the NC group, the HFD + MG and HFD + EGCG groups showed the same shift as the HFD group along PC1 whereas the HFD + NFEG group had almost the same intestinal microbiota as the NC group along both PC1 and PC2 (Figure 6B). At the genus level, high-abundance species

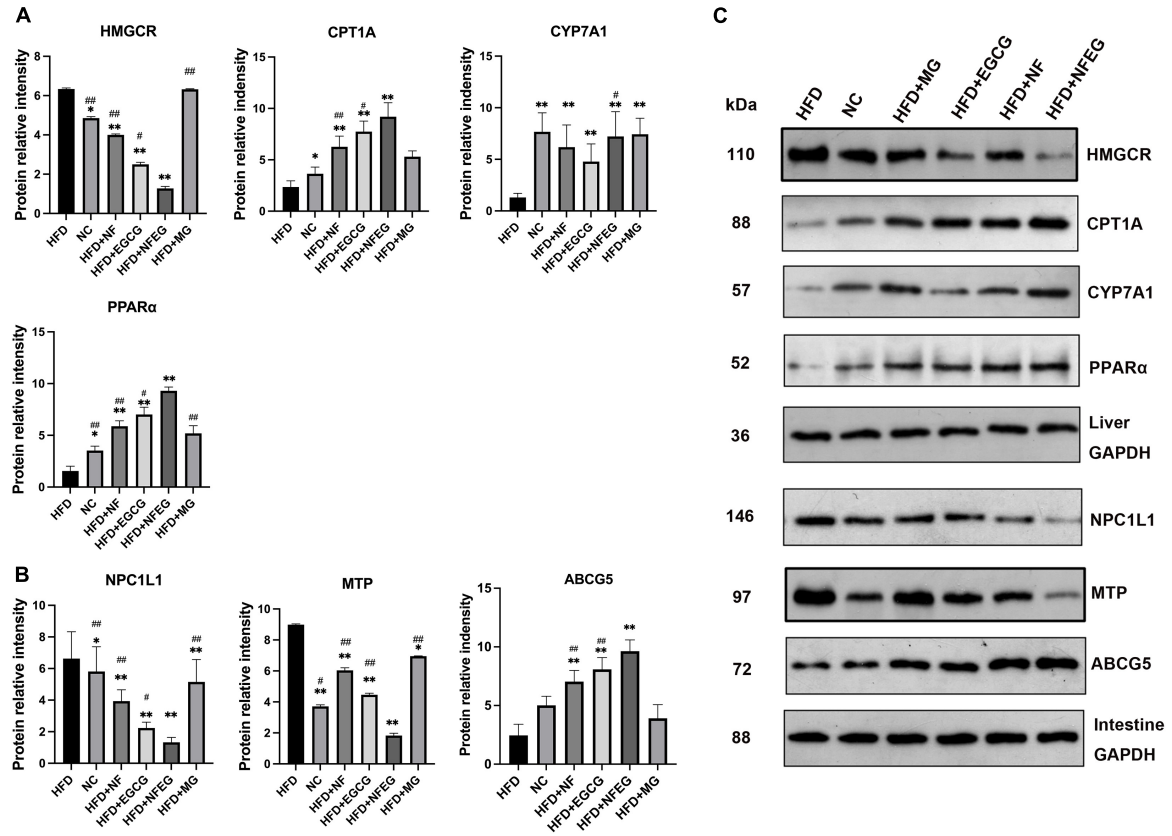


FIGURE 4
Protein expression of genes in the liver and small intestine; the results of Western blot were almost consistent with those of qRT-PCR. **(A)** Protein relative intensity expression of HMGCR, CYP7A1, CPT1A, and PPARα in rat liver. **(B)** Protein relative intensity expression of NPC1L1, MTP, and ABCG5 in rat small intestine. **(C)** WB bands. (*$P < 0.05$) and (**$P < 0.01$) vs. HFD group; ($^{\#}P < 0.05$) and ($^{\#\#}P < 0.01$) vs. HFD + NFEG group.

were selected to profile the expression of each group with the heatmap in Figure 6C. From a homoplastic viewpoint, linear discriminant analysis effect size (LEfSe) method was employed to identify statistically significant biomarkers and dominant microbiota among these groups (Figures 6D,E). Unexpectedly, Firmicutes was found to be the primary phylum of the gut microbiota in the HFD group, and this finding agreed with the study of Wang et al. (5). Meanwhile, Bacterioidetes was confirmed to be the dominating phylum of the gut microbiota in the HFD + MG group. The relative abundance of *Allobaculum, Blautia, Bacteroides, Butyricicoccus, Phascolarctobacterium, Faecalibaculum, Ruminococcaceae UCG-013*, and *Turicibacter* significantly increased ($p < 0.05$), and that of *Romboutsia, Erysipelotrichaceae*, and *Lachnospiraceae_NK4A136_group* significantly decreased ($p < 0.01$). No regulatory effect on *Lactobacillus* was found (Figures 7A–L). Experimental results showed that NF had a significant effect on *Allobaculum* (Figure 7A) and *Erysipelotrichaceae* (Figure 7J) because it only induced significant changes in the HFD + NFEG and HFD + NF groups. NFEG-microgel significantly enriched *Bacteroides, Butyricicoccus, Phascolarctobacterium, Faecalibaculum*, and *Ruminococcaceae UCG-013* and significantly reduced *Romboutsia* ($P < 0.01$) as which shown in Figures 7B,D–G,I. The relative abundances of these genera in the HFD + NFEG were significantly different from those in the HFD + NF, HFD + EGCG, HFD + MG, and HFD groups ($P < 0.05$).

Discussion

Lipid metabolism involves the regulation cycle of multi-tissues, multi-genes, and multi-molecules. Therefore, microgels must contain multiple active components to improve the synergistic lipid-lowering effect. Elucidate the possible mechanism of lipid metabolism regulation from multiple ways is necessary. Chitosan is the only positively charged edible food fiber found in nature, and MLPE are negatively charged. Thus, these two can self-assemble and combine into stable microgels through electrostatic interaction. Microgel is mainly used to coat hydrophilic compounds. In our study, we first loaded NF into liposomes with hydrophilic surface and then incorporated the liposomes into the microgels, thus allowing

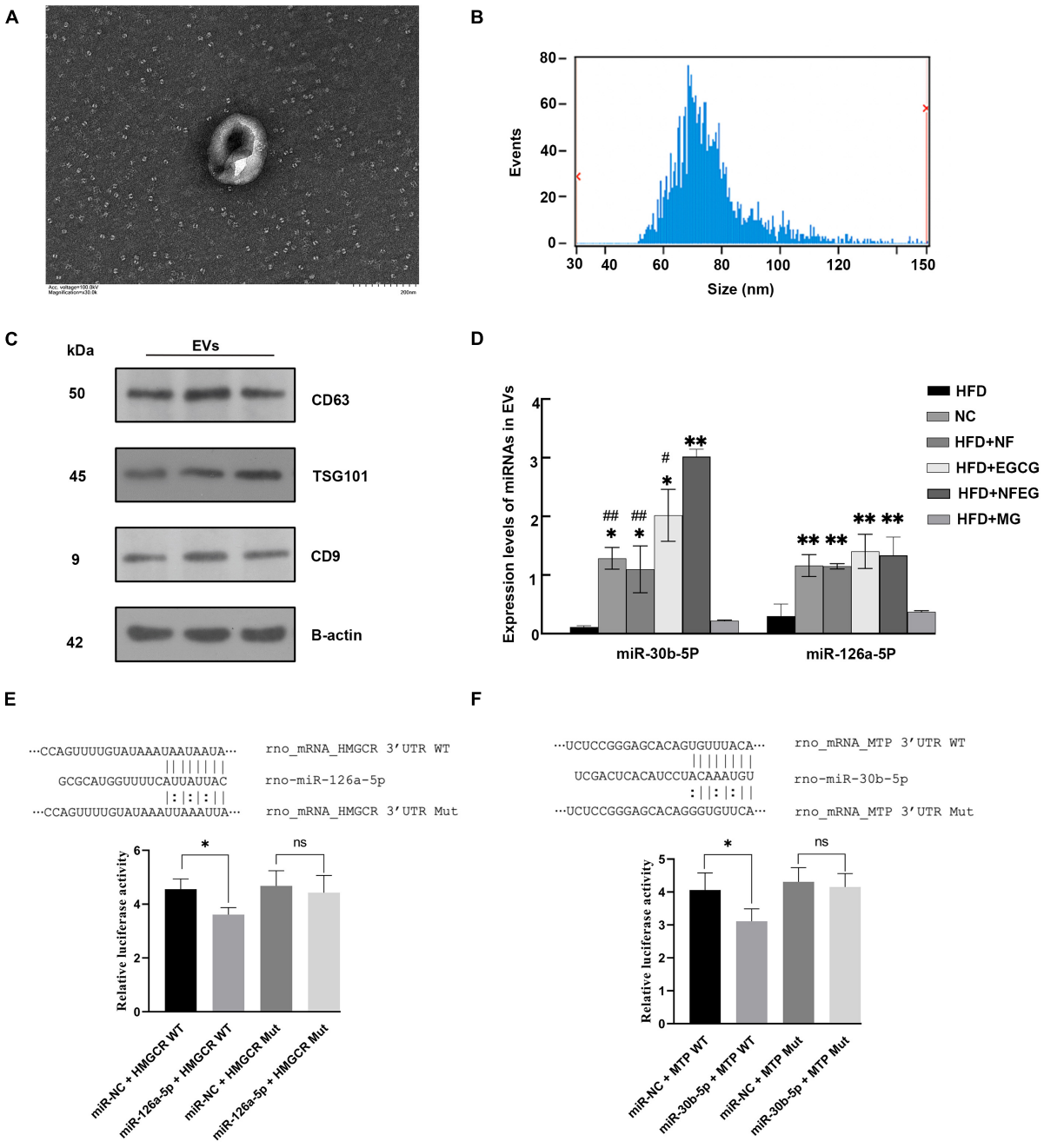


FIGURE 5
Identification and miRNA expression level of EVs and dual-luciferase reporter assay analysis. **(A)** Serum EVs of different groups observed by transmission electron microscopy (TEM) at the scale of 200 nm. **(B)** Particle size analysis showing that the particle size of each exosome ranged from 50 to 140 nm. **(C)** Expression levels of CD9, CD63, and TSG101 determined by Western blot. **(D)** miRNAs were extracted from rat serum EVs, and their expression levels were detected by qRT-PCR. Significant difference was indicated with the sign (*$P < 0.05$) and (**$P < 0.01$) vs. HFD group; ($^{\#}P < 0.05$) and ($^{\#\#}P < 0.01$) vs. HFD + NFEG group. Dual-luciferase reporter assay analysis: **(E)** the top portion of panel **(E)** shows the binding site of miR-126a-5p mimics and corresponding target genes HMGCR, and the bottom portion of panel **(E)** shows the relative fluorescence values after transfection and mutation, which suggested that significant interaction existed between miR-126a-5p and target gene HMGCR. Panel **(F)** shows similar results on the interaction of miR-30b-5p with MTP. Significant difference was indicated with the sign *$P < 0.05$.

the microgels to encapsule hydrophobic compounds. The HFD + NFEG group had significantly lower body weight, serum TC, TG, and LDL-C and significantly higher HDL-C compared with the HFD + NF and HFD + EGCG groups, proving that NF and EGCG were jointly involved in lipid regulation in the HFD rats. Most of the current research focuses on a single

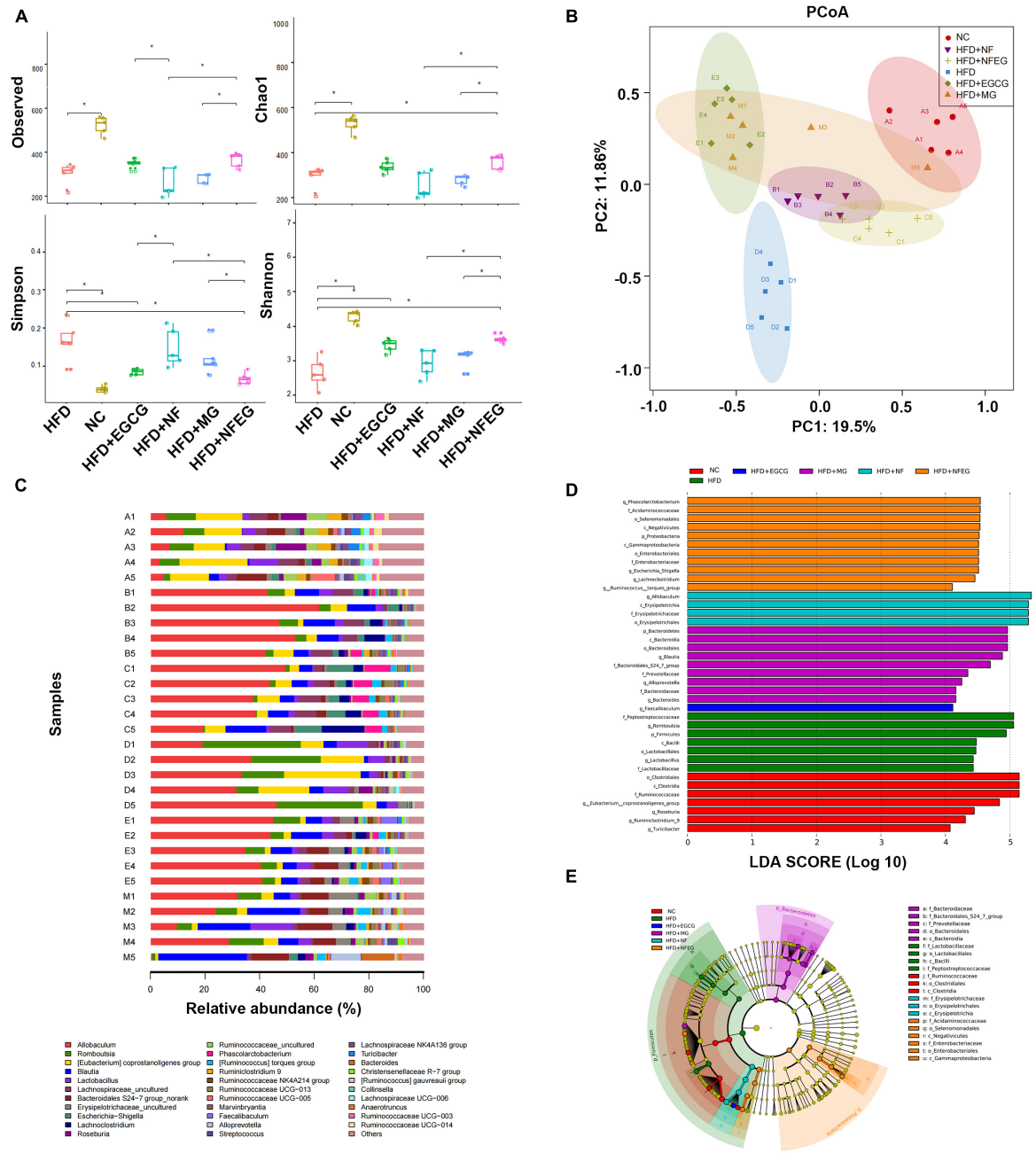


FIGURE 6
NFEG-microgel changed the diversity and composition of gut microbiota in HFD-fed rats. **(A)** α-diversity index (observed, Chao1, Shannon, and Simpson index). **(B)** Bray–Curtis PCoA plot based on the OTU abundance of each rat. **(C)** Heat map of bacterial taxonomic profiling at the genus level of intestinal bacteria based on each rat from different groups: NC group (A1–A5), HFD + NF group (B1–B5), HFD + NFEG group (C1–C5), HFD group (D1–D5), HFD + EGCG group (E1–E5), and HFD + MG group (M1–M5). Linear discriminant analysis (LDA) scores **(D)** and cladogram **(E)** generated from linear discriminate analysis effect size (LEfSe) analysis, showing the biomarker taxa (LDA score of > 4 and a significance of $P < 0.05$ determined by the Wilcoxon signed-rank test). Significant difference was indicated with the sign $^{*}P < 0.05$ and $^{**}P < 0.01$.

material carrier. The microgel prepared by using two natural materials in this study is safe, non-toxic, and has a certain lipid-lowering effect. Its preparation process is simple, and it has the advantages of embedding a variety of nutrients, which has potential application prospects in the field of food or nutrition.

Lipid accumulation *in vivo* is mainly related to the expression of lipid synthesis genes (such as HMGCR), lipid metabolism genes (such as PPARα, AMPK, ACC, CPT1A, and CYP7A1) in the liver, and lipid import genes (such as NPC1L1 and CD36) and lipid transporter genes (such as MTP, ABCG5,

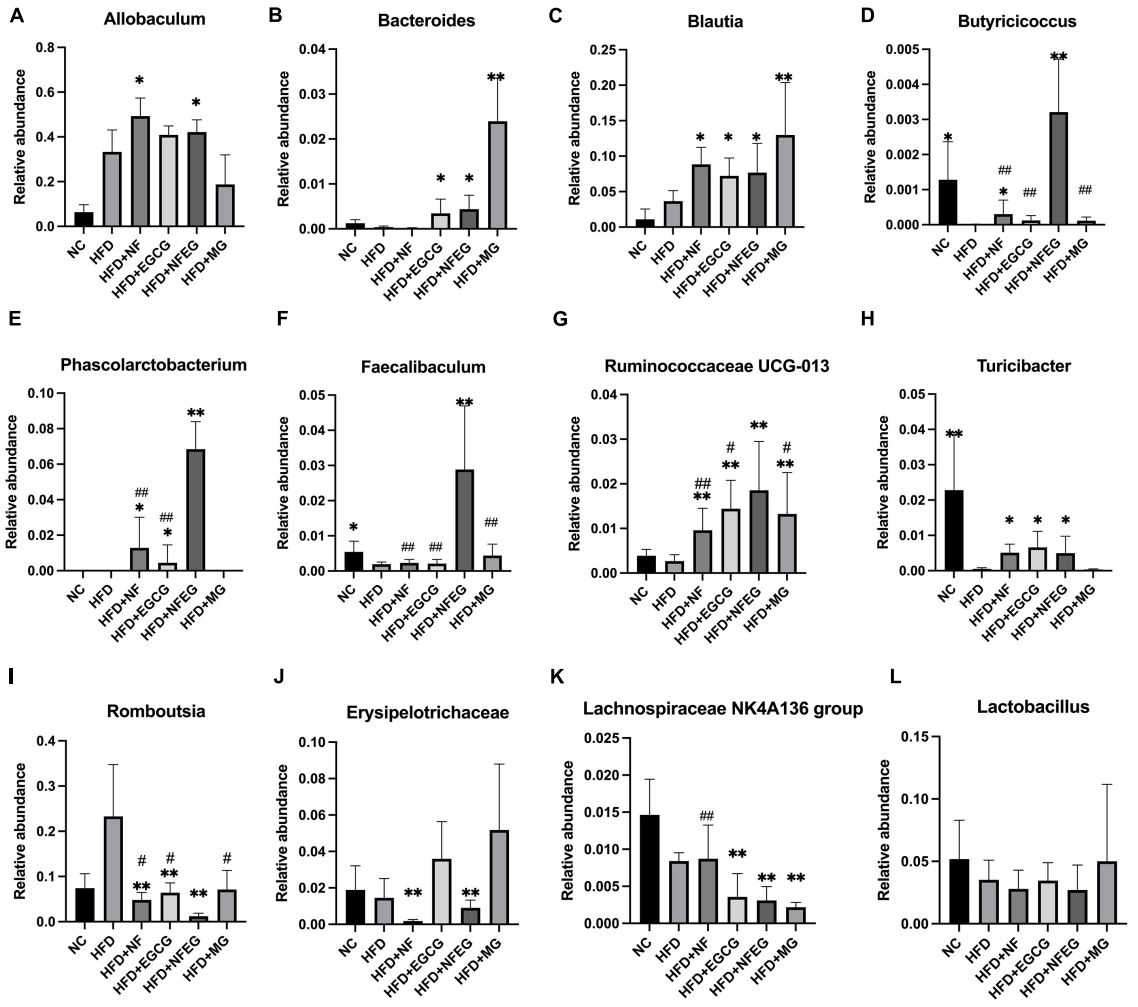


FIGURE 7
NFEG-microgel changed the relative abundance of gut microbiota in HFD-fed rats. Relative abundance of *Allobaculum* **(A)**, *Blautia* **(B)**, *Bacteroides* **(C)**, *Butyricicoccus* **(D)**, *Phascolarctobacterium* **(E)**, *Faecalibaculum* **(F)**, *Ruminococcaceae UCG-013* **(G)**, *Turicibacter* **(H)**, *Romboutsia* **(I)**, *Erysipelotrichaceae* **(J)**, *Lachnospiraceae_NK4A136_group* **(K)**, and *Lactobacillus* **(L)**. (*$P < 0.05$) and (**$P < 0.01$) vs. HFD group; ($^{\#}P < 0.05$) and ($^{\#\#}P < 0.01$) vs. HFD + NFEG group.

and FATP4) in the small intestine. Metabolism is regulated by reducing substrate overload to minimize the intake and transfer of metabolic substrates to metabolically active tissues. AMPK plays a central role in lipid metabolism by regulating the downstream gene ACC and CPT1 pathways. ACC catalyzes the production of malonyl-CoA, which is a major component of de novo adipogenesis and an allosteric inhibitor of CPT1 (key restriction enzyme in fatty acid β-oxidation and is also regulated by PPARα) (29). In our study, NFEG-microgel significantly increased AMPK level, decreased ACC level, and ultimately increased CPT1A function. The PPARα subtype performs its major functions in the liver, and the receptor is involved in all aspects of lipid metabolism including fatty acids (FA) transport, binding, absorption, synthesis, and oxidative degradation (30). HMGCR is a rate-limiting enzyme in cholesterol biosynthesis pathway, and CYP7A1 is a rate-limiting enzyme in the bile acid synthesis pathway; both play an important role in regulating the amount of cholesterol because bile acids provide an important excretory pathway for cholesterol metabolism (31). NPC1L1 mediates the intestinal uptake of dietary and biliary cholesterol. CD36 and FATP4 are major player in metabolic tissues and are among the proteins involved in FA uptake (32, 33). In this work, EGCG reversed the HFD-induced effects on intestinal substrate transporters CD36 and FATP4, and this finding was the same as that reported by Lu et al. (34). We found that NF and EGCG played a synergistic role in alleviating lipid accumulation. In particularly, NF mainly acted on lipid metabolism in the liver and regulated lipid transport and efflux in the intestine, and EGCG and blank microgel inhibited lipid absorption in the intestine.

As a series of post-transcriptional gene repressors, miRNAs are widely related to the regulation of gene expression, including almost all aspects of the system controlling metabolism. In regulating gene expression, miRNAs play an important role in communication. Yan et al. testified that the disruption of miR-126a in mice caused hepatocyte senescence, inflammation, and metabolism deficiency and revealed that the administration of miR-30b-5p antagonist attenuated liver inflammation in the injured liver. Zhang et al. (35) reported the increased expression of PPARα and decreased expression of lipid synthesis related gene SREBP-1 in miR-30b-5p overexpressed Huh-7 cells. Our detection results showed that the expression levels of miR-126a-5p and miR-30b-5p significantly differed among the six groups. Dual-luciferase reporter gene assay validated that miR-126a-5p and miR-30b-5p significantly interacted with their target genes HMGCR and MTP, respectively. The present study revealed that the miRNAs in the serum EVs target the genes associated with lipid metabolism as shown in the Graphical Abstract by Biorender.[4] Therefore, the NAFLD relieving mechanism of NFEG-microgel might be related to the regulation of miRNA signaling.

Gut microbes are considered "new organs" and play an important role in metabolic disorders. Wang et al. (5) demonstrated that the abundance of Lachnospiraceae_NK4A136_group and Erysipelotrichaceae decreased after supplementation, and this finding is consistent with our experimental results. Multiple studies revealed that Turicibacter was negatively correlated with inflammation in obesity (36, 37). Kaakoush clarified a strong association between Erysipelotrichaceae and host lipid metabolism. Wang et al. (38) revealed that HFD can reduce the abundance of *Lactobacillus, Faecalibaculum*, and *Blautia*. SCFAs may contribute to energy expenditure and appetite regulation. In addition to their role in gut health and signaling molecules, SCFAs may also affect substrate metabolism and peripheral tissue function by entering the systemic circulation. A growing body of evidence proves the benefits of SCFAs in adipose tissues and liver substrate metabolism and function (39). Zhang et al. confirmed that berberine can significantly enrich *Blautia* and *Allobaculum,* which produce SCFAs in the intestine, and consequently increase the amount of intestinal SCFAs (40).

According to the outcomes of PCoA and stratified cluster analysis, HFD feeding profound altered the composition and diversity of the gut microbiota, and NFEG-microgel significantly improved the intestinal microbiota dysbiosis in rats. NFEG-microgel significantly enriched the bacteria that produce SCFAs, including *Allobaculum, Blautia, Bacteroides, Butyricicoccus, Phascolarctobacterium* (41), *Romboutsia*, and *Faecalibaculum* (42), suggesting that these SCFA-producing bacteria play an important role in the efficacy of NFEG-microgel.

4 http://www.biorender.com

However, we only broadly illustrated the association, but not the causality, between the improved gut microbiota and the anti-obesity effects of NFEG-microgel. Further studies with feces transplantation experiments are necessary to clarify this association. Additionally, chitosan and proanthocyanidins were used to prepare microgels, which proved to be stable and effective, but the molecular mechanism of their binding still needs to be further explored. In addition, the source and production mechanism of EVs need further study.

In conclusion, NFEG-microgel could prominently inhibit the development of NAFLD and its related metabolic alterations by suppressing body weight gain, reducing serum lipids, ameliorating hepatic injury, and regulating the genes associated with lipid metabolism and miRNA increase. Furthermore, NFEG-microgel increases the diversity of gut bacterial community and the relative contents of beneficial bacteria and decreases the abundance of pathogenic bacteria. This generalizable strategy of encapsulating multiple nutrients in porous microgels using chitosan and MLPE can be applied in other systems to achieve the synergistic effects of multiple nutrients. NFEG-microgel offers an effective and innovative treatment of dyslipidemia.

Data availability statement

The original contributions presented in this study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA882230.

Ethics statement

This animal study was reviewed and approved by the Laboratory Animal Ethics Committee from China Jiliang University under the approval number: 2022-005.

Author contributions

SZ designed the experimental scheme, performed the experiment operation, analyzed the data, and finished the manuscript. WX completed parts of experiments including oral administration and took samples of the rats. JG guided and supervised the whole experimental process. JL, FG, AX, and JZ also provided some guidance and reviewed 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1069797/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Morphological observation of liver.

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Iron status and mental disorders: A Mendelian randomization study

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Background: Mental disorders account for an enormous global burden of disease, and has been associated with disturbed iron metabolism in observational studies. However, such associations are inconsistent and may be attributable to confounding from environmental factors. This study uses a two-sample Mendelian randomization (MR) analysis to investigate whether there is any causal effect of systemic iron status on risk of 24 specific mental disorders.

Methods: Genetic variants with concordant relations to 4 biomarkers of iron status (serum iron, ferritin, transferrin saturation, and transferrin) were obtained from a genome-wide association study performed by the Genetics of Iron Status (GIS) consortium. Summary-level data for mental disorders were obtained from the UK Biobank. An inverse-variance weighted (IVW) approach was used for the main analysis, and the simple median, weighted median and MR-Egger methods were used in sensitivity analyses.

Results: Genetically predicted serum iron, ferritin, and transferrin saturation were positively associated with depression and psychogenic disorder, and inversely associated with gender identity disorders. A higher transferrin, indicative of lower iron status, was also associated with increased risk of gender identity disorders and decreased risk of psychogenic disorder. Results were broadly consistent when using multiple sensitivity analyses to account for potential genetic pleiotropy.

Conclusion: Our findings offer a novel insight into mental health, highlighting a detrimental effect of higher iron status on depression and psychogenic disorder as well as a potential protective role on risk of gender identity disorders. Further studies regarding the underlying mechanisms are warranted for updating preventative strategies.

KEYWORDS

iron, ferritin, transferrin, mental disorders, UK Biobank, Mendelian randomization

Introduction

The essential element iron is closely involved in diverse fundamental biological processes, such as energy metabolism, oxygen transport, redox balance, and immunological modulation (1). Iron deficiency causes a number of pathological consequences, the most prominent being anemia. More than 2 billion individuals suffer from iron deficiency anemia worldwide, which may contribute to cognitive developmental defects in children (2). However, iron overload is also common and equally detrimental, as excess labile iron catalyzes the generation of reactive oxygen species (ROS) *via* the Fenton reaction and can trigger non-apoptotic cell death (3).

Disturbed iron homeostasis is related to a wide variety of chronic diseases, including mental disorders. In specific brain regions of patients with Alzheimer's disease (AD), significant iron accumulation has been clearly observed and correlated well with a decline in cognitive function (4, 5). Increased iron concentrations were also reported as characteristic of the degenerating substantia nigra of Parkinson's disease (PD), the second most prevalent neurodegenerative disease after AD (6). However, limited epidemiological evidence on the relationship between iron status and risk of other mental disorders is available.

Here, we conducted a Mendelian randomization (MR) study to robustly evaluate whether systemic iron status affects risk of multiple mental disorders. By exploiting genetic variants as instrumental variables of systemic iron status, the approach greatly avoided the risk of residual confounding as genetic variants are randomly allocated at conception and therefore irrelevant to environmental factors (7). Since disease development typically cannot alter the germline nuclear sequences, it may also overcome the problem of reverse causation (8). In this way, we employ MR to investigate the causal role for iron status in mental disorders, which would have important public health implications.

Materials and methods

In this present two-sample MR study, we used data from two different genome-wide association studies (GWASs)—one for the exposures and one for the outcome—to estimate the effects of exposure on outcome. Only summary-level data from these published studies were used. Appropriate patient consent and ethical approval were obtained in the original studies.

Genetic instrument selection

For unbiased detection of causal effects, the genetic variants used as genetic variants in this MR analysis must satisfy three key assumptions: (1) the used genetic variants should be robustly associated with iron status, (2) the used should not be associated with any confounders and (3) the genetic variants should influence the outcome (risk of specific mental disorder) only through iron status, rather than alternative pathways. The second and third assumptions are collectively known as independence from pleiotropy.

A large-scale meta-analysis of GWASs by the Genetics of Iron Status (GIS) Consortium was used to obtain single nucleotide polymorphisms (SNPs) proposed to be associated with systemic iron status. Data from 11 discovery cohorts and 8 replication cohorts were used in the meta-analysis, consisting of 48,972 individuals of European ancestry. It identified that 11 genome-wide-significant SNPs related to biomarkers of iron status, including increased serum iron, ferritin, transferrin saturation and decreased transferrin. Of these, three SNPs [rs1800562 and rs1799945 in the hemochromatosis (HFE) gene and rs855791 in the transmembrane serine protease 6 (TMPRSS6) gene] were robustly associated with concordant variance for each biomarker (**Supplementary Table 1**) (9). In addition, these SNPs are not in linkage disequilibrium and have been used as instrumental variables for systemic iron status in previous MR studies (10–12). Therefore, we included them in our MR analysis to predict systemic iron status.

F statistic of first-stage regression was employed to evaluate the strength of the instruments and was calculated using the following equation: $F = (R^2/k)/([1\text{-}R^2]/[n\text{-}k\text{-}1])$, where R^2 is the proportion of the iron status variability accounted for by the SNP, k is the number of instruments used in the model and n is the sample size (13). To minimize weak instrument bias, only SNPs with an F statistic > 10 were included in subsequent analyses.

Outcome data sources

Summary-level GWAS statistics for 24 specific mental disorders were extracted from the Pan-ancestry genetic analysis of the UK Biobank (14). This UK-based prospective cohort study was approved by the North West Multicentre Research Ethics Committee, and recruited around 500,000 genotyped adults aged 40–69 years. Detailed characteristics of cases and controls can be found in **Supplementary Table 2**. The summary-level data are publicly available at https://pan.ukbb.broadinstitute.org.

Statistical analysis

An inverse-variance weighted (IVW) method with random-effects was used to generate the main MR estimates for the causal associations between each measure of iron status and risk of mental disorders. A threshold of $P < 0.05$ was used to determine statistical significance. Odds ratios (ORs) with

95% confidence intervals (CIs) of mental disorders are per one standard deviation (SD) increase in genetically predicted serum iron, log10 ferritin, transferrin saturation and transferrin levels.

Cochran's *Q* test was applied to evaluate heterogeneity in IVW estimates across different instruments (interpreting $P < 0.05$ as evidence of heterogeneity) (15).

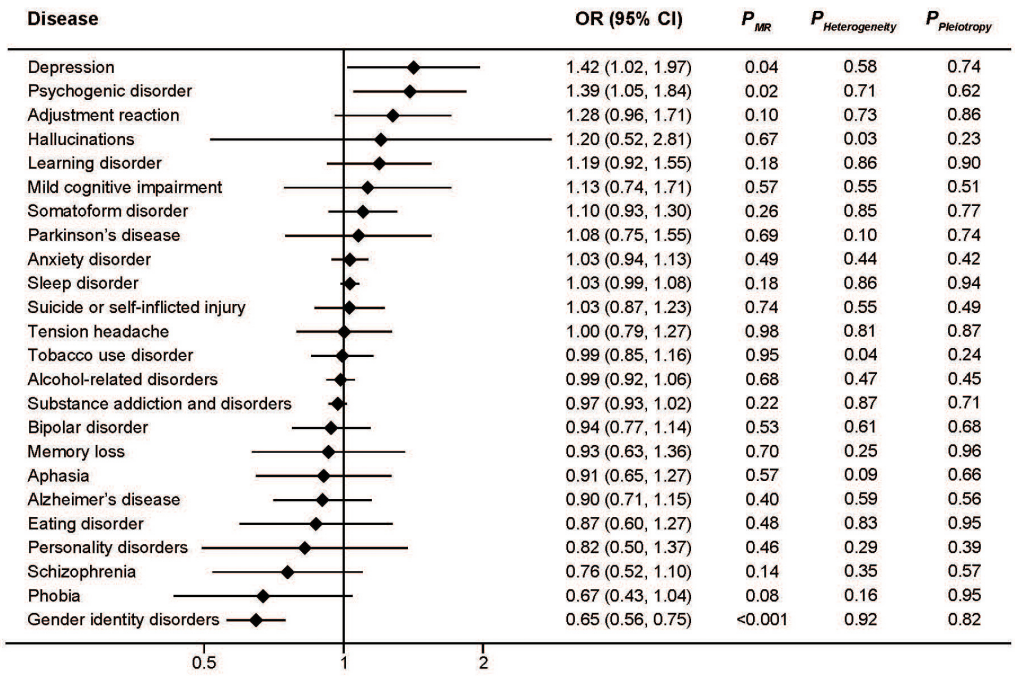

FIGURE 1
Associations between genetically predicted serum iron and risk of 24 specific mental disorders.

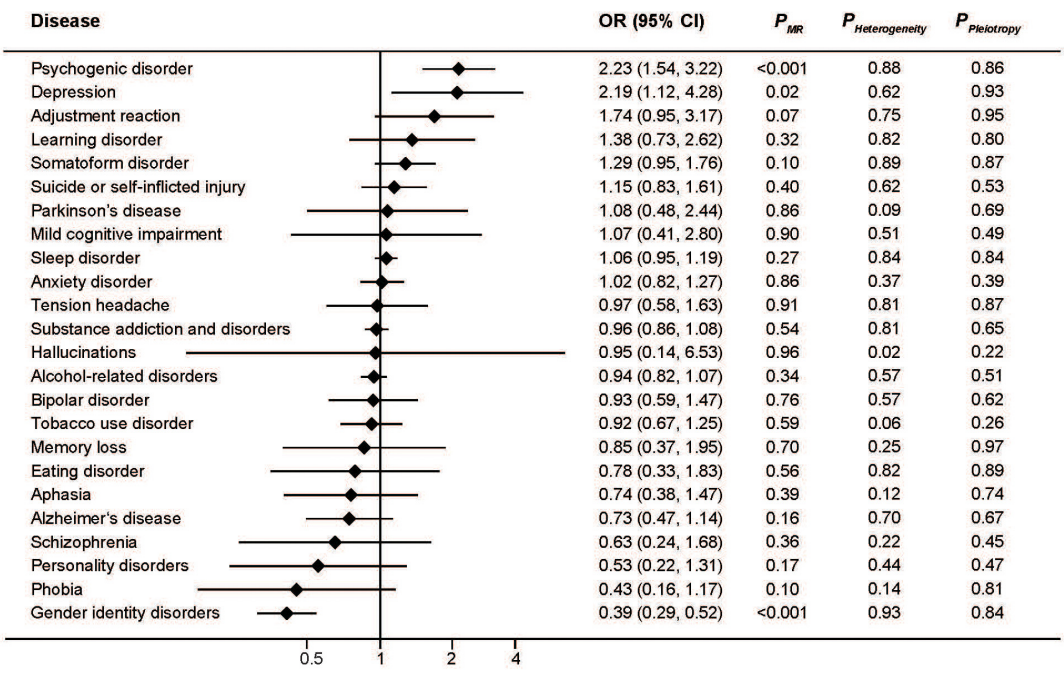

FIGURE 2
Associations between genetically predicted serum ferritin and risk of 24 specific mental disorders.

Pleiotropy refers to a genetic variant influencing the outcome of interest through pathways independent of the risk factor. We then conducted a range of sensitivity analyses, including the simple median, weighted median and MR-Egger methods, to address potential pleiotropy. The simple and weighted median methods provide consistent MR estimates even when up to 50% of the information comes from invalid instrumental variables (16). The MR-Egger method is able to assess whether genetic variants have pleiotropic effects on the outcome and provides a consistent estimate of the causal effect even if all genetic variants are invalid (17). Since the relatively low statistical power of these methods compared with the main IVW analysis, they were used solely to confirm a consistent effect estimate, rather than to ascertain statistical significance itself *via* a *p*-value threshold. We also searched the PhenoScanner database[1] to detect secondary phenotypes for the SNPs associated with the iron status biomarkers (18).

All MR analyses were performed using the TwoSampleMR package by R 4.0.2 software.[2]

Results

Three SNPs including rs1800562 and rs1799945 in the *HFE* gene and rs855791 in the *TMPRSS6* gene were employed for our main analysis. The *F* statistics for the three SNPs ranged from 47 for 2,127 across all four biomarkers of systematic iron status, as described previously (10, 12), making significant bias from use of weak instruments unlikely (13).

Genetically predicted high serum iron levels were positively associated with depression (OR: 1.41; 95%CI: 1.02, 1.97; $P = 0.04$) and psychogenic disorder (OR: 1.39; 95%CI: 1.05, 1.84; $P = 0.02$), but inversely associated with gender identity disorders (OR: 0.65; 95%CI: 0.56, 0.75; $P < 0.001$). No association was found in the other 21 mental disorders. We detected significant heterogeneity only in the analyses of hallucinations ($P = 0.03$) and tobacco use disorder ($P = 0.04$). Pooled MR estimates for the effect of increased serum iron on risk of different mental disorders are shown graphically in **Figure 1**.

Similar to serum iron, the MR analysis showed a protective effect of ferritin on gender identity disorders as well as a detrimental effect on depression and psychogenic disorder (**Figure 2**). The pooled ORs for per SD unit increase of $\log_{10}$ (ferritin) on psychogenic disorder, depression, and gender identity disorders were 2.23 (95%CI: 1.54, 3.22; $P < 0.001$), 2.19 (95%CI: 1.12, 4.28; $P = 0.02$), and 0.39 (95%CI: 0.29, 0.52; $P < 0.001$), respectively. Consistent associations were also found with transferrin saturation (**Figure 3**). The pooled ORs for per SD unit increase of transferrin on psychogenic disorder, depression, and gender identity disorders were 1.30 (95%CI: 1.13, 1.48); $P < 0.001$, 1.29 (95%CI: 1.03, 1.62; $P = 0.03$), and 0.73 (95%CI: 0.68, 0.78; $P < 0.001$), respectively.

1 http://www.phenoscanner.medschl.cam.ac.uk/phenoscanner

2 www.r-project.org

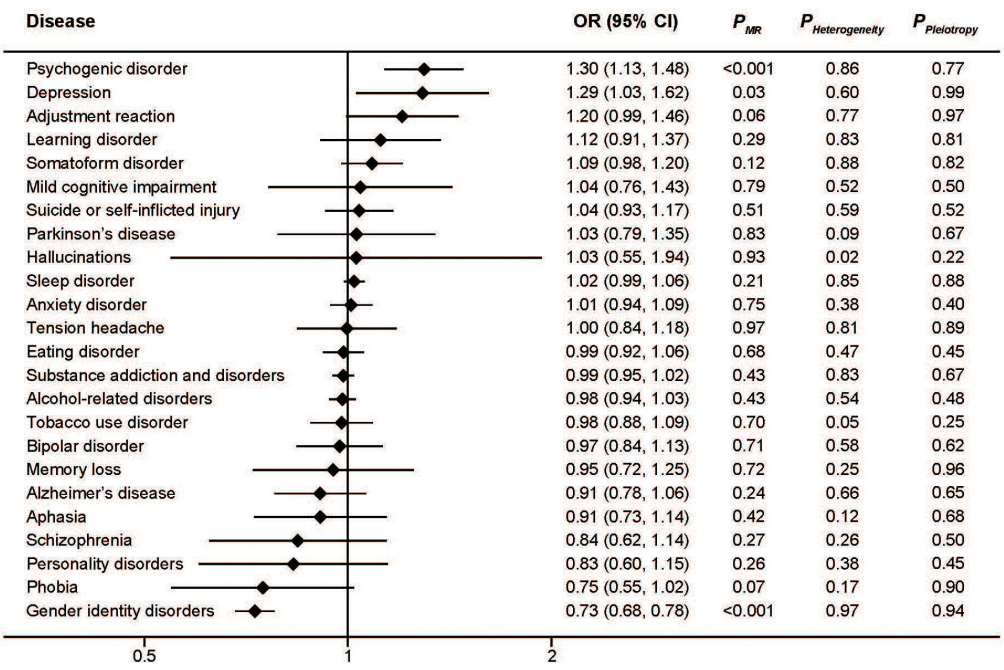

FIGURE 3
Associations between genetically predicted transferrin saturation and risk of 24 specific mental disorders.

In addition, higher transferrin levels, which are indicative of lower iron status, were associated with increased risk of gender identity disorders (OR: 1.53; 95%CI: 1.17, 2.00; $P < 0.005$) as well as decreased risk of somatoform disorder (OR: 0.87; 95%CI: 0.78, 0.98; $P = 0.02$) and psychogenic disorder (OR: 0.68; 95%CI: 0.59, 0.77; $P < 0.001$) (**Figure 4**). The effect of transferrin on depression seemed protective, but did not reach statistical significance (OR: 0.72; 95%CI: 0.48, 1.07; $P = 0.10$).

The MR-Egger approach did not produce evidence of pleiotropy in any of our analyses (**Figures 1–4**). We further examined potential pleiotropy using the PhenoScanner database. A potentially pleiotropic effect on risk of these mental disorders may be contributed by rs1800562 because of its association with reduced total and low-density lipoprotein cholesterol levels (19). Moreover, sensitivity analyses using simple median, weighted median, and MR Egger methods produced directionally consistent effects as the IVW estimates, supporting the consistency and robustness of our findings (**Table 1**).

Discussion

Mental disorders affected more than 1 billion people globally and lead to 19% of all years lived with disability (20). Observational epidemiological studies have associated altered iron levels with risk of AD or PD; however, these studies are susceptible to confounding and reverse causation, and thus it remains unclear whether these connections are true. More importantly, the effect of iron on most other mental disorders remains unknown. Therefore, it is necessary to better understand the causal relationship between iron status and mental disorders in humans, in order to provide evidence to support further preclinical or clinical studies.

Iron is generally assumed to be a risk factor for AD, in line with the well-known phenomenon of iron accumulation in specific brain regions of AD patients (4), but a number of epidemiological studies have questioned this conclusion (21–24). In this study, our results showed no causal association between genetically determined markers of systemic iron status and AD. One explanation for the lack of clear relationship between serum iron status and cognitive decline is that circulating iron/ferritin levels may not be a reliable indicator of brain iron in AD patients (25).

One previous MR study indicated that increased serum iron levels are causally associated with a decreased risk of developing PD (26). Epidemiological studies, however, provided strong evidence about significantly higher serum iron levels in PD patients than health controls (27). Our study failed to replicate any of these findings, as no significant associations between iron biomarkers and PD risk were found. These conflicting results illustrate the complexity of the problem, and more investigation is required.

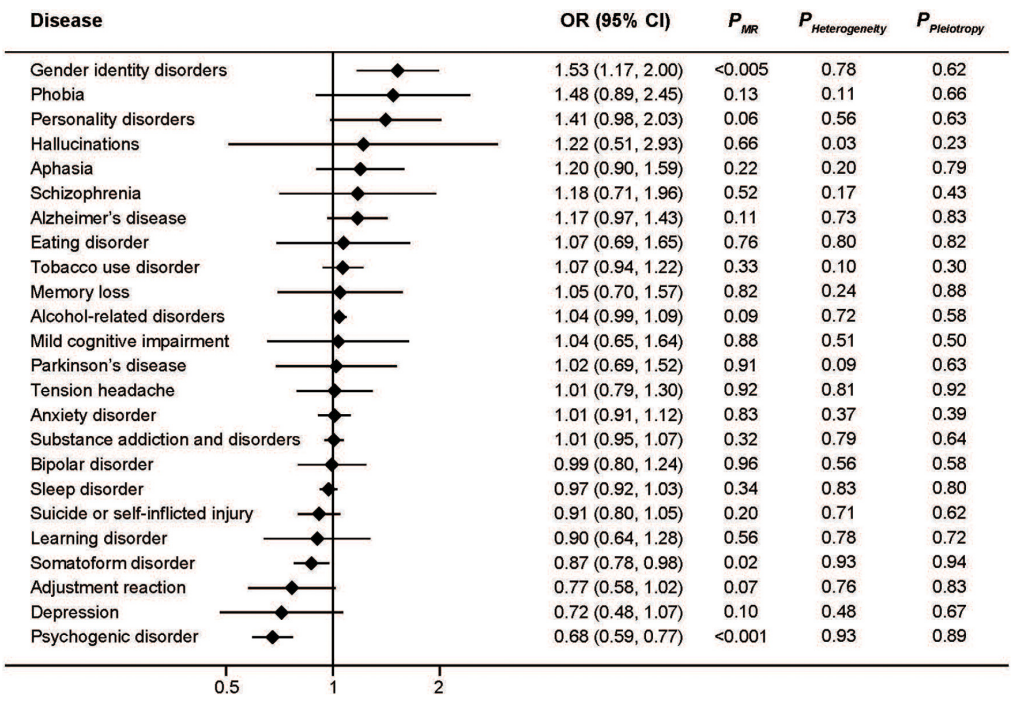

FIGURE 4
Associations between genetically predicted serum transferrin and risk of 24 specific mental disorders.

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TABLE 1 Sensitivity analyses of the associations between genetically predicted iron status and mental disorders.

Disease/methods	Serum iron			Ferritin			Transferrin saturation			Transferrin		
	β	*SE*	*P*	β	*SE*	*P*	β	*SE*	*P*	β	*SE*	*P*
Alzheimer's disease												
Weighted median	−0.12	0.20	0.54	−0.40	0.40	0.34	−0.12	0.13	0.36	0.17	0.18	0.35
Simple median	−0.08	0.20	0.70	−0.26	0.52	0.64	−0.08	0.17	0.66	0.20	0.39	0.60
MR-Egger	−0.65	0.69	0.52	−0.64	0.68	0.52	−0.24	0.26	0.53	0.20	0.24	0.55
Aphasia												
Weighted median	−0.14	0.14	0.31	−0.32	0.27	0.24	−0.12	0.090	0.20	0.19	0.12	0.10
Simple median	−0.24	0.15	0.12	−0.38	0.30	0.17	−0.14	0.10	0.17	0.16	0.15	0.28
MR-Egger	−0.59	0.84	0.61	−0.59	0.82	0.60	−0.25	0.31	0.57	0.24	0.26	0.53
Mild cognitive impairment												
Weighted median	0.13	0.34	0.71	0.0021	0.64	1.00	0.031	0.21	0.88	0.034	0.28	0.90
Simple median	0.22	0.34	0.51	0.73	0.99	0.43	0.21	0.29	0.47	−0.89	0.75	0.23
MR-Egger	−0.90	1.10	0.56	−0.89	1.09	0.56	−0.33	0.42	0.57	0.28	0.38	0.59
Memory loss												
Weighted median	−0.16	0.20	0.42	−0.24	0.41	0.55	−0.08	0.14	0.54	0.03	0.18	0.85
Simple median	−0.12	0.21	0.55	−0.20	0.44	0.65	−0.07	0.14	0.63	0.08	0.27	0.75
MR-Egger	−0.14	1.10	0.92	−0.13	1.09	0.93	−0.03	0.42	0.96	−0.0010	0.38	1.00
Hallucinations												
Weighted median	0.53	0.33	0.11	−0.08	0.58	0.88	0.05	0.19	0.79	0.25	0.24	0.29
Simple median	0.52	0.37	0.16	1.52	1.12	0.17	0.43	0.32	0.18	−0.87	0.78	0.26
MR-Egger	−2.18	0.93	0.26	−2.16	0.92	0.26	−0.84	0.36	0.26	0.75	0.32	0.26
Schizophrenia												
Weighted median	−0.19	0.22	0.37	−0.30	0.44	0.50	−0.12	0.15	0.42	0.14	0.20	0.47
Simple median	−0.23	0.23	0.32	−0.77	0.71	0.28	−0.22	0.21	0.28	0.96	0.61	0.11
MR-Egger	0.37	0.84	0.74	0.35	0.84	0.75	0.12	0.33	0.78	−0.09	0.31	0.82
Bipolar disorder												
Weighted median	−0.03	0.16	0.86	0.003	0.34	0.99	0.001	0.11	0.99	−0.03	0.15	0.85
Simple median	0.03	0.18	0.87	0.045	0.44	0.91	0.016	0.13	0.90	−0.02	0.26	0.94
MR-Egger	0.25	0.57	0.74	0.25	0.56	0.73	0.11	0.22	0.71	−0.11	0.20	0.69
Depression												
Weighted median	0.47	0.25	0.06	0.83	0.56	0.11	0.29	0.18	0.10	−0.31	0.23	0.18
Simple median	0.46	0.28	0.10	0.79	0.64	0.22	0.28	0.20	0.15	−0.34	0.35	0.33
MR-Egger	0.73	0.91	0.57	0.71	0.90	0.58	0.26	0.35	0.60	−0.21	0.33	0.64
Suicide or self-inflicted injury												
Weighted median	0.009	0.13	0.95	0.15	0.26	0.55	0.04	0.09	0.66	−0.09	0.12	0.44
Simple median	−0.04	0.14	0.79	−0.13	0.39	0.74	−0.04	0.12	0.75	0.16	0.28	0.56
MR-Egger	0.49	0.45	0.48	0.48	0.45	0.48	0.18	0.17	0.48	−0.16	0.16	0.49
Phobia												
Weighted median	−0.33	0.21	0.11	−0.62	0.41	0.10	−0.22	0.14	0.11	0.29	0.18	0.12
Simple median	−0.40	0.22	0.07	−0.65	0.50	0.19	−0.23	0.15	0.13	0.38	0.50	0.46
MR-Egger	−0.50	1.28	0.76	−0.52	1.26	0.75	−0.22	0.48	0.72	0.23	0.41	0.68

(Continued)

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TABLE 1 (Continued)

Disease/methods	Serum iron			Ferritin			Transferrin saturation			Transferrin		
	β	*SE*	*P*	β	*SE*	*P*	β	*SE*	*P*	β	*SE*	*P*
Personality disorders												
Weighted median	−0.16	0.29	0.58	−0.71	0.56	0.21	−0.21	0.17	0.24	0.40	0.25	0.11
Simple median	−0.04	0.31	0.90	−0.13	0.86	0.88	−0.04	0.25	0.88	0.16	0.55	0.77
MR-Egger	−1.50	0.94	0.36	−1.47	0.93	0.36	−0.56	0.36	0.36	0.48	0.32	0.38
Gender identity disorders												
Weighted median	−0.51	0.29	0.08	−0.89	0.61	0.14	−0.30	0.20	0.14	0.41	0.28	0.13
Simple median	−0.51	0.32	0.11	−1.00	0.79	0.20	−0.30	0.23	0.20	0.85	0.59	0.15
MR-Egger	−0.72	1.05	0.62	−0.72	1.04	0.61	−0.28	0.40	0.61	0.26	0.36	0.60
Psychogenic disorder												
Weighted median	0.41	0.28	0.14	0.82	0.57	0.15	0.29	0.18	0.12	−0.38	0.25	0.40
Simple median	0.42	0.30	0.16	0.85	0.70	0.22	0.30	0.21	0.16	−0.36	0.51	0.47
MR-Egger	0.98	0.97	0.50	0.97	0.96	0.49	0.39	0.37	0.49	−0.36	0.33	0.48
Somatoform disorder												
Weighted median	0.14	0.25	0.58	0.28	0.49	0.57	0.10	0.16	0.53	−0.14	0.23	0.53
Simple median	0.19	0.26	0.47	0.30	0.63	0.63	0.11	0.18	0.56	−0.13	0.43	0.77
MR-Egger	0.41	0.85	0.72	0.41	0.85	0.72	0.16	0.33	0.70	−0.16	0.29	0.69
Adjustment reaction												
Weighted median	0.24	0.31	0.44	0.46	0.62	0.46	0.16	0.20	0.43	−0.25	0.28	0.37
Simple median	0.30	0.33	0.36	0.48	0.76	0.53	0.17	0.24	0.48	−0.21	0.60	0.73
MR-Egger	0.48	1.07	0.73	0.48	1.05	0.73	0.20	0.41	0.71	−0.20	0.37	0.68
Eating disorder												
Weighted median	−0.18	0.49	0.71	−0.25	1.08	0.82	−0.086	0.34	0.80	0.04	0.47	0.93
Simple median	−0.12	0.55	0.83	−0.19	1.32	0.89	−0.07	0.38	0.86	0.08	0.93	0.93
MR-Egger	0.0013	1.83	1.00	0.018	1.81	0.99	0.03	0.70	0.98	−0.05	0.63	0.95
Tension headache												
Weighted median	−0.09	0.30	0.77	−0.14	0.59	0.81	−0.05	0.21	0.81	0.02	0.27	0.95
Simple median	−0.08	0.31	0.79	−0.13	0.78	0.86	−0.05	0.23	0.84	0.06	0.49	0.91
MR-Egger	−0.21	1.04	0.87	−0.20	1.03	0.88	−0.07	0.40	0.90	0.04	0.36	0.92
Learning disorder												
Weighted median	0.18	0.37	0.62	0.26	0.80	0.75	0.09	0.25	0.72	−0.07	0.35	0.84
Simple median	0.13	0.40	0.75	0.20	1.03	0.84	0.07	0.29	0.81	−0.09	0.64	0.89
MR-Egger	−0.04	1.37	0.98	−0.20	1.03	0.88	−0.03	0.53	0.96	0.05	0.47	0.94
Substance addiction and disorders												
Weighted median	−0.03	0.07	0.64	−0.04	0.14	0.79	−0.02	0.05	0.73	0.007	0.06	0.92
Simple median	−0.04	0.08	0.56	−0.14	0.19	0.46	−0.04	0.06	0.49	0.11	0.15	0.43
MR-Egger	0.09	0.24	0.78	0.09	0.24	0.78	0.03	0.09	0.79	−0.03	0.08	0.80
Alcohol-related disorders												
Weighted median	−0.01	0.05	0.82	−0.09	0.10	0.36	−0.03	0.03	0.40	0.05	0.04	0.30
Simple median	−0.01	0.06	0.83	−0.03	0.13	0.80	−0.01	0.04	0.81	0.02	0.09	0.82
MR-Egger	−0.20	0.16	0.44	−0.20	0.16	0.44	−0.08	0.06	0.43	0.07	0.06	0.43

(Continued)

TABLE 1 (Continued)

Disease/methods	Serum iron			Ferritin			Transferrin saturation			Transferrin		
	β	*SE*	*P*	β	*SE*	*P*	β	*SE*	*P*	β	*SE*	*P*
Tobacco use disorder												
Weighted median	0.02	0.06	0.68	−0.08	0.11	0.46	−0.01	0.03	0.70	0.07	0.04	0.13
Simple median	0.07	0.06	0.30	0.22	0.19	0.26	0.06	0.05	0.24	−0.18	0.13	0.19
MR-Egger	−0.43	0.17	0.25	−0.42	0.17	0.25	−0.16	0.07	0.25	0.14	0.06	0.25
Sleep disorder												
Weighted median	0.02	0.07	0.80	0.03	0.14	0.82	0.01	0.05	0.81	−0.02	0.06	0.80
Simple median	0.02	0.07	0.78	0.03	0.18	0.86	0.01	0.05	0.83	−0.03	0.14	0.81
MR-Egger	0.01	0.25	0.98	0.01	0.25	0.98	0.01	0.10	0.96	−0.01	0.09	0.94
Parkinson's disease												
Weighted median	0.05	0.15	0.73	0.00070	0.30	1.00	0.0017	0.10	0.99	0.05	0.13	0.68
Simple median	−0.04	0.17	0.83	−0.06	0.33	0.86	−0.02	0.11	0.85	0.03	0.18	0.89
MR-Egger	−0.34	0.96	0.79	−0.35	0.95	0.78	−0.15	0.36	0.75	0.16	0.31	0.70

Our study increases the probability that iron overload has a clinically relevant impact on the risk of depression. This finding is important since evidence on the association between serum iron levels and depression risk collected so far has been controversial. Iron deficiency was generally thought of as a risk factor for depression. Stewart and Hirani once studied about 2,000 elderly individuals, and found that anemia and low serum ferritin levels were linked with depressive symptoms (28). A meta-analysis also indicated that dietary iron intake is inversely associated with risk of depression, but its result is difficult to interpret owing to limited number of included studies and high heterogeneity (29). However, recent studies have shown effects of iron in opposite directions (30, 31). These epidemiological studies suffer from reverse causation, and their conclusions may be confounded by disease status. For instance, inflammation influences iron metabolism resulting in an increase in serum ferritin and a decrease in serum iron at the same time. Given neuroinflammation has been considered to impact the development of depression (32), it may drive both higher iron status and greater depression risk. Based on MR approach, we have used genetic variants as instrumental variables for iron status to overcome these limitations of observational studies and strengthen the evidence for a detrimental effect of iron status on depression risk. We also found that genetically high iron status was associated with higher risk of psychogenic disorder and lower risk of gender identity disorders. To the best of our knowledge, this is the first evidence linking iron status to these mental disorders.

There are very few studies investigating the molecular mechanisms involved in psychogenic disorder as well as gender identity disorders. And there's still no direct evidence that shows exactly how iron metabolism affects risk of these mental disorders. However, brain capture of blood iron are necessary for an appropriate synthesis of neurotransmitters, such as serotonin, dopamine, and noradrenaline. In addition, these neurotransmitters, involved in emotional behaviors, depend on neuron aromatic hydoxylases functioning with iron as essential cofactor (33). Iron deficient animals had significantly lower dopamine D2 receptor densities in the frontal cortex and caudate putamen. Meanwhile, serotonin (5-HT) transporter densities increased in the nucleus accumbens (34). Noradrenaline also has impact on neuroplasticity *via* brain-derived neurotrophic factor, which is key for prefrontal and hippocampus neurons playing a role in depression (35). Thus, abnormality of iron metabolism may cause changes in these mental disorders by affecting neurotransmitters. More population studies are needed to confirm the association; meanwhile, further mechanism studies are warranted to examine the biological connection between iron homeostasis and the diseases.

Key strengths of the study are primarily attributed to the MR design, which overcomes many unavoidable limitations introduced in observational studies. In addition, we systematically assessed the associations between four individual iron biomarkers and 24 mental disorders using summary-level data from large-scale genetic consortia and cohorts. Another strength is that the consistency between different MR approaches suggests the robustness of our findings.

There are also several limitations to this study. First, the statistical power was low in several analyses due to limited case number for some types of mental disorders. And it is also hard to perform stratified analyses by gender, age, or other categories. In addition, potential pleiotropy could not be completely ruled out. Although no evidence was found by the MR-Egger method, residual bias may still exist because the exact biological

function of the SNPs associated with iron status is not entirely clear. Last, we investigated the association between iron status within the normal range and health risk. Therefore, our findings may not be used to make inferences regarding the effect of pathological iron overload caused by primary or secondary hemochromatosis.

In summary, the present two-sample MR study is the first to systematically evaluate the causal role of iron status for a wide range of mental disorders. Our findings may highlight the potential therapeutic targets and preventative strategies. Future research is required to examine the causal associations in various populations with different ethnic backgrounds based on individual-level data, as well as the possible underlying mechanisms.

Data availability statement

The original contributions presented in this 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 Hangzhou Normal University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XF designed the research and wrote the manuscript. JQ and FL performed the statistical analyses. 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1084860/full#supplementary-material

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The modification of individual factors on association between serum 25(OH)D and incident type 2 diabetes: Results from a prospective cohort study

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Several epidemiological studies have suggested an association between low vitamin D status and increased risk for type 2 diabetes (T2D). This study aimed to explore the dose-response relationship of serum 25-hydroxyvitamin D [25(OH)D] concentrations with incident T2D and the interaction between serum 25(OH)D with individual factors on T2D risk. A total of 1,926 adults without diabetes (mean age: 52.08 ± 13.82 years; 42% men) were prospectively followed for 36 months. Cox proportional hazards model and restricted cubic spline analysis were performed to assess the association and dose-response relationship between serum 25(OH)D and T2D incidence. Both additive and multiplicative interactions were calculated between serum 25(OH)D and individual factors. The net reclassification index (NRI) was used to evaluate the improvement of risk prediction of T2D by adding serum 25(OH)D to traditional risk factors. There were 114 new T2D cases over a mean follow-up of 36 months. Serum 25(OH)D was not associated with T2D incidence, and no significant dose-response relationship was found in the total population. However, stratified analyses suggested a non-linear inverse relationship among individuals with baseline fasting plasma glucose (FPG) <5.6 mmol/L ($P_{overall}$ = 0.061, $P_{non\text{-}linear}$ = 0.048). And a significant multiplicative interaction was observed between serum 25(OH)D and FPG on T2D risk (P = 0.005). In addition, we found a significant additive interaction of low serum 25(OH)D with older age (RERI = 0.897, 95% CI: 0.080–1.714; AP = 0.468, 95% CI: 0.054–0.881), male (AP = 0.441, 95% CI: 0.010–0.871), and insufficient physical activity (RERI = 0.875, 95% CI: 0.204–1.545; AP = 0.575, 95% CI: 0.039–1.111) on T2D risk. Significant additive interactions were also observed between vitamin D deficiency/insufficiency with male, overweight/obesity, and insufficient physical activity on T2D risk.

Moreover, adding low serum 25(OH)D to a model containing established risk factors yielded significant improvements in the risk reclassification of T2D (NRI = 0.205, 95% CI: 0.019–0.391). Our results indicated a non-linear relationship of serum 25(OH)D concentrations with T2D risk among individuals with normal FPG and additive interactions of serum 25(OH)D with gender, overweight/obesity, and physical activity on T2D risk, suggesting the importance of outdoor exercise.

KEYWORDS

vitamin D, type 2 diabetes, physical activity, interaction, risk reclassification

1. Introduction

Type 2 diabetes (T2D) is a metabolic disease characterized by insulin resistance and pancreatic β-cell dysfunction. It was estimated that 537 million adults were living with diabetes in 2021, with an anticipated increase to 783 million by 2045 (1). The increasing incidence of T2D has prompted an urgent need for innovative methods to reverse this trend. Recently, vitamin D has attracted increasing public health interest because of its extra-skeletal effects (2, 3), including its association with the risk of developing T2D. It is biologically plausible that vitamin D may play a role in the pathogenesis of T2D, as insulin resistance and systematic inflammation have been reported with vitamin D deficiency *in vivo* and *in vitro* studies (4–6).

Several meta-analyses (7–9) summarized observational studies and suggested a negative association of blood 25-hydroxyvitamin D [25(OH)D] concentrations with T2D risk. However, the results of vitamin D supplementation for preventing T2D were inconsistent across intervention studies (2, 3, 10). For instance, the Diabetes Prevention with active Vitamin D (DPVD) randomized controlled trial indicated that active vitamin D treatment did not significantly decrease the incidence of diabetes among individuals with pre-diabetes (10). However, researchers did *post hoc* analyses and found that vitamin D supplementation significantly decreased the risk of diabetes in adults with a baseline body mass index (BMI) <30 kg/m^2 (2). We have previously shown that gender and baseline BMI modified the effect of vitamin D supplementation on metabolic profile among patients with T2D (11). These results suggested that individual factors might modify the relationship between blood 25(OH)D concentrations and T2D risk.

Several previous studies investigated the modifying effects of BMI, gender, and intensive exercise (12–15). For instance, one study reported that baseline hypovitaminosis D_3 successfully predicted hyperglycemia in the control group, but not in athletes, suggesting that physical activity might modify the association between vitamin D_3 levels and metabolic risk (15). Nevertheless, few studies explored the potential additive interactions between these risk factors and serum 25(OH)D on T2D risk. It is possible that the joint effect of low vitamin D levels with established risk factors on T2D may exceed expectations based on the individual effect. Data from the National Health and Nutrition Examination Survey (NHANES) indicated that the interaction of insufficient 25(OH)D and high BMI explained 47% of the increased odds of insulin resistance (16). Since vitamin D deficiency and insufficiency are easy to be diagnosed and cured, exploring the additive interactions of serum 25(OH)D with established risk factors on T2D risk may be helpful to identify the susceptible population who can be greatly benefited from improving vitamin D status.

Moreover, the changes in biological effects as serum 25(OH)D increases are S-shaped rather than linear (17). Several studies investigated the dose-response relationship between serum 25(OH)D and T2D risk and yielded inconsistent results (18–21). However, most previous studies were conducted in Europe where vitamin D supplements are commonly used, and the dose-response relationship of serum 25(OH)D with T2D risk has not been studied among the Chinese population.

In addition, previous studies reported that adding serum 25(OH)D to the Framingham Risk Score could improve coronary heart disease risk prediction in patients with essential hypertension or patients with T2D (22, 23). However, little was known about whether baseline serum 25(OH)D could refine risk prediction for T2D.

Thus, we conducted a prospective cohort study in Chinese adults to assess the dose-response relationship between serum 25(OH)D concentrations and the risk of T2D, and to explore the interactions of serum 25(OH)D with established risk factors on T2D risk. We also investigated whether serum 25(OH)D might improve the predictive value of T2D beyond conventional risk factors.

2. Materials and methods

2.1. The ethics board approval

The current study conformed to the principles set by the Declaration of Helsinki and was approved by the

Ethics Committee of Soochow University (ESCU-20160001). Written informed consent was obtained from all participants included in the study.

2.2. Study population

From 1 September to 14 October 2013, a total of 2,072 adults from the Liandu district in Lishui city, in east-central China, of Han ethnicity were invited to participate in the study. Liandu district consists of 16 blocks, and approximately 400,000 people live there. A multistage sampling method was applied to the present study. First, 4 residence communities in each block were sampled by simple random sampling. Then, systematic sampling was adopted to select 33 households in each residence community. Finally, one adult who had lived in the Liandu district for at least 2 years, was randomly selected from each household without replacement. In total, 2,072 residents were enrolled. Of these invited residents, 1,926 individuals met the inclusion criteria for the study: without diabetes, without malignancy, without chronic liver or renal diseases, and without using vitamin D supplementation (based on a self-assessment questionnaire). A follow-up survey was conducted for all participants in October 2016 to collect data on fasting plasma glucose (FPG) and the development of T2D.

2.3. Primary endpoint

The development of T2D was the primary endpoint in this study. New-onset T2D was defined as a self-reported history of a physician diagnosis during the follow-up period, and/or receiving pharmacological treatment for T2D, and/or an FPG $\geq$7.0 mmol/L (24).

2.4. Baseline measurements

At the baseline visit, standardized questionnaires were used to collect information regarding participants' demographics [i.e., age, gender, and residential district (rural or urban)], socio-economic status (including education level), smoking status, alcohol consumption, physical activity, and family history of diabetes]. Education level was categorized into four groups: no school, primary school, middle school, and junior college or higher. For smoking status, the research population was categorized into three groups: current smokers (smoking during the last 12 months or quit smoking less than 6 months ago), former smokers (quit smoking more than 6 months ago), and non-smokers (never smoked). For alcohol consumption, the research population was categorized as current drinkers (at least one time per week), former drinkers (quit drinking more than 6 months ago), and non-drinkers (never drank). According to the WHO recommendations, sufficient physical activity was defined as engaging in at least 150 min of moderate-intensity activity per week or equivalent (25). Family history was judged as positive when at least one of the first-degree relatives had diabetes.

Height (in cm) was measured with a wall-mounted stadiometer and weight (in kg) was measured with a balance-beam scale. BMI was calculated as body weight divided by height squared (kg/m^2). BMI $\geq$28.0, 24.0–27.9, and $<$24.0 kg/m^2 was classified as obesity, overweight, and normal weight (26). Blood pressure was measured three times while participants were in the relaxed sitting position after 15 min of rest. There was a 5-min rest period between each measurement and the mean value of the three measurements was used for analysis. Hypertension was defined as mean systolic blood pressure (SBP) $\geq$140 mmHg and/or diastolic blood pressure (DBP) $\geq$90 mmHg and/or the use of antihypertensive medications.

2.5. Data collection and definitions

Participants were instructed to take all regular medications, but not aspirin or non-steroidal anti-inflammatory drugs for 48 h before the visit. Participants were further requested to refrain from smoking for 1 h and from vigorous physical activity for 12 h before the visit. A venous blood sample was collected from each participant following an 8-h overnight fast for biochemical analysis. Aliquots of serum, plasma, and buffy coat were frozen and shipped on dry ice to Lishui Center for Disease Control and Prevention and stored at $-80°$C for future assays. Serum 25(OH)D concentration was determined using an automatic chemiluminescence immunoassay analyzer (ADVIA Centaur XP, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA). According to the Endocrine Society clinical practice guideline (27), vitamin D deficiency, insufficiency, and sufficiency were defined as serum 25(OH)D $<$20, 20–29.9, and $\geq$30 ng/ml. FPG concentration was determined by the hexokinase method using an automatic biochemical analyzer (COBAS c702, Roche Diagnostics Gmbh, Mannheim, Germany). Based on one of the diagnostic criteria for prediabetes proposed by the American Diabetes Association (24), impaired fasting glucose (IFG) was defined as 5.6 mmol/L $\leq$ FPG $<$ 7.0 mmol/L. The concentrations of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured by an enzymatic method on the automatic biochemistry analyzer (COBAS c702). Dyslipidemia was defined as TC $\geq$6.2 mmol/L and/or TG $\geq$2.3 mmol/L and/or HDL-C $<$1.0 mmol/L according to Chinese guideline for the management of dyslipidemia in adults (28).

2.6. Statistical analysis

All data were analyzed by using SAS version 9.4 (SAS Institute, Cary, NC, USA) and STATA version 16 (StataCorp, TX, USA), and statistical significance was defined as a two-sided P-value < 0.05. Data were presented as mean ± standard deviation or median (interquartile range: IQR) for continuous variables and as n (%) for categorical variables. Participants were divided into two groups using the median of serum 25(OH)D. The differences in quantitative variables between the two groups were compared by using independent Student's t-tests or Wilcoxon test as appropriate. Qualitative variables were tested by Chi-square analysis. The Cox proportional hazard regression model was performed to determine the hazard ratio (HR) for the risk of developing T2D and serum 25(OH)D concentration with T2D incidence as the dependent variable. Serum 25(OH)D was analyzed as a continuous variable or categorical variable which was categorized using median, thresholds of vitamin D deficiency and insufficiency, quartiles, and quintiles. In model 1, only serum 25(OH)D was included as the independent variable. Then age, gender, and BMI were included in model 2. In model 3, the following variables were added to model 2: residential district, education level, FPG, hypertension, dyslipidemia, smoking status, alcohol use, physical activity, and family history of diabetes. Tests for trends were performed by entering the median value of each category of serum 25(OH)D as a continuous variable in the models. We further calculated the HRs for serum 25(OH)D and T2D across strata of age, gender, residential district, BMI, physical activity, and baseline FPG. To further explore the possible dose-response or non-linear relationship between serum 25(OH)D concentrations and T2D risk, we used a restricted cubic spline model with three knots located at 10, 20, and 35 ng/ml (the reference: 20 ng/ml) (29). Both additive and multiplicative interactions between serum 25(OH)D (categorized using median or the threshold of vitamin D deficiency/insufficiency) and some established risk factors on the risk of T2D were calculated. Additive interaction better reflects the presence of biological interaction (30). To evaluate additive interaction, the relative excess risk due to interaction (RERI), attributable proportion of interaction (AP), and synergy index (S) were calculated as follows: RERI = HR_{11}-HR_{10}-HR_{01} + 1, AP = RERI / HR_{11}, S = (HR_{11}-1) / ((HR_{10}-1) + (HR_{01}-1)), where the subscripts indicate the presence (1) or absence (0) of the two risk factors (31). In the absence of additive interaction, RERI and AP are equal to 0, and S is equal to 1. A cross-product interaction term was set in the Cox proportional hazard regression model to assess multiplicative interaction. The improvement of T2D risk prediction by adding serum 25(OH)D (categorized using median) to traditional risk factors (variables adjusted in model 3) was assessed using the net reclassification index (NRI) and integrated discrimination improvement (IDI) (32, 33). The calibration of each model was evaluated by using Gronnesby and Borgan χ^2 statistic. Akaike and Bayesian information criteria were calculated to assess the global goodness of fit for each model. Likelihood ratio test was used to compare the global fit of the two models. The discrimination power of each model was evaluated by using Harrell's C index.

3. Results

3.1. Baseline characteristics of the study population

Table 1 summarized the baseline characteristics of the study populations. The mean age of the study population was 52.08 ± 13.82 years, and 808 (41.95%) were men. The median serum 25(OH)D concentration was 25.415 ng/ml (IQR: 21.14–29.55 ng/ml). Of 1,926 participants, percentage of vitamin D deficiency (<20 ng/ml) and insufficiency (20–29.9 ng/ml) was 19.37 and 57.84%, respectively.

As shown in Table 1, participants were divided into two groups according to the median of serum 25(OH)D. Subjects with serum 25(OH)D ≥25.415 ng/ml tended to be men, current smokers, current drinkers, live in rural areas, and have lower BMI and FPG (all P-values < 0.01), but were less likely to have dyslipidemia, sufficient physical activity, and family history of diabetes (all P-values < 0.05). There was no significant difference in age, education level, proportions of IFG, and hypertension between the two groups.

3.2. Associations of serum 25(OH)D concentrations with T2D incidence

As shown in Table 2, during the 36 months of follow-up, 114 participants (5.9%) developed incident T2D. Taking subjects with serum 25(OH)D ≥25.415 ng/ml as reference, those with 25(OH)D <25.415 ng/ml did have an increased risk of incident T2D in model 1 (HR = 1.48, 95% CI: 1.02–2.16; P = 0.039) and model 2 (adjusted HR = 1.49, 95% CI: 1.02–2.20; P = 0.042). However, after further adjustments for potential confounders, this association did not reach statistical significance in model 3 (adjusted HR = 1.36, 95% CI: 0.92–1.99; P = 0.120). Compared with vitamin D-sufficient participants, those with vitamin D deficiency and insufficiency had a higher risk of developing T2D in model 1 and model 2. But these associations were not significant in model 3. The HRs (95% CIs) were 1.83 (0.96–3.47) and 1.60 (0.92–2.79) for vitamin D deficient and insufficient subjects. No significant linear trend was observed in model 3 (P = 0.076). When considered as a continuous variable, a 10-ng/ml higher 25(OH)D concentration was not significantly associated with T2D incidence in models

TABLE 1 Baseline characteristics of the study population according to serum 25(OH)D concentrations (n = 1,926).

Characteristic	Total (n = 1,926)	Serum 25(OH)D concentrations (ng/ml)		P
		<25.415 (n = 963)	≥25.415 (n = 963)	
25(OH)D, median (IQR)	25.415 (21.14–29.55)	21.14 (17.93–23.55)	29.55 (27.37–32.45)	
Age (years)	52.08 ± 13.82	51.65 ± 14.18	52.52 ± 13.44	0.164
Sex (men), n (%)	808 (41.95)	294 (30.53)	514 (53.37)	<0.001
District (rural), n (%)	754 (39.15)	328 (34.06)	426 (44.24)	<0.001
Education, n (%)				0.118
No school	327 (16.98)	164 (16.93)	163 (17.03)	
Primary school	524 (27.21)	256 (26.58)	268 (27.83)	
Middle school	938 (48.70)	461 (47.87)	477 (49.53)	
Junior college or higher	137 (7.11)	82 (8.52)	55 (5.71)	
Body mass index (kg/m^2)	23.73 ± 3.32	23.94 ± 3.43	23.53 ± 3.18	0.007
Normal (<24)	1,091 (56.65)	516 (53.58)	575 (59.71)	0.009
Overweight (24–28)	640 (33.23)	334 (34.68)	306 (31.78)	
Obesity (≥28)	195 (10.12)	113 (11.73)	82 (8.52)	
FPG (mmol/L)	5.65 ± 0.59	5.69 ± 0.58	5.61 ± 0.60	0.003
IFG, n (%)	996 (51.71)	516 (53.58)	480 (49.84)	0.101
Hypertension, n (%)	632 (32.81)	318 (33.02)	314 (32.61)	0.846
Dyslipidemia, n (%)	832 (43.20)	471 (48.91)	361 (37.49)	<0.001
Current smoker, n (%)	415 (21.55)	168 (17.45)	247 (25.65)	<0.001
Current drinker, n (%)	585 (30.37)	225 (23.36)	360 (37.38)	<0.001
Sufficient physical activity, n (%)	429 (22.27)	235 (24.40)	194 (20.15)	0.025
Family history of diabetes, n (%)	54 (2.8)	35 (3.63)	19 (1.97)	0.027

IQR, interquartile range; FPG, fasting plasma glucose; IFG, impaired fasting glucose.

1–3 (Table 2). When categorized using quartiles and quintiles, serum 25(OH)D concentrations was not significantly associated with T2D incidence in model 3 (Table 2).

3.3. The dose-response relationship between serum 25(OH)D concentrations with T2D risk

Restricted cubic spline analyses were conducted to explore the possible dose-response or non-linear relationship of serum 25(OH)D concentrations with the risk of T2D. As shown in Figure 1, no significant dose-response relationship between serum 25(OH)D and T2D risk was observed among the total population ($P_{overall}$ = 0.1640, $P_{non\text{-}linear}$ = 0.1508). Stratified analysis suggested a non-linear inverse relationship among individuals with baseline FPG <5.6 mmol/L ($P_{overall}$ = 0.061, $P_{non\text{-}linear}$ = 0.048; Figure 1). When the serum 25(OH)D concentrations was between approximately 28.5 and 40 ng/ml, HR decreased rapidly with the increase of serum 25(OH)D (Supplementary Table 1). And when the serum 25(OH)D concentrations reached or exceeded approximately 40 ng/ml, the reducing trend of HR value tended to be flat. For participants with baseline FPG <5.6 mmol/L, the adjusted HRs of T2D were 0.38 (95% CI: 0.16–0.92) at 30 ng/ml, 0.05 (95% CI: 0.00–0.60) at 40 ng/ml, 0.01 (95% CI: 0.00–0.42) at 50 ng/ml compared with 20 ng/ml (Table 3). No significant dose-response relationship between serum 25(OH)D concentrations and T2D risk was observed among other subgroups (Supplementary Figures 1, 2).

For individuals with baseline FPG <5.6 mmol/L, those in the second quartile had a higher risk of T2D than those in the highest quartile (adjusted HR = 8.38, 95% CI: 1.82–38.60; Supplementary Table 2), and those in the second (adjusted HR = 5.96, 95% CI: 1.22–29.03) and third (adjusted HR = 5.14, 95% CI: 1.06–25.08) quintile had an increased T2D risk than those in the highest quintile (Supplementary Table 3).

3.4. Interaction analysis of serum 25(OH)D with conventional risk factors on T2D risk

Participants with serum 25(OH)D concentrations less than median (25.415 ng/ml) were classified as low 25(OH)D

TABLE 2 Associations of serum 25(OH)D concentrations with incident type 2 diabetes.

25(OH)D (ng/ml)	Events (%)	Model 1		Model 2		Model 3	
		HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
High (≥25.415)	46 (4.78)	1.00	–	1.00	–	1.00	
Low (<25.415)	68 (7.06)	1.48 (1.02–2.16)	0.039	1.49 (1.02–2.20)	0.042	1.36 (0.92–1.99)	0.120
Without vitamin D deficiency (≥20)	88 (5.67)	1.00	–	1.00	–	1.00	–
Vitamin D deficiency (<20)	26 (6.97)	1.23 (0.79–1.90)	0.356	1.18 (0.75–1.84)	0.470	1.26 (0.80–1.97)	0.319
Vitamin D sufficiency (≥30)	16 (3.64)	1.00	–	1.00	–	1.00	–
Vitamin D deficiency or insufficiency (<30)	98 (6.59)	1.82 (1.07–3.09)	0.026	1.83 (1.07–3.14)	0.028	1.66 (0.96–2.84)	0.068
Vitamin D sufficiency (≥30)	16 (3.64)	1.00	–	1.00	–	1.00	–
Vitamin D insufficiency (20–29.9)	72 (6.46)	1.79 (1.04–3.07)	0.036	1.81 (1.05–3.14)	0.034	1.60 (0.92–2.79)	0.094
Vitamin D deficiency (<20)	26 (6.97)	1.92 (1.03–3.58)	0.040	1.89 (1.00–3.60)	0.051	1.83 (0.96–3.47)	0.065
P for trend*		0.049		0.068		0.076	
Q4 (≥29.55)	20 (4.15)	1.00	–	1.00	–	1.00	–
Q3 (25.415–29.55)	26 (5.41)	1.30 (0.73–2.33)	0.379	1.30 (0.72–2.33)	0.381	1.23 (0.68–2.22)	0.499
Q2 (21.14–25.415)	36 (7.47)	1.81 (1.05–3.13)	0.034	1.85 (1.06–3.22)	0.030	1.56 (0.89–2.72)	0.121
Q1 (<21.14)	32 (6.65)	1.60 (0.92–2.80)	0.098	1.59 (0.89–2.83)	0.117	1.48 (0.83–2.63)	0.185
P for trend*		0.064		0.079		0.146	
Q5 (≥30.61)	16 (4.12)	1.00	–	1.00	–	1.00	–
Q4 (26.92–30.61)	19 (4.96)	1.20 (0.62–2.34)	0.587	1.23 (0.63–2.40)	0.544	1.10 (0.56–2.16)	0.781
Q3 (23.98–26.92)	22 (5.71)	1.39 (0.73–2.65)	0.314	1.44 (0.75–2.75)	0.273	1.23 (0.64–2.36)	0.539
Q2 (20.17–23.98)	30 (7.79)	1.91 (1.04–3.50)	0.037	1.92 (1.03–3.58)	0.040	1.55 (0.83–2.90)	0.172
Q1 (<20.17)	27 (7.01)	1.70 (0.92–3.16)	0.091	1.71 (0.90–3.24)	0.099	1.59 (0.84–3.01)	0.152
P for trend*		0.033		0.045		0.078	
Continuous#	114 (5.92)	0.75 (0.56–1.00)	0.052	0.76 (0.57–1.03)	0.072	0.80(0.59–1.09)	0.155

Model 1: unadjusted; model 2: adjusted for age, gender, and body mass index; model 3: adjusted for model 2 and district, education level, fasting plasma glucose, hypertension, dyslipidemia, smoking status, alcohol drinking, physical activity, and family history of diabetes. HR, hazard ratio; CI, confidence interval.

*Test for trend based on the variable containing the median value for each group.

#HR was scaled to 10-ng/ml higher serum 25(OH)D concentrations.

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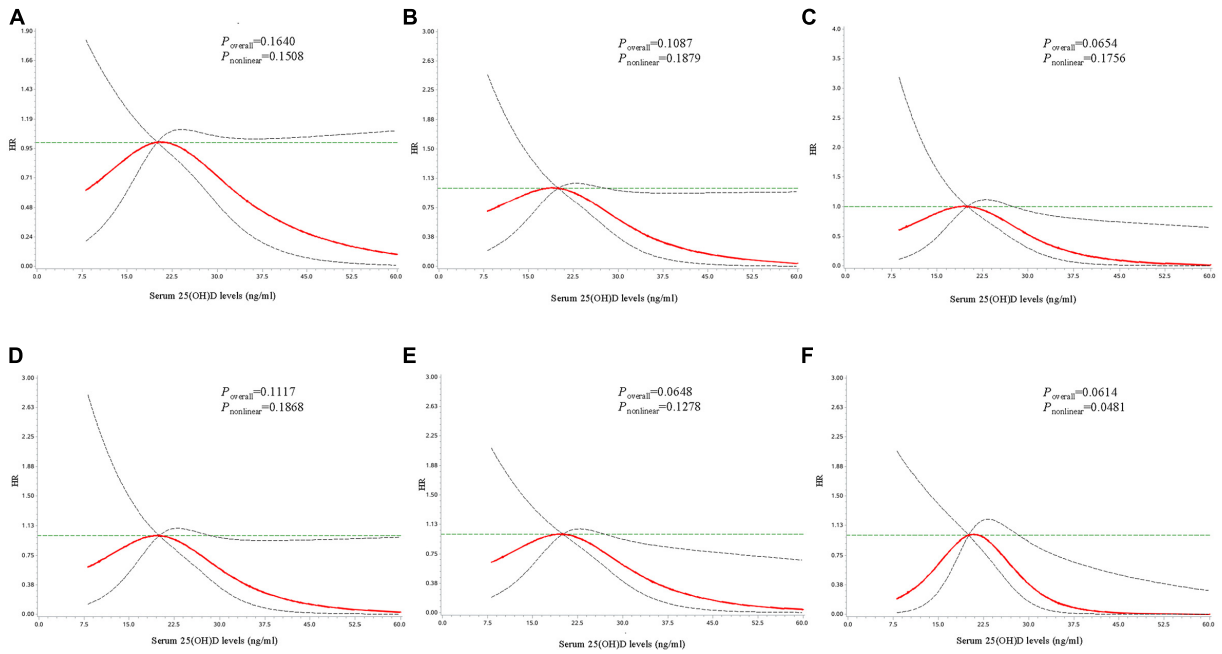

FIGURE 1
Dose-response curves for the associations between serum 25(OH)D concentrations with risk of developing type 2 diabetes. Dose-response curves for the associations between serum 25(OH)D with risk of developing type 2 diabetes in the total population **(A)**, among individuals with age ≥52 years **(B)**, with male gender **(C)**, with overweight/obesity **(D)**, with insufficient physical activity **(E)**, and with fasting plasma glucose <5.6 mmol/L at baseline **(F)**. The solid line and dashed line represent the adjusted estimated HRs and 95% confidence intervals. Analyses were adjusted for age, gender, body mass index, district, education level, fasting plasma glucose, hypertension, dyslipidemia, smoking status, alcohol drinking, physical activity, and family history of diabetes.

group, and others were defined as high 25(OH)D group. As shown in Table 4, stratified analyses indicated that the significant relationship of low 25(OH)D with increased risk of T2D was limited to men (adjusted HR = 1.82, 95% CI: 1.03–3.21; P = 0.039), individuals with overweight/obesity (adjusted HR = 1.64, 95% CI: 1.00–2.68; P = 0.048), insufficient physical activity (adjusted HR = 1.58, 95% CI: 1.03–2.42; P = 0.037), FPG <5.6 mmol/L at baseline (adjusted HR = 2.78, 95% CI: 1.18–6.51; P = 0.019). The RERI and AP for the interaction of low serum 25(OH)D with older age was 0.651 (−0.095 to 1.398) and 0.464 (−0.050 to 0.977), while S was not statistically significant, suggesting a potential additive interaction between these two factors on T2D risk. And about 46.4% of the HR of being T2D was attributable to the interaction between these two risk factors. The AP for the interaction of low serum 25(OH)D with male was 0.441 (95% CI: 0.01–0.871), while RERI and S were not statistically significant (Table 4), suggesting a potential additive interaction between these two factors. For additive interaction between low serum 25(OH)D and insufficient physical activity (Table 4), the estimated RERI and AP were 0.875 (95% CI: 0.204–1.545) and 0.575 (95% CI: 0.039–1.111), respectively, while S was not statistically significant, suggesting that there might be a potential additive interaction between low serum 25(OH)D and insufficient physical activity on T2D risk. A significant multiplicative interaction effect was observed between low serum 25(OH)D and FPG on T2D risk (P = 0.005).

When stratified by the threshold of vitamin D deficiency/insufficiency [serum 25(OH)D <30 ng/ml], a significant association of vitamin D deficiency/insufficiency with increased risk of T2D was found among men (adjusted HR = 2.26, 95% CI: 1.05–4.86; P = 0.037), individuals with overweight/obesity (adjusted HR = 2.42, 95% CI: 1.04–5.63; P = 0.040), individuals with insufficient physical activity (adjusted HR = 1.92, 95% CI: 1.03–3.57; P = 0.039), and individuals with FPG <5.6 mmol/L at baseline (adjusted HR = 4.93, 95% CI: 1.14–21.35; P = 0.033; Table 5). And the RERI for the interactions of vitamin D deficiency/insufficiency with male, overweight/obesity, and insufficient physical activity were 0.857 (95% CI: 0.104–1.609), 1.382 (95% CI: 0.294–2.470), and 0.877 (95% CI: 0.200–1.554), respectively, while the corresponding AP and S were not statistically significant, except the AP for the interaction of vitamin D deficiency/insufficiency with overweight/obesity (0.570, 95% CI: 0.116–1.024; Table 5).

No evidence suggested significant additive or multiplicative interactions of serum 25(OH)D with residential district (Tables 4, 5).

TABLE 3 Hazard ratios (95% CIs) of type 2 diabetes by serum 25(OH)D concentrations.

	HR (95% CI)*			
	20 ng/ml	30 ng/ml	40 ng/ml	50 ng/ml
Total	1.00 (reference)	0.73 (0.50–1.05)	0.37 (0.14–1.03)	0.19 (0.03–1.06)
Age				
<52 years	1.00 (reference)	1.14 (0.60–2.16)	0.82 (0.16–4.23)	0.58 (0.03–9.76)
≥52 years	1.00 (reference)	0.60 (0.37–0.97)	0.25 (0.06–0.94)	0.10 (0.01–0.94)
Gender				
Male	1.00 (reference)	0.53 (0.30–0.92)	0.17 (0.04–0.77)	0.05 (0.00–0.70)
Female	1.00 (reference)	1.02 (0.62–1.67)	0.89 (0.22–3.54)	–
District				
Rural	1.00 (reference)	0.71 (0.36–1.42)	0.29 (0.05–1.82)	0.11 (0.00–2.62)
Urban	1.00 (reference)	0.77 (0.50–1.20)	0.46 (0.14–1.56)	0.27 (0.04–2.14)
BMI				
<24 kg/m^2	1.00 (reference)	0.95 (0.55–1.63)	0.79 (0.20–3.19)	0.65 (0.06–6.79)
≥24 kg/m^2	1.00 (reference)	0.58 (0.34–0.98)	0.21 (0.05–0.94)	0.13 (0.02–0.94)
Physical activity				
Sufficient	1.00 (reference)	1.40 (0.58–3.35)	1.79 (0.18–17.80)	–
Insufficient	1.00 (reference)	0.61 (0.40–0.93)	0.25 (0.08–0.81)	0.10 (0.01–0.73)
FPG				
<5.6 mmol/L	1.00 (reference)	0.38 (0.16–0.92)	0.05 (0.00–0.60)	0.01 (0.00–0.42)
≥5.6 mmol/L	1.00 (reference)	0.92 (0.61–1.38)	0.77 (0.25–2.37)	0.64 (0.10–4.32)

BMI, body mass index; FPG, fasting plasma glucose; HR, hazard ratio; CI, confidence interval.
*Adjusted for age, gender, body mass index, district, education level, fasting plasma glucose, hypertension, dyslipidemia, smoking status, alcohol drinking, physical activity, and family history of diabetes.
The symbol "–"indicates the maximum value of serum 25(OH)D concentrations in these two subgroups were less than 50 ng/ml.

3.5. Prediction value of serum 25(OH)D for type 2 diabetes

The category-free NRI of the model including serum 25(OH)D status improved by 0.205 (95% CI: 0.019–0.391, P = 0.034) for predicting T2D compared to the conventional risk model (Table 6). When serum 25(OH)D status was added to the conventional risk model, 19% more cases were correctly reclassified. Other analysis results, including the calibration, global goodness of fit, and discrimination of each model, can be found in Supplementary Table 4.

The added predictive value of including serum 25(OH)D were more prominent in adults with age ≥52 years (NRI = 0.349, 95% CI: 0.123–0.576; P = 0.004), men (NRI = 0.473, 95% CI: 0.199–0.747; P = 0.001), adults with overweight/obesity (NRI = 0.249, 95% CI: 0.020–0.478; P = 0.041), adults with insufficient physical activity (NRI = 0.293, 95% CI: 0.089–0.497; P = 0.006) or with FPG <5.6 mmol/L at baseline (NRI = 0.503, 95% CI: 0.171–0.835; P = 0.008). Serum 25(OH)D correctly reclassified 26, 30, 25, and 45% more cases among those with age ≥52 years, with overweight/obesity, with insufficient physical activity, or with FPG <5.6 at baseline (Table 6).

4. Discussion

The relationship between serum 25(OH)D concentrations and T2D risk have attracted extensive attention during the past decades, and several previous studies, but not all, reported a significant inverse correlation between serum 25(OH)D and T2D risk. Several meta-analyses summarized these studies and concluded that low vitamin D levels might be associated with an increased risk of T2D (7–9). For instance, Song et al performed a meta-analysis on the data from 21 prospective studies and found that the summary risk ratio for T2D was 0.62 (95% CI: 0.54–0.70) when comparing the highest to the lowest category of 25(OH)D concentrations (9). The China Kadoorie Biobank (CKB) study indicated that a 10-ng/ml higher 25(OH)D concentration was associated with a 9% (95% CI: 0–18%) lower risk of incident diabetes after adjustment for age, gender, latitude, season, SBP, physical activity, and body fat percentage (34). While the Hong Kong Osteoporosis Study reported no association between serum vitamin D and the risk of incident diabetes in Hong Kong Chinese (35).

TABLE 4 Interactions between serum 25(OH)D and established risk factors on type 2 diabetes risk.

	Events (%)	HR (95% CI)*	*P**	RERI (95% CI)	AP (95% CI)	S (95% CI)	$P_{interaction}$ #
Total	114 (5.92)	1.36 (0.92–1.99)	0.120				
Age				0.897 (0.080 to 1.714)	0.468 (0.054 to 0.881)	42.688 (0 to NA)[b]	0.138
<52 years	38 (3.93)	0.95 (0.49–1.84)	0.870				
≥52 years	76 (7.92)	1.56 (0.96–2.53)	0.074				
Gender				0.766 (−0.064 to 1.596)	0.441 (0.010 to 0.871)[a]	−26.391 (NA)[b]	0.134
Male	53 (6.56)	1.82 (1.03–3.21)	0.039				
Female	61 (5.46)	1.04 (0.62–1.76)	0.877				
District				0.042 (−1.003 to 1.088)	0.024 (−0.571 to 0.619)	1.059 (0.245 to 4.574)	0.908
Rural	36 (4.77)	1.32 (0.65–2.67)	0.446				
Urban	78 (6.66)	1.34 (0.84–2.14)	0.219				
BMI				0.943 (−0.120 to 2.005)	0.372 (−0.021 to 0.764)	2.585 (0.415 to 16.117)	0.114
<24 kg/m^2	40 (3.67)	1.10 (0.57–2.13)	0.784				
≥24 kg/m^2	74 (8.86)	1.64 (1.00–2.68)	0.048				
Physical activity				0.875 (0.204–1.545)[a]	0.575 (0.039 to 1.111)[a]	−1.471 (NA)[b]	0.087
Sufficient	21 (4.90)	0.79 (0.31–2.07)	0.637				
Insufficient	93 (6.21)	1.58 (1.03–2.42)	0.037				
FPG				−1.729 (−4.031 to 0.574)	−1.643 (−3.442 to 0.156)	0.029 (0–1.08 × 10^6)	0.005
<5.6 mmol/L	29 (3.12)	2.78 (1.18–6.51)	0.019				
≥5.6 mmol/L	85 (8.53)	1.07 (0.69–1.66)	0.763				

BMI, body mass index; FPG, fasting plasma glucose; HR, hazard ratio; CI, confidence interval; RERI, relative excessive risk due to interaction; AP, attributable proportion due to interaction; S, the synergy index.

*Serum 25(OH)D was categorized using median (25.415 ng/ml) and the reference group was serum 25(OH)D ≥25.415 ng/ml, the HRs were adjusted for age, gender, body mass index, district, education level, fasting plasma glucose, hypertension, dyslipidemia, smoking status, alcohol drinking, physical activity, and family history of diabetes.

#*P* for multiplicative interaction.

[a]Statistically significant with RERI >0 and AP >0 indicating additive interaction.

[b]The 95% CI of S was not available because the values of S were less than 0.

In the current study, the association between serum 25(OH)D concentrations and incident T2D was non-significant in an adult population from east-central China. The failure to detect significance might be due to the small sample size, short follow-up time, or high prevalence of vitamin D deficiency/insufficiency (77.21%). Vitamin D deficiency/insufficiency was prevalent in the Chinese population, especially among the elderly population and women (34, 36, 37). Due to the high prevalence of vitamin D deficiency/insufficiency, the sample size of the vitamin D sufficiency group was less than that of the vitamin D deficiency/insufficiency group, which might limit the ability to detect a significant association between vitamin D status and T2D risk. For example, the association of vitamin D deficiency/insufficiency with T2D risk was marginally significant after complete adjustments (adjusted HR = 1.66, 95% CI: 0.96–2.84; *P* = 0.068). Another possible explanation could be the study population who were at high risk for T2D (38). Vitamin D might be only beneficial in individuals with normal glucose tolerance because the development of T2D consisted of progressive insulin resistance, which was initially compensated by enhanced insulin secretion. At the onset of T2D, the β cell mass was reduced by 25–50% (39). When IFG is already present, the effect of vitamin D might be not strong enough to reverse the deterioration of glucose metabolism. In the current study, about half (51.71%) of the study participants had IFG at baseline. Stratified analysis showed that the effect of low serum 25(OH)D on T2D risk was significant in individuals with baseline FPG <5.6 mmol/L, but not among those with FPG ≥5.6 mmol/L at baseline. However, in a Swedish middle-aged population, high serum 25(OH)D predicted a lower risk of T2D in persons with prediabetes, but not among those with normal glucose tolerance (40). In participants with FPG <5.6 mmol/L, we found a non-linear inverse relationship between serum 25(OH)D and T2D with risk decreased rapidly when the serum 25(OH)D was between approximately 28.5 and 40 ng/ml, and when the serum 25(OH)D reached or exceeded approximately 40 ng/ml, the reducing trend of HR value tended to be flat.

TABLE 5 Interactions between vitamin D deficiency/insufficiency and established risk factors on type 2 diabetes risk.

	HR (95% CI)*	*P**	RERI (95% CI)	AP (95% CI)	S (95% CI)	$P_{interaction}$ #
Total	1.66 (0.96–2.84)	0.068				
Age			0.686 (−0.466 to 1.839)	0.284 (−0.271 to 0.838)	1.937 (0.210 to 17.838)	0.541
<52 years	1.66 (0.63–4.40)	0.305				
≥52 years	1.61 (0.83–3.11)	0.161				
Gender			0.857 (0.104 to 1.609)[a]	0.535 (−0.037 to 1.108)	−2.346 (NA)[b]	0.144
Male	2.26 (1.05–4.86)	0.037				
Female	1.09 (0.51–2.30)	0.826				
District			0.212 (−1.062 to 1.486)	0.101 (−0.538 to 0.739)	1.237 (0.251 to 6.089)	0.969
Rural	1.57 (0.63–3.91)	0.336				
Urban	1.63 (0.83–3.21)	0.160				
BMI			1.382 (0.294 to 2.470)[a]	0.570 (0.116 to 1.024)[a]	33.084 (0 to NA)[b]	0.112
<24 kg/m^2	0.98 (0.46–2.13)	0.967				
≥24 kg/m^2	2.42 (1.04–5.63)	0.040				
Physical activity			0.877 (0.200 to 1.554)[a]	0.605 (−0.189 to 1.399)	−1.056 (NA)[b]	0.181
Sufficient	0.88 (0.26–3.01)	0.840				
Insufficient	1.92 (1.03–3.57)	0.039				
FPG			−3.346 (−9.746 to 3.054)	−1.566 (−3.170 to 0.039)	0.254 (0.078 to 0.828)	0.022
<5.6 mmol/L	4.93 (1.14–21.35)	0.033				
≥5.6 mmol/L	1.19 (0.66–2.14)	0.569				

BMI, body mass index; FPG, fasting plasma glucose; HR, hazard ratio; CI, confidence interval; RERI, relative excessive risk due to interaction; AP, attributable proportion due to interaction; S, the synergy index.

*Serum 25(OH)D was categorized using 30 ng/ml and the reference group was serum 25(OH)D ≥30 ng/ml, the HRs were adjusted for age, gender, body mass index, district, education level, fasting plasma glucose, hypertension, dyslipidemia, smoking status, alcohol drinking, physical activity, and family history of diabetes.

P for multiplicative interaction.

[a]Statistically significant with RERI > 0 and AP > 0 indicating additive interaction.

[b]The 95% CI of S was not available because the values of S were less than 0.

Previous studies reported that T2D risk increased significantly below the cutoff of 10 ng/ml (19), 16 ng/ml (21), and 18 ng/ml (20). Our results showed a significant multiplicative interaction between serum 25(OH)D and FPG on T2D risk, but the test of additive interaction was non-significant. The modification of baseline glucose metabolism status on the association between vitamin D and T2D risk deserves further investigation.

Stratified analyses also showed that the association between serum 25(OH)D and T2D risk was limited to men, adults with overweight/obesity, and individuals with insufficient physical activity. In agreement with our study, the MONICA 10 study showed that a significant inverse association of serum 25(OH)D concentrations with T2D risk was only evident among individuals with overweight/obesity (14). The Nurses' Health Study also reported that the inverse association was stronger among women with overweight/obesity than women with normal BMI (13). However, results from the Copenhagen City Heart Study did not observe any effect modification by BMI (41). Interaction analysis between 25(OH)D and BMI was conducted in some studies. Most of them only did multiplicative interaction and reported negative results (12, 13, 41). For instance, no significant multiplicative interaction was observed between vitamin D biomarkers and BMI on diabetes incidence among older adults from the ESTHER study (18). But the MONICA 10 study reported significant multiplicative interaction between 25(OH)D and BMI as well as WC (14). Data from the NHANES indicated that there is no multiplicative or additive interaction between overweight/obesity and insufficient 25(OH)D on T2D (17). Our results suggested a potential additive interaction between vitamin D deficiency/insufficiency and overweight/obesity on T2D risk. And about 55.5% of the HR of being T2D was attributable to the interaction between these two factors. As more than half of the Chinese adult population is overweight/obesity and vitamin D deficiency/insufficiency is highly prevalent among persons with obesity (42, 43), our finding might have an important impact on public health.

As for gender and physical activity, two prospective studies reported that the inverse association of 25(OH)D with incident T2D did not differ by gender or physical activity, and no significant multiplicative interaction existed (14, 41). Our results suggested a potential additive interaction between serum 25(OH)D and male on T2D risk. Future large-scale studies

TABLE 6 Reclassification of type 2 diabetes risk after addition of serum 25(OH)D to conventional risk model.

	NRI (95% CI)*	*P**	% of events correctly reclassified	*P*
Total	0.205 (0.019 to 0.391)	0.034	19%	0.039
Age				
<52 years	0.006 (−0.318 to 0.330)	0.973	−5%	0.746
≥52 years	0.349 (0.123 to 0.576)	0.004	26%	0.022
Gender				
Male	0.473 (0.199 to 0.747)	0.001	17%	0.216
Female	−0.017 (−0.270 to 0.235)	0.896	−21%	0.096
BMI				
<24 kg/m^2	0.062 (−0.254 to 0.378)	0.701	0	1
≥24 kg/m^2	0.249 (0.020 to 0.478)	0.041	30%	0.011
Physical activity				
Sufficient	0.146 (−0.292 to 0.584)	0.515	5%	0.827
Insufficient	0.293 (0.089 to 0.497)	0.006	25%	0.017
FPG				
<5.6 mmol/L	0.503 (0.171 to 0.835)	0.008	45%	0.016
≥5.6 mmol/L	0.076 (−0.145 to 0.297)	0.501	11%	0.329

BMI, body mass index; FPG, fasting plasma glucose; NRI, net reclassification index; CI, confidence interval.
*Serum 25(OH)D was categorized using median (25.415 ng/ml), conventional risk factors included age, sex, body mass index, district, education level, fasting plasma glucose, hypertension, dyslipidemia, smoking status, alcohol drinking, physical activity, and family history of diabetes.

are needed to confirm this gender difference. Our results also revealed an additive interaction between serum 25(OH)D and physical activity on T2D risk. The joint effects of low serum 25(OH)D and insufficient physical activity on T2D risk were greater than would be expected from the effects of the individual risk factor alone. This finding may have large public health significance because the burden of T2D among persons with insufficient physical activity may be decreased by making improvements in serum 25(OH)D concentrations. Solar exposure is a key determinant of serum 25(OH)D concentrations, as skin exposure to solar ultraviolet is a primary source of vitamin D. Therefore, increased outdoor physical activities during the daytime may lead to more solar exposure, which increases vitamin D synthesis. Thus, targeting lifestyle through sufficient outdoor physical activity may be the first option that will correct vitamin D deficiency/insufficiency and insufficient exercise. The mechanism underlying the additive interaction of serum 25(OH)D with physical activity on T2D risk is not clearly understood. In adults at high risk of T2D, moderate to vigorous physical activity was associated with lower concentrations of trimethylamine N-oxide (TMAO) (44), a novel gut-derived metabolite that was associated with insulin resistance and impaired glucose tolerance (45, 46). Animal studies showed vitamin D supplementation greatly reduced plasma TMAO levels in mice (47), and a cross-sectional study reported high TMAO concentrations were associated with vitamin D deficiency (48). It is plausible to speculate that higher TMAO due to insufficient physical activity may be aggravated by low serum 25(OH)D. Further studies are needed to clarify our results and explore the underlying mechanism.

In addition, we calculated the category-free NRI to measure the incremental predictive value of adding serum 25(OH)D status to conventional risk factors for the development of T2D. The addition of low serum 25(OH)D significantly improved the NRI for T2D by 0.205 in the total population. The improvement in the prediction of T2D, above conventional risk factors, demonstrated the potential of serum 25(OH)D as a biomarker for T2D risk. Stratified analyses indicated that the added predictive value of including serum 25(OH)D status was most evident among men, adults with older age, overweight/obesity, insufficient physical activity, or normal FPG. Few studies previously evaluated the impact of adding baseline serum 25(OH)D on the net reclassification of T2D risk beyond conventional risk factors. Researchers found that the addition of serum 25(OH)D to the Framingham Risk Score could improve coronary heart disease risk estimation in patients with essential hypertension or patients with T2D (22, 23). However, adding serum 25(OH)D in addition to established risk factors only marginally added prognostic value of mortality risk in patients undergoing coronary catheterization (49). Considering that vitamin D deficiency and insufficiency are easy to be diagnosed and cured, future studies are needed to evaluate the cost-benefit of adding serum 25(OH)D into the T2D risk estimation and

then determine the feasibility of measuring serum 25(OH)D for T2D prevention, especially in high-risk populations.

To the best of our knowledge, the present study is the first to report an additive interaction between serum 25(OH)D concentrations and physical activity on T2D risk. Nevertheless, there are several limitations in the present study. First, the relatively small sample size, which limited our statistical power, and the short-term follow-up. The follow-up period might not be long enough to detect a modest effect and the association of serum 25(OH)D with T2D may be underpowered, especially within subgroup analysis. Second, we did not have data on oral glucose tolerance tests or HbA1c, which might lead to misclassification of the diagnosis of T2D. Third, a lack of information on serum 25(OH)D concentrations at follow-up might be a potential confounder and result in insufficient confirmation of our results. For instance, the Vitamin D and Type 2 Diabetes (D2d) study did not find a significant effect of vitamin D supplementation on diabetes risk, but a secondary analysis found that adults who maintained higher intratrial serum 25(OH)D levels during follow-up had a reduced risk of diabetes (50).

5. Conclusion

Our results suggest a non-linear reverse relationship between serum 25(OH)D and T2D risk among individuals with normal FPG at baseline, and a significant multiplicative interaction was detected between serum 25(OH)D and FPG on T2D risk. Significant additive interaction of serum 25(OH)D with male, overweight/obesity, and physical activity was observed. For T2D risk prediction, adding low serum 25(OH)D to the model with established risk factors might offer incremental predictive power. Our data are of significance to public health, and future studies are needed to confirm these findings and identify susceptible populations who can be greatly benefited from outdoor physical activity.

Data availability statement

The original contributions presented in this 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 Soochow University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZH, XZ, and ZZ: conceptualization, project administration, and funding acquisition. XZ, YM, and JW: methodology and data analyses. ZH and JZ: investigation and data collection. XZ, JL, and BL: manuscript writing and editing and final approval. 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.1077734/full#supplementary-material

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Recent advances in the application of ionomics in metabolic diseases

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Trace elements and minerals play a significant role in human health and diseases. In recent years, ionomics has been rapidly and widely applied to explore the distribution, regulation, and crosstalk of different elements in various physiological and pathological processes. On the basis of multi-elemental analytical techniques and bioinformatics methods, it is possible to elucidate the relationship between the metabolism and homeostasis of diverse elements and common diseases. The current review aims to provide an overview of recent advances in the application of ionomics in metabolic disease research. We mainly focuses on the studies about ionomic or multi-elemental profiling of different biological samples for several major types of metabolic diseases, such as diabetes mellitus, obesity, and metabolic syndrome, which reveal distinct and dynamic patterns of ion contents and their potential benefits in the detection and prognosis of these illnesses. Accumulation of copper, selenium, and environmental toxic metals as well as deficiency of zinc and magnesium appear to be the most significant risk factors for the majority of metabolic diseases, suggesting that imbalance of these elements may be involved in the pathogenesis of these diseases. Moreover, each type of metabolic diseases has shown a relatively unique distribution of ions in biofluids and hair/nails from patients, which might serve as potential indicators for the respective disease. Overall, ionomics not only improves our understanding of the association between elemental dyshomeostasis and the development of metabolic disease but also assists in the identification of new potential diagnostic and prognostic markers in translational medicine.

KEYWORDS

trace element, ionome, ionomics, metabolic disease, bioinformatics

1. Introduction

Metabolic diseases are well-known as a major threat to global public health, which include diabetes mellitus (DM), obesity, metabolic syndrome (MetS), non-alcoholic fatty liver disease (NAFLD), hyperlipidemia, hyperuricemia (or gout), bone metabolic disease (such as osteoporosis and osteomalacia), and some other diseases that are caused by endocrine dysfunction (e.g., thyroid diseases) or nutrient imbalance (such as Wilson's disease) (1, 2). In the past several decades, much effort has been made to understand the underlying mechanisms and to develop rational treatments for these diseases and disorders. However, due to the complex pathogenesis and characteristics of most metabolic diseases, the progress still remains quite limited. Thus, effective strategies of early diagnosis and treatment of metabolic diseases are urgently needed.

Among known risk factors and possible molecular mechanisms associated with metabolic diseases, dyshomeostasis of various elements has been implicated in the onset and progression of many types of them (3–6). Except for a small number of elements that are required in large amounts (called macroelements or macronutrients), such as potassium (K), sodium (Na), calcium (Ca), and magnesium (Mg), the majority of them are trace elements. Biological trace elements (or micronutrients, mostly metals) are needed in small quantities but are essential for

growth, development, and various physiological processes of all living organisms, which include iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), molybdenum (Mo), nickel (Ni), cobalt (Co), chromium (Cr), vanadium (V), selenium (Se), iodine (I), and several other elements (7, 8). They serve as critical cofactors for the assembly of metalloproteins, which participate in a very wide range of biological processes such as enzymatic reaction, cellular signaling transduction, redox regulation, mitochondrial function, immunological response, and hormone and vitamin biosynthesis (9, 10). In addition, several environmental toxic elements, such as cadmium (Cd), mercury (Hg), arsenic (As), lead (Pb), and aluminum (Al), have detrimental effects on human health (11, 12).

It has been known that the cell has developed complex regulatory mechanisms (such as uptake, excretion, and storage) to tightly control the metabolism and homeostasis of various trace elements in order to ensure appropriate supply while preventing accumulation to toxic levels (13). Previously, a large number of studies have reported that deficiency or overload of certain trace elements are related to a variety of metabolic diseases and disorders (14–18). For example, Fe deficiency is significantly associated with obesity, whereas excessive Fe accumulation in adipose tissue and liver is often seen in type 2 diabetes mellitus (T2DM) and NAFLD (14, 15). Furthermore, some elements may interact and interfere with the absorption, bioavailability, and even normal functions of others (19–21). On the other hand, trace element levels may be altered in various clinical conditions, which may be associated with the prognosis and efficacy of the treatment (22). Although numerous studies have been performed to evaluate the status of single or several elements in biological samples of different types of metabolic diseases, it is still unclear how disruption of the balance of various elements are involved in the development and progression of these diseases. A more systematic understanding of the metabolism and homeostasis of trace elements and their relationships with metabolic diseases is urgently needed.

In the recent 20 years, with the explosive growth of multi-omics data (such as genome, transcriptome, and proteome) and a corresponding increase in data analysis methods, the complex relationship between trace elements and human health or diseases has been examined comprehensively. The concept of metallome (the ensemble of all metal ions in an organism) and its extension ionome (representing the composition of all metals, metalloids, and non-metals found in a living system) have also been introduced (23, 24). Ionomics, the study of the ionome, involves simultaneous and quantitative analyses of elemental composition in living systems (such as cells, tissues, and organs) and changes in this composition under different physiological and pathological conditions by using high-throughput elemental profiling techniques (24–26). This approach has been extensively applied in plants, yeast, and most recently mammals (including humans), which provides a very useful platform for not only identifying new characteristics and components of trace element metabolism and homeostasis but also gaining information about how the complex ionomic network is affected by different factors and states (26–28). In addition, isotopic and speciation analysis of elements is becoming an inherent or complementary part of ionomics, which is important for a more accurate assessment of the status of elements present in biological samples (29, 30). In recent years, ionomics approach has been applied to study the global effect of a large amount of elements for various human diseases, which offers a great opportunity to discover new ion-based mechanisms of these diseases (especially complex disease) and to enable the possibility to develop new diagnostic tools and therapeutic strategies (31, 32).

In this review, we present a comprehensive survey on the complex relationship between ionomic profiles in diverse human biological samples and several major types of metabolic diseases mainly based on recent large-scale ionomic/metallomic studies, such as case-control studies, cohort- and population-based studies, and meta-analyses. Such information may provide a holistic and integrated understanding of the critical roles trace elements play in metabolic diseases.

2. Ionomics technology and its application in human health and diseases

The major purpose of ionomics is to measure the ion contents in a biological system and to elucidate their correlations to phenotypes of interest. Nowadays ionomics has been widely used for studies on environmental monitoring, nutrient utilization, biofortification, food safety, and forward and reverse genetics, particularly in plant developmental and stress biology which has been extensively reviewed in the literature (23–25, 33–35). In addition, the rapid expansion of ionomics in human health and diseases has provided new evidence for the relationship between trace elements and a variety of common diseases, which may be helpful for the early detection of disease and the innovation of new drugs against imbalanced elements.

2.1. Techniques for elemental quantification and related data resources

In early times, elemental profiling analysis in various samples was mainly used in geoscience, environmental science, and ecological studies. In the last decade, ionomics has grown in popularity in different areas of life science as a powerful tool for studying the metabolism and homeostasis of diverse trace elements and minerals in different organisms, including plants and humans. The major techniques for the quantification of chemical elements include inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma atomic/optical emission spectroscopy (ICP-AES/ICP-OES), X-ray fluorescence (XRF), atomic absorption spectrometry (AAS), neutron activation analysis (NAA), and several other methods (23, 33). Currently, ICP-MS and ICP-AES are the two most widely used high-throughput analytical tools. ICP-MS has been the dominant technique for the analysis of multi-elemental concentrations due to its high accuracy, high sensitivity, wide measurement capability, and the ability for isotopic analysis (36). Although ICP-AES/ICP-OES is less sensitive than ICP-MS, it offers the advantages of lower cost and simplicity of operation (37). Both methods have been used for ionomics studies in yeast, plants, and animals, which demonstrate the potential benefits of ionomics in the exploration of new aspects of trace elements in biology and medicine (26, 27, 38–40). Other techniques, although used less frequently, may provide alternative approaches for determining various elements from different samples (41, 42).

TABLE 1 Major ionomics results for different types of metabolic diseases.

Disease	Population (subgroup)	Number of subjects (case/control)	Sample type	Elevated elements	Decreased elements	Significant correlations (–, $p < 0.05$ and |correlation coefficient| > 0.3) or significantly changed ratios (/, $p < 0.05$) between elements in patients	Potential confounders adjusted or matched	References
T2DM	Moscow, Russia (women)	93/1,236	Hair	Hg, K, Na	Ca, Co, Mg, Zn	–	–	(60)
T2DM	Guanajuato, Mexico	76/12	Serum	Al, Cd, Cu, Hg, Mn, Ni	Co, Cr, V	Mn–Cu, Mn–Zn, Se–Cu, Se–Mn, Se–Zn, Zn–Cu	Smoking status, other chronic diseases	(61)
		76/12	Urine	As, Cr, Cu, Zn	Cd, Co, Pb, Mn, Mo, Ni, Se	Se–As, Se–Cd, Se–Ni		
T2DM	Shanghai, China	122/854	Plasma	Cr, Cu, P, S, Se	Mg, Re	–	Age, sex, BMI	(62)
T2DM	Tanta, Egypt	40/36	Serum	Cu	As, Cr, Fe, Mg, Mn, Se, Zn	***As–Fe***[a], ***Cu–Fe***, ***Cu–Mg***, ***Cu–Zn***, Fe–Mg, Se–Cr, Se–Fe, Zn–Fe, Zn–Mg	Age, sex, weight	(63)
T2DM	Suzhou, China	122/429	Plasma	As, Ba, Cd, Cr, Cs, Cu, Mn, Pd, Se, Sr, Zn, V	–	–	Age, sex, BMI, family history of diabetes, smoking status, drinking status	(64)
T2DM	Nord-Trøndelag county, Norway	128/755	Blood	Ag, Cd, Cr, Fe, Ni, Zn	Br	–	Age, sex, BMI, education, waist-to-hip ratio, income, smoking status, family history of diabetes, seafood intake, alcohol consumption	(65)
T2DM	Winston-Salem, USA	21/19	Toenail	Al, Ni, V, Zn	Cs	–	–	(66)
T2DM	Nanning, China	223/302	Serum	Ba, Ca, Fe, Se, Sr	V	–	Age, sex, BMI, smoking status, drinking status, physical activity	(67)
T2DM	Wuhan, China	94/94	Plasma	Al, Ba, Cu, Rb, Se, Sr, Ti, Tl, Zn	Mo	–	Age, sex, BMI, smoking status, drinking status, regular exercise, education, and family history of diabetes.	(68)
T2DM	Jinan, China	100/40	Blood	Co, Cr, Cu, Fe, Mn, Mo, V, Zn	Li	–	–	(69)
T2DM complication	Cartagena, Spain	31/43	Saliva	–	Co	–	–	(81)
		31/43	Plasma	Sr	–			
T2DM CVD complication	Wuhan, China	1,114/2,783	Plasma	As, Co, Cr, Mn, Mo, Ni, Sb, Sn, Sr, Ti, V, W	Cd, Cu, Pb, Se, Zn	–	Age, sex, BMI, education, smoking status, drinking status, physical activity, family history of CVD, hypertension, hyperlipidemia, baseline fasting plasma glucose, antidiabetic, duration of diabetes, eGFR	(82)
T2DM complication (microvascular)	Ankara, Turkey	118/40	Blood	–	Mg, Cr	–	–	(83)

(Continued)

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TABLE 1 (Continued)

Disease	Population (subgroup)	Number of subjects (case/control)	Sample type	Elevated elements	Decreased elements	Significant correlations (–, $p < 0.05$ and |correlation coefficient| > 0.3) or significantly changed ratios (/, $p < 0.05$) between elements in patients	Potential confounders adjusted or matched	References
T2DM	Sardinia region, Italy	68/59	Blood	–	Cr, Mn, Ni	–	–	(84)
T1DM	Sardinia region, Italy	192/59	Blood	–	Cr, Mn, Ni, Pb, Zn	–	–	
T1DM	Miami, USA	63/65	Serum	Cu, Mo	Mn, Se, Zn	Cu/Se, Cu/Zn	Age, sex, BMI	(85)
GDM	Padova, Italy	28/19	Placenta	Se	Cd	–	Age, birth weight, gestational week of GDM diagnosis, gestational week of delivery	(87)
GDM	Padova, Italy	36/36	Placenta	Hg, Si	Cd, Cu, Fe, Mn	–	Age, pre-pregnancy BMI, birth weight, gestational week of delivery	(88)
		35/30	Blood (maternal)	Cu	Al, Co, K, Rb, Sb			
		35/34	Umbilical cord blood	Al, Ca, Co, Cu, Mo, Na, Ti, Zn	Fe, K, Mn, Rb, Si			
GDM	Wuhan, China	241/1,849	Urine	As, Co, Ni, Sb, V	–	–	Fetal sex, pre-pregnancy BMI, smoking status, physical activity, maternal age, gravidity, occupational status, income, family history of diabetes	(89)
GDM	Yozgat, Turkey	60/52	Serum	Cd, Cu, Pb, Sb	Cr, Se, Zn	–	–	(90)
GDM	Beijing, China	335/343	Hair (maternal)	Hg, Sn	–	–	Age, pre-pregnancy BMI, education, occupation, parity, passive smoking, hair dye, hypertension during pregnancy, activity, folic acid supplementation	(91)
GDM	Wuhan, China	305/305	Plasma	Cu, Fe	Ca, Zn	–	Age, pre-pregnancy BMI, parity, income, education, passive smoking	(92)
Obesity	Moscow, Russia (women)	141/1,236	Hair	Hg, K	Ca, I, Mg, Zn	–	–	(60)
Obesity/overweight	Shanghai, China	516/460	Plasma	Cu, Fe, P, S, Sr	Mg	–	Age, sex, BMI	(62)
Obesity	Ankara, Turkey (children)	34/33	Serum	–	Co, V	–	Age, sex, pubertal stage	(103)
Obesity	Konya, Turkey (women)	45/50	Serum	Cu	Fe, Zn	–	Age	(104)
Obesity	Poznan, Poland (adolescents)	78/20	Serum	–	Ca, Mg, Zn	–	Age, sex	(105)
Obesity	Erzurum, Turkey (children)	85/24	Serum	Cu, Mn, Se	Zn	–	Age, sex	(106)

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TABLE 1 (Continued)

Disease	Population (subgroup)	Number of subjects (case/control)	Sample type	Elevated elements	Decreased elements	Significant correlations (–, $p < 0.05$ and |correlation coefficient| > 0.3) or significantly changed ratios (/, $p < 0.05$) between elements in patients	Potential confounders adjusted or matched	References
Obesity	Zagazig City, Egypt (children)	80/80	Serum	Cu	Fe, Se, Zn	–	Age, sex	(107)
Obesity	Makkah, Saudi Arabia	47/70	Hair	Cu, Se	Fe, Mn, Zn	Cu/Fe, Cu/Zn	–	(108)
Obesity	NHANES project, USA	1,127/5,475	Urine	Ba, Tl	Cd, Co, Pb	–	Age, sex, race/ethnicity, income, serum cotinine, television, video game, computer usage	(109)
Obesity	Reus, Spain (women)	42/51	Serum	As, Ba, Cu, Se, Sr	Ca, Fe, Mg, Na, Zn	–	Age	(110)
Obesity/overweight	Moscow, Russia	112/106	Hair	Al, As	Co, Cu, I, Mg, Mn, Ni, Zn	–	Age, smoking status, alcohol consumption, history of acute cardiovascular events, mineral supplementation, metal implants, occupational exposure to environmental toxins, environmental exposure to trace elements, acute inflammatory diseases	(111)
Obesity	Sonapur, Bangladesh	100/100	Serum	Na	Ca, Fe, K, Zn	–	Age, sex	(112)
Obesity (abdominal)/ MetS	Wuhan, China	816/576	Urine	Ba, Cu, Fe, U, Zn	–	Cu/Zn	Age, sex, BMI, smoking status, drinking status, income, education level, physical activity, family history of diabetes	(113)
Obesity	Dhaka, Bangladesh (women)	70/70	Serum	Na	Ca, Fe, K, Zn	Na–K	Age	(114)
Obesity	Yaroslavl, Russia	196/199	Serum	Se	Cr, V	–	Age, sex	(115)
			Hair	Cr, Se, V	Zn			
			Urine	V	–			
Obesity	Poznan, Poland (women)	40/40	Serum	Cu	Ca, Fe, Mg, Se, V, Zn	–	Age, BMI, and metabolic parameters	(116)
			Hair	–	Fe, Mn, V			
			Urine	Fe	Se, V			
Obesity	Kerman, Iran (children and adolescents)	48/38	Urine	As, Pb, Zn	–	–	Age, sex, BMI, physical activity, physical and clinical examinations	(117)
MetS	Shanghai, China	399/577	Plasma	Cr, Cu, P, S, Se, Sr	Mg	–	Age, sex, BMI	(62)
MetS	Seoul, Korea	161/295	Hair	As, Hg, K, Na, Pb	Ca, Mg, Zn	Ca/K, Ca/P, Na/Mg	Age, smoking status, alcohol use	(142)

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TABLE 1 (Continued)

Disease	Population (subgroup)	Number of subjects (case/control)	Sample type	Elevated elements	Decreased elements	Significant correlations (–, $p < 0.05$ and |correlation coefficient| > 0.3) or significantly changed ratios (/, $p < 0.05$) between elements in patients	Potential confounders adjusted or matched	References
MetS	Szczecin, Poland (aging men)	161/152	Serum	Zn	Mg	–	–	(143)
MetS	Beijing, China	80/65	Blood	Cd, Cu, Pb, Se	–	–	Age	(144)
MetS	Kaohsiung, Taiwan	826/1,618	Blood	Pb	–	–	Age, sex	(145)
			Urine	As, Cu, Ni	–			
MetS	China	628/1,481	Plasma	Ti	–	–	Age, sex, BMI, smoking status, drinking status, education, income, physical activity	(146)
			Urine	–	Fe, Se, V			
MetS	Ann Arbor, USA (women)	173/774	Urine	As, Co, Zn	–	–	Age, BMI, race, study site, education, smoking status, alcohol drinking, physical activity, total energy intake, menopausal status	(147)
MetS	Nanning, China (men)	254/1,716	Serum	As, Ba, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Rb, Se, Sn, Sr, V, Zn	–	–	Age, smoking, alcohol drinking, physical activity, education, family history	(148)
MetS	Taipei, Taiwan	40/110	Plasma	B, Cd, Cu, Li, Mg, Ni, Sr, Zn	–	–	Age, sex, BMI, smoking status	(149)
MetS	Shenzhen, China	149/1,128	Plasma	–	Mg, Mn	–	Age, sex, smoking status, drinking status, estimated glomerular filtration rate	(150)
NAFLD	Athens, Greece	119/70	Plasma	Cs, Fe, Tl	Ba, Zn	–	Age, sex, BMI	(180)
NAFLD	Nanning, China (men)	330/1,264	Serum	Ba, Ca, Fe, Mg, Mn, Ni, Pb, Sn, V, Zn	Mo	–	Age, marital status, education, smoking status, drinking status, MetS, insulin resistance	(181)
Hyperlipidemia	Konya, Turkey	46/37	Serum	Cd, Co, Ni	Cr, Fe, Mn, Mo, Se	–	Age, sex, blood pressure	(224)
Hyperuricemia	Shenzhen, China	344/1,062	Plasma	As, Cu, Mg, Se, Tl, Zn	Co	–	Age, sex, BMI, smoking status, drinking status, other diseases (hyperlipidemia, hypertension, DM, chronic kidney disease)	(247)
Hyperuricemia	USA	751/3,175	Urine	As, Cd	Co, I, Mn	–	Age, gender, BMI, smoking status, drinking status, income, education, fish/shellfish consumption, other diseases (hypertension, hypercholesterolemia, DM)	(248)

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TABLE 1 (Continued)

Disease	Population (subgroup)	Number of subjects (case/control)	Sample type	Elevated elements	Decreased elements	Significant correlations (–, $p < 0.05$ and |correlation coefficient| > 0.3) or significantly changed ratios (/, $p < 0.05$) between elements in patients	Potential confounders adjusted or matched	References
Osteoporosis	Banjarmasin, Indonesia (postmenopausal women)	15/23	Bone	As, B	Pd, Rb, Se	–	Age, history of previous disease	(267)
Osteoporosis	Izmir, Turkey (postmenopausal women)	491/237	Serum	-	Cu, Fe, Mg, Zn	–	Age, BMI, duration of menopause, family history of fractures, medical conditions, smoking status, medication use	(268)
Osteoporosis	Shanghai, China	37/14	Bone	–	Cu, Mn, Zn	–	Age, sex, BMI, serum albumin, biochemical blood indices, bone turnover markers	(269)
Osteomalacia	Rio de Janeiro, Brazil	8/603	Hair	Al, Ba, Ca, Cd, Fe, Mg, Mn, Sr	Hg	–	–	(278)
Rickets	Van, Turkey	30/30	Serum	–	Zn		Age, sex, medical history, physical examination	(279)

[a]Bold and italic: negative correlation.

With the increase in volume of data generated by ionomics studies, it becomes more and more important to develop appropriate information management systems for ionomics data acquisition, storage, retrieval, and analysis. However, publicly accessible platforms and databases that allow researchers to obtain such resources are very rare so far. The iHUB system is the first integrated workflow system, which contains ionomics data of yeast and important plants such as Arabidopsis thaliana, maize, rice, and soybean, and provides open access for raw data management, data mining and analysis, and knowledge discovery (43). The OPTIMAS Data Warehouse (OPTIMAS-DW) is a comprehensive and integrated data resource for maize, which contains ionomics and other omics data such as transcriptome, proteome, metabolome, and phenome, and allows users to extract information of particular interest (44). Unfortunately, an ionomics database associated with human disease is still absent.

On the other hand, with the rapid progress in the experimental and computational identification of novel trace element-related genes and their functions, relevant information should be well-maintained for further utilization. A known ionome gene (KIG) database was recently developed, which is a curated list of genes known to affect uptake, accumulation, and distribution of elements in several plant species (45). New systems and annotation tools are clearly required to obtain a better understanding of the function and regulation of genes affecting the ionome on a genome-wide scale.

2.2. Bioinformatics for ionomics data analysis

The recent explosion of ionomics data has prompted the development of computational strategies and methods for investigation of the associations between ionomic profiles and complex traits. Bioinformatics approaches for ionomics data analysis include both basic statistical methods (such as statistical significance testing and analysis of variance) and advanced multivariate statistical methods (e.g., principal component analysis and logistic regression analysis), which could also be applied for other types of omics data (31). Moreover, network-based systems biology approaches and machine learning algorithms have been used to clarify the dynamic interplay between elements under different physiological or metabolic conditions and to build ionome-based prediction models or markers for a variety of diseases (46–49). Very recently, an integrated R-based tool called IonFlow was developed for the analysis of ionomics data sets, which may help researchers to process, explore, and interpret their data *via* multiple approaches (50).

2.3. Application of ionomics in human disease

Before the concept of ionomics was introduced, ICP-MS had been used to evaluate the concentrations of certain trace elements in biofluids, hair, nails, and some other human tissues for different diseases such as cancer, neurodegenerative diseases, and coronary heart disease, demonstrating that imbalance of trace elements may be one of the risk factors of many complex or common diseases (51–53). Most of these preliminary studies only focused on single or a very limited number of trace elements and lacked deep analysis

of the interactions between elements. In recent years, ionome-based studies have been performed for a variety of complex diseases, which may assist in diagnosis and prognosis evaluation of disease. Considering that most ionomics results have been derived from cross-sectional or case-control studies, it is uncertain whether disrupted ion homeostasis is a potential cause of these diseases. Large-scale prospective, longitudinal, and interventional studies are necessary to solve these questions. In addition, the intrinsic limitation of current elemental analytical techniques and the validity and reliability of using ionomic information in the prediction of diseases imply that there is still a long way ahead of us before ionomics can be widely used in medical clinics. However, the application of ionomics in several types of diseases has provided evidence for its potential benefits, which suggests significant correlations between trace elements and the development of different diseases and may help early detection and improve therapeutic regimens for patients in the future.

3. Recent progress in ionomics of metabolic diseases

It has been known for a long time that trace elements and minerals play a critical role in the onset and progression of metabolic diseases (32, 54, 55). Previously, lots of studies have been conducted to explore the linkages between single or multiple elements and various metabolic diseases, such as the association between intake of certain elements and the incidence of DM and osteoporosis and the implication of heavy metal exposure in the epidemiology of metabolic diseases (56–58). In recent years, ionomics approaches have been used to examine the variations of elemental profiles in different body fluids and tissues from patients with metabolic diseases, providing new clues for not only better understanding of the relationship between trace element dyshomeostasis and these diseases but also the development of novel biomarkers for detection, diagnosis, and prognosis of them. In the following text, we will discuss recent progress on ionomics studies of several major types of metabolic diseases. A summary of the main ionomics results obtained from the literature is given in Table 1.

3.1. Diabetes mellitus

DM comprises a group of metabolic diseases characterized by chronic hyperglycemia resulting from insufficient insulin secretion and/or insulin resistance in target tissues, which may lead to multi-organ dysfunction and failure (59). There are three main types of DM: type 1 DM (T1DM), T2DM, and gestational DM (GDM). T2DM accounts for more than 90% of all diabetic cases, which has become the most prevalent metabolic disease worldwide. It has been suggested that ion homeostasis may play an important role in T2DM and some other types of DM (3).

To date, the ionomes in blood (including whole blood, plasma, and serum), urine, hair, and even teardrops have been examined in different cohorts of patients with T2DM, implying that changes in elemental profiles represent a potential source of biomarkers of this complex disease (60–70). The majority of significantly changed elements in different biofluids (especially in the blood samples) are positively associated with T2DM, including several heavy metals (such as Cu and Cd) and Se whose accumulation in the body may be a high risk for T2DM. In contrast, concentrations of Zn and Mg in the blood and hair/nail samples were observed to generally have a negative association with the prevalence of T2DM. These trends are consistent with previous observations that environmental heavy metals and Se might affect insulin sensitivity and normal insulin regulatory function and cause pathoglycemia or even more serious illness such as T2DM, whereas deficiency of Zn, an essential trace metal for synthesis and secretion of insulin, may have a major impact on the pathogenesis of T2DM (3, 64, 67). Thus, such variations could be used for differentiating patients with T2DM from healthy controls. Moreover, significant correlations ($|\text{correlation coefficient}| > 0.3$ and $p < 0.05$) between elements and increased Cu/Zn ratio were also reported, suggesting possible benefits of the use of such information in detection and prognosis evaluation of T2DM (61, 63). By using machine learning algorithms [such as discriminate analysis, support vector machine (SVM), and random forest], several new approaches and models for the early detection of T2DM based on multi-elemental contents have been developed, which exhibit good prediction performance and may serve as a valuable tool of its diagnosis (66, 70–72). Very recently, the blood levels of several essential trace elements (such as V, Cr, Mn, and Se) were found to be negatively correlated with hemoglobin A1c (HbA1c) in diabetic subjects, especially in those with HbA1c $\geq$ 7.0%, suggesting mutual effects between them and metabolic abnormalities of blood glucose during the onset and progression of T2DM (69). On the other hand, although a significant number of studies only examined the concentrations of single or a small set of trace elements in biofluids of T2DM patients, similar or more complex patterns of changes were obtained for some of them in different regions of the world (73–80). For example, serum Cu, Zn, and Se levels were found to vary with the effect of glycemic control in T2DM patients, indicating a potential relationship between their homeostasis and the prognosis and treatment of T2DM (73). Long-term Ni and As exposures were also observed to be associated with elevated prevalence of T2DM in Chinese elderly and Mexican women, respectively, implying the harmfulness of more heavy metal contamination in the pathogenesis of T2DM (74, 75). Although González de Vega et al. reported lower plasma concentrations of total Se found in Spanish patients with T2DM which might be due to decreased levels of selenoprotein P (76), the majority of other studies on Se and DM suggest that Se may increase the risk of T2DM across a wide range of exposure levels (77). In addition, several recent ionomics studies have found that trace element imbalance is involved in the development of diabetic complications. Marín-Martínez et al. reported that decreased Co level in saliva and increased strontium (Sr) level in plasma were associated with the presence of chronic complications (both microvascular and macrovascular) in patients with T2DM, which provides some useful enlightenment for predicting diabetic complications (81). Other ionomics studies revealed that increased Sr and decreased Zn and Se levels in plasma have a significant association with incident cardiovascular disease (CVD) risk in patients with T2DM, whereas decreased Mg and Cr levels in blood might be involved in the progression of microvascular complications (such as diabetic retinopathy and diabetic nephropathy) (82, 83).

Ionomics studies have also been conducted for several other types of DM (84–87). One Italian group measured the blood levels of multiple trace elements in patients with T1DM and T2DM from Sardinia, and observed similar alternations of significantly changed

elements between them (Cr, Mn, and Ni deficiency) in that area (84). Similar to T2DM, elevated serum level of Cu and lower levels of Zn and/or Mg were also found in subjects with T1DM from different regions, suggesting that perturbation in the balance of these essential metals may be related to the pathology of both types of DM (80, 85). Peruzzu et al. reported that whole blood levels of Cr and Cu were strongly correlated with glycemic control whereas Zn, Fe, and Se were associated with lipid metabolism in patients with T1DM, suggesting different effects of these elements on metabolic control in this disease (86). With regard to GDM, Roverso et al. carried out the first ionomics study of the placentas from women affected by GDM, and showed that a decreased level of Cd and increased level of Se might be linked to the molecular pathways of GDM (87). They further measured the ionomes of placenta, maternal whole blood, and umbilical cord blood samples from a larger cohort of GDM and normal pregnant women, which revealed that a number of elements detected in the umbilical cord blood of fetuses were significantly associated with the occurrence of GDM (such as increased Cu and decreased Fe levels) (88). This may indicate that elemental profiles in the cord blood provide more evidence for understanding the biochemical processes occurring during GDM. Moreover, several recent case-control and cohort studies as well as meta-analyses reported that exposure to toxic heavy metals [such as Ni, antimony (Sb), Cd, and Cu] and insufficient Zn content in the body in early pregnancy are associated with an increased risk of GDM, either individually or as a metal mixture (89–96). Overall, these studies demonstrate high complexity and tissue specificity of the ionomes in patients with different types of DM. However, elevated Cu and Se concentrations, decreased Zn concentrations, and increased Cu/Zn ratio in various biofluid samples might be one of the common risk factors for DM. Further effort is needed to investigate the molecular mechanisms underlying dyshomeostasis of trace elements in patients with different types of DM.

3.2. Obesity

Obesity is a chronic and complex disease characterized by excessive amounts of fat within adipocytes, which may result from insulin resistance, dysregulation in lipid and other metabolic balances, and inflammatory and/or hormonal processes (97). It may increase the risk of other diseases and health problems, such as T2DM, hypertension, CVD, and certain cancers. Both genetic susceptibility and environmental factors contribute significantly to the development of obesity (98). Among them, trace element and mineral dyshomeostasis has already been known to be associated with the pathogenesis of this disease (99). On the other hand, obese patients may be at a greater risk of developing imbalance of trace elements.

Before the use of ionomics in obesity research, many studies have shown a possible relationship between obesity and disturbance of homeostasis of certain trace elements, such as Zn or Fe deficiency and Cu overload, although the exact metabolic roles of these elements remain unclear (100–102). In the recent decade, a variety of studies have been carried out to examine the concentrations of more elements or ionomes in different biological samples of patients with obesity and healthy controls, which demonstrate more complicated changes linked to the prevalence of this disease (60, 62, 103–117). Consistent with previous observations, significantly increased levels of Cu and decreased levels of Zn and Fe in multiple samples (especially in serum) appear to be the most common features for obesity. However, the relationship between obesity and the circulating levels of Se is still controversial. A recent meta-analysis revealed that overweight/obese individuals might have lower levels of Se in the urine and nails but higher levels in the hair (18). Compared to DM, deficiency of essential trace elements may play a more important role in the pathogenesis of obesity and obesity-related metabolic dysfunction, especially in women and children. The Cu/Zn ratio was also mainly increased in several types of samples of the obese groups (in particular in diabetic obese individuals), and was negatively associated with increased risk of metabolic abnormalities in the urine (108, 113). In addition, exposure to environmental toxic metals in the body may significantly influence the ionome and other metabolic parameters of obesity. For instance, Skalny et al. reported that Hg-exposed overweight/obese subjects had quite different hair elemental profiles (such as elevated Se and Zn and decreased Mn and Mg levels) and a more adverse effect on metabolic parameters when compared to unexposed obese adults, suggesting that metabolic alterations observed in obesity may be partially related to Hg-associated disturbances in trace element metabolism (118).

A significant number of studies with larger cohorts have been performed recently to improve our understanding of the relationship between obesity and single or few essential/non-essential elements in various biofluids and tissues, which showed generally similar changes in the concentrations of some of them in different populations, such as increased serum and hepatic tissue Cu levels (119, 120), decreased serum, hair, and salivary Zn levels (121–123), decreased fingernail Se levels (124), and increased toxic heavy metal (such as Al, Hg, Pb, and As) contents (125–128). Many of these significantly changed elements are positively or negatively correlated with body mass index (BMI) and waist circumference, which are commonly used to estimate the risk of overweight, obesity, and some other metabolic diseases such as MetS (128, 129). Moreover, levels of some elements are associated with insulin resistance in obese adults. Kim and Song examined the concentrations of several trace elements in the hair of viscerally obese adults, and found that Cr and Se levels were inversely associated with insulin resistance, whereas Cu level has a positive correlation with insulin resistance (130). Thus, imbalance of these micronutrients might be involved in the development of obesity, which could be used as potential indicators for its incidence. Interestingly, although serum levels of Cu and Zn are thought to be significantly related to obesity in both children and adults, a recent study showed that they did not differ significantly between pre-obese and obese children, implying that alternations of the two metals are earlier than the occurrence of obesity (131). Nevertheless, further research is needed to confirm these findings prospectively in larger study populations and to extend the understanding of the roles of different elements in the prevalence of obesity.

3.3. Metabolic syndrome

MetS is a group of metabolic disorders, including abdominal obesity, hypertension, high blood sugar levels (insulin resistance or glucose intolerance), and dyslipidemia [high triglyceride and low high-density lipoprotein (HDL)-cholesterol levels]. It may raise the

risk of CVD, T2DM, stroke, and other serious health problems (4). The cause of MetS has been intensively studied, and many factors (such as genetic and metabolic susceptibility) may contribute to the development of MetS (132). Among these, an imbalance in trace element status is an important risk factor and potential biomarkers for MetS (133). The important roles of several micronutrients (such as Fe, Zn, and Cr) in carbohydrate and lipid metabolism and their alterations in MetS have been discussed in multiple reviews, which provide evidence for the involvement of trace element dyshomeostasis in the pathology of MetS (4, 134–136).

In early years, a limited number of studies have been conducted to explore the distribution and relationships of selected trace elements in different samples of patients with MetS and normal controls, which revealed that some of these elements might be related to the incidence and course of MetS (137–140). For instance, elevated circulating Cu levels and body Fe stores as well as reduced serum levels of Zn were reported in patients with MetS in different areas. On the other hand, controversy exists with regard to the relationship between MetS and Se levels in body, probably due to differences in study design, methodology, and population characteristics (141).

Several ionomic profiling studies with large cohorts of patients were carried out in the recent decade (62, 142–150), which basically support previous observations that accumulations of Cu and Fe in the body are strongly associated with the increased prevalence of MetS. Moreover, imbalance of additional elements were detected in different biological samples of MetS cases, implying a high degree of variability of the ionomic patterns in patients with MetS. Generally, the majority of significantly changed elements have positive associations with MetS, especially environmental toxic metals (such as Pb, Cd, and As), implying that exposure to these elements affects the body composition and metabolic profiles and further exacerbates the risk of MetS (142, 144–149). In contrast, decreased Mg levels were often observed in the blood (including plasma and serum) and hair samples from patients from different regions (62, 142, 143, 150), which could influence glucose metabolism and insulin sensitivity and action as well as promote chronic inflammation, linking Mg deficiency with developing MetS and other metabolic disorders such as T2DM and obesity (151). So far the ionomic information available for assessing the potential relationship between Zn status and the incidence of MetS is inconsistent and may vary by gender. It seems that increased Zn levels in the serum/plasma samples may lead to the occurrence of MetS in men but have a protective effect in women (143, 148, 152). In addition, some of the elements were found to be significantly associated with different MetS components. For example, Wen et al. have shown that urine Cu and blood Pb levels were positively associated with obesity-related indices such as body roundness index and body/visceral adiposity index, and urine Ni was positively correlated with lipid accumulation product in a large cohort of MetS cases (145). Increased Ca/Mg ratio in the hair was also found to have a positive correlation with insulin resistance in Korean adult males (153). Very recently, Zhang et al. investigated the relationship between the blood concentrations of several essential metals and MetS components in Chinese children, which revealed that different metals correlate with MetS components individually, such as Cu (positively associated with elevated waist and triglyceride levels) and Mg (positively associated with reduced HDL-cholesterol levels) (154). Such information may suggest a potential use of ionomic data in monitoring the progression of MetS components and helping determine the proper treatment strategies.

Besides the ionome-level studies mentioned above, more case-control studies and meta-analyses recently focused on single or several trace elements and explored their differences between MetS patients and healthy controls in different biological samples (17, 155–164). Although some of these results appear to be contradictory and ambiguous, it appears that elevations of Se, Cu, Zn (in males), and toxic metals (such as Cd and Hg) and reduction of Mg in blood samples are associated with increased risk of MetS and its components. Besides Zn, gender difference may affect the correlation between MetS and some other elements such as Se (165). Moreover, interactions between different elements may modify their own roles in MetS, e.g., the harmful effects of Hg exposure on MetS could be significantly attenuated by high levels of Se in toenails (166). Future studies should aim to unravel the mechanisms underlying the associations between these elements and the development of MetS to assist in the prevention and early intervention of this disease and its components.

3.4. Non-alcoholic fatty liver disease

NAFLD is the most common chronic liver disease worldwide, which is characterized by excessive fat accumulation (≥5%) in hepatocytes in the absence of excessive alcohol consumption (167). It encompasses a broad histological spectrum ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma (HCC) that may develop further (168). Because of the lack of efficient diagnostic methods and therapeutic targets, early detection and treatment of NAFLD still face many challenges. The underlying mechanism for the pathogenesis and progression of NAFLD is complex, and disturbance in trace element and mineral metabolism and interactions have been identified as one of the key risk factors of this disease (169, 170). As NAFLD is closely associated with the components of MetS, obesity, and T2DM, a change in nomenclature from NAFLD to metabolic-associated fatty liver disease (MAFLD) has been recently proposed, which may better reflect the underlying pathophysiology of this disease (171). Here, we still use NAFLD in the following sections.

The association between certain trace elements and NAFLD risk has been reported since 1990s, such as hepatic Fe overload and lower liver and serum Cu concentrations in different types of NAFLD patients (including pronounced hepatic steatosis, NASH, and even NASH-related cirrhosis with HCC) (172–177). The serum ferritin, a marker of Fe storage, has been considered as an independent predictor of histologic severity and fibrosis in patients with NAFLD, which may also indicate that changes in Fe speciation [say, the conversion between Fe(II) and Fe(III) species] are related to the pathogenesis of this disease (178). However, children with NASH exhibit decreased serum Fe levels with no significant Fe accumulation in the liver, implying a new mechanism for Fe deficiency in pediatric NASH (179). To date, very few ionomics studies have been carried out for NAFLD, which mainly used blood samples from patients. For the first time, Asprouli et al. measured plasma ionomes in different stages (mild, moderate, and severe) of NAFLD subjects in Greece, which demonstrated that Zn had a negative association with the severity of the disease whereas cesium (Cs) showed a positive correlation (180). Li et al. examined the relationship between NAFLD and serum contents of a variety of metals based on cross-sectional

and longitudinal analyses in Chinese men, and found that depressed Mo and elevated Zn levels were associated with an increased risk of NAFLD (181). It should be admitted that ionomics in NAFLD is still at the very beginning of its development. So far ionomic profiling of other biological samples in NAFLD patients is not yet available.

In the last few years, a large number of studies have explored the correlation between single or several elements and the development of NAFLD. First, consistent with previous findings, Fe accumulation appears to be the most significant and common marker for NAFLD, and indicators of Fe status (e.g., serum Fe and ferritin levels) could be used for the prediction of disease severity and adverse outcomes (such as the risk of advanced hepatic fibrosis and HCC) in different populations (182–186). Second, decreased Cu and Mn concentrations in hepatic tissues, serum, and/or hair were reported in different cohorts of NAFLD patients, and higher Cu or Mn levels achieved significant protective effect against NAFLD, especially in men (187–192). In addition, recent Cu isotopic composition analysis showed that serum 65Cu/63Cu ratio was significantly lower in NAFLD patients and remained stable during disease progression, suggesting its potential for early diagnosis of NAFLD (193). Third, although the relationship between Zn deficiency and the incidence of NAFLD is still uncertain, low serum levels of Zn have been proposed to serve as an independent risk factor for poor prognosis in patients with NAFLD (194–197). In contrast, the roles of Se in different types of NAFLD are complex and conflicting (198). It seems that increased plasma/serum Se levels are positively associated with elevated prevalence of liver steatosis but negatively associated with that of hepatic fibrosis or HCC (198–201). Finally, exposure to toxic heavy metals (such as Pb, Cd, Hg, and Ni) was significantly associated with the onset and progression of NAFLD in different regions of the world, which is probably a causative factor of hepatic steatosis and fibrosis in some patients (202–208). Moreover, increased serum Ca and phosphorus (P) levels were also reported as independent risk factors for fatty liver in Korean population (209). Although not at the ionomic level, these single/few element-based studies might still provide useful information regarding the changes of trace elements in biological samples of NAFLD patients. Further investigation is necessary to evaluate the value of ionomic changes in the early detection of NAFLD individuals.

3.5. Hyperlipidemia and hyperuricemia

Hyperlipidemia (also referred to dyslipidemia) represents the condition of abnormally elevated levels of one or more lipids and/or lipoproteins in the blood, mainly including high levels of total cholesterol (hypercholesterolemia), triglycerides (hypertriglyceridemia), and low-density lipoprotein (LDL)-cholesterol or low levels of HDL-cholesterol. It is a significant risk factor for a variety of diseases such as fatty liver, CVD, DM, neurodegenerative diseases, and acute pancreatitis (210). Hyperuricemia (serum uric acid level $\geq$6.8 mg/dL), a key risk factor for the development of gout, is caused by increasing production and/or decreasing clearance of uric acid (the end product of purine metabolism) (211). In addition, hyperuricemia may also increase the risk of chronic kidney disease, CVD, metabolic disorders such as hyperlipidemia, obesity, and other insulin resistance-related syndromes (212). In recent years, the incidence of hyperlipidemia and hyperuricemia has increased significantly, which have become two of the most common health threatening disorders worldwide. Therefore, the prevention and treatment of the two pathological conditions are important for a reduced risk of more severe outcomes such as atherosclerosis, coronary heart disease, and renal disease (213, 214). It has been proposed that trace elements and minerals play an important role in the regulation of lipid and purine metabolism. Thus, investigation of the association between trace element imbalance and the occurrence of hyperlipidemia and hyperuricemia might be helpful to study the etiology and pathogenesis of the two metabolic diseases and to develop new lipid- and uric acid-lowering drugs.

The relationship between hyperlipidemia and trace element homeostasis has been analyzed in early times. Deficiency of several essential elements (Zn, Fe, Cr, and Se), Cu excess, and significant urinary excretion of Fe, Zn, and Cu were observed to be associated with the prevalence of hyperlipidemia (215–219). However, Cu deficiency and hepatic Fe overload were reported to contribute to hypertriglyceridemia and hypercholesterolemia in rats, and the genetic mechanisms driven to hypertriglyceridemia might favor Fe deposits (220–222). Moreover, some lipid-lowering agents (e.g., statins) could greatly influence the serum levels of multiple trace elements such as Zn and Cu (223). In the last decade, very few ionomics studies have been conducted for hyperlipidemia patients. Yerlikaya et al. found that lower levels of essential elements (Cr, Fe, Mn, Mo, and Se) as well as higher levels of Co, Ni, and Cd in serum are associated with an increased risk of primary hyperlipidemia, suggesting an important role of these elements in the regulation of lipid homeostasis (224). On the other hand, several studies have either confirmed previous observations or revealed additional elements that are associated with hyperlipidemia. For example, high Mn exposure could decrease the risk of high triglycerides (225), while elevated blood/serum levels of toxic heavy metals (such as Hg, As, and Pb) may increase the risk of different types of hyperlipidemia (226–228). Very recently, Barragán et al. examined the associations of plasma essential trace elements with hyperlipidemia in a Mediterranean population, which demonstrated that plasma Mg concentrations and combined effect of plasma Zn, Cu, and Se levels had a positive association with hypercholesterolemia whereas plasma Mn concentrations would have negative effects on the amount of plasma lipids (229, 230). A U-shaped link was suggested to exist between plasma Se levels and hypercholesterolemia, indicating that both low and high Se concentrations might have negative effects on plasma lipids (230).

Dyshomeostasis of trace elements has also been implicated in the incidence and progression of hyperuricemia for a long time. In general, circulating levels of several essential metals (such as Ca, Fe, and Cu) and toxic metals (Pb and As) showed positive correlations with the prevalence of hyperuricemia in different populations (231–238). In contrast, concentrations of Mg in serum and I in urine were inversely associated with the prevalence of hyperuricemia (239, 240). Although the relationship between Zn levels and hyperuricemia has not been explored, dietary Zn intake was reported to be inversely correlated with hyperuricemia in different regions, suggesting that Zn deficiency might increase the susceptibility of hyperuricemia (241, 242). Interestingly, there was a discrepancy of the association between blood Cd, serum uric acid levels, and hyperuricemia in different genders. Several studies have shown that blood Cd levels had a positive correlation with increasing risk of hyperuricemia in men (243, 244) or a negative correlation in women in Chinese

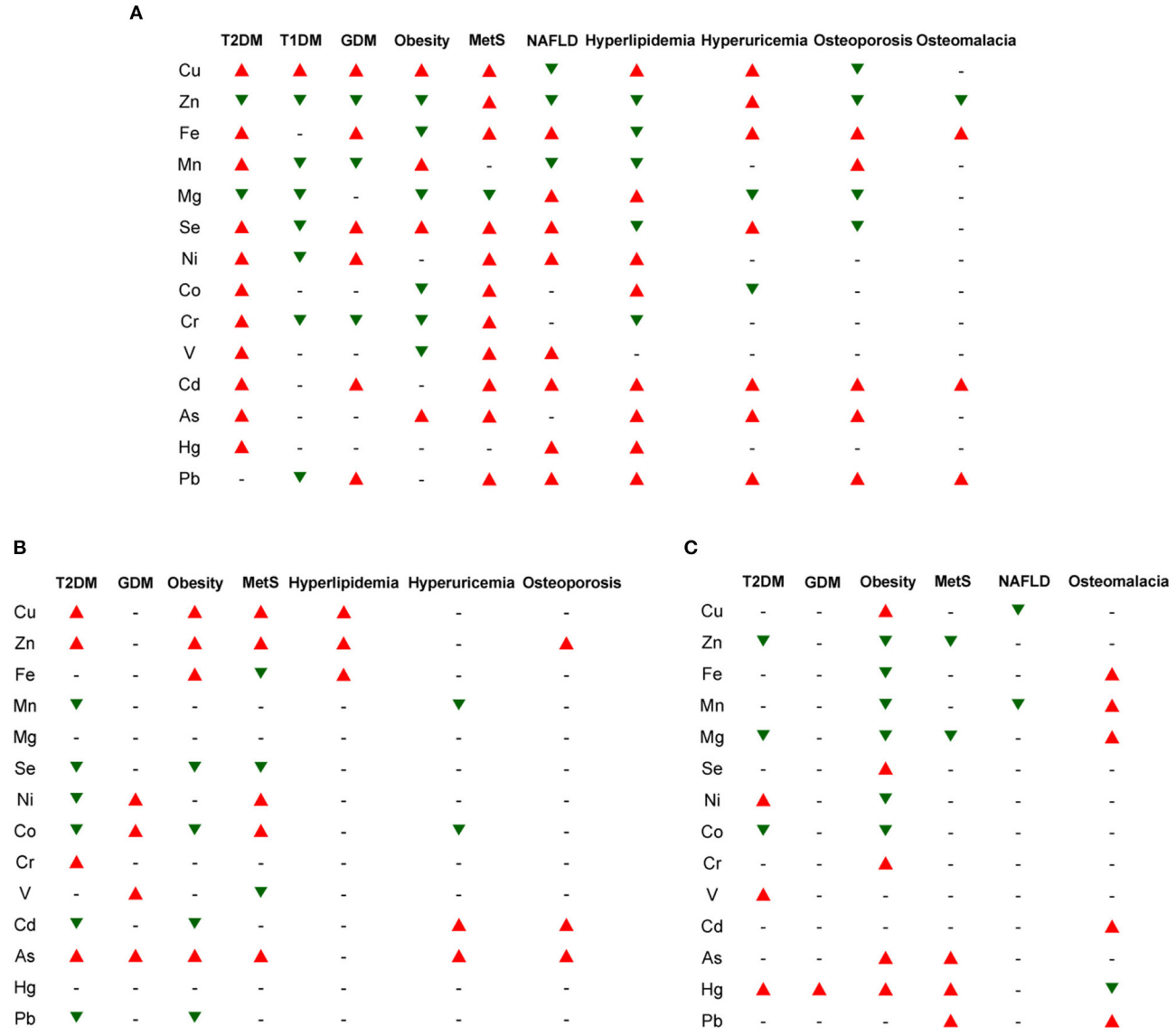

FIGURE 1
The general trends in the changes of major elemental concentrations in different biological samples for several types of metabolic diseases. **(A)** blood (including whole blood, serum, and plasma); **(B)** urine; **(C)** hair/nails. The red and green triangles indicate increased and decreased trends of elemental levels, respectively, which have been reported in the majority of ionomic and other related studies using the corresponding samples from patients. The short line (–) represents unclear or no significant change. T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; GDM, gestational diabetes mellitus; MetS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease.

and Korean populations (237). However, an opposite pattern was observed among populations in the U.S., in which serum Cd levels (in the acceptable range) were positively associated with increased prevalence of hyperuricemia in women, but inversely associated in men (245). This may suggest that different compositions of blood, race, and different ranges of blood Cd can affect the effect of Cd exposure on uric acid levels (246). So far the ionomics study on hyperuricemia is almost a blank. Wang et al. measured the plasma levels of 13 metals in hyperuricemia patients and controls in Chinese Han adults and found that higher plasma levels of several metal ions (particularly Zn and As) and lower levels of Co might increase hyperuricemia risk (247). Ma et al. reported that increased urinary levels of toxic metals (As and Cd) and decreased levels of essential metals (Co, Mn, and I) might have a positive combined effect on hyperuricemia (248). A systematic meta-analysis has been recently conducted to clarify the relationship between exposure to certain metals and the risk of hyperuricemia, which revealed that exposure to As, Ca, Cd, and Pb is associated with an increased risk of hyperuricemia whereas Mo exposure appears to be associated with a decreased prevalence of this disease (249). All these findings reinforce the importance of heavy metal (especially environmental toxic metal) accumulations in the risk of hyperlipidemia and hyperuricemia in general population. Moreover, alternations specific for each of the two metabolic diseases (e.g., elevated serum Co levels in hyperlipidemia and decreased plasma Co and urinary I levels in hyperuricemia) might help to provide potential markers for distinguishing them before clinical symptoms appear. Further ionome-level studies are required to validate the above results

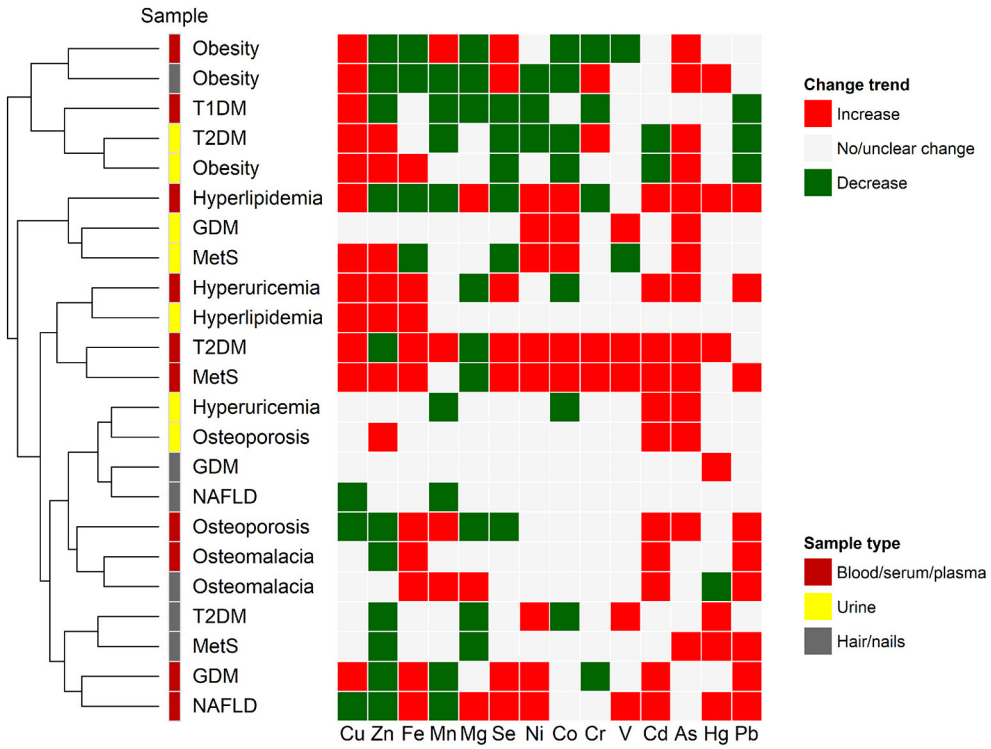

FIGURE 2
Clustering of ionomic profiles for metabolic disease. Alternations in the concentrations of major elements in several types of biological samples of patients with different metabolic diseases were analyzed using hierarchical clustering approach. Red and green colors represent increased and decreased trends of elemental concentrations, respectively, when compared with healthy controls. The gray color represents no significant or unclear change. Abbreviations of different diseases are the same as those in Figure 1.

and to explore new patterns of elemental profiles in more biological samples and larger cohorts.

3.6. Osteoporosis and osteomalacia

Osteoporosis is a systemic bone metabolic disease characterized by bone mass reduction and bone microstructure destruction, which increases the risk of fracture, particularly in older postmenopausal women. The pathogenesis of osteoporosis is complex and involves the interaction of genetic, hormonal, environmental, and nutritional factors (250). Osteomalacia (or rickets in children) is a metabolic bone disorder of decreased mineralization of newly formed bone due to the lack of available Ca, P, or vitamin D. The major causative factors of osteomalacia include nutritional deficiency, impaired absorptive capabilities, and renal insufficiency or dialysis (251). Trace elements have been known to be essential for maintaining bone health, whose imbalance may be significantly associated with the disorders of bone metabolism and further lead to osteoporosis and/or osteomalacia (252, 253). Analysis of the relationship between trace element contents and osteoporosis/osteomalacia may assist in the prevention, diagnosis, and treatment of bone metabolic diseases.

The correlation between certain trace element and mineral levels within the body and osteoporosis has been examined in a large number of population-based studies, case-control cohort studies, and meta-analyses since the early 1970s. In general, reduced circulating levels and/or elevated urinary excretion of a variety of essential metals [such as Ca, Cu, Zn, Mg, Se, and boron (B)] as well as accumulation of Fe, Mn, and toxic heavy metals (Cd, Pb, and As) are strongly related to the prevalence of osteoporosis (254–266). In the recent decade, with the widespread use of ionomics approaches in various complex diseases, several studies have been carried out to provide evidence of the relationship between trace elements in blood and bone tissues and osteoporosis. For the first time, Noor et al. analyzed the difference of the bone concentrations of 27 elements from Indonesian postmenopausal women with and without osteoporosis, which showed that increased B and As and decreased Se levels in bone tissues might directly contribute to microarchitectural abnormalities in osteoporotic bones (267). Another ionomics study suggested that low serum levels of Cu, Zn, Fe, and Mg are associated with the incidence of osteoporosis in Turkish postmenopausal women, implying that Fe deficiency is also one of the risk factors for the development of osteoporosis (268). Lin et al. measured multi-elemental contents in human bone tissues of older Chinese adults and found a significant correlation between decreased Cu, Zn, and Mn levels and increased risk of osteoporosis, indicating that both Mn exposure and deficiency (although very rare in human body) might lead to abnormal bone metabolism, osteoporosis, and even fractures (269). A SVM-based prediction model for bone mineral density loss and osteoporosis was recently proposed by using urinary concentrations of several toxic metals (such as As and Cd) and some other features, suggesting the possibility to develop new methods for the early detection of osteoporosis based on elemental distribution in

biofluids (270). Additionally, lowering excessive toxic metals and/or supplementation of deficient essential elements might be valuable for the therapy of osteoporosis.

In the past several decades, besides low serum levels of Ca and P that have been proposed as a screening test in patients who have clinical symptoms suggestive of osteomalacia (271), elevated concentrations of several other elements have been observed in different biological samples of osteomalacia patients, such as Al, Fe, Cd, and Sr in bone or serum and Pb in blood or hair (childhood rickets) (272–275). In contrast, Fe deficiency was reported in autosomal dominant hypophosphatemic rickets, a rare form of rickets caused by mutations in the fibroblast growth factor 23 (FGF23) gene, implying that monitoring the Fe status is helpful for diagnosis, prognosis, and treatment of this genetic disease (276). Moreover, Ca/Zn ratio in bone was found to be related to the degree of osteomalacia in Cd-exposed subjects, indicating that interactions among these metals might be involved in the pathogenesis of osteomalacia (277). To date, ionomics studies on osteomalacia/rickets are extremely rare. Previously, Miekeley et al. reported that elevated concentrations of Ca, Cd, Al, Fe, and several other elements in hair were associated with the incidence of osteomalacia, which could be used as a complementary tool for its early detection (278). Dogan et al. examined multiple trace element levels in serum and found significantly decreased Zn concentrations in children with nutritional rickets associated with vitamin D deficiency, suggesting an important role of Zn in the early steps of bone maturation (279). However, the correlations between many other trace elements and the prevalence of osteomalacia/rickets are still unclear. Future investigations are necessary to identify more ionome-level variations for bone metabolic diseases.

4. Development of integrated ionomic fingerprints for metabolic disease

Comparison of the integrated ionomic profiles in different biological samples for major types of metabolic diseases has demonstrated common/specific trends in the changes of elemental contents (Figure 1). Considering that the number of ionomics studies for each subtype of blood samples (i.e., whole blood, serum, and plasma) is very limited for most metabolic diseases, in this review, we combined different subtypes of blood samples to try to give a general trend of the changes of elemental concentrations in the blood as previously adopted for cancer blood metallomics (280).

Among all examined ions, Cu levels are significantly increased in almost all examined samples (except hair/nails) from patients with various metabolic diseases (except NAFLD and bone metabolic diseases), indicating that Cu accumulation is one of the common risk factors for a wide variety of metabolic diseases and might act as a general marker for diagnosis and poor prognosis of them. Elevated concentrations of Se and environmental heavy metals (such as Ni, Cd, As, and Pb) and reduced levels of Zn and Mg are often observed in the blood/serum/plasma and hair/nails in patients with metabolic diseases, suggesting that alternations in the homeostasis of these elements may also serve as indicators of these diseases. In contrast, higher excretion of Zn and lower excretion of Se, Cd, and Pb in the urine were observed in several major types of metabolic diseases (especially T2DM, obesity, and MetS), which may partially contribute to the imbalance of these elements within the patients. In addition, the Cu/Zn ratio is also increased in the serum/hair from patients with T2DM and obesity, indicating its potential as a general circulating marker for these diseases.

Although the elemental contents are distinct and highly dynamic in different samples from patients with same/different metabolic diseases, each sample type appears to have specific ionomic pattern, which could be further used to develop novel biomarkers or prediction models for the respective disease. Clustering analysis revealed that same sample types from patients with different metabolic diseases may have quite similar or correlated ionomic patterns (Figure 2), such as blood (including whole blood, serum, and/or plasma) and hair/nails from T2DM and MetS as well as urine samples from T2DM and obesity, which might be associated with common pathogenetic mechanisms (say, insulin resistance) of these diseases (281). On the other hand, although most sample types from same/different diseases showed diverse elemental profiles, certain samples from patients with the same disease were clustered together, suggesting a potential relationship between them (Figure 2). For example, blood and hair/nails from obesity patients have same trends in the changes of major elemental concentrations (say, decreased levels of Zn, Fe, and Mg and increased levels of Se), whereas the urine samples of obesity have somewhat different elemental patterns (such as increased Zn and Fe, decreased Se and Cd, and unchanged Mg levels). Previous human biomonitoring programs have shown significantly different elemental contents between hair and urine samples in physiological conditions (282). It has been suggested that urine can be used for the monitoring of very low levels of elements and the assessment of recent exposure of heavy metals due to its rapid clearing and metabolism, whereas hair and nail samples may serve as indicators for long term exposure of environmental chemicals and toxic metal accumulation in various populations (282–284). Moreover, the elemental ratio or correlation analysis (shown in Table 1) may offer additional clues with respect to the complex crosstalk between elements in different types of metabolic diseases.

Although ionomics provides valuable insights into the mineral and trace element composition in a living organism and their relationship with different physiological and pathological conditions, there are still limitations for its potential role in diagnosis and prognosis of various diseases including metabolic diseases. One major limitation of current ionomics studies is the relatively small number and the varying characteristics (e.g., age, gender, and history of smoking and drinking) of patients and controls enrolled in different projects, which might restrict the power of ionomic evaluation when considering the high variability of trace element contents in biological samples. Another limitation is that ionomics study could not provide a definite cause and effect relationship between dyshomeostasis of trace elements and the development of metabolic diseases. Moreover, studies on the connection between alternations of elemental profiles and the levels of element-binding proteins (say, metalloproteins) and the dynamic interplay between elements are still lacking for most metabolic diseases. Nevertheless, ionomic analyses of biofluids, hair, nails, and other related tissues may be useful to give a comprehensive overview of the complex interactions between trace elements and minerals and metabolic diseases and help to generate new fingerprints for diagnosis, prognosis, and even for evaluation of therapeutic efficacy in these diseases, which cannot be solved only by the assessment of single or very few element levels.

5. Conclusions and perspectives

Disruption of ion metabolism and homeostasis plays a significant role in various diseases including cancer, metabolic diseases, neurological diseases, CVD, and many more. In the recent decade, ionomics has become a powerful tool for systematically investigating the composition and distribution of trace elements and minerals in different biological systems. However, our understanding of the relationship between ionome and the pathogenesis of metabolic diseases is limited so far.

This review focuses on several major types of metabolic diseases and introduces recent progress in the application of ionomics in the study of these diseases, which may raise the possibility of using such information for early detection and prognosis of patients with metabolic disease. Increased Cu concentrations in the majority of biological samples appear to be the most significant marker for various metabolic diseases. Elevated circulating levels of Se and environmental toxic metals and reduced Zn levels have also been demonstrated to be the common risk factors for the majority of metabolic diseases. Other elemental and inter-elemental biomarkers may also reflect the imbalance of ion contents that occurs in different samples of same/different types of metabolic diseases. In spite that the use of ionomics in the field of human disease remains at the initial stage, it may help to depict the crosstalk among different ions in disease pathogenesis and provide a potential source of diagnostic, prognostic, and predictive biomarkers, particularly in the context of metabolic disease. Future research with larger and more representative samples and/or more complex elemental composition (including chemical speciation and isotope analyses) is encouraged to improve our current understanding of the association between the ionomic network and the onset and progression of different types/subtypes of metabolic diseases.

Author contributions

YZ and HY conceived and wrote the manuscript. BH, JJ, and YX 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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Quantitative omics analyses of NCOA4 deficiency reveal an integral role of ferritinophagy in iron homeostasis of hippocampal neuronal HT22 cells

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Introduction: Neurons require iron to support their metabolism, growth, and differentiation, but are also susceptible to iron-induced oxidative stress and cytotoxicity. Ferritin, a cytosolic iron storage unit, mediates cellular adaptation to fluctuations in iron delivery. NCOA4 has been characterized as a selective autophagic cargo receptor facilitating the mobilization of intracellular iron from ferritin. This process named ferritinophagy results in the degradation of ferritin and the consequent release of iron into the cytosol.

Methods: Here we demonstrate that NCOA4 is important for the adaptation of the HT22 mouse hippocampal neuronal cell line to cellular iron restriction. Additionally, we determined the pathophysiological implications of impaired ferritinophagy *via* functional analysis of the omics profile of HT22 cells deficient in NCOA4.

Results: NCOA4 silencing impaired ferritin turnover and was cytotoxic when cells were restricted of iron. Quantitative proteomics identified IRP2 accumulation among the most prominent protein responses produced by NCOA4 depletion in HT22 cells, which is indicative of functional iron deficiency. Additionally, proteins of apoptotic signaling pathway were enriched by those responsive to NCOA4 deficiency. Transcriptome profiles of NCOA4 depletion revealed neuronal cell death, differentiation of neurons, and development of neurons as potential diseases and bio functions affected by impaired ferritinophagy, particularly, when iron was restricted.

Discussion: These findings identify an integral role of NCOA4-mediated ferritinophagy in the maintenance of iron homeostasis by HT22 cells, and its potential implications in controlling genetic pathways of neurodevelopment and neurodegenerative diseases.

KEYWORDS

ferritinophagy, proteomics, RNA-seq, ferritin, iron deficiency, IRP2

1. Introduction

Iron is an essential nutrient required for a variety of molecular and metabolic processes, including cell development, DNA replication, respiration, and energy production. In humans, iron deficiency remains among the most prevalent nutrient disorders worldwide and causes anemia, impaired motor and cognitive function, increased risk of significant psychopathologies, including depression, anxiety, schizophrenia, and autism spectrum disorder, and other health defects (1–3). On the other hand, excess iron within cells can facilitate the formation of harmful reactive oxygen species (ROS), and thus cause cytotoxicity (4). Hence, the supply, storage, and distribution of iron must be tightly regulated to maintain cellular and systemic iron homeostasis within an ideal range that is physiologically adequate and not imposing harm.

Ferritin is a cytosolic protein for iron storage, composed of 24 ferritin-H (FTH) and ferritin-L (FTL) subunits, which form a highly stable spherical configuration accommodating up to 4,500 atoms of iron. FTH and FTL protein translation responds to the cytosolic labile iron pool (LIP), which is constantly gauged by iron responsive element (IRE)-binding proteins, IRP1 and IRP2 (4). IRPs sterically hinder the translation of ferritin transcripts, particularly, when cellular iron levels become limited. When the LIP expands, IRPs dissociate and promote the translation of ferritin subunits. IREs are also present in the transcripts of iron importers transferrin receptor 1 (*TFRC*) and divalent metal transporter 1 (*SLC11A2*), and the iron exporter ferroportin 1 (*SLC40A1*), which allows a coordinated regulation of iron import, export, and storage for maintenance of cellular iron homeostasis.

Recently, an IRP-independent mechanism regulating ferritin iron release *via* selective autophagy has been identified. This process, called ferritinophagy, is initiated by the binding of an autophagic cargo receptor protein, nuclear receptor coactivator 4 (NCOA4), to iron-laden ferritin (5, 6). The transfer of NCOA4-ferritin to the lysosome by ferritinophagy results in the proteolysis of ferritin, and, in turn, the release of its iron content. NCOA4 is upregulated in response to cellular iron restriction (7, 8). The transcript of NCOA4 does not contain an IRE and is not regulated by IRP activity (6), and NCOA4 is post-translationally controlled by iron status (7, 8).

The physiological implications of NCOA4-mediated ferritinophagy have been determined using various *in vitro* and *in vivo* models of NCOA4 deficiency, particularly those recapitulating cell-types and tissues integral to systemic iron metabolism. Of relevance are enterocytes (9), hepatocytes (10), macrophages (5, 11, 12), and erythroid progenitors (7, 13, 14), which function in tissues carrying out iron absorption, storage, recycling, and utilization, respectively. NCOA4 in these cell-types are essential for maintaining cellular iron availability and for supporting nutritional and physiological demand for iron elevated by either dietary iron restriction or red blood cell production. Systemic deletion of *Ncoa4* in mice leads to functional iron deficiency, manifested with hematological signatures of iron deficiency anemia despite iron overload in tissues (15).

Dysregulation of ferritin and intracellular iron in neurons have been implicated in neurological disorders (16). Moreover, a potential role of NCOA4 in neuropathy has been recently proposed (17). Thus, we investigated the regulation and roles of NCOA4 in a hippocampal neuronal cell line model, HT22 cells. HT22 is an immortalized mouse hippocampus-derived neuronal cell line extensively studied as a model of not only cholinergic neurons, but also glutamate excitotoxicity and endogenous oxidative stress. The latter has been implicated in various neurodegenerative diseases, thus making the HT22 cell line of physiological relevance (18, 19). Our findings confirmed the requirement of NCOA4-mediated ferritin turnover for the survival of HT22 cells under iron restriction. Moreover, functional analyses of the omics profiles of NCOA4 deficiency in HT22 cells revealed enrichment of gene responses associated with diseases and biological functions of brain cells. These support a possible role of ferritinophagy in the pathophysiology of neurodegenerative diseases, previously hypothesized (17).

2. Materials and methods

2.1. Cell culture and chemical treatments

HT22 cells (a generous gift from Dr. Phu Tran from the University of Minnesota, MN, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose and L-glutamine without sodium pyruvate, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Corning, Manassas, VA). Cells were maintained at a confluency of less than 70% in 5% CO_2 at 37°C. To produce cellular iron overload and deprivation, cell cultures were treated with ferric ammonium citrate (FAC; Sigma-Aldrich, St. Louis, MO) and deferoxamine (DFO; Sigma-Aldrich, St. Louis, MO), respectively. Final concentrations of each chemical are provided in the section "3. Results" and corresponding figure legends.

2.2. siRNA-mediated gene silencing

NCOA4 levels were manipulated by siRNA-mediated gene silencing. Cells were plated to maintain confluency at less than 70% and transfected using Lipofectamine RNAiMAX following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Briefly, siRNA stock solutions at 20 μM were diluted with Opti-MEM to 150 nM, and then added to an equal volume of a solution prepared by mixing Lipofectamine RNAiMAX and Opti-MEM at a 1:50 ratio. After incubation at room temperature for 5 min, the siRNA-Lipofectamine complexes were administered to cells to yield a final siRNA concentration of 12.5 nM. Cells were then incubated in 5% CO_2 at 37°C for up to 48 h. *Ncoa4* siRNA (Silencer Select ID s77517, Ambion, Waltham, MA) and negative control siRNA (4390847) (Ambion, Waltham, MA) were used in all knockdown experiments as described and validated previously (8, 13).

2.3. RNA isolation and quantitative PCR (qPCR)

Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and centrifuged at 200 × *g* for 10 min at 4°C. RNA was isolated using the TRI reagent (Sigma-Aldrich, St. Louis, MO) following the manufacture's protocol. Briefly, cells were lysed with TRI reagent at room temperature for 5 min and treated with 0.1 volume of 1-bromo-chloropropane. The top clear RNA phase was collected after separation by centrifugation at 12,000 × *g* for 15 min

at 4°C. RNA was precipitated with 2-propanol by centrifugation at 12,000 × *g* for 10 min at 4°C, washed with 75% ethanol, and centrifuged at 7,500 × *g* for 5 min at 4°C. RNA yield and purity were determined spectrophotometrically using a Beckman Coulter DU 730 Life Science UV/Vis spectrophotometer. Equal amounts of total RNA (150 ng) were reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA), and 1/10 dilutions of cDNA samples were PCR amplified using PowerUP SYBR Green Master Mix reagent (Applied Biosystems, Waltham, MA) combined with gene-specific primers designed using the NCBI Primer-BLAST tool (20). Relative gene expression levels were determined by the ΔΔCt method and normalized to *Tbp* levels. Primers for each gene are listed in **Supplementary Table 1**.

2.4. Protein extraction and western blot analyses

Cell pellets were lysed using an NP-40-based lysis buffer [100 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.1% NP40, 5.0% glycerol, water, and protease inhibitor cocktail (Roche, Basel, Switzerland)], centrifuged at 19,000 × *g* for 5 min at 4°C, and the supernatants were collected for cellular protein analyses. Protein contents of cell lysates were determined by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA). For western blot analyses, equal amounts of protein (15–30 μg) were denatured by boiling in Laemmli buffer supplemented with 2.5% 2-mercaptoethanol for 10 min. Proteins were separated by SDS-PAGE using 4–15% TGX gels (Bio-Rad, Hercules, CA), and transferred to nitrocellulose membranes using a Trans-Blot Turbo Transfer system (Bio-Rad, Hercules, CA). Protein transfer was confirmed *via* Ponceau Red staining. For detection of specific proteins, membranes were blocked with 5% milk in PBS for 45 min at room temperature and incubated with primary antibodies anti-NCOA4 (A302-272A, Bethyl Laboratories, Montgomery, TX) at 1:1,000, anti-ferritin (F502, Sigma-Aldrich, St. Louis, MO) at 1:2,000, and anti-GAPDH (12004167, Bio-Rad, Hercules, CA) at 1:10,000. The anti-IRP2 IgG was a generous gift from Dr. Betty Leibold at the University of Utah and was used at 1:1,000. After washes with PBS containing 0.1% Tween-20, proteins were visualized using relevant near-infrared fluorescence-conjugated secondary antibodies applied at 1:10,000 and an Odyssey Fc imager (Li-Cor, Lincoln, NE). Protein abundance from western images was quantified using the Li-Cor Image Studio Lite software and normalized to corresponding GAPDH values.

2.5. Cell viability and bioenergetics assays

Iron is critical for neuronal viability and to support their high energy production. Cell density and neuronal viability were determined *via* trypan blue exclusion and a dehydrogenase-activity-mediated colorimetric assay Cell Counting Kit-8 (CCK-8; Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. In brief, equal number of cells were seeded and treated on a 96-well plate. CCK-8 solution (10 μL) was added to each well and cells were incubated for 2 h in 5% CO_2 at 37°C. Absorbance at 450 nm was determined using a BioTek Synergy H1 microplate reader. For cellular bioenergetics measures, HT22 cells were transfected with siRNA as described above. Iron deficiency compromises cellular respiration. Forty-eight hours after siRNA treatment, real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were simultaneously measured using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent Technologies; Santa Clara, CA). Measurements were taken at baseline and after treatments with 1 μM oligomycin (ATP synthase inhibitor), 3 μM FCCP (eliminates mitochondrial proton gradient allowing maximal ETC electron flow and oxygen consumption), and 1 μM antimycin A combined with 1 μM rotenone (inhibitors of electron transport chain complex III and I, respectively) as described (21, 22). Mitochondrial-specific basal respiration, ATP-coupled respiration, maximal respiration, spare respiratory capacity, coupling efficiency were calculated as described (22). Prior to bioenergetics assays, cultures were treated with 1μM Hoechst 33342 and nuclei were imaged with fluorescent microscopy using a ZEISS Celldiscoverer 7 as we have described (23). The OCR and ECAR data were then normalized to cell density for each well.

2.6. Quantitative proteomics analysis

Snap-frozen cells were processed at the University of Minnesota Center for Mass Spectrometry and Proteomics (CMSP) for proteomic analyses using the Tandem Mass Tag (TMT) system (Thermo Fisher Scientific, Waltham, MA). At the CMSP, cell pellets were reconstituted with extraction buffer [7 M urea, 2 M thiourea, 0.4 M triethylammonium bicarbonate at pH 8.5, 20% acetonitrile and 4 mM tris(2-carboxyethyl)phosphine], sonicated, and processed using a Barocycler NEP2320 (Pressure Biosciences, Inc., South Easton, MA). After treatment with 8 mM iodoacetamide, protein contents were determined by Bradford assay. Subsequently, samples were digested with trypsin (Promega, Madison, WI) at a 1:40 ratio of total protein for 16 h at 37°C. Digested samples were freeze-dried, cleaned using an Extract Clean C18 SPE cartridge (Grace-Davidson, Deerfield, IL), and resuspended in 0.1 M triethylammonium bicarbonate, pH 8.5, to yield 1 μg protein/μL. Equal amount of each sample (20 μg) were labeled with TMT Isobaric Label Reagent (Thermo Scientific, Watham, MA) per manufacturer's protocol. Labeled samples were resuspended in 20 mM ammonium formate, pH 10, in 98:2 water:acetonitrile and fractionated offline by high pH C18 reversed-phase chromatography as previously described (24). Peptide-containing fractions were dried *in vacuo*, resuspended in 2% acetonitrile and 0.01% formic acid, and analyzed using an Orbitrap Fusion mass spectrometer (Thermo Scientific, Watham, MA). Final analysis of proteomics data was carried out using Scaffold 5 (Proteome Software, Portland, OR).

2.7. RNA-sequencing (RNA-seq)

Total RNA for RNA-seq was isolated from TRI-reagent-treated cells using the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA) following the manufacturer's instructions. Sample quality assessment, library creation, and next-generation sequencing were carried out at the University of Minnesota Genomics Center. Total RNA was quantified using a fluorometric RiboGreen assay, and RNA integrity was assessed using an Agilent BioAnalyzer 2100. The RIN of all samples were within the range of 7.9 to 8.9. Total RNA samples were converted to sequencing libraries using Takara Bio's SMARTer Stranded Total RNA-Seq – Pico Mammalian Kit following the manufacturer's protocol. Then, indexed libraries were normalized and pooled for sequencing. Libraries were sequenced on an Illumina

NextSeq 550 instrument to achieve more than 10 million 75-bp paired-end reads per sample. The edgeR software package (25) was used for normalization and differential expression analysis of the RNA-seq data. The sequencing dataset reported in this paper has been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE211931).

2.8. Bioinformatic and statistical analyses

Functional enrichment analyses of the quantitative proteomics data were conducted using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional annotation tools (26) and Gene Set Enrichment Analysis (GSEA) (27). Pathway analyses of differential expressions revealed by the transcriptome analyses were conducted using the Ingenuity Pathway Analysis (IPA) software package (Qiagen). All data are presented as mean ± standard deviation. Based on the experimental design and number of comparisons, statistical significance was determined using either Student's *t*-tests, or with *post-hoc* tests of one-way or two-way ANOVA. Dunnett's post-test and Bonferroni post-test were performed for one-way and two-way ANOVA, respectively. Statistical analyses were performed using the JMP Pro 14 software (SAS Institute, Cary, NC), and *P*-values lower than 0.05 were considered to indicate statistically significant differences.

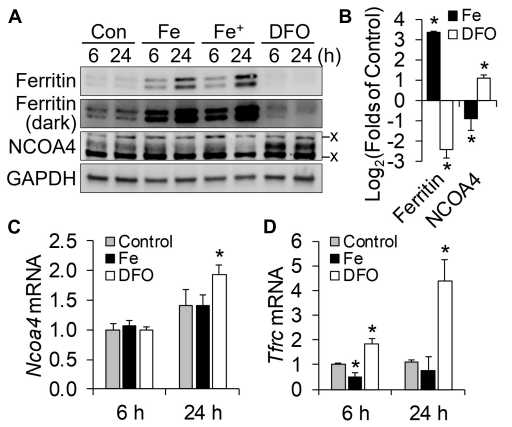

FIGURE 1
NCOA4 post-transcriptionally responds to iron availability in HT22 mouse hippocampal neuronal cells. HT22 cells were treated with ferric ammonium citrate (Fe, 200 μM; Fe^{+}, 400 μM) and desferrioxamine (DFO, 100 μM) for cellular iron overload and restriction, respectively. **(A)** Responses of ferritin and NCOA4 to iron availability, measured by western blot analyses. x, non-specific bands. **(B)** Quantitation of ferritin and NCOA4 western blots. HT22 cells were treated with Fe or DFO for 24 h (n = 4 independent experiments). Protein abundance was normalized to GAPDH. **(C,D)** Effects of cellular iron status on transcript abundance of *Ncoa4* **(C)** and *Tfrc* **(D)**, normalized to *Tbp* levels in HT22 cells (n = 3 biological replicates). Data are presented as mean ± SD (n = 3 biological replicates). *P < 0.05, compared with control levels.

3. Results

3.1. NCOA4 responds to iron status in the HT22 mouse hippocampal neuronal cell line

The responsiveness of NCOA4 to iron status can vary by cell-type and developmental stage (8). Thus, we first assessed whether iron status regulates NCOA4 in the immortalized HT22 mouse hippocampal cell line. Cellular iron restriction by the iron chelator DFO increased NCOA4 protein levels in HT22 cells as early as 6 h after treatment (**Figures 1A, B**). Supplemental iron at either 200 or 400 μM reduced NCOA4 abundance after 24 h, but not after 6 h. Notably, ferritin levels increased within 6 h of iron supplementation, i.e., without a change in NCOA4 levels. After 24 h of iron treatment, NCOA4 responded to changes in iron levels in a direction opposite to that of ferritin. Because iron added at 200 and 400 μM were producing comparable effects on NCOA4 and ferritin expression, we selected the lower dose of iron treatment for our subsequent studies. *Ncoa4* mRNA abundance remained stable (**Figure 1C**) despite changes in *Tfrc* mRNA levels by cellular iron restriction and overload (**Figure 1D**). These results support a role of NCOA4 in facilitated ferritin turnover, and its regulation by neuronal iron status at a post-transcriptional level (7, 8).

3.2. NCOA4 facilitates ferritin turnover and survival of HT22 cells during iron deficiency

Deferoxamine is a cell-impermeable iron chelator and thus produces iron deficiency by limiting iron for cellular import. We hypothesized that NCOA4-mediated ferritinophagy would function as an alternative iron source when neuronal iron uptake is limited. To test this, HT22 cells were transfected with siRNA to silence NCOA4. Successful depletion of NCOA4 by RNAi was confirmed at mRNA (**Figure 2A**) and protein levels (**Figure 2B**). As in untransfected cells, a progressive decline in ferritin and a corresponding increase in NCOA4 in response to DFO treatment occurred in control cells (cells receiving scrambled siRNA) (**Figure 2C**). NCOA4 deficiency *per se* resulted in a ~50% drop in ferritin abundance (**Figure 2B**). However, the response of ferritin to iron restriction was absent in NCOA4-depleted cells (**Figure 2C**). Accordingly, ferritin levels in NCOA4-deficient cells ended up being higher than those in control cells when both groups of cells were treated with DFO for 24 h. While DFO treatment or NCOA4 deficiency alone did not influence cell viability, the combination of the two led to morphological changes (**Figure 2D**) and compromised viability of HT22 cells (**Figure 2E**). Overall, these data indicate that NCOA4 plays a larger role in ferritin turnover in neurons under iron depletion, and ferritinophagy constitutes a primary route of iron supply when neuronal iron import is restricted.

3.3. Quantitative proteomic analysis reveals the role of NCOA4 in cellular iron homeostasis and mitochondrial function, with potential implications for neurodegenerative disease pathology

Bioinformatic analysis of omics data permits prediction of the pathophysiological and organismal implications of *in vitro* treatments. To evaluate the roles of NCOA4 in the HT22 neuronal cell line, we performed proteomics analysis to determine differential

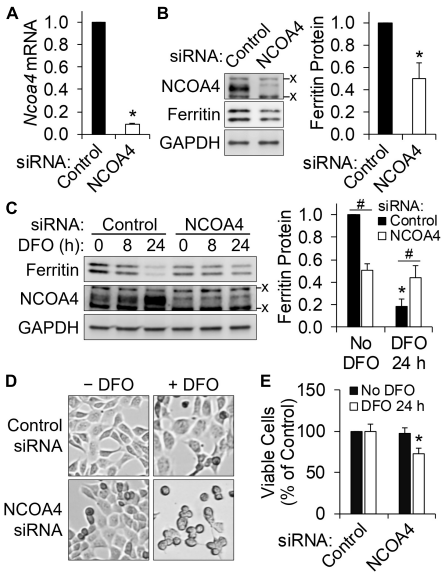

FIGURE 2
HT22 neuronal cells require NCOA4 for ferritin turnover and survival during iron restriction. NCOA4 was knocked-down *via* liposome-mediated siRNA delivery, and DFO (100 μM) was added to induce iron deficiency. **(A)** siRNA-induced *Ncoa4* knockdown confirmed by qPCR, normalized to *Tbp* levels. **(B)** Loss in ferritin protein by NCOA4 depletion. Ferritin quantitation was normalized to GAPDH. **(C)** NCOA4 deficiency impairs the repression of ferritin by iron restriction. $^{*}P < 0.05$ by DFO; $^{\#}P < 0.05$ by NCOA4 depletion. **(D,E)** Morphological changes **(D)** and reduced viability **(E)** of NCOA4-depleted HT22 neuronal cells by iron restriction. Data presented as mean ± SD of $n = 3–6$ independent experiments. $^{*}P < 0.05$ compared with control levels. x, non-specific band. siRNA, small interfering RNA; qPCR, quantitative real-time PCR.

expression patterns induced by the loss of NCOA4. Employment of quantitative proteomics as our initial omics analysis concerned the post-transcriptional regulatory mechanisms for genes involved in iron homeostasis and storage (4, 7, 8). Of relevance are NCOA4 and ferritin, which primarily respond to iron status at the protein but not the transcript level (7, 8). Remarkably, the iron regulatory protein IRP2 (IREB2_MOUSE) was identified as one of the proteins upregulated the most in response to NCOA4 depletion (**Figure 3A**). The turnover of IRP2 is facilitated by cytosolic iron in a proteasome-dependent manner (4). Thus, elevated IRP2 abundance is indicative of functional iron deficiency. Additionally, the decline in ferritin expression induced by NCOA4 deficiency, which was initially identified by western blot analysis (**Figure 2B**), was confirmed by lower counts of FTH and FTL peptides in the proteomics dataset (**Supplementary Dataset 1**).

To ascertain the pathophysiological implications of the differential expressions by NCOA4 deficiency, proteins with significant increases of more than 1.5-fold were subjected to bioinformatic pathway analyses. Over-representation analysis using the DAVID tools identified significant enrichment of gene sets associated with mineral absorption, ribosomes, and the neurodegenerative disorders, Alzheimer's disease, and Parkinson's disease (**Figure 3B** and **Supplementary Table 2**). Functional class sorting by the Gene Set Enrichment Analysis (GSEA) of upregulated proteins revealed "endoplasmic reticulum membrane" and "apoptotic signaling pathway" as the enriched gene sets in NCOA4-deficient cells (**Figure 3C** and **Supplementary Figure 1**). The reactome of nervous system development and positive regulation of locomotion were among the gene sets enriched in the analysis of proteins significantly downregulated by NCOA4 depletion (**Figure 3C** and **Supplementary Figure 1**).

One of the main roles of iron in cell biology is regulation of mitochondrial oxidative phosphorylation by providing prosthetic groups for TCA cycle and electron transport chain enzyme protein subunits. The list of proteins significantly upregulated by the loss of NCOA4 included proteins integral to mitochondrial function and respiration (**Supplementary Dataset 1**). Of relevance were the proteins DnaJ homolog subfamily A member 3 (DNAJA3), cytochrome c oxidase subunit 2 (COX2), dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex (DLAT), and the mitochondrial fission 1 protein (FIS1). Thus, we determined if the expression of NCOA4 affects real-time cellular oxygen consumption rate (OCR) in HT22 cells (**Figure 3D**). DFO treatment decreased all measures of mitochondrial-specific respiration (**Supplementary Figure 2**). Combining DFO treatment with *Ncoa4* knockdown did not alter the magnitude or direction of the DFO effects (**Supplementary Figure 2**). However, *Ncoa4* knockdown alone increased all aspects of mitochondrial respiration including basal, ATP-coupled and maximal respiration (**Figure 3E**), demonstrating the functional relevance of the increased mitochondrial protein abundance quantified by proteomics. The spare respiratory capacity of HT22 cells was also increased by NCOA4 knockdown, indicating an elevated ability to respond to metabolic stress (**Figure 3E**). Non-mitochondrial respiration, coupling efficiency, and proton leak levels were not altered.

3.4. Loss of NCOA4 impairs the response of IRP2 to iron restriction in HT22 cells

The accumulation of IRP2 by NCOA4 depletion, which had been initially identified by quantitative proteomic analyses (**Figure 3A**), was confirmed by western analyses (**Figure 4A**). An E3 ubiquitin ligase, FBXL5, promotes IRP2 turnover when the cytosolic LIP expands (28). Conversely, a reduction in LIP results in the upregulation of IRP2, which represses the translation of ferritin transcripts by binding to their IRE. Thus, the net reduction in ferritin (despite impaired ferritinophagy) by NCOA4 depletion could be attributed to the concomitant IRP2 activity (**Figure 4B**). Next, we determined how DFO-mediated restriction of iron import influences IRP2 in NCOA4-deficient cells. DFO treatment induced a progressive accumulation of IRP2 in control cells (treated with scrambled siRNA), reaching a 5-fold increase after 24 h of treatment (**Figure 4C**). However, IRP2 abundance did not change in response to DFO treatment in NCOA4-deficient cells. The mRNA of *Tfrc* contains five IREs at its 3′-UTR, and thus is stabilized by increased IRP activity. In agreement with the lack of IRP2 regulation by DFO in NCOA4-deficient cells, *Tfrc* transcript abundance no longer responded to iron restriction when cells were deficient of NCOA4 (**Figure 4D**). Notably, *Tfrc* transcript abundance was not affected by NCOA4 depletion when the cells did not receive DFO (**Figure 4D**), despite their higher IRP2 levels (**Figure 4A**). Unlike iron restriction, iron supplementation produced a change in IRP2 levels in both control and NCOA4-depleted HT22 cells (**Figure 4E**).

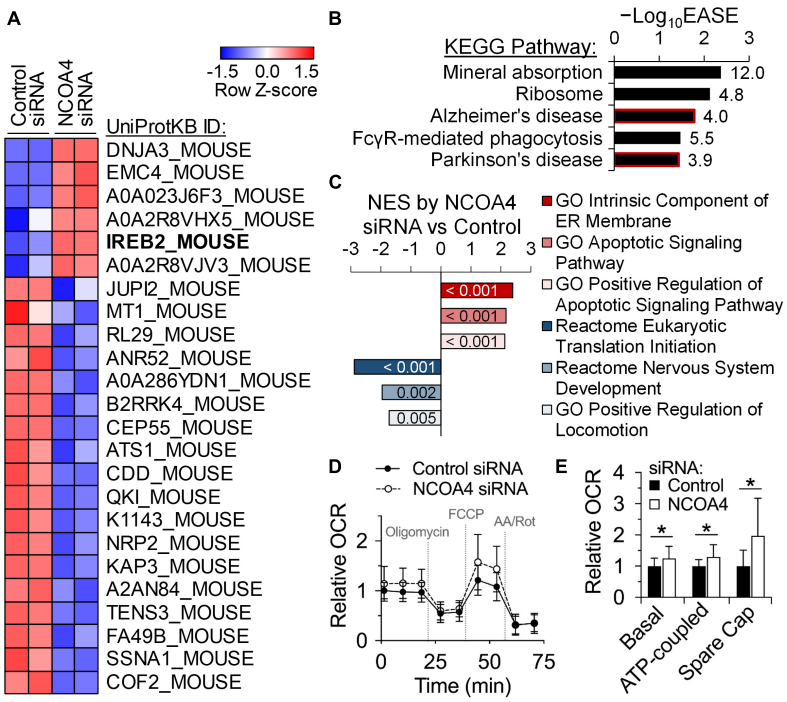

FIGURE 3
Quantitative proteomics identify roles of NCOA4 in apoptosis, translation, and neuronal functioning and development. Protein expression profiles of control and NCOA4-depleted HT22 cells were compared using the tandem mass tag quantitative proteomics approach. **(A)** Expression of proteins with FC values above 2.0 or below -2.0, and $P < 0.05$. Heatmap was generated with Z-scores of Log_2-transformed normalized expression values. **(B)** KEGG Pathways enriched by DE proteins of NCOA4-depleted cells with $|FC| > 1.5$ and $P < 0.05$, which were identified using the DAVID functional annotation analysis. EASE Score is a modified Fisher's exact *P*-value from the DAVID tool. Numbers next to each bar indicate fold enrichment for DE proteins. **(C)** Normalized enrichment scores (NES) of gene sets significantly enriched by proteins DE by NCOA4 depletion ($P < 0.05$) from GSEA. Numbers in each bar indicate *P*-value of enrichment by the DE proteins. **(D)** Effects of NCOA4 depletion on real-time OCRs after treatments of oligomycin, FCCP, and antimycin A/rotenone. Individual well OCR values were normalized to cell density. Data from $n = 3$ independent cultures and are presented as mean ± SD. **(E)** Basal respiration, ATP-coupled respiration, and spare respiratory capacity (Spare Cap) calculated from OCR measurements **(D)**. $^*P < 0.05$ compared with control levels. FC, fold-change; KEGG, Kyoto Encyclopedia of Genes and Genomes; DE, differentially expressed; DAVID, Database for Annotation, Visualization, and Integrated Discovery; GSEA, Gene Set Enrichment Analysis; OCR, oxygen consumption rate.

3.5. Functional enrichment analyses of the transcriptome of NCOA4- and iron-deficient HT22 cells reveal neurological implications of ferritinophagy during iron deficiency

For a more comprehensive assessment of the pathophysiological implications of the interactions between ferritinophagy and neuronal iron status, the transcriptome of HT22 cells deficient in NCOA4, iron import, or both were profiled *via* RNA-seq and analyzed bioinformatically. Initially, we compared the transcriptome and proteome profiles of control and NCOA4-deficient cells to identify 64 genes responsive to NCOA4 depletion at both transcript and protein levels (**Supplementary Figure 3**). Functional network analyses of transcriptome data revealed neurological disease and nervous system development and function as part of the functional networks associated with NCOA4 deficiency (**Supplementary Figure 4**).

Among the entire RNA-seq dataset, the set of genes differentially regulated in HT22 cells deficient in both ferritinophagy and iron import, a condition which led to cytotoxicity, was of particular interest. A sum of 304 transcripts with fold-changes (FC) above 2 and adjusted FDR *q*-values below 0.05 were identified exclusively in cells treated with *Ncoa4* siRNA and DFO together (**Figure 5A**). The expression trend of these genes suggests that ferritinophagy can support neurons in attenuating the magnitude of gene responses produced by restricted iron import, and vice versa (**Figure 5B**). Neuronal cell death, apoptosis, and differentiation of neurons were among the diseases and biofunctions predicted as activated by the simultaneous losses in ferritinophagy and iron import in HT22 cells (**Figure 5C**). Neuronal development pathways of neurons were affected in the opposite manner, indicating NCOA4-mediated ferritinophagy is necessary for gene programs important for normal iron-dependent neuron development.

The normalized gene expression values of iron homeostasis genes determined by RNA-seq indicated higher expression values of genes involved in cytosolic iron storage, *Fth1* and *Ftl1*, than those involved in transmembrane transport of the metal in HT22 cells. Among these, the transcript levels of iron importers *Tfrc*, *Slc11a2*, and *Slc29a14* were found to be iron responsive (**Figure 5D**). Notably, the iron exporter gene *Slc40a1* did not produce detectable amounts of transcripts in HT22 cells regardless of the cellular iron status. The gene *Slc11a2* encodes the divalent metal transporter 1 (DMT1) protein which transports iron across cellular membranes into the cytosol. Like *Tfrc*, *Slc11a2* transcripts carry 3′-UTR IREs and thus are stabilized by iron restriction *via* IRP binding. Supporting our previous observation on the lack of IRP2 regulation by DFO in NCOA4-deficient cells (**Figure 4C**), the transcripts of both *Tfrc* and *Slc11a2* were identified less responsive to iron restriction by the RNA-seq data of NCOA4-depleted HT22 cells (**Figure 5E**).

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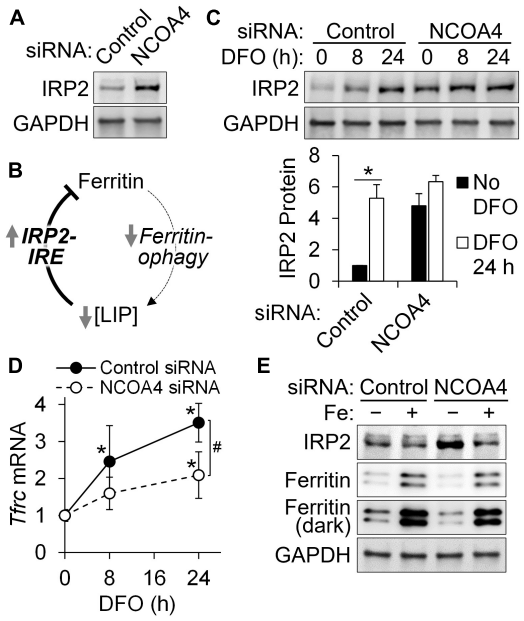

FIGURE 4
NCOA4 depletion impairs the adaptive responses of iron genes to cellular iron restriction in HT22 cells. HT22 cells were treated with *Ncoa4* siRNA to knockdown NCOA4 expression. **(A)** Accumulation of IRP2 protein by NCOA4 depletion. **(B)** Schematic model of IRP2-mediated ferritin repression by NCOA4 depletion. **(C)** Loss in IRP2 responses to iron restriction in NCOA4-depleted cells. DFO was added at 100 μM. IRP2 quantitation was normalized to GAPDH. **(D)** NCOA4 depletion represses the responses of *Tfrc* mRNA to iron restriction (DFO, 100 μM). Transcript abundance is relative to control siRNA-treated cells without DFO, and was normalized to that of *Tbp*. **(E)** IRP2 and ferritin remain responsive to supplemental iron in NCOA4-depleted HT22 cells. Iron was added as ferric ammonium citrate at 200 μM for 24 h. Data in panels **(C,D)** are presented as mean ± SD, and are from $n = 3–5$ independent experiments. $^{*}P < 0.05$ by DFO; $^{\#}P < 0.05$ by NCOA4 depletion. DFO, desferrioxamine; LIP, labile iron pool.

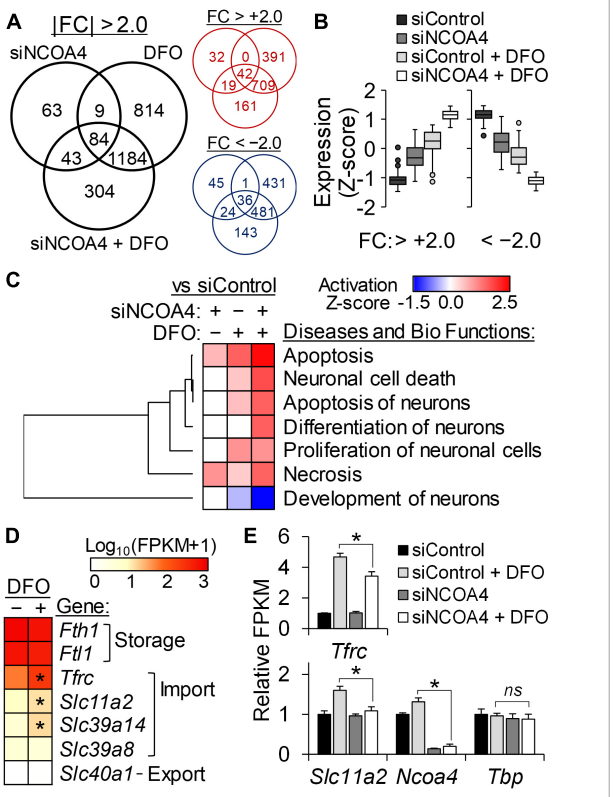

FIGURE 5
Transcriptome analysis reveal a protective role of NCOA4 against apoptosis induced by neuronal iron deficiency. Transcripts responsive to NCOA4 depletion by siRNA (siNCOA4), iron deficiency (DFO), and a combination of the two (siNCOA4 and DFO) were identified by RNA-seq ($n = 3$ biological replicates). DFO was treated at 100 μM for 24 h. **(A)** Numbers of differential expressions shared by or exclusive to each treatment are shown in the black Venn diagram. Transcripts with $|FC| > 2.0$ and FDR-adjusted $P < 0.05$ vs control siRNA (siControl) were considered DE. Venn diagrams in red and blue indicate the numbers of upregulated and downregulated genes, respectively, by each treatment. **(B)** Expression pattern of DE genes exclusively identified in cells treated with siNCOA4 plus DFO. For each gene, expression values of all treatment groups were standardized by Z-score transformation to yield a mean of zero and SD of one. **(C)** Comparisons among the IPA activation Z-scores of neuron-related Diseases and Bio Functions by genes responsive to siNCOA4, DFO, and siNCOA4 plus DFO. IPA core analyses was performed for DE by each treatment (vs siControl), and further analyzed by comparison analysis. Neuron-related Diseases and Bio Functions with significant activation Z-scores by siNCOA4 plus DFO vs siControl are shown ($P < 0.05$). **(D)** Relative transcript abundance of genes for cellular iron homeostasis determined by RNA-seq FPKM. Gene expression data was ranked by mean FPKM values of siControl cells, and presented as $Log_{10}(FPKM + 1)$. $^{*}P < 0.05$ by DFO. **(E)** Less *Tfrc* and *Slc11a2* transcript responses to iron restriction in NCOA4-depleted cells. Data are shown as mean ± SD of FPKM from $n = 3$ biological replicates. $^{*}P < 0.05$ by NCOA4 depletion; ns, not significantly different; siNCOA4, *Ncoa4* siRNA; siControl, control siRNA; DFO, desferrioxamine; DE, differentially expressed; FC, fold-change; FDR, false discovery rate; vs, versus.

4. Discussion

Iron is an essential nutrient crucial for neurodevelopment and lifespan brain health (29). Conversely, excessive accumulation of iron and ferritin in the brain has been associated with the pathogenesis of neurodegeneration and neuronal loss (30, 31). Thus, neurons need a regulatory system which allows them to efficiently balance their iron availability with their biological demand for the metal nutrient. Using HT22 cells as an *in vitro* cell line model of mouse hippocampal neurons, we identified ferritinophagy as a potential mechanism by which neurons maintain a balance between their supply and demand for iron, especially when iron becomes scarce. The importance of ferritin at the cellular level is twofold. First, it sequesters excess iron, which can be potentially toxic, and stores it for future use. The second function of ferritin as an iron resource is strongly supported herein, and the elevated IRP2 and increased sensitivity to cellular iron restriction by NCOA4 depletion are of particular relevance.

The transcriptomic and proteomic profiles of NCOA4 deficiency in HT22 cells revealed enrichment of gene responses functionally associated with neurobiology and neurological disorders, and their interaction with cellular iron status. These included neuronal cell death, neurodevelopment, neuron proliferation, and neurodegeneration. The bioinformatic association between impaired ferritinophagy and molecular pathways of neurodegenerative conditions agree with previous preclinical observations linking functional iron deficiency of neurons to neurodegeneration. Particularly, *Irp2*-null mice feature progressive neurodegeneration attributed to neuronal iron restriction (32). The genetic loss of IRP2 misinforms neurons as if they are in iron excess. This leads

to changes in iron homeostatic gene expressions driving a decline in the cellular import and more removal *via* export and storage (4), and thus functional iron deficiency. Additionally, conditional deletion of *Tfrc* in dopaminergic neurons of mice resulted in neurological and behavioral phenotypes recapitulating symptoms of Parkinsonism in humans (33). These and our *in vitro* data collectively inform the need for future studies using primary neurons and an *in vivo* model of neuronal *Ncoa4* deficiency, testing whether functional iron deficiency by impaired ferritinophagy similarly influences the central nervous system and produces phenotypes of neurodegeneration.

Early iron deficiency has been shown to impair learning and memory, which has been attributed to disrupted mitochondrial function and neuronal loss (21). Our quantitative omics analyses revealed differential expressions associated with mitochondrial function and apoptosis by NCOA4 deficiency. Of particular relevance were DNAJA3, COX2, and apoptotic protease-activating factor 1 (APAF). DNAJA3 modulates apoptotic signal transduction within the mitochondrial matrix and has been characterized as a metalloprotein (34). Known for its role in mitochondrial energy transduction, cytochrome c oxidase has been suggested to play a role in stress-induced apoptosis and degenerative diseases (35). APAF1 is involved in apoptosis due to its binding and subsequent activation of procaspase-9, and studies suggest that cytochrome c and APAF1 work together to activate the apoptotic pathway (36). NCOA4 is a multifunctional protein; thus, the differential expression patterns induced by its loss in HT22 neurons cannot not be attributed solely to its role in ferritinophagy. However, morphological changes, compromised cell viability, and transcriptome profiles of neurodegeneration by NCOA4 depletion were augmented or introduced by coexisting iron deficiency. These indicate a clear nutrient-gene interaction pattern where cellular dependence on NCOA4 becomes more apparent under conditions of insufficient nutrient supply. On the other hand, NCOA4 depletion and iron restriction by DFO had opposite effects on mitochondrial function measured by OCR. Future mechanistic studies with primary neurons need to be performed to specifically determine whether neuronal iron mediates regulation of mitochondrial function by NCOA4.

Ferroportin can contribute to the removal of iron from the cytosol as an iron exporter on the plasma membrane. Interestingly, the transcripts for the iron exporter ferroportin (*Slc40a1*) were undetectable by our RNA-seq experiments, which was in contrast to the relatively higher counts for the ferritin (*Fth1* and *Ftl1*) transcripts. Ferroportin is the sole non-heme iron exporter on the plasma membrane. Thus, the lack of ferroportin expression in HT22 cells suggests that sequestration of iron by ferritin serves as the primary mode of protection against excess cytosolic iron in neurons. Alternatively, excess iron might be removed from HT22 cells *via* a ferroportin-independent route, such as exosomal ferritin as recently described (37). These are in agreement with previous *in vivo* findings where *Slc40a1* was identified dispensable for the survival and function of dopaminergic neurons in mice (33).

Collectively, our studies using HT22 cells identify how NCOA4 is a critical nexus regulating the tight control of intracellular iron to maintain cell viability. NCOA4-mediated ferritinophagy was identified to be crucial for adaptation to fluctuations in iron levels, particularly when cellular iron supply is restricted. Loss of NCOA4 led to molecular marker changes implying functional iron deficiency and differential gene expressions associated with development of neurons, mitochondrial function, apoptosis, and neurodegenerative disorders. Our *in vitro* data suggest that the cellular iron pool and NCOA4-mediated ferritinophagy could serve as potential molecular targets for prevention of neurodegenerative disorders and facilitate the development of therapeutic strategies for their treatment. Future studies on primary neurons and the *in vivo* brain are needed to understand the requirement of ferritinophagy for brain development and function throughout the lifespan, and to decipher the mechanisms underlying the genetic and nutritional regulation of NCOA4 expression and activity in the central nervous system.

Data availability statement

The original contributions presented in this study are included in this article/**Supplementary material**, further inquiries can be directed to the corresponding author. The RNA-seq data presented in this study are deposited in the NCBI Gene Expression Omnibus (GEO) repository, accession number GSE211931 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE211931).

Author contributions

M-SR: conceptualization and supervision. EB, TB, and M-SR: methodology and investigation. EB and M-SR: formal analysis, data curation, original draft preparation, and visualization. CG: validation. MG and M-SR: resources and project administration. CG, TB, MG, and M-SR: review and editing. All authors have read and agreed to the published version of 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1054852/full#supplementary-material

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Association between dietary minerals and glioma: A case-control study based on Chinese population

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Background: As one of the essential nutrients for the human body, minerals participate in various physiological activities of the body and are closely related to many cancers. However, the population study on glioma is not sufficient.

Objective: The purpose of this study was to evaluate the relationship between five dietary minerals and glioma.

Methods: A total of 506 adult patients with glioma and 506 healthy controls were matched 1:1 according to age (±5 years) and sex. The food intake of the subjects in the past year was collected through the food frequency questionnaire, and the intakes of calcium, magnesium, iron, zinc, and copper in the diet were calculated. The logistic regression model was used to estimate the odds ratio (OR) and 95% confidence interval (95% CI) for dietary minerals to gliomas.

Results: After adjusting for confounders, higher intakes of calcium (OR = 0.65, 95% CI: 0.57–0.74), magnesium (OR = 0.18, 95% CI: 0.11–0.29), iron (OR = 0.04, 95% CI: 0.02–0.11), zinc (OR = 0.62, 95% CI: 0.54–0.73), and copper (OR = 0.22, 95% CI: 0.13–0.39) were associated with a significantly decreased risk of glioma. Similar results were observed in gliomas of different pathological types and pathological grades. The restriction cubic spline function suggested significant linear dose-response relationships between intakes of five minerals and the risk of glioma. When the dietary minerals exceeded a particular intake, the risk of glioma stabilized.

Conclusion: Our study suggests that higher dietary intakes of calcium, magnesium, iron, zinc, and copper are associated with a decreased risk of glioma. However, the results of this study require further exploration of potential mechanisms in the future better to elucidate the effects of mineral intake on gliomas.

KEYWORDS

minerals, glioma, case-control study, Chinese population, dose-response relationship

Introduction

Gliomas are the most common type of primary central nervous system tumor, accounting for about 80.7% of malignant brain tumors (1). Although the incidence of glioma is very low, only 5.47/100,000 (1), due to the high mortality rate of glioma and the large population base in China, it caused serious disease burden and economic burden to families.

Therefore, exploring modifiable factors in the etiology of glioma was essential to provide substantial scientific support for primary prevention.

Compared with other cancers, the etiology of gliomas was largely uncertain and complex. Although gliomas have long been reported to be related to head trauma (2), allergies (3), use of mobile phones (4), and occupational exposure (5). Ionizing radiation was still the only apparent environmental risk factor (6). In recent years, the relationship between diet and glioma has attracted more and more attention (7). Existing studies have found that dietary patterns (8), food groups (9), and nutrients (10) all had certain effects against gliomas. In particular, the vitamins and phytochemicals in these foods had certain antioxidant effects, protecting healthy tissues from oxidative stress-induced damage and inhibiting the occurrence and development of glioma (7, 11–13). However, previous studies on diet and glioma ignored minerals with similar effects. These elements also played an antioxidant (14), anti-inflammatory (15), and anti-tumor (16) effect on the body. Therefore, several common minerals, such as calcium (Ca), iron (Fe), and zinc (Zn), may also affect gliomas. Yekta et al. found a significant negative association between dietary Ca intake and glioma (OR = 0.23, 95% CI: 0.08–0.65) in an Iranian hospital-based case-control study (17). This association was also found in the San Francisco Bay Area Adult Glioma Study but was only significant in women (18). Chen et al. also found that the consumption of intracellular Ca ions affected the abnormal growth of C6 glioma cells by affecting the signal transduction of the endoplasmic reticulum (19). Studies on Fe and gliomas had similar findings. Ward et al. followed up for 14.1 years in a European prospective cohort study to explore the effect of meat and heme Fe on gliomas but found no association between them [Hazard ratio (HR) = 0.96, 95% CI: 0.73–1.26] (20). However, *in vivo* exposure, a higher concentration of toenail Fe was found to have a protective effect against gliomas (OR = 0.42, 95% CI: 0.19–0.95) (21). Although cell experiments found that low Zn can inhibit the cell growth of rat glioma C6 cells (22), Dimitropoulou et al. did not find any significant association between dietary Zn and glioma in the nutritional epidemiological study (23). The influence of magnesium (Mg) and copper (Cu) on glioma was far less than that of other elements, and their related studies mainly focused on metalloproteins. Bioinformatics studies have found differential expressions of various copper-related proteins in gliomas and normal tissues (24). The overexpression of Mg transporter 1 was also associated with the occurrence and progression of gliomas (25).

Although experimental data on the role of minerals in the prevention of glioma were promising, the vast majority of studies have focused on *in vitro* assays. Epidemiological studies on minerals and gliomas were insufficient. On the one hand, no studies have reported their dose-response relationship. On the other hand, relevant studies were mainly based on the population of European and American populations, with geographical limitations. Therefore, to further explore the association between dietary minerals and glioma, we investigated the association of five typical dietary mineral intakes with the risk of glioma in the case-control study based on a Chinese population and attempted to delineate the dose-response relationship between the two aim to provide some epidemiological evidence for the prevention of glioma by five minerals.

Methods

Study population

One thousand and twelve subjects (506 cases and 506 controls) participated in the diet and glioma case-control study at Beijing Tiantan Hospital, Capital Medical University, between 2021 and 2022. The case group consisted of patients with glioma who were recently diagnosed by neuro-oncologists and pathologists according to the 2021 neuro-tumor diagnostic criteria (26). Among them, there were 104 cases of astrocytoma, 67 cases of oligodendroglioma, 237 cases of glioblastoma, and 98 cases of other gliomas (including 18 cases of diffuse midline glioma). The control groups were recruited from healthy individuals in the community and matched 1:1 with cases by age and sex. All participants were ≥18 years of age. Among them, 15 people in the case group refused to participate, with a response rate of 97.2%, and 41 people in the control group refused to participate, with a response rate of 92.7% (Supplementary Figure 1). On this basis, they were excluded according to certain conditions, including suffering from digestive, neurological, and endocrine system diseases, suffering from other cancers, significant dietary behavior changes such as dieting before the investigation, abnormal energy intake (>5,000 or <400 kcal/d), pregnant women and nursing mothers, and taking drugs such as hormones. All participants provided informed consent, and the study protocol was approved by the Institutional Review Board of Beijing Tiantan Hospital, Capital Medical University (No.KY2022-203-02).

Data collection

The required information was obtained through questionnaires. Through face-to-face interviews, investigators collected data on demographics, lifestyle habits, disease history, and dietary intake and measured some anthropometric indicators. Demographic data included date of birth, sex, occupation, education level, and household income. Lifestyle habits included living conditions in high-risk areas, smoking status, drinking status, and physical activity. Living near electromagnetic fields and broadcast antennas in the past 10 years have been defined as high-risk residential areas (4). Physical activity was assessed using the International Physical Activity Questionnaire (27). Disease histories were collected for diseases potentially associated with glioma, including allergies, head trauma, and other cancers.

Anthropometric data mainly consisted of height and weight, which were collected by trained staff using standardized techniques and calibrated equipment. Body mass index (BMI) was calculated by dividing weight (in kilograms) by the square of height (in meters), and the result was accurate to two decimal places.

Dietary intakes were assessed through the 114-item food frequency questionnaire (Supplementary Table 1). The questionnaire has been validated in previous studies (28). According to the foods reported in the literature that may affect the risk of glioma, several foods were added and deleted on this basis to make the food frequency questionnaire more suitable for the research needs. To improve the accuracy of the dietary survey,

the investigators collected the dietary intake information of the study subjects in the past year through face-to-face interviews by providing pictures of different food volumes and qualities. The study subjects need to fill in the intake of each food according to their conditions, including whether the food was consumed, the frequency of intake (number of intakes per day/week/month), and the average intake per time. In order to further verify the reproducibility and validity of the questionnaire in this study, after about 1 year, we investigated 30 healthy controls again, collected the dietary information of the subjects through the food frequency questionnaire and 24-h recall (two working days and one rest day), calculated the food consumption and nutrient intake, and evaluated the reproducibility and validity of the questionnaire by the mean and correlation coefficient. For reproducibility, the correlation coefficients of food group were 0.502–0.847, and that of nutrients were 0.437–0.807. For validity, the correlation coefficients of food group were 0.381–0.779, and the correlation coefficients of nutrients were 0.380–0.804 (Supplementary Tables 2–5).

The study involved five common minerals, including Ca, Mg, Fe, Zn, and Cu. The intakes of all minerals were calculated based on the information of each food item and the Chinese Food Composition Table (29). The daily intakes of various foods were calculated according to the frequency of food intake and the amount of each intake filled in by the study subjects. The "Chinese Food Composition Table" provided the content of five minerals per unit of food. It was calculated by multiplying the daily intake of various foods and the unit content of minerals in the food. Then the sum of the intakes of all minerals in different foods was calculated as the total intake. Energy intake polyunsaturated fatty acids (PUFAs) were calculated similarly.

Statistical analysis

Demographics, lifestyle habits, and disease history were characterized using descriptive analysis. The *t*-test was used for normally distributed continuous variables, and the chi-square test was used for categorical variables to compare general characteristics between pathological subtypes and controls. The Mann-Whitney *U*-test was used to compare mineral intakes between case and control groups, and Spearman's correlation coefficient was used to evaluate the correlation between the five minerals. We used logistic regression models to estimate ORs and 95% CI between mineral intake and the risk of glioma, adjusting for potential confounders. In this analysis, each mineral intake was divided into tertiles, with the lowest tertile as the reference group. In addition, mineral intake was also brought into the model as a continuous variable.

Potential confounding variables included age, BMI, occupation (manual workers, mental workers, or others), educational levels (primary school and below, middle school, or university and above), household income (below 3,000 ¥/month, 3,000–10,000 ¥/month, or above 10,000 ¥/month), high-risk residential areas (yes or no), smoking status (never smoking, former smoking, or current smoking), drinking status (non-drinker, occasional drinker, or frequent drinker), history of allergies (yes or no), history of head trauma (yes or no), family history of cancer (yes or no), physical activity (low, moderate, or violent), PUFAs intake, and energy intake.

Age, sex, BMI, occupation, education level, household income, smoking status, history of allergies, family history of cancer, and physical activity were also used as the basis for subgrouping, and subgroup analyses were performed by logistic regression after adjusting for confounding factors. In addition, to overcome the inherent limitations of elemental analysis as a grade variable, the dose-response relationship was analyzed using the restricted cubic spline function in the logistic regression model after adjusting for confounders, with nodes distributed in the 20th, 40th, 60th, and 80th percentiles, the reference value (OR = 1) was set at the 10th percentile (30).

All statistical analyses were performed using SPSS 26.0 and R 4.1.1. A two-sided *P*-value <0.05 was used to determine the statistical significance.

Results

Study population and mineral characteristics

The patients with glioma of different pathological subtypes and their corresponding control groups were completely identical in sex composition and similar in age distribution. Compared with controls, glioma patients differed in BMI ($P < 0.001$), occupation ($P = 0.024$), education levels ($P < 0.001$), household income ($P < 0.001$), smoking status ($P = 0.039$), drinking status ($P < 0.001$), physical activity ($P < 0.001$), history of allergies ($P < 0.001$), and family history of cancer ($P = 0.001$). Patients with various pathological subtypes of glioma had higher BMI, slightly lower education levels, lower household income, more drinkers, and more physical activity, which was consistent with the overall population. In addition, only the population with glioblastoma had a higher family history of cancer ($P = 0.006$). Other glioma populations had more manual workers ($P = 0.002$) and a lower history of allergies ($P = 0.004$). In other respects, there were no significant differences (Table 1).

In terms of dietary intakes, compared with controls, cases had higher intakes of refined grains ($P < 0.001$) and alcohol ($P < 0.001$), and lower intakes of whole grains ($P < 0.001$), legume and products ($P < 0.001$), tubers ($P < 0.001$), vegetables ($P < 0.001$), fungi and algae ($P < 0.001$), fruits ($P < 0.001$), fish and seafood ($P < 0.001$), and dairy products ($P = 0.025$). For other food groups, there was no significant difference (Supplementary Table 6).

In terms of mineral intakes, as shown in Table 2, the intakes of Ca, Mg, Fe, Zn, and Cu in the control group were all significantly higher than those in the case group. In addition, there were significant correlations between individual mineral intakes (Spearman coefficients ranged from 0.709 to 0.919) (Supplementary Table 7).

TABLE 1 Basic characteristics of the study participants.

	Astrocytoma			Oligodendroglioma			Glioblastoma			Others			P^b
	Case	Control	P^a	Case	Control	P^a	Case	Control	P^a	Case	Control	P^a	
Age (years)	39.32 ± 13.43	38.01 ± 13.01	0.477	39.52 ± 9.75	37.66 ± 9.67	0.268	45.27 ± 13.07	43.75 ± 12.89	0.202	41.83 ± 13.58	40.58 ± 13.27	0.517	0.072
Sex (%)			1.000			1.000			1.000			1.000	1.000
Male	58.7	58.7		61.2	61.2		55.3	55.3		52.0	52.0		
Female	41.3	41.3		38.8	38.8		44.7	44.7		48.0	48.0		
BMI	24.02 ± 3.05	22.89 ± 3.34	0.012	24.37 ± 2.91	23.27 ± 3.64	0.056	23.99 ± 3.30	23.13 ± 3.12	0.004	23.90 ± 3.56	22.89 ± 3.33	0.042	<0.001
High-risk residential area (%)			0.222			0.662			0.729			0.319	0.534
Yes	23.1	16.3		17.9	20.9		19.0	20.3		27.6	21.4		
No	76.9	83.7		82.1	79.1		81.0	79.7		72.4	78.6		
Occupation (%)			0.406			0.931			0.119			0.002	0.024
Manual workers	23.1	17.3		32.8	31.4		22.4	21.1		35.7	14.3		
Mental workers	61.5	61.5		55.2	58.2		51.0	59.5		43.9	63.3		
Others	15.4	21.2		12.0	10.4		26.6	19.4		20.4	22.4		
Education level (%)			0.013			0.009			<0.001			<0.001	<0.001
Primary school and below	2.9	4.8		6.0	0		6.3	2.5		1.3	2.0		
Middle school	43.3	24.0		40.3	23.9		41.8	26.2		39.8	24.5		
University and above	53.8	71.2		53.7	76.1		51.9	71.3		46.9	73.5		
Household income (%)			0.007			<0.001			<0.001			0.024	<0.001
<3,000 ¥/month	11.5	18.3		10.4	19.4		7.2	19.8		13.3	13.3		
3000–10,000 ¥/month	72.1	51.0		83.6	47.8		77.6	46.8		70.4	54.0		
>10,000 ¥/month	16.3	30.8		6.0	32.8		15.2	33.4		16.3	32.7		
Smoking status (%)			0.615			0.405			0.382			0.079	0.039
Never smoking	70.2	73.1		64.2	74.6		73.0	74.7		66.3	79.6		
Former smoking	10.6	6.7		16.4	10.4		13.1	9.3		12.3	5.1		
Current smoking	19.2	20.2		19.4	15.0		13.9	16.0		21.4	15.3		
Drinking status (%)			0.010			0.003			<0.001			0.003	<0.001
Non-drinker	61.5	58.7		59.7	58.3		64.6	55.3		68.4	55.1		
Occasional drinker	10.6	25.0		11.9	31.3		13.9	30.8		13.2	33.7		

(Continued)

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TABLE 1 (Continued)

	Astrocytoma			Oligodendroglioma			Glioblastoma			Others			P^b
	Case	Control	P^a	Case	Control	P^a	Case	Control	P^a	Case	Control	P^a	
Frequent drinker	27.9	16.3		28.4	10.4		21.5	13.9		18.4	11.2		
History of allergies (%)			0.122			0.171			0.167			0.004	<0.001
Yes	7.7	14.4		7.5	14.9		8.0	11.8		7.1	21.4		
No	92.3	85.6		92.5	85.1		92.0	88.2		92.9	78.6		
History of head trauma (%)			0.675			0.069			0.451			0.345	0.474
Yes	13.5	11.5		13.4	4.5		9.3	11.4		12.2	8.2		
No	86.5	88.5		86.6	95.5		90.7	88.6		87.8	91.8		
Family history of cancer (%)			0.080			0.395			0.006			0.616	0.001
Yes	30.8	20.2		23.9	17.9		33.3	21.9		25.5	22.4		
No	69.2	79.8		76.1	82.1		66.7	78.1		74.5	77.6		
Physical activity, (%)			0.003			<0.001			<0.001			<0.001	<0.001
Low	16.3	35.6		13.4	50.7		14.3	47.7		9.2	49.0		
Moderate	40.4	37.5		44.8	40.3		41.8	35.4		38.8	34.7		
Violent	43.3	26.9		41.8	9.0		43.9	16.9		52.0	16.3		

[a] *P*-values were derived from Student's *t*-tests for continuous variables according to the data distribution and the chi-square test for the classified variables.

[b] Results of the overall case group and the overall control group.

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TABLE 2 Dietary minerals intakes of study participants.

Minerals		Q1	Q2	Q3	Q4	*P*-value
Ca (mg/d)	Case	233.07 ± 61.02	411.04 ± 52.37	573.68 ± 51.32	941.45 ± 290.89	<0.001
	Control	203.90 ± 79.60	406.71 ± 51.36	592.04 ± 55.34	1,028.21 ± 358.82	
Mg (mg/d)	Case	161.51 ± 35.32	241.55 ± 19.45	318.91 ± 31.33	502.50 ± 140.23	<0.001
	Control	145.72 ± 43.22	241.06 ± 20.13	331.08 ± 31.10	529.23 ± 135.73	
Fe (mg/d)	Case	9.30 ± 1.81	13.29 ± 1.11	17.27 ± 1.37	27.01 ± 8.74	<0.001
	Control	8.24 ± 2.45	13.11 ± 1.04	17.40 ± 1.35	27.00 ± 7.37	
Zn (mg/d)	Case	5.09 ± 1.09	7.39 ± 0.65	9.79 ± 0.68	14.58 ± 4.23	0.002
	Control	4.50 ± 1.33	7.54 ± 0.66	9.84 ± 0.72	15.07 ± 3.83	
Cu (mg/d)	Case	0.76 ± 0.17	1.14 ± 0.11	1.57 ± 0.17	2.77 ± 0.91	<0.001
	Control	0.66 ± 0.21	1.15 ± 0.11	1.62 ± 0.15	2.80 ± 0.87	

Q1, Q2, Q3, and Q4 represented the quartiles of mineral intake.

TABLE 3 Adjusted ORs and 95% CIs for the association between dietary minerals and glioma.

	T1	T2	T3	Continuous[c]	P_{-trend}
Ca	≤381.39	381.39–611.85	>611.85		
Case/control	211/127	187/157	108/222		
Model 1[a]	1	0.70 (0.51–0.97)	0.27 (0.19–0.39)	0.84 (0.80–0.88)	<0.001
Model 2[b]	1	0.46 (0.23–0.90)	0.11 (0.05–0.25)	0.65 (0.57–0.74)	<0.001
Mg	≤229.28	229.28–341.01	>341.01		
Case/control	191/147	197/140	118/219		
Model 1[a]	1	1.01 (0.74–1.38)	0.41 (0.30–0.57)	0.77 (0.70–0.84)	<0.001
Model 2[b]	1	0.45 (0.22–0.90)	0.06 (0.02–0.16)	0.18 (0.11–0.29)	<0.001
Fe	≤12.56	12.56–18.05	>18.05		
Case/control	184/154	200/137	122/215		
Model 1[a]	1	1.19 (0.87–1.63)	0.50 (0.37–0.68)	0.67 (0.57–0.80)	<0.001
Model 2[b]	1	0.35 (0.18–0.71)	0.07 (0.03–0.17)	0.04 (0.02–0.11)	<0.001
Zn	≤7.06	7.06–10.14	>10.14		
Case/control	191/147	176/161	139/198		
Model 1[a]	1	0.82 (0.61–1.11)	0.53 (0.39–0.73)	0.95 (0.93–0.98)	<0.001
Model 2[b]	1	0.32 (0.16–0.64)	0.07 (0.02–0.18)	0.62 (0.54–0.73)	<0.001
Cu	<1.08	1.08–1.67	>1.67		
Case/control	194/144	179/158	133/204		
Model 1[a]	1	0.83 (0.61–1.15)	0.49 (0.36–0.67)	0.76 (0.65–0.88)	<0.001
Model 2[b]	1	0.36 (0.18–0.71)	0.09 (0.04–0.22)	0.22 (0.13–0.39)	<0.001

T1, T2, and T3 represented the tertiles of mineral intake.
[a] Model 1: Unadjusted model.
[b] Model 2: Adjusted for age, BMI, occupation, education level, household income, high-risk residential areas, smoking status, drinking status, history of allergies, history of head trauma, family history of cancer, physical activity, PUFAs intake, and energy intake.
[c] Ca, Mg per 100 mg/d increment, Fe per 10 mg/d increment, Zn, Cu per 1 mg/d increment.

Association between dietary minerals and glioma

The results of the association between minerals with glioma are shown in Table 3. After adjustment for confounding variables (Model 2), the results for the mineral categorical variable showed that individuals with the highest Ca intake was associated with a 90% decreased risk of glioma compared with the first tertile (OR = 0.11, 95% CI: 0.05–0.25), individuals with the highest Mg intake was associated with a 95% decreased risk of glioma (OR = 0.06, 95% CI: 0.02–0.16), and individuals with the highest Fe intake was associated with a 93% decreased risk of glioma (OR = 0.07,

95% CI: 0.03–0.17), and individuals with the highest Zn intake was associated with an 89% decreased risk of glioma (OR = 0.07, 95% CI: 0.02–0.18), and individuals with the highest Cu intake was associated with an 87% decreased risk of glioma (OR = 0.09, 95% CI: 0.04–0.22).

The results of the analysis of the continuous variables showed that for each 100 mg/d increase in Ca intake, the risk of glioma decreased by 35% (OR = 0.65, 95% CI: 0.57–0.74), and for each 100 mg/d increase in Mg intake, the risk of glioma decreased by 82% (OR = 0.18, 95% CI: 0.11–0.29), and for each 10 mg/d increase in Fe intake, the risk of glioma decreased by 96% (OR = 0.04, 95% CI: 0.02–0.11), and for each 1 mg/d increase in Zn intake, the risk of glioma decreased by 38% (OR = 0.62, 95% CI: 0.54–0.73), and for each 1 mg/d increase in Zn intake, the risk of glioma decreased by 78% (OR = 0.22, 95% CI: 0.13–0.39).

Minerals and pathological classification and grade of glioma

The analysis of pathological classifications of glioma showed that all five minerals were associated with decreased significantly risks of glioblastoma. The results were consistent with those of the overall population of gliomas. But for astrocytoma, the results of Fe and Cu were significant. Due to the small sample size of oligodendroglioma, no further analysis was carried out (Table 4).

The results of minerals and different grades of gliomas showed that Mg, and Zn significantly were associated with a significantly decreased risk of low-grade gliomas. In contrast, the results of Ca, Fe, and Cu were not statistically significant. For high-grade gliomas, Ca, Mg, Fe, Zn, and Cu were associated with a significantly decreased risk (Table 5).

Subgroup analysis

In the subgroup analysis by age, sex, BMI, occupation, education level, household income, smoking status, history of allergies, family history of cancer, and physical activity, we observed that most of the results in the subgroup analysis were consistent with the main results. Very few subgroups had no significant results due to the small sample size (Supplementary Table 8).

Dose-response relationship

In Figure 1, we used restricted cubic splines to describe the relationship between minerals and the risk of glioma. There were linear dose-response relationships between the intakes of five minerals and the risk of glioma. For Ca, when the intake exceeded 398.02 mg/d, the risk of glioma decreased significantly with the increase in intake. When the intake exceeded 870.29 mg/d, the risk of glioma was relatively stable ($P_{-\text{nonlinearity}} = 0.6182$). For Mg, when the intake exceeded 151.29 mg/d, the risk of glioma decreased significantly with the increase in intake. When the intake exceeded 310.40 mg/d, the risk of glioma was relatively stable ($P_{-\text{nonlinearity}} = 0.5374$). For Fe, when the intake exceeded 8.80 mg/d, the risk of glioma decreased significantly with the increase in intake. When the intake exceeded 17.55 mg/d, the risk of glioma was relatively stable ($P_{-\text{nonlinearity}} = 0.0974$). For Zn, when the intake exceeded 7.46 mg/d, the risk of glioma decreased significantly with the increase in intake. When the intake exceeded 12.55 mg/d, the risk of glioma was relatively stable ($P_{-\text{nonlinearity}} = 0.2470$). For Cu, when the intake exceeded 1.28 mg/d, the risk of glioma decreased significantly with the increase in intake. When the intake exceeded 2.65 mg/d, the risk of glioma was relatively stable ($P_{-\text{nonlinearity}} = 0.0636$).

Discussion

Our study assessed the relationships between five common minerals and gliomas in the Chinese population. The results showed that the intakes of Ca, Mg, Fe, Zn, and Cu were significantly negatively associated with the risk of glioma. Similar results were observed in several subgroups, indicating that the association was relatively robust, especially in different pathological subtypes of gliomas and different grades of gliomas for the first time. It showed that this association was unlikely to be confused between different glioma subtypes. The restricted cubic spline model further confirmed a significant linear dose-response relationship between the five minerals and the risk of glioma, and with the increase in intake, the risk of glioma tended to be stable.

Ca is the most abundant mineral element in the human body, of which about 99% is concentrated in bones and teeth and plays a crucial role in bone mineralization and a wide range of biological functions (31). As an essential element for the human body, people can only get it from Ca-rich food sources, including milk and soybeans (31). Based on the physiological effects of Ca, studies showed that it was closely related to osteoporosis and cardiovascular disease, and cancer was no exception (32). In contrast, studies on dietary Ca and glioma were rare. Yekta et al. found a significant negative association between dietary Ca intakes in 128 glioma patients and 256 healthy individuals in a case-control study based on an Iranian hospital (OR = 0.23, 95% CI: 0.08–0.65) (17). Due to differences in dietary Ca sources between the Chinese population and the Middle East, although Ca intake in this study was higher than ours, our study found similar results. Higher dietary Ca intake was associated with a significantly decreased risk of glioma (OR = 0.11, 95% CI: 0.05–0.25) with a significant linear-dose-response relationship, which complemented the evidence for low Ca intake. For glioma subtypes, dietary Ca also had the same protective effect against glioblastoma (OR = 0.69, 95% CI: 0.53–0.89). This was similar to earlier results from the San Francisco Bay Area Adult Glioma Study. Tedeschi-Blok et al. compared dietary Ca intakes in 337 astrocytoma patients and 450 controls and found that dietary Ca intake was inversely associated with astrocytomas in a female-only population. Ca in our study population was inversely associated with astrocytoma, and the Ca intake of this population was closer to our study (18). In addition, a meta-analysis of the dose-response relationship showed that each 100 mg/d increase in Ca intake reduced the risk of glioma by 7% (OR = 0.93, 95% CI: 0.88–0.98), which was similar to our results. However, this meta-analysis included only four studies with high heterogeneity, and the results still needed to

TABLE 4 Adjusted ORs and 95% CIs for the association between dietary minerals and glioma of different pathological classifications.

Pathological classification[c]	Model 1[a]	*P*-value	Model 2[b]	*P*-value
Astrocytoma				
Ca	0.83 (0.75–0.92)	<0.001	0.01 (0.001–1.47)	0.072
Mg	0.76 (0.63–0.92)	0.004	0.002 (0.001–2.46)	0.086
Fe	0.62 (0.43–0.89)	0.011	–*	0.014
Zn	0.95 (0.89–1.01)	0.070	0.15 (0.01–1.67)	0.124
Cu	0.71 (0.52–0.98)	0.037	0.02 (0.001–0.43)	0.014
Glioblastoma				
Ca	0.85 (0.80–0.92)	<0.001	0.69 (0.53–0.89)	0.004
Mg	0.75 (0.65–0.87)	<0.001	0.14 (0.05–0.41)	<0.001
Fe	0.72 (0.57–0.92)	0.009	0.19 (0.05–0.64)	0.008
Zn	0.95 (0.91–0.99)	0.034	0.69 (0.53–0.88)	0.003
Cu	0.74 (0.59–0.92)	0.008	0.12 (0.03–0.47)	0.002

Due to the small sample size of oligodendroglioma, no further analysis was conducted.
[a]Model 1: Unadjusted model.
[b]Model 2: Adjusted for age, BMI, occupation, education level, household income, high-risk residential areas, smoking status, drinking status, history of allergies, history of head trauma, family history of cancer, physical activity, PUFAs intake, and energy intake.
[c]Ca, Mg per 100 mg/d increment, Fe per 10 mg/d increment, Zn, Cu per 1 mg/d increment.
*The lower limit of the confidence interval of the result was too small to be represented normally.

TABLE 5 Adjusted ORs and 95% CIs for the association between dietary minerals and glioma of different grades.

Glioma grading[c]	Model 1[a]	*P*-value	Model 2[b]	*P*-value
Low grade				
Ca	0.80 (0.70–0.90)	<0.001	0.02 (0.001–2.44)	0.111
Mg	0.77 (0.62–0.95)	0.014	0.01 (0.001–0.22)	0.006
Fe	0.58 (0.39–0.87)	0.008	–*	0.085
Zn	0.94 (0.88–1.01)	0.080	0.30 (0.12–0.77)	0.012
Cu	0.84 (0.61–1.14)	0.254	0.53 (0.13–2.22)	0.386
High grade				
Ca	0.86 (0.81–0.91)	<0.001	0.67 (0.55–0.81)	<0.001
Mg	0.78 (0.70–0.88)	<0.001	0.13 (0.06–0.30)	<0.001
Fe	0.74 (0.60–0.91)	0.003	0.09 (0.03–0.30)	<0.001
Zn	0.96 (0.93–0.99)	0.037	0.67 (0.55–0.82)	<0.001
Cu	0.79 (0.66–0.94)	0.009	0.12 (0.04–0.32)	<0.001

[a]Model 1: Unadjusted model.
[b]Model 2: Adjusted for age, BMI, occupation, education level, household income, high-risk residential areas, smoking status, drinking status, history of allergies, history of head trauma, family history of cancer, physical activity, PUFAs intake, and energy intake.
[c]Ca, Mg per 100 mg/d increment, Fe per 10 mg/d increment, Zn, Cu per 1 mg/d increment.
*The lower limit of the confidence interval of the result was too small to be represented normally.

be further confirmed in the future (33). Most of its mechanisms were currently considered to be related to the regulation of parathyroid hormone. Increased Ca levels in the body can reduce the release of parathyroid hormone (34), which was thought to play a promoting role in the development of cancer (35, 36). In gliomas, parathyroid hormone-related proteins were also found to regulate the transcriptional activation of glioma-related oncogenes (37), and immunohistochemical results showed that parathyroid hormone-related proteins were present in astrocytomas, suggesting that parathyroid hormone-related proteins may be related to the imbalance of growth or differentiation of astrocytoma cells (38). In addition, intracellular Ca and Ca signal pathways were closely related to gliomas (19). Elevated intracellular Ca^{2+} can activate nitric oxide synthase to generate nitric oxide, which impacted tumorigenesis (39), but it was difficult to directly link dietary Ca intake with Ca signaling channels.

Fe is involved in various metabolic processes in the body, including oxygen transport, DNA synthesis, and electron transport,

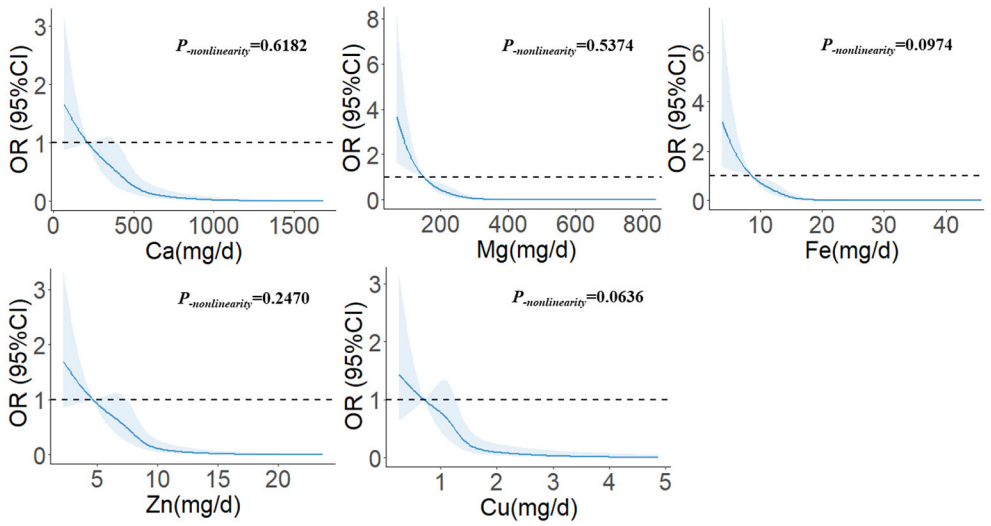

FIGURE 1
The restricted cubic spline for the associations between dietary minerals and glioma. The lines represent adjusted odds ratios based on restricted cubic splines for the intake in the regression model. Knots were placed at the 20th, 40th, 60th, and 80th percentiles of the dietary minerals intake, and the reference value was set at the 10th percentile. The adjusted factors were the same as in Model 2.

and is an essential element in almost all organisms (40). There are two main dietary Fe forms—heme Fe and non-heme Fe (41). Among them, heme Fe mainly comes from meat. In the western diet, heme Fe accounted for 10% of the total dietary Fe, but because the body more easily absorbed it, it accounted for nearly 2/3 of Fe absorption (42). Non-heme Fe exists mainly in plants. Nutritional disorders caused by Fe deficiency, such as anemia, infection, liver disease, and nervous system disease, have become public health issues of great concern (43). There have been many reports of dietary Fe and cancer in recent years. But studies on Fe and glioma were rare. Parent et al. found a non-significant association between Fe in occupational exposure and glioma in a population-based multicenter case-control study (OR = 1.10, 95% CI: 0.80–1.50). Still, the exposure route in this study was mainly the respiratory system (44). Ward et al. followed up for 14.1 years in the European Prospective Investigation into Cancer and Nutrition and found that total dietary Fe (HR = 0.94, 95% CI: 0.71–1.24) and heme Fe (HR = 0.96, 95% CI: 0.73–1.26) were not associated with the risk of glioma (20). This was not consistent with our results. We found that higher dietary Fe intake had a protective effect against gliomas (OR = 0.07, 95% CI: 0.03–0.17), and similar results were observed in astrocytomas and glioblastomas as well as in high-grade gliomas. In addition, because Fe was not only an essential element of the body but also potentially toxic to cells, it was significant to describe its dose-response relationship. We found a linear dose-response relationship between dietary Fe and glioma, and when the intake exceeded 17.55 mg/d, the risk of glioma did not change ($P_{-\text{nonlinearity}} = 0.0974$). Although dietary Fe has not shown a protective effect against gliomas in previous studies, studies on internal exposure seemed to support our results. Anic et al. determined the toenail Fe concentration by neutron activation analysis and found a significant negative association between toenail Fe and the risk of glioma (OR = 0.42, 95% CI: 0.19–0.95) (21). The relationship and mechanism between Fe and glioma were relatively complex. Bioinformatics studies have found that Fe metabolism-related genes can be used as prognostic indicators of low-grade gliomas (45). Some studies also found that Fe played an essential role in treating gliomas. In animal experiments, it was found that after intravenous injection of Fe complex into male nude mice with glioma, the tumor growth of nude mice was significantly inhibited after 3 weeks, and the possible mechanism was apoptosis (46). Eales et al. also found that verteporfin can radically and selectively kill anoxic glioma cells by binding free Fe (47).

Zn is the second most abundant transition metal ion in organisms, second only to Fe, and is indispensable to the growth and development of plants, animals, and microorganisms (48, 49). It can not only be used as a cofactor of more than 300 enzymes (50) but also play a key role in oxidative stress, immunity, and aging (49), and it has been reported that dietary Zn has protective effects against depression, type 2 diabetes and some cancers (51). Dietary Zn reduced the risk of cancer, mainly in the digestive system (52, 53). There have been few studies on Zn and gliomas. Dimitropoulou et al. observed only a slight protective effect of dietary Zn against meningioma (OR = 0.62, 95% CI: 0.39–0.99) in the study of adult brain tumors in the UK, but the association with glioma was not significant (OR = 0.92, 95% CI: 0.66–1.28) (23). However, in our study, higher dietary Zn intake significantly reduced the risk of glioma (OR = 0.07, 95% CI: 0.02–0.18), and there was a significant linear-dose response relationship. When the intake was 7.46–12.55 mg/d, the risk of glioma decreased with increased intake. The risk did not change beyond 12.55 mg/d. The mechanism of Zn involved in the development of glioma may be various. On the one hand, Zn, as a component of superoxide dismutase (54), had a strong antioxidant effect and played an essential role in oxidative stress and repairing DNA damage (49,

55). Along with the depletion of Zn in the body, this can lead to DNA damage and the production of free radicals, which can lead to the formation of tumors (56, 57). This was no exception in glioma (58). On the other hand, appropriate Zn can induce apoptosis of glioma cells. Haase et al. found in C6 rat glioma cells that Zn can promote proliferation and growth at a concentration of 50–100 μM, but too low (<50 μM) or too high (>200 μM) can induce apoptosis, especially when it exceeded 300 μM, it seemed to cause the necrosis of glioma cells (59). In addition, Zn may act as an epigenetic regulator of gliomas by promoting proper DNA folding, protecting genetic material from oxidative damage, and controlling the activation of enzymes involved in epigenetic regulation (60).

Mg is the fourth most abundant mineral in the body and the second most abundant intracellular divalent cation (61, 62). Whole grains, green vegetables, and nuts are rich dietary sources of Mg, but the loss of Mg during cooking and processing partly explains Mg deficiency (63). Because Mg is involved in many biological processes in the body, including energy production, glycolysis, oxidative phosphorylation, nucleic acid, and protein synthesis (62, 64), it was closely related to muscle health, asthma, cardiovascular disease, and mental illness (62). But the impact on cancer was currently inconsistent. In animal models, low Mg status was found to have a dual effect against tumors—inhibiting primary tumor growth and promoting metastatic tumor engraftment (65). However, epidemiological evidence still suggested that Mg deficiency may increase the risk of certain cancers. The results of an earlier meta-analysis showed that higher dietary Mg intake was associated with a significant reduction in overall cancer risk (RR = 0.80, 95% CI: 0.66–0.97) (66). Our study provided some evidence of the association between dietary Mg and gliomas, with higher dietary Mg having a protective effect against gliomas (OR = 0.06, 95% CI: 0.02–0.16), especially in low-grade gliomas. Mg has shown some anti-inflammatory effects in preclinical and epidemiological studies. Both dietary Mg intake and serum Mg levels were associated with increased levels of low-grade systemic inflammation, pro-inflammatory factors, and inflammatory markers (62, 67–69). This may create a microenvironment that was conducive to tumor invasion and metastasis (70). Since the presence of inflammatory cells and the release of inflammatory mediators also promoted glioma proliferation, angiogenesis, and attack, it seemed impossible to ignore the effect of Mg on gliomas (71). In addition, it may also be related to Mg transporter 1, which was highly selective for Mg transport (72). Recent studies have also found that the overexpression of Mg transporter 1 promoted the growth of glioma cells through the up-regulation of PD-L1 expression mediated by the ERK/MAPK signaling pathway (73), but whether dietary Mg was involved remains to be further explored.

Although Cu is also an essential trace element for the body, compared with the previous minerals, the demand for Cu is deficient, with only about 100 mg of Cu in the human body (74). Animal offal, corn products, certain vegetables, and individual fruits are good sources of dietary Cu (74). Since Cu was a cofactor for many oxidoreductases, it was involved in the body's antioxidant defense, neuropeptide synthesis, and immune function (75, 76). It also played an important role in fetal development (77), cardiovascular disease, and cognitive function (74). Studies on Cu and cancer were rare, and most studies have found no significant association between dietary Cu and some cancers. However, our study found that higher dietary Cu significantly reduced the risk of glioma (OR = 0.09, 95% CI: 0.04–0.22). Still, its effect was not as significant as that of the other four minerals. Moreover, dietary Cu seemed to have a protective effect only on high-grade gliomas (OR = 0.12, 95% CI: 0.04–0.32). No similar result was observed in low-grade gliomas (OR = 0.53, 95% CI: 0.13–2.22). Since both Cu deficiency and Cu excess were harmful to health, some scholars proposed that the dose-response curve between Cu and health was *U*-shaped (78). This was similar to the results of our study. Although there was a linear dose-response relationship between dietary Cu and glioma in this study, the risk leveled off when the intake exceeded a certain level. Due to the relatively narrow range of dietary Cu intake, we suspected that the current dose-response curve might be the left half of the *U*-shaped curve. In addition, some studies also found that Cu played a role in the treatment of glioma. *In vitro* studies, Trejo-Solis et al. found that Cu compounds induced autophagy and apoptosis of glioma cells by increasing the production of intracellular reactive oxygen species and the activity of c-junNH2-terminal kinase (79). Castillo-Rodriguez et al. found anti-proliferation, pro-apoptosis, and anti-invasion effects of Cu coordination compounds on U373 human glioma cells and significantly reduced tumor volume, cell proliferation, and mitotic index in mice transplanted with U373 glioma cells, and apoptosis index was increased (80).

The limitation of this study was that we could not explore the association between different forms and valence minerals in diet and glioma. Since the food composition table only provided the total amount of minerals without differentiating their form and valence, different forms and valence of minerals may have different effects on the body, we cannot analyze the results of the form and valence of these minerals on glioma in detail. Secondly, we only evaluated dietary sources for the relationship between minerals and glioma, and we could not comprehensively assess other sources, such as air. However, the sources of minerals were mainly dietary, so the results of this study were still of certain significance. In addition, because the study was a case-control study, we could not verify the causal relationship between the two and avoid inherent bias. In order to reduce the impact of information bias on this study, all questionnaires were completed face to face by investigators with medical education background. These investigators can participate in the survey only after receiving unified training and strict assessment before conducting the survey. In addition, in order to improve the accuracy of the dietary survey, the investigators assisted participants in estimating the amount of food in detail through food picture flip books containing different food volumes and qualities. However, the study still had some advantages. First, we explored the association between five common dietary minerals and gliomas. The results were consistent with existing *in-vitro* studies, especially for Cu and Mg, which lacked clinical studies and explored the association between gliomas of different pathological subtypes and pathological grades and minerals. Moreover, this was the first time that these dose-response relationships between dietary mineral intake and the risk of glioma were described, and the significant linear dose-response relationships provided further population evidence for mineral prevention and treatment of glioma.

Conclusion

In summary, we observe that higher intakes of Ca, Mg, Fe, Zn, and Cu were associated with a decreased risk of glioma. Therefore, we may not be able to ignore the influence of dietary minerals on glioma. In the future, further prospective studies should be conducted to verify their relationship.

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 Beijing Tiantan Hospital, Capital Medical University (No. KY2022-203-02). The patients/participants provided their written informed consent to participate in this study.

Author contributions

WL and WZ contributed to the conception or design of the work and wrote the manuscript. WZ, YH, XK, CW, and FC contributed to data collection and analysis. ZK, SY, RZ, and YP contributed to data collection and management. All authors have 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1118997/full#supplementary-material

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The I148M *PNPLA3* variant mitigates niacin beneficial effects: How the genetic screening in non-alcoholic fatty liver disease patients gains value

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Background: The PNPLA3 p.I148M impact on fat accumulation can be modulated by nutrients. Niacin (Vitamin B3) reduced triglycerides synthesis in *in vitro* and *in vivo* NAFLD models.

Objectives: In this study, we aimed to investigate the niacin-I148M polymorphism crosstalk in NAFLD patients and examine niacin's beneficial effect in reducing fat by exploiting hepatoma cells with different *PNPLA3* genotype.

Design: We enrolled 172 (Discovery cohort) and 358 (Validation cohort) patients with non-invasive and histological diagnosis of NAFLD, respectively. Dietary niacin was collected from food diary, while its serum levels were quantified by ELISA. Hepatic expression of genes related to NAD metabolism was evaluated by RNAseq in bariatric NAFLD patients (n = 183; Transcriptomic cohort). Hep3B (148I/I) and HepG2 (148M/M) cells were silenced (siHep3B) or overexpressed (HepG2^{I148+}) for *PNPLA3*, respectively.

Results: In the Discovery cohort, dietary niacin was significantly reduced in patients with steatosis $\geq$ 2 and in I148M carriers. Serum niacin was lower in subjects carrying the G at risk allele and negatively correlated with obesity. The latter result was confirmed in the Validation cohort. At multivariate analysis, the I148M polymorphism was independently associated with serum niacin, supporting that it may be directly involved in the modulation of its availability. siHep3B cells showed an impaired NAD biosynthesis comparable to HepG2 cells which led to lower niacin efficacy in clearing fat, supporting a required functional protein to guarantee its effectiveness. Conversely, the restoration of PNPLA3 Wt protein in HepG2^{I148+} cells recovered the NAD pathway and

improved niacin efficacy. Finally, niacin inhibited *de novo lipogenesis* through the ERK1/2/AMPK/SIRT1 pathway, with the consequent *SREBP1*-driven *PNPLA3* reduction only in Hep3B and HepG2^{I148M+} cells.

Conclusions: We demonstrated a niacin-PNPLA3 I148M interaction in NAFLD patients which possibly pave the way to vitamin B3 supplementation in those with a predisposing genetic background.

KEYWORDS

niacin, *PNPLA3* I148M, nutrigenomic, NAD, dietary supplementation

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the chronic liver disorder with the highest prevalence worldwide whose pathogenesis is mainly related to the presence of obesity and type 2 diabetes (T2D). NAFLD comprises a plethora of clinical conditions ranging from simple steatosis to necroinflammation and ballooning which together define non-alcoholic steatohepatitis (NASH) that in turn may evolve to fibrosis and then to cirrhosis and hepatocellular carcinoma (HCC).

The environment is not the only predisposing factor to NAFLD which has a strong hereditable component and we previously demonstrated that hepatic fat is the main driver of the progression to end-stage liver damages in genetically predisposed individuals (1). In the last decade, the rs738409 (p.I148M; c.C444G) missense variation in *Patatin-like phospholipase domain-containing 3* (*PNPLA3*) has been consistently associated with the entire spectrum of NAFLD in different populations-based studies (2–4). PNPLA3 is the strongest genetic predictor of NAFLD, showing a high prevalence among NASH patients of whom 34% carry the mutant allele in homozygous. Heterozygous CG carriers have showed a prevalence of NASH and fibrosis higher than CC patients but lower than those carrying the GG genotype. Moreover, the homozygosity for PNPLA3 risk allele has been associated with more than 2-fold greater risk to develop NASH and cirrhosis, with up to a 12-fold increased risk for HCC and with an 18-fold increase in liver-related mortality (5, 6), thus suggesting a dose-dependent allele risk.

PNPLA3 protein localizes on lipid droplets (LD) surface and has triacylglycerol hydrolase and acyltransferase activities, which promote LDs remodeling in hepatocytes and hepatic stellate cells (7). The 148M substitution leads to a reduced fatty acid hydrolysis yielding an impaired mobilization of triglycerides (TG) which accumulate in the liver (8, 9). It has been showed that the methionine-isoleucine substitution at residue 148 in the PNPLA3 protein does not impact on the orientation of the catalytic dyad. However, the longer side chain of methionine blocks the access of fatty acids to the catalytic site, thus hampering PNPLA3 hydrolytic activity (10). Kumari et al. demonstrated that the I148M polymorphism increases the hepatic lipogenic activity and TG synthesis (7). Consistently, in a murine model, the overexpression of the mutated PNPLA3 (I148M) in the liver increased fatty acids and TG formation, impaired TAG hydrolysis and weakened the remodeling of TAG long-chain polyunsaturated fatty acids (PUFAs) (11). Moreover, it has been demonstrated that I148M variant disrupts ubiquitylation and proteasomal degradation of PNPLA3 thus resulting in the accumulation of mutated protein on lipid droplets and impaired lipids mobilization (12). Finally, it has been shown that carbohydrates may induce the accumulation of mutant PNPLA3 on LD surface thus worsening hepatic fat content (13).

To date there are no approved pharmacological treatments for the management of NAFLD and the clinical recommendations rely on lifestyle changes including daily exercise and healthy diet. Several drugs (anti-inflammatory, anti-fibrotic agents, and metabolic modulators), partially improving NASH activity and fibrosis, are under investigations even though they have produced marginally positive results (14). Although advanced stages of NAFLD are irreversible, isolated hepatic steatosis and early NASH offer a "therapeutic window" for targeted interventions based on nutritional and lifestyle modifications. Indeed, effective and sustained weight loss has been associated with marked improvement in glycemic control, hepatic insulin sensitivity, transaminases and liver histology (15).

It has been demonstrated that the response to diet may differ accordingly to the individual genetic background (15). A crosstalk between *PNPLA3* rs738409 variant and nutrition has been already assessed. A nutrigenetic analysis revealed that the hepatic fat fraction in GG carriers is strongly influenced by carbohydrates and dietary sugar whose consumption may induce sterol-regulatory element binding protein-1C (SREBP1c) and, in turn, the expression of PNPLA3 mutated protein thus exacerbating fat deposition (16). In addition, hepatic fat accumulation can be modulated by the interaction between PNPLA3 I148M variant and dietary omega 6/omega 3 PUFAs and diet supplemented with *n*-3 respect to *n*-6 PUFA could provide a targeted therapy in NAFLD subjects who are homozygous for the *PNPLA3* G allele (17).

It has also been demonstrated that a higher intake of several micronutrients including niacin (nicotinic acid or vitamin B3) could modulate hepatic steatosis. Pharmacological doses of niacin have favorable effects on lipid parameters, increasing high-density lipoprotein cholesterol (HDL-C) and decreasing low-density lipoprotein cholesterol (LDL-C), TG and lipoprotein (a) (18). Niacin administration in Sprague–Dawley rats fed high-fat diet significantly reduced hepatic and serum TG, lipid peroxidation thus ameliorating steatosis (19). In HepG2 cells treated with palmitic acid (PA) to mimic steatosis, niacin supplementation reduced the expression of *acyl-CoA diacylglycerol acyltransferase 2 (DGAT2)* responsible for the committing step of TG synthesis, the production of ROS and inflammation by inhibiting interleukin-8 (IL-8) (20). Li et al. revealed that *DGAT2* inhibition reduced nuclear localization of *SREBP1*c, which is involved in *de novo* lipogenesis (DNL) and in the transcriptional regulation of *PNPLA3*, thus providing a possible mechanism through which niacin could ameliorate hepatic

fat accumulation (21). Additionally, niacin may improve steatosis by inhibiting DNL through the activation of ERK1/2/AMPK/SIRT1 pathway. SIRT1 is involved in the transcriptional regulation of *SREBP1c* paralleling the epigenetic regulation of *PNPLA3* gene and together with the NAD driven-AMPK activation downregulate DNL, suggesting a possible gene-nutrient interplay and providing another strategy across which niacin could exert its beneficial role (22, 23).

Consistently, patients with hypertriglyceridemia and treated with niacin (Niacin ER, trade name: Niaspan) showed a significant reduction of liver and visceral fat whereas the *DGAT2* rs3060 and rs101899116 variants were associated with a smaller decrease in liver fat content in response to niacin (24). Finally, Linder et al. evaluated whether dietary niacin intake predicts change of liver fat content during a lifestyle intervention. Among fat compartments, the hepatic one showed the largest decrease and about half of NAFLD patients reached a steatosis resolution thus suggesting that niacin-fortified foods may represent a valuable strategy to treat the disease, taking into consideration the individual genetic background (25).

Therefore, this study aimed to assess the dietary and circulating niacin levels in NAFLD patients stratified according to the presence of the I148M variant which represents the stronger genetic predictor related to steatosis whose impact on fat deposition may be modulated by nutrients. Moreover, we examined the efficacy of niacin in reducing fat accumulation by exploiting Hep3B and HepG2 cells, which are wild-type and homozygous for the I148M mutation, respectively.

Materials and methods

Discovery cohort

We enrolled patients affected by NAFLD (n = 172; Discovery cohort), of whom the presence of steatosis was non-invasively evaluated through ultrasound echography using a convex 3.5 MHz probe and by FibroScan®, at the Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milano. FibroScan® is a rapid and painless ultrasound (US)-based technique which emits low-frequency (50 mHz) vibrations into the liver, creating a propagating shear wave. This is detected by a pulse-echo acquisition that calculates its velocity which, in turn, is proportional to the stiffness of the tissue passed through. The Controlled attenuation parameter (CAP) value $\geq$ 248 estimated the presence of liver steatosis, whereas the liver stiffness measurement (LSM) value $\geq$ 7.0 and $\geq$ 6.2 kPa defined a significant liver fibrosis (26, 27). Patients were genotyped to assess the presence of the rs738409 C > G (p.I148M) *PNPLA3* variant as previously described (28–30), and the population was consistent with Hardy-Weinberg equilibrium (p = 0.13). Moreover, in order to assess dietary habits, we requested patients to carefully compile a food diary for 3 weeks. Kilocalories (kcal) of micro- and macro-nutrients were calculated with MètaDieta software[1] and are listed in **Supplementary Table 1**. Dietary products containing niacin are shown in **Supplementary Table 2**. Demographic, anthropometric and clinicopathological features of the Discovery cohort are shown in **Supplementary Table 3**.

Validation cohort

The Validation cohort includes 358 NAFLD unrelated patients of European descent who were consecutively enrolled at the Metabolic Liver Diseases outpatient service at Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico, Milan, Italy. Inclusion criteria were the availability of liver biopsies performed for suspected NASH or severe obesity, DNA samples, and clinical data. Individuals with excessive alcohol intake (men, > 30 g/day; women, > 20 g/day), viral and autoimmune hepatitis, or other causes of liver disease were excluded. Patients were stratified according to both the presence of the rs738409 C > G (p.I148M) *PNPLA3* variant and the population was not consistent with Hardy-Weinberg equilibrium (p = 0.03). The study conformed to the Declaration of Helsinki and was approved by the Institutional Review Board of the Fondazione Ca' Granda IRCCS of Milan and relevant Institutions. All participants gave written informed consent. Patients' genotyping and histological evaluation are presented in the **Supplementary materials and methods**. Demographic, anthropometric and clinicopathological features of the Discovery cohort are shown in **Supplementary Table 4**.

Transcriptomic cohort

RNA-seq was performed in a subset of 183 severely obese patients (31 without and 152 with NAFLD) in whom a percutaneous liver biopsy was performed during bariatric surgery at Fondazione IRCCS Cà Granda, Ospedale Policlinico, Milan, Italy (31). The study was conformed to the Declaration of Helsinki and approved by the Institutional Review Boards and their Ethics Committees. All participants gave written informed consent. Clinical characteristics of patients of whom RNA-seq data was available are presented in **Supplementary Table 5**. RNA-seq mapping descriptive statistics, detailed protocol, data analysis approach, patients' genotyping and histological assessment are described in the **Supplementary materials and methods**.

Measurement of circulating niacin

Niacin levels were evaluated in sera of both Discovery (n = 172) and Validation (n = 358) cohorts, collected at the time of NAFLD diagnosis. Niacin was quantified through the "ID-Vit Niacin" assay (Immunodiagnostik AG., Germany), based on a microbiological method which measures the total free niacin contained in the serum. The assay exploits microtiter plates covered with *Lactobacillus plantarum*, which uses niacin to grow. Serum samples were incubated at 37°C for 48 h and then turbidity was measured at λ = 610–630 nm with a spectrophotometer. The amount of niacin in the serum was directly proportional to the bacterial growth.

1 https://www.meteda.it/en/product/metadieta

In vitro models and treatments

To investigate the possible interaction between *PNPLA3* and niacin metabolism, we compared two human hepatoma cell lines with a different genetic background (Hep3B, HepG2) and commonly used to study liver metabolism *in vitro*. The Hep3B cells are wild-type for the *PNPLA3* gene, while the HepG2 cells carry the rs738409 C > G (I148M) *PNPLA3* polymorphism in homozygosity. Both cell lines were cultured in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/L penicillin, 100 U/L streptomycin and 1% L-glutamine (Life Technologies-ThermoFisher Scientific, Waltham, MA, USA) and maintained at 37°C and 5% CO_2. To mimic human steatosis, both cell types were exposed to PA at 0.25 mM, whereas to assess the niacin efficacy on fat accumulation in hepatocytes, they were supplemented with a mixture of PA and niacin (PA + NIA) at 0.5 mM for 24 hours. Treatments were freshly prepared and administered when appropriate.

RNA interference

Hep3B cells were transiently transfected for 48 h by pooling three different target-specific siRNA oligo duplexes directed against the exons 2, 4, and 5 of the human *PNPLA3* (siHep3B) in order to improve the gene-silencing efficiency and at a final concentration of 10 μM (MyBioSource, Inc., San Diego, CA, USA). Cyclophilin B (10 μM) was used as a scramble negative control (Horizon Discovery, Waterbeach, UK).

PNPLA3 llentiviral overexpression

PNPLA3 was stably overexpressed in HepG2 cells through pLenti-C-mGFP lentiviral vector which were engineered to express a complete Open Reading Frame (ORF) fused with green fluorescent protein (GFP) tag (henceforth HepG2$^{I148^+}$). We seeded 3×10^4 cells in 24-well plates and incubated at 37°C and 5% CO_2 overnight. Multiplicity of infection (MOI) was set at 2.5 and the amount of lentiviral particles for the transduction were calculated according to the manufactures'instruction (OriGene, Rockville, USA). Lentiviral particles were added to pre-warmed cultured media for 24 h. To introduce PNPLA3-GFP tagged protein, HepG2 cells were transduced with *PNPLA3* Human Tagged ORF Clone Lentiviral Particle (OriGene, Rockville, USA) containing a molecular sequence which aligns with the PNPLA3 mRNA (gene accession number: NM_025225).

Statistical analysis

For descriptive statistics, continuous variables were reported as mean and standard deviation or median and interquartile range for highly skewed biological variables. Variables with skewed distribution were logarithmically transformed before analysis. Differences between two groups were calculated with non-parametric Wilcoxon test, followed by *post hoc* pairwise comparison. One-way non-parametric ANOVA (Kruskal–Wallis) followed by *post hoc* Dunn's multiple comparison test was used to compare multiple groups adjusted for the number of comparisons. *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed using JMP 16.0 (SAS, Cary, NC) and Prism software (version 6, GraphPad Software).

Results

The *PNPLA3* I148M variant modulates both dietary and serum niacin levels in NAFLD patients and more so in the presence of obesity

Genetic variations may modulate the response to therapeutic approaches and vitamins in NAFLD patients. Among the latter, niacin has been reported to protect against severe steatosis although its interplay with genetics remains to be elucidated. The assessment of food diary in 172 patients with a non-invasive assessment of NAFLD revealed that niacin was the micronutrient with the lowest dietary levels in carriers of the PNPLA3 I148M variation ($p = 0.04$ CG/GG vs CC; **Supplementary Table 1**). Therefore, in the attempt to investigate the impact of the rs738409 C > G *PNPLA3* genotype on niacin metabolism, we assessed the vitamin absorption in the serum of NAFLD patients belonging to the Discovery cohort. Alimentary and circulating niacin levels were then correlated with clinical-pathological features of NAFLD subjects in order to evaluate a possible gene-environment interaction.

In the Discovery cohort, 114/172 patients (66.27%) had severe steatosis (grade 2-3) and 104/172 (60.46%) carried the *PNPLA3* p.I148M variant (**Supplementary Table 3**). At bivariate analysis, niacin intake was lower in NAFLD subjects with steatosis ≥ 2 compared to those with low-grade or no steatosis ($p < 0.05$ at Wilcoxon, adj $p = 0.02$ *vs* steatosis < 2, **Figure 1A**) and in *PNPLA3* CG/GG carriers ($p < 0.05$ at Wilcoxon, adj $p = 0.02$ vs *PNPLA3* CC, **Figure 1B**).

Similarly, serum niacin was reduced in individuals carrying the *PNPLA3* CG/GG mutation ($p < 0.05$ at Wilcoxon, adj $p = 0.03$ *vs* *PNPLA3* CC, **Figure 1C**) although no significant differences in its levels emerged among patients with steatosis < 2 and ≥ 2 (**Supplementary Figure 1A**), possibly suggesting that the presence of the rs738409 C > G *PNPLA3* genotype rather than hepatic fat accumulation may influence niacin absorption or metabolism. Moreover, at correlation analysis, serum niacin negatively associated with body mass index (BMI) ($p < 0.01$, **Figure 1D**) supporting the hypothesis that niacin availability may be even swayed by environmental risk factors as obesity.

Therefore, to deepen the impact of obesity on niacin metabolism, we stratified the Discovery cohort according to BMI ≥ 30 kg/m^2 and to the presence of the I148M variant. We found that dietary niacin showed a trend of reduction in patients carrying the *PNPLA3* at-risk G allele and BMI ≥ 30 kg/m^2 (**Figure 1E**) whereas by stratifying patients according to the presence of severe steatosis the lower levels of niacin in subjects with the *PNPLA3* CG/GG mutation didn't vary across BMI (**Figure 1F**). We next assessed circulating niacin and we observed that its serum levels tended to be lower in G allele carriers and

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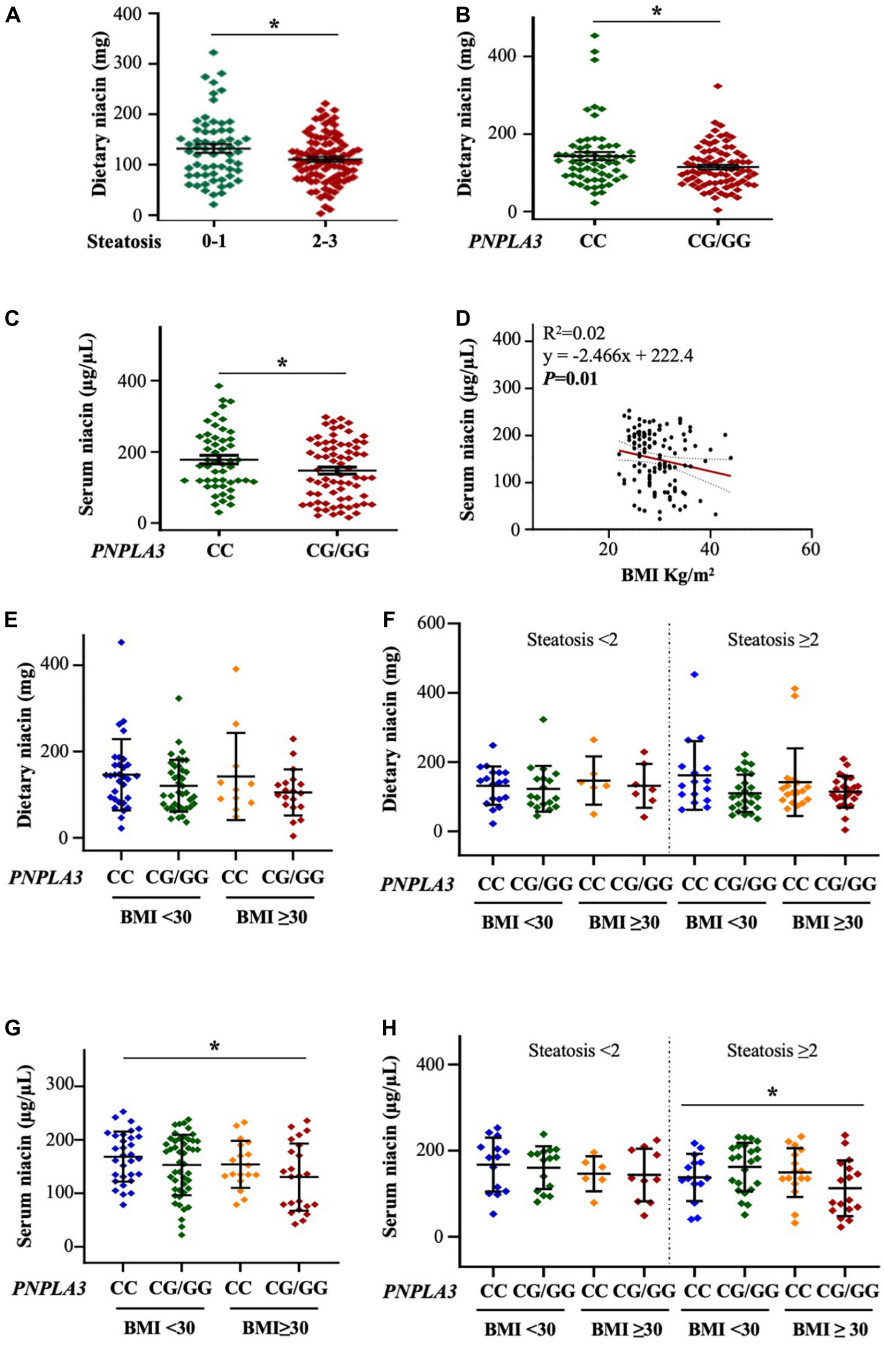

FIGURE 1
The PNPLA3 I148M variant affects alimentary and serum niacin levels in the discovery cohort. **(A,B)** Niacin intake was reduced in NAFLD patients with steatosis ≥ 2 and *PNPLA3* CG/GG mutation at bivariate analysis (*$p < 0.05$ at Wilcoxon test, *vs* steatosis < 2 and *vs PNPLA3* CC). **(C)** Circulating niacin levels were lower in PNPLA3 I148M carriers at bivariate analysis compared to non-carriers (*$p < 0.05$ at Wilcoxon test). **(D)** Negative correlation between serum niacin concentration and body mass index (BMI). **(E,F)** Bivariate analysis shows the trend of dietary niacin intake in NAFLD patients stratified according to both the *PNPLA3* G at-risk allele and obesity. In panel **(F)**, NAFLD patients were even subcategorized by the presence of steatosis < 2 and steatosis ≥ 2. **(G,H)** The *PNPLA3* at-risk genotype affected niacin absorption in a subgroup of obese NAFLD subjects and with steatosis ≥ 2 ($p = 0.005$ at ANOVA, *$p < 0.05$ *vs* BMI < 30 CC; $p = 0.04$ at ANOVA, *$p < 0.05$ *vs* steatosis ≥ 2 with BMI < 30 CC).

the reduction became significant in those with BMI ≥ 30 kg/m^2 ($p = 0.005$ at ANOVA, adj $p = 0.03$ *vs* BMI < 30 CC, Figure 1G). This effect was highly emphasized in the subcategory of patients with steatosis ≥ 2 ($p = 0.04$ at ANOVA, adj $p = 0.03$ *vs* steatosis ≥ 2 with BMI < 30 CC, Figure 1H), probably due to the additive weight of PNPLA3 I148M variant and obesity in niacin absorption.

At generalized linear model adjusted for sex, age, steatosis ≥ 2 and alimentary niacin, the presence of the *PNPLA3* CG/GG mutation was independently associated with serum niacin in NAFLD individuals with BMI ≥ 30 kg/m^2 (β = −0.21, 95% CI: −0.41–0.004, $p = 0.04$, Table 1), thereby supporting that PNPLA3 p.I148M aminoacidic substitution may be directly involved in

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TABLE 1 Association among serum niacin (μg/μL) levels and the *PNPLA3* rs738409 C > G (p.I148M) variant in the discovery cohort (n = 172), stratified according to BMI ≥ 30 kg/m^2.

	Circulating niacin (μ g/μ L)					
	BMI < 30			BMI ≥ 30		
	β	95% CI	*P*-value*	β	95% CI	*P*-value*
Sex, M	−0.02	−0.14 to 0.10	0.73	0.03	−0.19 to 0.26	0.74
Age, years	−0.006	−0.014 to 0.002	0.15	0.0002	−0.01 to 0.01	0.97
Steatosis ≥ 2, yes	−0.03	−0.12 to 0.07	0.57	−0.12	−0.31 to 0.07	0.21
Dietary niacin (kcal)	−0.12	−0.29 to 0.04	0.15	−0.19	−0.44 to 0.05	0.12
PNPLA3 G allele, yes	−0.03	−0.13 to 0.06	0.46	−0.21	−0.41 to 0.004	**0.04**

Analysis was performed in 172 NAFLD patients stratified according to body mass index (BMI) ≥ 30 kg/m^2, of whom the serum niacin dosage was available. Bold values were obtained at generalized linear model adjusted for sex, age, steatosis ≥ 2, dietary niacin and the presence of *PNPLA3* G allele. Both alimentary and circulating niacin levels were log-transformed. Serum niacin was considered as an independent variable. CI, confidence interval. *Statistically significant relationship between each predictor variable and the response variable in the linear regression analysis.

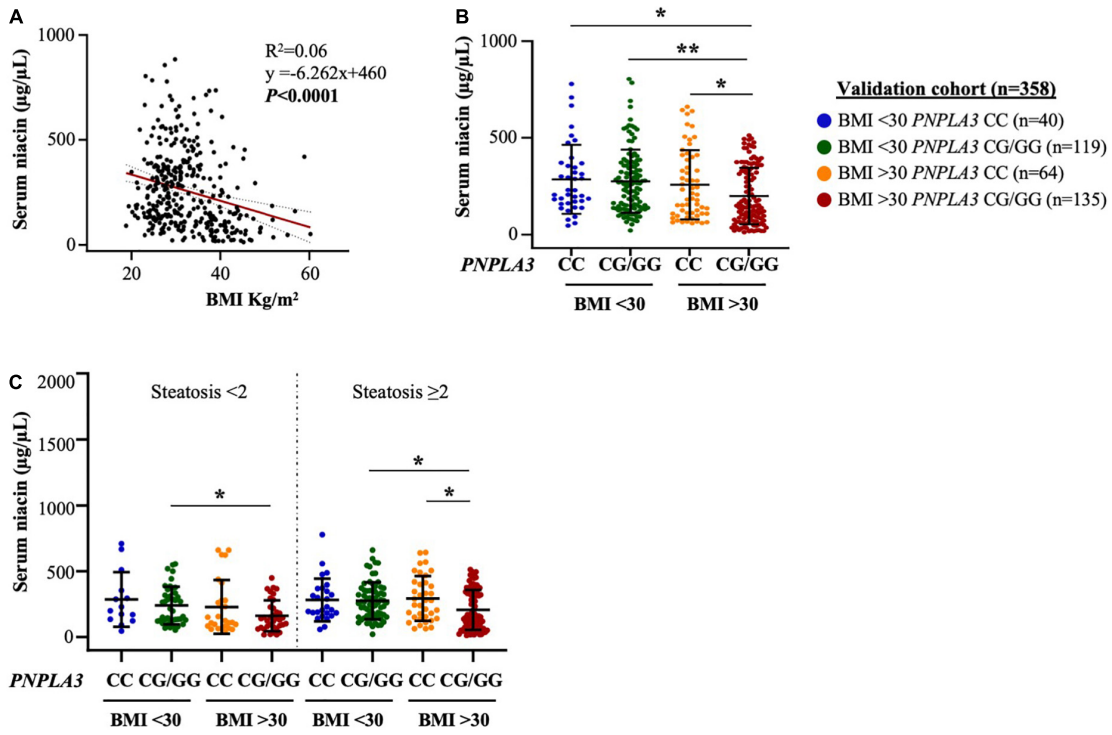

FIGURE 2
Obesity heightens PNPLA3 genetic risk on niacin absorption in the validation cohort. **(A)** Negative correlation between serum niacin concentration and body mass index (BMI). **(B,C)** The presence of the PNPLA3 I148M variant combined with obesity reduced niacin absorption in biopsied NAFLD subjects, independently of steatosis severity (p = 0.004 at ANOVA, *p < 0.05 *vs PNPLA3* CG/GG with/without obesity and *vs PNPLA3* CC with BMI ≥ 30). p = 0.0001 at ANOVA, **p < 0.01 *vs. PNPLA3* CG/GG without obesity.

the alteration of systemic niacin availability and that the effect attributable to this mutation may be amplified by adiposity.

The additive effect of the *PNPLA3* I148M mutation and obesity impacts on circulating niacin in patients with biopsy-proven NAFLD

To validate the results obtained in the Discovery cohort, we measured serum niacin in a larger cohort including n = 358 subjects with histological assessment of NAFLD, of whom dietary niacin was not available (Validation cohort). In this cohort, steatosis ≥ 2 was diagnosed in 232/358 patients (64.80%), whereas obesity (BMI ≥ 30 kg/m^2) and the PNPLA3 I148M variant were identified in 194/358 (54.18%) and 255/358 (71.2%), respectively (Supplementary Table 4). Circulating niacin concentration was inversely correlated with BMI (p < 0.0001, Figure 2A) and the lowest levels were observed in individuals affected by obesity and carrying the *PNPLA3* CG/GG mutation thus confirming the results obtained in the Discovery cohort (p = 0.0001 at ANOVA, adj p < 0.05 *vs PNPLA3* CC with either BMI < 30 or BMI ≥ 30; adj p < 0.01 *vs PNPLA3* CG/GG with BMI < 30, Figure 2B).

TABLE 2 Association between serum niacin (μg/μL) levels and the *PNPLA3* rs738409 C > G (p.I148M) variant in the validation cohort (n = 358), stratified according to BMI ≥ 30 kg/m^2.

	Circulating niacin (μg/μL)					
	BMI < 30			BMI ≥ 30		
	β	95% CI	*P*-value*	β	95% CI	*P*-value*
Sex, M	−0.02	−0.11 to 0.06	0.60	−0.02	−0.22 to 0.17	0.80
Age, years	0.0001	−0.006 to 0.007	0.96	0.0002	−0.01 to 0.015	0.97
Steatosis ≥ 2, yes	0.07	−0.009 to 0.16	0.08	−0.09	−0.26 to 0.08	0.29
PNPLA3 G allele, yes	−0.02	−0.11 to 0.06	0.56	−0.24	−0.43 to 0.06	**0.009**

Analysis was performed in 358 biopsied patients stratified according to BMI ≥ 30 kg/m^2, of whom the serum niacin dosage was available. Bold values were obtained at generalized linear model adjusted for sex, age, steatosis ≥ 2 and the *PNPLA3* G allele, yes. The circulating niacin was log-transformed and considered as an independent variable. CI, confidence interval. *Statistically significant relationship between each predictor variable and the response variable in the linear regression analysis.

Moreover, serum niacin levels were decreased in presence of both obesity and the *PNPLA3* G risk allele, thereby resembling what observed in the Discovery cohort although we didn't observe any variance between patients with steatosis < 2 and ≥ 2 (p = 0.004 at ANOVA, adj p < 0.05 *vs PNPLA3* CG/GG with/without obesity and *vs PNPLA3* CC with BMI ≥ 30, Figure 2C). In addition, no relevant differences were found in serum niacin levels by stratifying patients according to the severity of necroinflammation, fibrosis and NAS (Supplementary Figures 1B–D), suggesting that whole spectrum of NAFLD *per se* may not influence niacin absorption.

At multivariate analysis adjusted for sex, age and steatosis ≥ 2 the association between the *PNPLA3* I148M variant and lower serum niacin remained strongly significant in NAFLD individuals who belonged to the Validation cohort with BMI ≥ 30 kg/m^2 (β = −0.24, 95% CI: −0.43–0.06, p = 0.009, Table 2), thus corroborating the hypothesis that the *PNPLA3* p.I148M missense variation may be a genetic modifier of vitamin B3 metabolism.

The *PNPLA3* I148M variation modulates hepatic enzymes of NAD metabolism in NAFLD patients

The results obtained in the Discovery and Validation cohorts have suggested that niacin availability, the primary source of NAD synthesis, may be affected in patients with the *PNPLA3*-driven genetic predisposition to develop NAFLD and more so in obese ones. In order to evaluate whether the presence of the *PNPLA3* I148M variant may even interfere with hepatic NAD metabolism, we assessed the expression of genes involved in NAD biosynthetic pathways as well as NAD/NADH-dependent enzymes through RNA-seq analysis performed in 183 biopsied NAFLD patients (Transcriptomic cohort) who underwent bariatric surgery.

At bivariate analysis, the hepatic *nicotinate phosphoribosyl-transferase 1* (*NAPRT1*) mRNA levels, the main enzyme involved in niacin conversion into NAD precursors, were lower in carriers of the *PNPLA3* G allele compared to wild-type group (p < 0.01 at Wilcoxon, adj p = 0.0026 *vs PNPLA3* CC, Figure 3A). Conversely, the expression of *NAD synthetase 1* (*NADSYN1*) and *nicotinamide phosphoribosyl-transferase* (*NMNAT1*), alternatively producing NAD from tryptophan and the salvage pathway, was increased in I148M *PNPLA3* carriers compared to non-carriers (p < 0.05 at Wilcoxon, adj p = 0.01 and adj p = 0.03,*vs PNPLA3* CC, respectively, Figures 3B, C) possibly due to a compensatory mechanism to provide NAD in the liver.

Similarly to what observed in the Discovery and Validation cohorts stratified according to steatosis grade, the mRNA levels of *NAPRT1*, *NADSYN1* and *NMNAT1* were not affected by the severity of steatosis (p = 0.0004 at ANOVA, adj p = 0.003 and p = 0.02 *PNPLA3* CG/GG with steatosis < 2 and ≥ 2 *vs PNPLA3* CC with steatosis < 2 and ≥ 2, respectively; Figures 3D; P = 0.0001 at ANOVA, adj p = 0.004 and p = 0.03 *PNPLA3* CG/GG with steatosis < 2 and ≥ 2 *vs PNPLA3* CC with steatosis < 2 and ≥ 2, respectively Figures 3E, F), supporting that the *PNPLA3* genetic variant more than hepatic fat accumulation influences the niacin-dependent NAD metabolism.

Moreover, the hepatic mRNA levels of the NAD/NADH-dependent enzymes as the *malate dehydrogenase 1/2* (*MDH1/2*), the *isocitrate dehydrogenase [NAD] subunit beta* (IDH3B), the *pyruvate dehydrogenase E1 subunits alpha A1 and beta* (*PDHA1/B*) and the *oxoglutarate dehydrogenase* (*OGDH*) were significantly reduced in carriers of the *PNPLA3* G allele (adj p < 0.05 and adj p = 0.0001 at Wilcoxon *vs PNPLA3* CC genotype, Figures 3G–L), suggesting that the rs738409 C > G *PNPLA3* at-risk genotype may affect vitamin B3 metabolism by modulating the expression of NAD/NADH-consuming genes.

The *PNPLA3* loss-of-function impairs NAD metabolism in hepatoma cells

Evidence in NAFLD patients highlighted that niacin availability and the hepatic NAD biosynthesis are altered by the presence of the I148M polymorphism. Therefore, to explore the possible interaction between *PNPLA3* and niacin metabolism, we compared NAD biosynthetic rate in Hep3B and HepG2 cells, which carried the *PNPLA3* CC and GG genotype, respectively. Moreover, we induced the *PNPLA3* silencing in Hep3B cells (siHep3B) in order to evaluate whether its loss-of-function may impair NAD production. Finally, we overexpressed the *PNPLA3* Wt gene (HepG2$^{I148^+}$) in HepG2 cells attempting to elucidate whether the re-introduction of the *PNPLA3* Wt form may restore vitamin B3 efficacy in reducing fat accumulation.

As expected, *PNPLA3* mRNA and protein levels were reduced in siHep3B by around 50% (p < 0.05 and p < 0.01 *vs* scramble, Figure 4A), while they were significantly increased after the

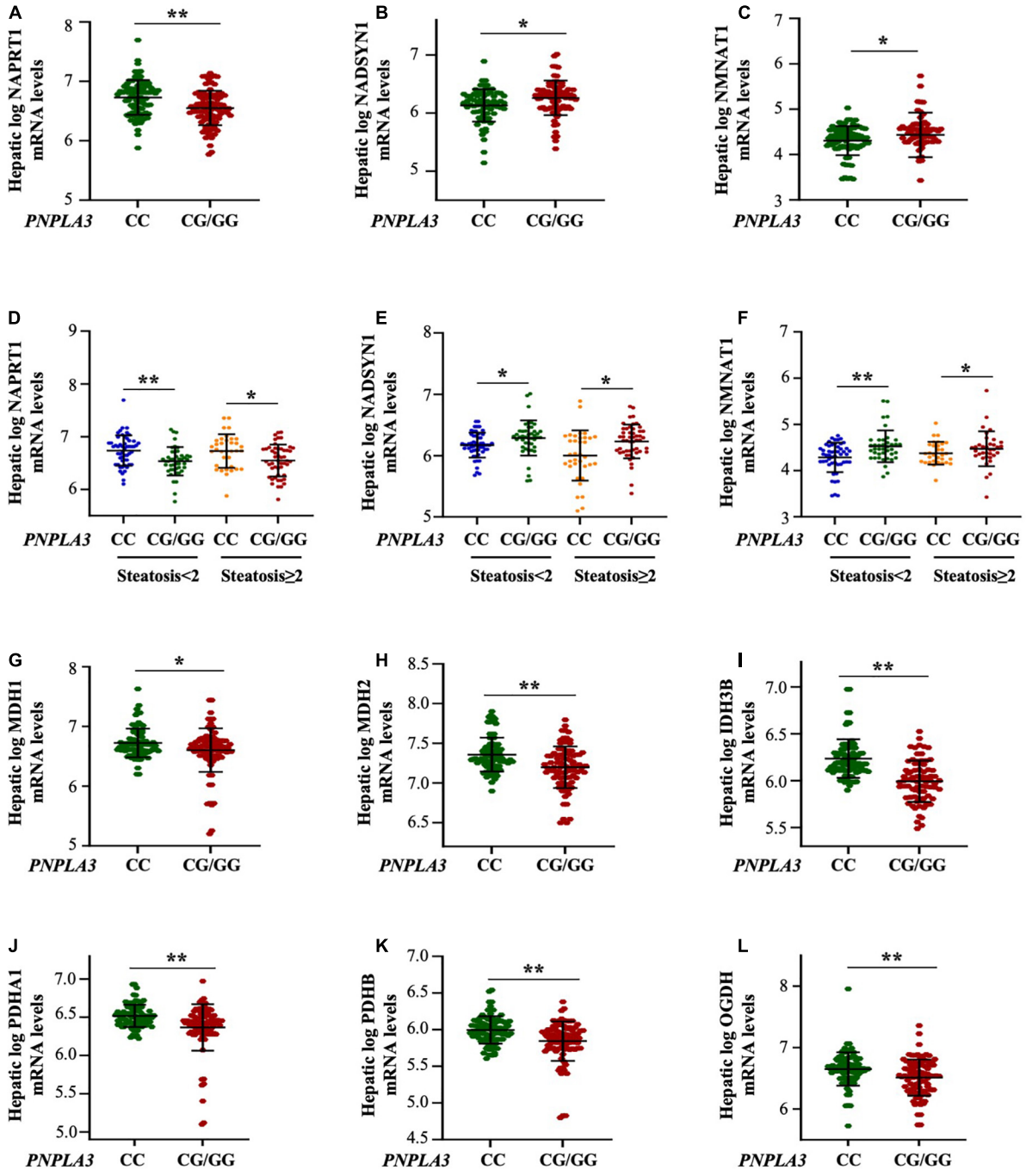

FIGURE 3
The PNPLA3 G allele impairs hepatic NAD metabolism in the transcriptomic cohort independently of steatosis severity. **(A–C)** Hepatic *NAPRT1* mRNA levels were reduced in the presence of the *PNPLA3* CG/GG mutation (**$p < 0.01$ at Wilcoxon test *vs PNPLA3* CC), while *NADSYN1* and *NMNAT1* expression were upregulated in *PNPLA3* CG/GG carriers (*$p < 0.05$ at Wilcoxon test *vs PNPLA3* CC). **(D–F)** Hepatic *NAPRT1, NADSYN1*, and *NMNAT1* expression were modulated in response to *PNPLA3* CG/GG mutation, but regardless of steatosis grade (NAPRT1: $p = 0.0004$ at ANOVA, **$p < 0.01$ *vs PNPLA3* CC with steatosis < 2; *$p < 0.05$ *vs PNPLA3* CC with steatosis ≥ 2; NADSYN: $p = 0.0001$ at ANOVA, *$p < 0.05$ vs *PNPLA3* CC with steatosis < 2 and ≥ 2; NMNAT1: $p = 0.0001$ at ANOVA, **$p < 0.01$ *PNPLA3* CC with steatosis < 2, *$p < 0.05$ *vs PNPLA3* CC with ≥ 2). **(G–L)** The mRNA levels of NAD-utilizing complexes (MDH1/2. IDH3B, PDHA1/B and OGDB) were decreased in presence of the *PNPLA3 G* at-risk allele (*$p < 0.05$ and **$p < 0.01$ at Wilcoxon *vs PNPLA3* CC).

lentiviral transduction in HepG2$^{I148^+}$ cells ($p < 0.01$ *vs* HepG2, Figure 4B).

At baseline, Hep3B cells showed higher NAD content compared to siHep3B ones ($p < 0.01$ *vs* siHep3B; Figure 4C), indicating that the *PNPLA3* deficiency may affect NAD production. Likewise, HepG2 cells exhibited lower NAD concentration, matching the levels measured in siHep3B cells, whereas in HepG2$^{I148^+}$ model it was comparable to Hep3B. After niacin exposure, NAD concentration increased in Hep3B cells but not in siHep3B ones ($p < 0.05$ *vs* Hep3B untreated, Figure 4C)

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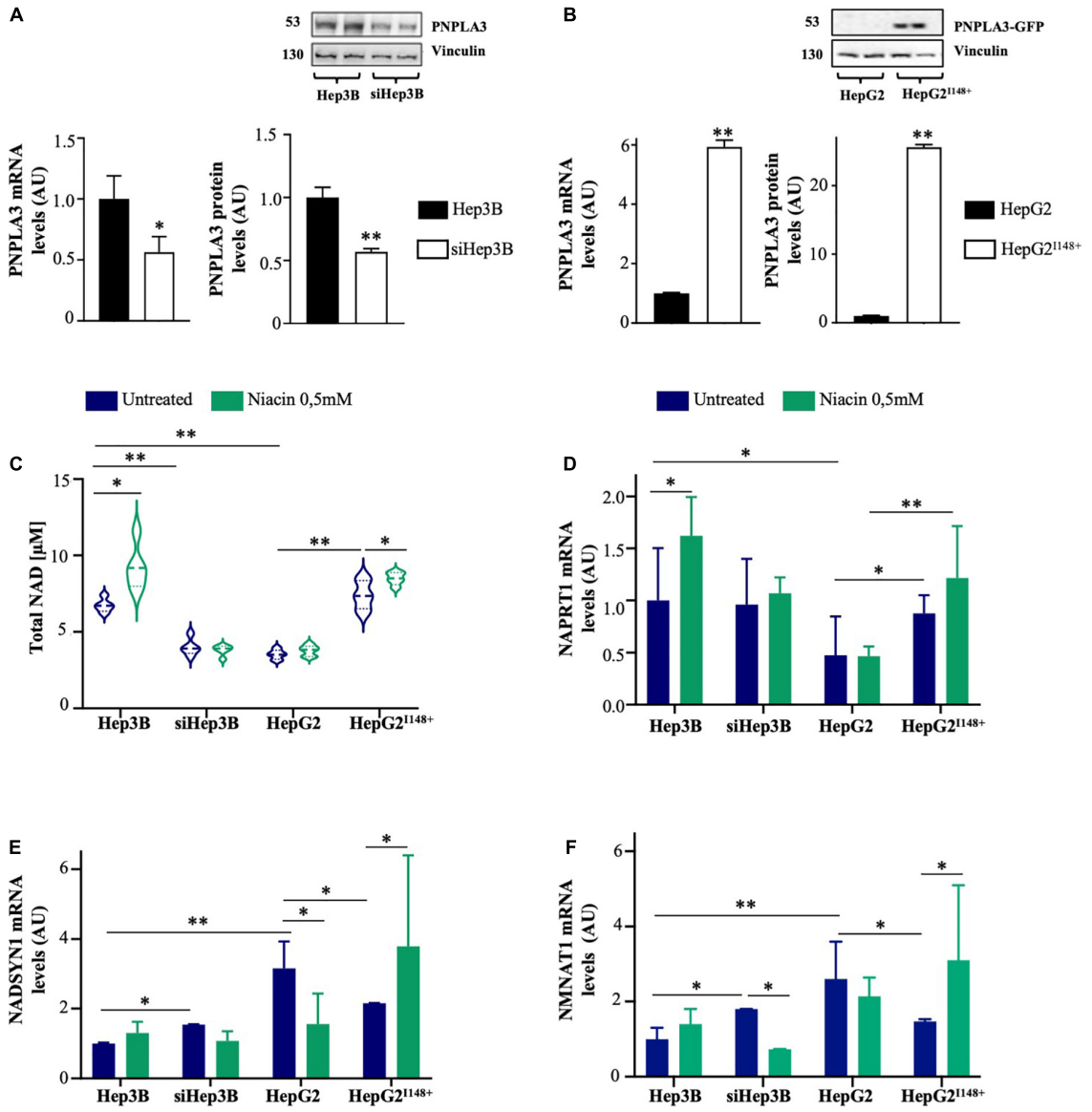

FIGURE 4
PNPLA3 loss-of-function dampens NAD synthesis in hepatocytes after niacin administration. **(A,B)** PNPLA3 mRNA and protein levels were assessed by qRT-PCR and Western blot, respectively, in hepatoma cells. **(C)** NAD content was assessed in Hep3B, siHep3B, HepG2 and HepG2^{I148+} cells through NAD/NADH Colorimetric/Fluorometric Assay Kit at both baseline and after niacin exposure. **(D–F)** *NAPRT1, NADSYN* and *NMNAT1* mRNA levels evaluated by qRT-PCR in hepatoma cells (Hep3B, siHep3B, HepG2 and HepG2^{I148+}) with or without niacin treatment. For gene expression, data were normalized to *ACTB* housekeeping gene and expressed as fold increase (Arbitrary Unit-AU) compared to control group. For Western blot, data were normalized to vinculin housekeeping protein and expressed as fold increase (AU) compared to control group. For violin plot, data were expressed as median concentration (thick dashed lines) and interquartile range (dotted lines). Adjusted $^{*}p < 0.05$ and $^{**}p < 0.01$.

supporting that *PNPLA3* silencing may modify the response to niacin supply. Furthermore, intracellular NAD content remain unchanged in HepG2 cells after niacin administration, showing a similar range of that observed in siHep3B ones, while it was increased in HepG2^{I148+} model ($p < 0.05$ *vs* HepG2^{I148+} untreated, Figure 4C).

Furthermore, in HepG2 cells the *NAPRT1* expression was reduced compared to both HepG2^{I148+} and Hep3B Wt models ($p < 0.01$ and $p < 0.05$ *vs* HepG2^{I148+}, $p < 0.01$ and $p < 0.05$ *vs* Hep3B; Figures 4C, D), thereby sustaining that the *PNPLA3* I148M variation may be involved in the impairment of the canonical NAD synthesis. Consistently with the increased NAD content upon niacin treatment, *NAPRT1* expression was induced in Hep3B cells ($p < 0.05$ *vs* Hep3B untreated, Figure 4D), whereas it was not modified in siHep3B ones. Similarly, HepG2 cells didn't show an increment of *NAPRT1* mRNA levels after niacin exposure, while its expression was slightly induced in the *PNPLA3* overexpressed cells, resembling the results obtained in Hep3B.

Moreover, both siHep3B and HepG2 cells showed higher basal expression of *NADSYN1* and *NMNAT1* than Hep3B and HepG2^{I148+} cells ($p < 0.01$ *vs* Hep3B; $p < 0.05$ *vs* HepG2^{I148+}; Figures 4E, F), possibly to compensate NAD shortage and corroborating the results obtained in the Transcriptomic cohort.

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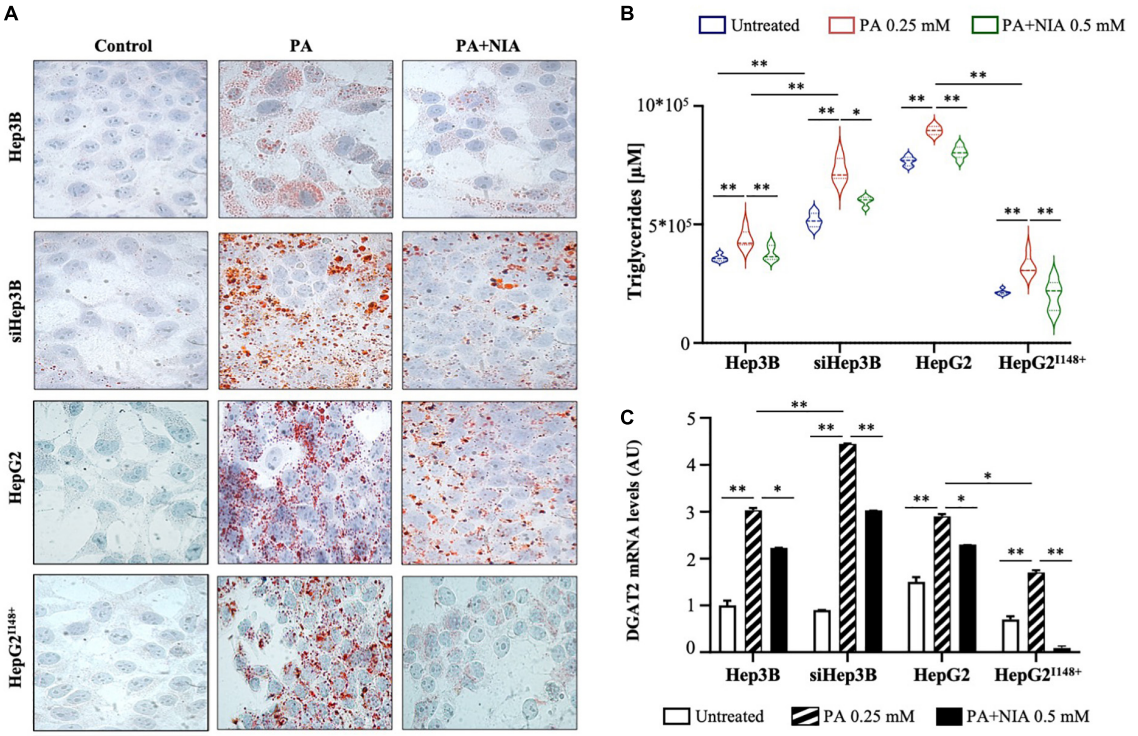

FIGURE 5

(A) Evaluation of LDs formation was assessed in hepatoma cells (Hep3B, siHep3B, HepG2, and HepG2^{I148+}) after PA challenge and NIA treatment by ORO staining (100× magnification). Nuclei were counterstained by hematoxylin. **(B)** TG content was measured in cell lysates (Hep3B, siHep3B, HepG2, and HepG2^{I148+}) through Triglycerides Colorimetric/Fluorometric Assay Kit. **(C)** *DGAT2* mRNA levels were quantified in hepatoma cells with or without niacin treatment after PA challenge (Hep3B, siHep3B, HepG2, and HepG2^{I148+}) by qRT-PCR. For gene expression, data were normalized to *ACTB* housekeeping gene and expressed as fold increase (Arbitrary Unit-AU) compared to control group. For violin plot, data were expressed as median concentration (thick dashed lines) and interquartile range (dotted lines). Adjusted $*p < 0.05$ and $**p < 0.01$.

As concern the alternative pathways of NAD biosynthesis, both Hep3B and HepG2^{I148+} displayed high *NADSYN1* and *NMNAT1* mRNA levels after niacin exposure ($p < 0.05$ *vs* HepG2^{I148+} untreated, Figures 4E, F). Contrariwise, siHep3B and HepG2 cells reduced the expression of *NADSYN1* and *NMNAT1* enzymes (*NADSYN1*: $p < 0.05$ *vs* HepG2 untreated, Figure 4E; *NMNAT1*: $p < 0.01$ *vs* siHep3B untreated, Figure 4F) probably due to a negative feedback loop exerted by niacin or its metabolites on the alternative pathways.

Thus, these findings may support that the presence of the *PNPLA3* loss-of-function induced by the silencing or the I148M variant may impair the canonical *via* of NAD biosynthesis at both baseline and after niacin supplementation. In support of this hypothesis, our *in vitro* results have suggested that the re-establishment of the PNPLA3 functional protein seems to rescue the vitamin B3 metabolism in hepatocytes, thereby sustaining the link between PNPLA3 and NAD availability.

The *PNPLA3* loss-of-function mitigates the beneficial role of niacin in reducing triglycerides synthesis

It has been previously demonstrated that niacin improves hepatic steatosis by downregulating *DGAT2* and reducing TG synthesis which, in turn, lead to a decreased oxidative stress (19, 20). In order to evaluate whether the presence of the I148M polymorphism may disturb niacin effectiveness on intracellular fat content, we treated hepatoma cells with PA alone or combined with NIA for 24 h.

At ORO staining, both Hep3B and HepG2^{I148+} cells accumulated less lipid droplets in response to PA administration rather than siHep3B and HepG2 cell lines, possibly due to the efficient PNPLA3 hydrolytic activity (Figure 5A). PA exposure enhanced the intracellular TG content and induced *DGAT2* upregulation in all experimental models ($p < 0.01$ *vs* Hep3B, siHep3B, HepG2 and HepG2^{I148+} untreated, Figures 5B, C). However, siHep3B and HepG2 cells exhibited an exacerbated lipid accumulation and *DGAT2* induction as a consequence of low lipid clearance induced by *PNPLA3* silencing or I148M variant, respectively ($p < 0.01$ and $p < 0.05$ *vs* Hep3B + PA or *vs* HepG2^{I148+} + PA, Figures 5B, C).

After niacin administration, lipid overload was reduced in all *in vitro* models, albeit this effectiveness was mitigated in siHep3B and HepG2 cells, supporting that the presence of a non-functional PNPLA3 protein may interfere with niacin protective role (Figure 5A). In keeping with the ORO staining, niacin administration strongly reduced the intracellular TG content ($p < 0.01$ *vs* Hep3B + PA, siHep3B + PA, HepG2 + PA and HepG2^{I148+} + PA, Figure 5B) and the mRNA levels of *DGAT2* in all cell lines ($p < 0.05$ *vs* Hep3B + PA and HepG2 + PA, $p < 0.01$ *vs* siHep3B and HepG2^{I148+} + PA, Figure 5C), showing

the greatest effect in Hep3B and HepG2$^{I148^+}$ models (Figures 5B, C) and sustaining that the presence of PNPLA3 in the WT form may improve the niacin efficacy on fat overload clearance.

Consistently with the worsened fat accumulation, siHep3B and HepG2 cells showed higher ER-oxidative injury compared to those induced by PA in Wt cellular models, by increasing the mRNA levels of *Activating Transcription Factor 4-6 (ATF4, ATF6)* and *Glucose-regulated protein 78 (GRP78)* and enhancing the production of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) ($p < 0.05$ and $p < 0.01$ *vs* Hep3B + PA and HepG2$^{I148^+}$ + PA; Supplementary Figures 2A–E).

Moreover, we found that niacin treatment strongly counteracted the negative effects of PA on *ATF4* ($p < 0.05$ *vs* PA, $p < 0.01$ *vs* PA, Supplementary Figure 2A), *ATF6* ($p < 0.05$ *vs* PA, $p < 0.01$ *vs* PA, Supplementary Figure 2B), *GRP78* expression ($p < 0.01$ vs PA, Supplementary Figure 2C) and oxidative injury (H_2O_2: $p < 0.05$ *vs* PA, $p < 0.01$ *vs* PA, Supplementary Figure 2D; MDA: $p < 0.05$ *vs* PA, $p < 0.01$ *vs* PA, Supplementary Figure 2E) in all the *in vitro* models. Although these findings have suggested that the effect of niacin in reducing TG synthesis may differ accordingly to the *PNPLA3* genetic background in our experimental models, we could speculate that the impact on hepatocellular toxicity may be more a consequence of the reduced fat accumulation rather than dependent by PNPLA3.

The *PNPLA3* loss-of-function promotes *de novo* lipogenesis by altering niacin-induced ERK1/2/AMPK/SIRT1 pathway

Few studies underlined that niacin could inhibit DNL through the activation of extracellular regulated kinase 1/2, AMP-activated protein kinase and sirtuin1 (ERK1/2/AMPK/SIRT1) pathway (20, 32). SIRT1, whose activity is dependent of NAD+ availability, is involved in the transcriptional regulation of *SREBP-1c* and in the epigenetic regulation of *PNPLA3* gene by nutritional factors (33). Therefore, we investigated whether niacin beneficial effects on DNL may be even affected by the loss of PNPLA3 hydrolytic activity.

In Hep3B and HepG2$^{I148^+}$ cells, niacin exposure promoted a marked phosphorylation of ERK1/2 (pERK1/2; $p < 0.05$ *vs* Hep3B + PA, $p < 0.01$ *vs* HepG2$^{I148^+}$ + PA, Figures 6A, B) and AMPK (pAMPK; $p < 0.01$ *vs* Hep3B + PA, $p < 0.01$ *vs* HepG2$^{I148^+}$ + PA, Figures 6A–C), while they were mildly activated in siHep3B and HepG2 models (Figures 6A–C). Consistently, *SIRT1* mRNA levels were upregulated in Hep3B and HepG2$^{I148^+}$ cells ($p < 0.05$ *vs* Hep3B + PA, $p < 0.05$ *vs* HepG2$^{I148^+}$ + PA, Figure 6D), whereas its expression was not significantly changed between siHep3B and HepG2 models at baseline and after treatment with PA or PA + NIA (Figure 6D).

Consequently to the induction of *SIRT1*, Hep3B and HepG2$^{I148^+}$ cells showed a huge downregulation of genes involved in DNL after niacin administration, as acetyl-CoA carboxylase (*ACC*, $p < 0.05$ *vs* Hep3B + PA, $p < 0.05$ *vs* HepG2$^{I148^+}$ + PA, Figure 6E), fatty acid synthase (*FASn*, $p < 0.05$ *vs* Hep3B + PA, $p < 0.05$ *vs* HepG2$^{I148^+}$ + PA, Figure 6F), and *SREBP1* ($p < 0.05$ *vs* Hep3B + PA, $p < 0.05$ *vs* HepG2$^{I148^+}$ + PA, Figure 6G). In contrast, siHep3B and HepG2 cells showed a mild or no reduction DNL-related genes after niacin exposure (Figures 6E–G), thereby strengthening the theory that niacin efficacy on lipid clearance may be impaired by a non-functional PNPLA3 protein.

In keeping with *SREBP1* downregulation, the mRNA levels of *PNPLA3* were reduced after niacin supplementation in Hep3B and HepG2$^{I148^+}$ models ($p < 0.01$ *vs* Hep3B + PA, $p < 0.01$ *vs* HepG2$^{I148^+}$ + PA, Figure 6H) but not in siHep3B and HepG2 ones, corroborating that the possible gene-nutrient interaction between *PNPLA3* and vitamin B3 occurs *via* the ERK1/2/AMPK/SIRT1 signaling and the subsequent *PNPLA3* transcriptional regulation mediated by SREBP1 (Figure 6I).

Discussion

Environmental risk factors, among which obesity, and genetic variations such as the I148M *PNPLA3* polymorphism strongly contribute to NAFLD pathogenesis. It has been established that PNPLA3 may be modulated by nutrients thus affecting the response to dietary interventions (34, 35). Niacin has been proposed for NAFLD management as it reduces TG synthesis thus improving steatosis in both mice and patients (19, 20). Therefore, we investigated the interplay between the I148M polymorphism and niacin absorption/metabolism in NAFLD patients and *in vitro* models.

We firstly examined food diary in 172 subjects with a non-invasive NAFLD diagnosis (Discovery cohort). We identified a cluster of micronutrients, mostly foods containing fibers and proteins, which appeared less ingested in I148M carriers, thereby offering the possibility to introduce them for a personalized nutritional intervention. Among them, niacin, enriched in fruits, vegetables, meat, and fish, resulted the least consumed. Accordingly, we found that serum niacin was lower in presence of I148M *PNPLA3* variation independently of dietary niacin, suggesting that it may be implied in niacin systemic availability, possibly affecting its absorption and metabolism.

Notably, serum niacin was inversely correlated with body weight and the lowest levels were observed in patients carrying the I148M variant with a BMI $\geq$ 30 kg/m^2 both in the Discovery and Validation cohorts, thus supporting a cumulative effect of obesity and the mutation in impairing niacin availability. A relation between niacin intake and BMI changes was previously described by Linder et al. who observed a higher niacin-dependent reduction of hepatic steatosis in NAFLD patients who lost weight during a period of diet counselling and physical activity (25). Consistently, an increased diet-related adiposity was associated with an amplified *PNPLA3* genetic risk of fatty liver. In particular, carbohydrates up-regulate mutant *PNPLA3* on lipid droplets surfaces, thus hindering their hydrolysis (34).

To deepen whether the I148M variant may influence niacin metabolism, we evaluated the hepatic NAD biosynthesis and we found that *NAPRT1*, involved in the canonical NAD pathway, was reduced in subjects with the *PNPLA3* G risk allele independently of steatosis grade, mirroring the low serum niacin levels observed in both Discovery and Validation cohorts. Conversely, the *NADSYN1* and *NMNAT1* mRNA levels, implicated in the alternative and recovery NAD signaling, respectively, increased in I148M carriers

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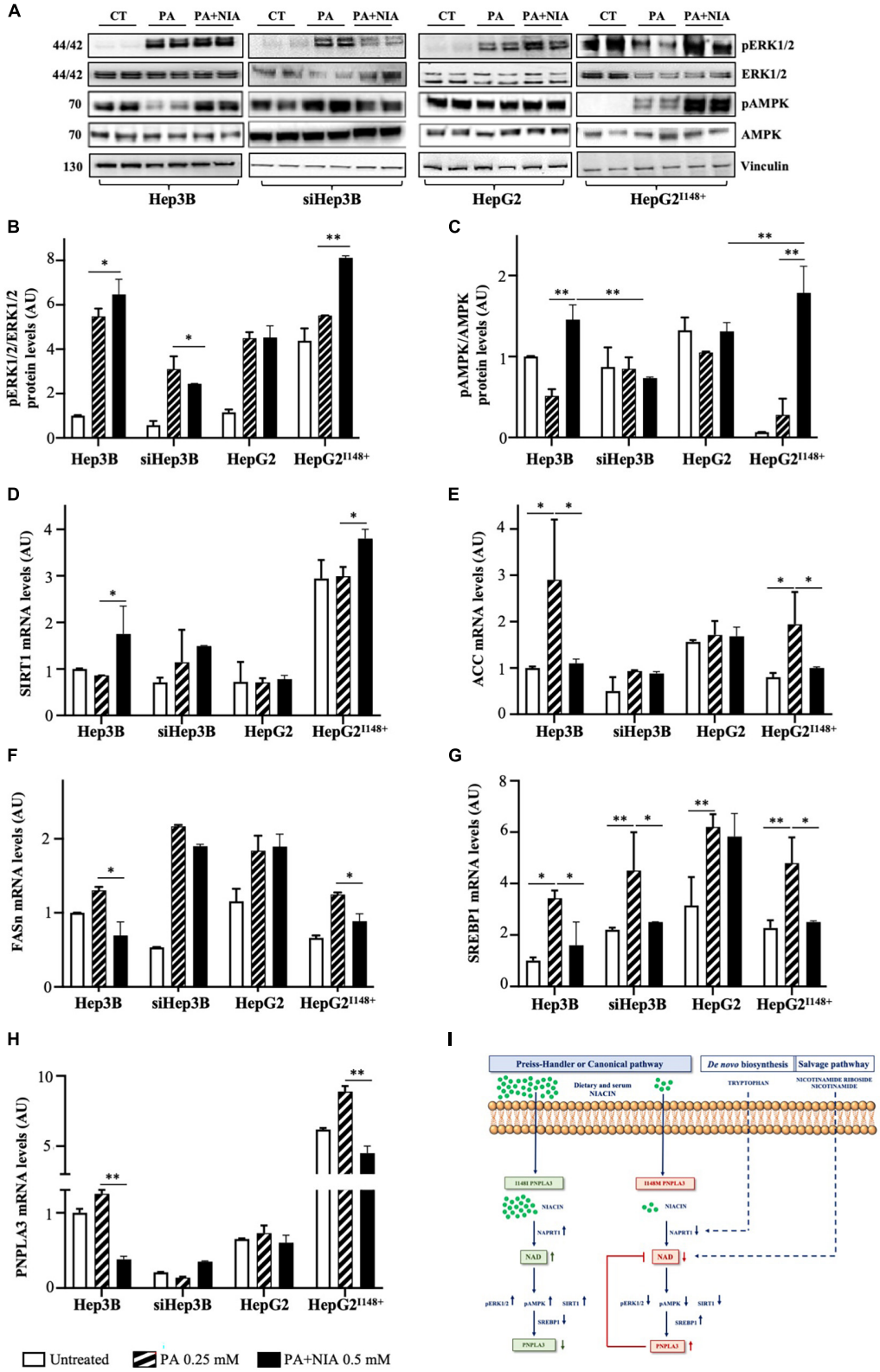

FIGURE 6
The PNPLA3-niacin crosstalk may occur via ERK1/2/AMPK/SIRT1 pathway. **(A–C)** Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204), p44/42 MAPK (ERK1/2), Phospho-AMPK and AMPK protein levels were evaluated by Western blot in hepatoma cells (Hep3B, siHep3B, HepG2 and HepG2^{I148+}) after PA challenge and niacin treatment. **(D–H)** *SIRT1*, *ACC*, *FASn*, *SREBP1* and *PNPLA3* mRNA levels were assessed in hepatoma cells by qRT-PCR. **(I)** Schematic figure of the putative interplay between the canonical pathway of NAD biosynthesis, which is dependent of niacin availability, and *PNPLA3* genotype. For gene expression, data were normalized to *ACTB* housekeeping gene and expressed as fold increase (Arbitrary Unit-AU) compared to control group. For Western blot, data were normalized to vinculin housekeeping protein and expressed as fold increase (AU) compared to control group. Adjusted $^{*}p < 0.05$ and $^{**}p < 0.01$.

as a possible compensatory mechanism to provide hepatic NAD. Alterations of NAD metabolism have been previously associated with NAFLD although no evidence is available regarding the role of genetics in this pathway. According to our findings, Penke et al. have shown that the increased NAD salvage pathway is involved in hepatic steatosis and supplementation with NAD precursors may aid to attenuate disease progression by promoting the NAD^+-dependent *Sirt1* activation thus highlighting the importance to maintain a sufficient hepatic NAD availability (23).

Our findings in NAFLD patients have suggested that the I148M variation may possibly influence niacin canonical turnover in the liver. To assess the mechanisms through which the PNPLA3 loss-of-function impacts on niacin metabolism, we exploited Hep3B (Wt) and HepG2 (I148M) cells, in which we silenced PNPLA3 or overexpressed the Wt protein, respectively. Hep3B cells exhibited higher levels of NAD which, as expected, increased after niacin exposure. Conversely, siHep3B and HepG2 cells showed lower NAD concentration compared to Hep3B cells and its content did not increase after niacin administration, suggesting an aberrant response to niacin supplementation in presence of the *PNPLA3* loss-of-function. The *PNPLA3* Wt overexpression in $HepG2^{I148^+}$ model restored both basal and niacin-induced NAD production, suggesting the requirement of a functional PNPLA3 protein to rescue the NAD synthesis. In keeping with the increased NAD levels, niacin exposure promoted the canonical *via* by upregulating *NAPRT1* mRNA in both Hep3B and $HepG2^{I148^+}$ cells, but not in siHep3B and HepG2 ones in which we observed an upregulation of the alternative pathways of NAD biosynthesis, thus resembling the transcriptomic data. In sum, our results have suggested that the PNPLA3 loss-of-function inhibits NAD production from niacin paralleled by the increase of NAD-related alternative pathways.

Niacin can reduce fat accumulation by downregulating TG synthesis *via* DGAT2 in cell cultures, rodents, and humans (24, 36, 37). DGAT2 inhibition was even associated with lower DNL due to less SREPB1-1c nuclear translocation and, consequently, transcriptional regulation of its lipogenic targets, among which *PNPLA3* (21). We found that niacin supplementation improved the intracellular fat content by targeting DGAT2 in all experimental models and it may be due to a combination of reduced TG synthesis and DNL. Such findings were consistent with those reported by Ganji et al. and Blond et al., who demonstrated that niacin treatment directly inhibits DGAT2 activity and reduced TG content in HepG2, HuH7 and primary hepatocytes, and with those of Li et al. who provided the link among DGAT2 inhibition and DNL (21, 24, 36, 37). Another study has pointed out that 39 patients with dyslipidemia improved lipid profile, exhibited lower visceral/subcutaneous fat and ameliorated hepatic fat content after niacin treatment (24, 38). Here, we showed that *PNPLA3* deficiency may affect niacin efficacy on TG synthesis as the siHep3B and HepG2 cells displayed less reduction of hepatocellular TG compared to Hep3B and $HepG2^{I148^+}$ models.

Furthermore, it has been demonstrated that niacin reverses oxidative stress and inflammation, in cells and animal models, possibly due to its effect in clearing lipids (38). We observed greater ER-oxidative stress in siHep3B and HepG2 cells than Hep3B and $HepG2^{I148^+}$ models after PA challenge, likely exacerbated by the presence of a non-functional PNPLA3 protein. Another mechanism by which niacin could reduce fat accumulation foresees the activation of the ERK1/2/AMPK/SIRT1 signaling (32, 39). Several studies revealed that phospho-AMPK and treatments with NAD^+ precursors promote *SIRT1* activation, which, in turn, decrease DNL (22, 23, 32, 39). Ye et al. have demonstrated that niacin treatment in obese HFD-fed mice hampered the transcriptional activity of *SREBP1* through ERK1/2/AMPK activation with the consequent reduction of hepatic and plasma TG content (40). We found that niacin promoted the ERK1/2 and AMPK phosphorylation paralleled by SIRT1 mRNA upregulation more in Hep3B and $HepG2^{I148^+}$ rather than siHep3B and HepG2 cells, suggesting that the PNPLA3 loss-of-function may reduce the inhibition of DNL induced by niacin. Consistently, niacin promoted a significant reduction of *SREBP1, ACC* and *FASn* expression in Hep3B and $HepG2^{I148^+}$ cells, but not in siHep3B and HepG2 models. Recently, it has been proven that the AMPK/SIRT1 may hamper the SREBP1 binding to PNPLA3 promoter (33) and as above mentioned SREBP1 is involved in the transcriptional regulation of PNPLA3 after carbohydrates loading (34). We found that *PNPLA3* mRNA levels were decreased in Hep3B and $HepG2^{I148^+}$ after niacin supplementation, and this effect may be ascribable to the low *SREBP1* expression. Conversely, niacin did not alter *PNPLA3* expression in HepG2 cells possibly suggesting that the crosstalk between PNPLA3 and niacin may occur through SREBP1.

To date, niacin was proposed at pharmacologic doses in the range of 1,500–2,000 mg (Niaspan®) for the treatment of dyslipidemia and prevention of cardiovascular complications (37). A clinical trial carried out in 39 hyper-triglyceridemic patients with steatosis has showed a reduction of liver fat by 47% and liver enzymes when treated with Niaspan for 6 months (37).

Lifestyle interventions have shown a great efficacy to improve hepatic steatosis and they currently represent a valid approach for NAFLD management. Preclinical studies have demonstrated that dietary intake of NAD precursors may ameliorate fatty liver by boosting the hepatic NAD metabolism. Accordingly, our study revealed a novel nutrigenetic regulation of the *PNPLA3* gene by niacin and underlined how the genetic screening which is useful in terms of costs and non-invasiveness gains value for a personalized approach in NAFLD patients. By looking at a translational perspective, either the nutritional supplementation with niacin or the increased consumption of niacin-fortified foods may represent an alternative option to overcome the low niacin levels observed in genetically predisposed NAFLD patients, even more with the co-occurrence of obesity.

Conclusions

The I148M *PNPLA3* variant, the strongest genetic predictor of NAFLD onset and progression, may undergo nutritional regulation and its deleterious effect could be worsened by environmental factors as obesity. Niacin, belonging to Vitamin B class, has been suggested for NAFLD management as it reduces hepatic fat content and inflammation. In this study, we highlighted a potential interaction between the presence of mutant PNPLA3 and niacin metabolism. Dietary evaluation through food diary pointed out that NAFLD subjects, among which around 60% carried the I148M variant and > 40% was affected by obesity, exhibited an unbalanced diet with low intake of vitamins, fibers, and proteins. In patients, our results have supported that the PNPLA3 CG/GG genotype

was associated with lower niacin availability in the serum and, at hepatic levels with altered expression of enzymes involved in NAD biosynthesis promoting more the alternative pathways than the canonical *via*. Even in hepatocytes, the presence of PNPLA3 loss-of-function limited the NAD production through the canonical pathway after niacin supplementation as well as it dampened niacin efficacy on fat accumulation and oxidative stress, thus sustaining the possible gene-nutrient crosstalk. In sum, vitamin B3 supplements or niacin-fortified foods should be recommended for NAFLD patients with a predisposing genetic background, amplified by adiposity.

Data availability statement

The ethical approval of the study does not allow to publicly share individual patients' genetic data. All data, code and materials used in the analysis are available upon reasonable request for collaborative studies regulated by materials/data transfer agreement (MTA/DTA) to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Fondazione IRCCS Cà Granda CE: 164_2019. The patients/participants provided their written informed consent to participate in this study.

Author contributions

EP and ML: study design, data analysis and interpretation, and manuscript drafting. MMe: data analysis and interpretation and manuscript drafting. GT: data analysis. AC, RL, SB, and MMa: patients recruitment and characterization. AF: discussion and manuscript revision. PD: study design, manuscript drafting, data analysis and interpretation, study funding, supervision, and has primary responsibility for final content. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1101341/full#supplementary-material

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Single and combined associations of blood lead and essential metals with serum lipid profiles in community-dwelling adults

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Background: Although several studies have examined the relationships between lead (Pb) exposure and serum lipid profiles, the associations of the metal mixture, including lead (Pb) and essential metals with lipid profiles, remain unclear.

Objective: To investigate the associations of the metal mixture including Pb and essential metals [magnesium (Mg), manganese (Mn), copper (Cu), iron (Fe), zinc (Zn), and calcium (Ca)] with serum lipid profiles [total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C)], as well as the potential interactions among the metals.

Methods: Nine hundred and ninety-eight Chinese community-dwelling adults completed a questionnaire and underwent checkups of anthropometric parameters, serum lipid profile levels (TC, TG, LDL-C, and HDL-C), and blood metal concentrations (Pb, Mg, Mn, Cu, Fe, Zn, and Ca). The multivariable linear regression, weighted quantile sum (WQS) regression, and Bayesian kernel machine regression (BKMR) were applied to evaluate the single and combined associations of blood Pb and essential metals with serum lipid profiles.

Results: In the multivariable linear regression model, the blood Pb was positively associated with TC, LDL-C, and HDL-C ($p<0.05$, all), and the blood Mg were positively associated with serum TC, LDL-C, and Ln TG ($p<0.05$, all). In the WQS regression and BKMR models, the metal mixture of blood Pb and the essential metals was positively associated with all of the serum lipid profiles. In addition, an inverse U-shaped association of Pb with Ln TG and the positive interactive effect between blood Pb and Mg levels on TC and LDL-C were found.

Conclusion: The levels of blood Pb, together with the essential metals, especially Mg levels, are suggested to be considered when assessing dyslipidemia risk. However, more evidence is still needed to validate the conclusions.

KEYWORDS

lead, magnesium, lipid profiles, metal mixture, Bayesian kernel machine regression

Introduction

Dyslipidemia is characterized by an imbalance of lipid profiles, including higher levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), or lower levels of high-density lipoprotein cholesterol (HDL-C), which has become a significant public health problem in global (1). The prevalence of high TC, high LDL-C, low HDL-C, and high TG in Chinese adults aged 35 to 75 was 7.1, 4.0, 15.6, and 16.9%, respectively (2). Moreover, ischemic heart disease and stroke are closely related to dyslipidemia, which accounts for over one-third of fatalities associated with these conditions (1). Thus, it is imperative to investigate the risk factors of dyslipidemia to prevent and treat it at an early stage.

In recent years, growing evidence has suggested that environmental metal exposures, including nonessential and essential metals, are associated with dyslipidemia (3, 4). Lead (Pb) is a nonessential toxic metal considered an environmental endocrine disruptor. Chinese are still exposed to low levels of Pb, despite government controls on Pb pollution (5, 6). Our previous studies have demonstrated that the blood Pb level was positively associated with fasting plasma glucose level, the prevalence of metabolic fatty liver disease, and diabetic complications (7–9). However, limited studies have investigated the associations between blood Pb and serum lipid profiles. In addition, the associations of essential metals with serum lipid profiles found in previous studies were inconsistent. For instance, a negative correlation was observed between serum magnesium (Mg) and lipid profiles (TC, LDL-C, and TG) among patients with diabetes (3). However, another study reported a positive association of plasma Mg with TC and LDL-C among Mediterranean adults (10).

Generally, humans are exposed to a variety of metals simultaneously. Mixtures of metals were considered to have different effects on human health than a single metal, since multiple metals may interact synergistically, antagonistically, or in other ways (11, 12). The association of metal exposure with serum lipids was not identical when different exposure profiles were considered (13). One cohort study demonstrated that the metal mixture, including Pb, aluminum, arsenic, barium, vanadium, and zinc (Zn), was associated with an increased risk of incident dyslipidemia (14). However, one recent cohort study with 573 manganese (Mn)-exposed workers did not find a significant cumulative effect of the metal mixture, including Pb and the other nine metals, on changes for TC, TG, or LDL-C (15). Currently, more and more Chinese supplement multivitamin tablets, which include various essential metals; however, the studies investigated the associations between a metal mixture including Pb and essential metals that are often tested clinically and the lipid profiles were limited. Moreover, the potential interactions of Pb and essential metals with lipid profiles have yet to be studied.

Thus, considering the detection frequency of essential metals in clinical work, we finally selected six essential metals in the current study, including Mg, Mn, copper (Cu), iron (Fe), Zn, and calcium (Ca). The current study aimed to examine the associations of a metal mixture including Pb and essential metals including Mg, Mn, Cu, Fe, Zn, and Ca with serum lipid profiles in Chinese community-dwelling adults. Furthermore, we also investigated the potential non-linear exposure-response relationship of Pb and essential metals with serum lipid profiles and the interactions among the metals.

Methods

Study design and population enrollment

Participants were enrolled from Lecong, Shunde District, Foshan, China, in 2021. The criteria for inclusion included being over 18 years old and not pregnant, as well as living in Shunde for at least 6 months. Among 1,111 potential participants, 4 were excluded for lack of blood metal data and 109 for taking lipid-lowering drugs. As a result, this study included 998 participants (Supplementary Figure 1).

The current study has been registered at[1] (ChiCTR2100054130). Based on the 1975 Helsinki Declaration, the Ethics Committee of Shunde Hospital of Southern Medical University approved the study protocol (20211103). Consent was obtained from all participants in an informed and written manner.

Measurements

A standard questionnaire was administered by trained study personnel to collect sociodemographic characteristics, lifestyle characteristics, and medication information from participants (16). We measured the height and weight of the subjects according to a standard protocol, and body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared (kg/m^2) (17). Blood pressure was measured by an automated electronic device (HEM-752 FUZZY, Omron, China) on the nondominant arm twice with at least a 1-min interval following a 5-min rest (18). We calculated the average systolic and diastolic blood pressure based on these two readings.

The fasting blood samples were collected from all participants from 08:00 to 10:00 after an overnight fast of at least 10 h. The whole blood samples were collected in vacuum tubes containing heparin sodium and used to measure blood Pb and the essential metal levels by a quadrupole inductively coupled plasma mass spectrometer (ICP-MS) equipped with a concentric glass nebulizer and a cyclonic spray chamber (7,700x ICP-MS system, Agilent Technologies, CA, USA). A total of 200 µL blood samples were diluted 1:20 (v/v) with a solution containing 0.1% triton X-100 (Sigma-Aldrich, France) and 0.1% nitric acid (69%, Merck, Germany). ICP-MS was used to quantify the diluted samples after vortexing them in a table-top vortexer for a minute. The detailed ICP-MS operating conditions and analysis are consistent with the previous study (19). The performances of the ICP-MS methods, including a low limit of detection (LoD), low limit of quantification (LoQ), accuracy, and precision, were provided in the supplementary materials.

Serum lipid profiles (including TC, TG, HDL-C, and LDL-C) and plasma glucose levels were conducted by BS800 (Mindray, Shenzhen, China). The blood samples for the fasting plasma glucose (FPG) and 2-h postprandial plasma glucose (PPG) levels after carrying out an oral 75 g glucose tolerance test were collected into vacuum tubes with the anticoagulant sodium fluoride. Among people with self-reported diabetes, only fasting plasma glucose and HbA1c were measured. Glycated hemoglobin (HbA1c) was assessed by high-performance

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liquid chromatography (HLC-723G8, TOSOH, Japan). All the samples were tested (including blood metal, serum lipid profiles, etc.) in Da-An Clinical Laboratory Center in Foshan, Guangdong, a certified laboratory by the College of American Pathologists, within 2 h under cold chain management. Moreover, all the testing items participated in the External Quality Assessment (EQA) of the National Center for Clinical Laboratories and achieved good results.

Outcome definitions

We categorized the ages into three categories (≤40, 41–60, and > 60 years) as the previous study (20). Education was categorized into three levels: below high school, high school, and beyond high school. Smoking status was classified as non-smokers (past consumption of fewer than 100 cigarettes), former smokers (over 100 cigarettes consumed in the past and more than six months without smoking), and current smokers (past consumption of over 100 cigarettes and smoking currently or within six months) (21). BMI categories were defined as obesity (BMI ≥28 kg/m^2), overweight (BMI ≥24, <28 kg/m^2), and normal weight (BMI <24 kg/m^2) according to Chinese criteria (22). Alcohol consumption was reported as standard drinks and converted to grams by multiplying by 14, and abused drink was defined as >30 g/day for men and > 20 g/day for women (23). The definition of hypertension was systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg, and/or self-reported previous diagnosis of hypertension by physicians (24). Diabetes was defined as FPG level ≥ 7.0 mmol/L, PPG ≥11.1 mmol/L, or HbA1c ≥6.5% and/or having a self-reported diagnosis of diabetes as the previous study (25). Higher TC (≥ 6.22 mmol/L), higher TG (≥ 2.26 mmol/L), higher LDL (≥ 4.14 mmol/L), and lower HDL (< 1.04 mmol/L) were defined as before (16).

Statistical analysis

The baseline characteristics of participants were summarized as mean ± standard deviation for continuous variables and frequencies for categorical variables (%) according to sex. Data of TG, blood Pb, and essential metal concentrations were transformed into a natural logarithm for further analysis.

Using Pearson's correlation analysis, the relationships between blood metal concentrations were classified as strong ($r > 0.8$), medium (> 0.3), and weak ($r \leq 0.3$) according to the correlation coefficients (r) (26). The multivariable linear regression, weighted quantile sum (WQS) regression, and Bayesian kernel machine regression (BKMR) models were used to identify the associations of the blood metals with the serum lipid profiles. To further validate the potential non-linear relationships of Pb and lipid profiles, restricted cubic spline (RCS) analysis with three knots was performed (at the 10th, 50th, and 90th percentiles).

The multivariable linear regression: blood metal levels were grouped as quartiles to assess potential linear or non-linear associations, with the lowest quartile as the reference group. The multivariable linear regression was performed to evaluate the associations of quartiles of Pb and essential metals with TC, LDL, HDL, and Ln TG, considering multiple-metal analysis (including all metals simultaneously). Age, sex, education, smoking status, abused drink, BMI categories, hypertension, and diabetes were adjusted in the full model of all the analyses. Covariates were tested for collinearity based on the variance inflation factor (VIF) < 5 (27).

WQS regression: We split the data into a training dataset and a testing dataset (40:60), with 1,000 boot-strap samplings in WQS, where variable weights were estimated on the training dataset, and mixture significance was determined on the testing dataset (28, 29). Metals with estimated weights greater than 0.143 (1/7) were considered to have a significant impact on the score of the WQS, which is calculated by dividing the sum of the absolute values of the weights by the total number of metals in the mixture (28, 29). Both positive and negative WQS scores were implemented and evaluated. The R package gWQS was used.

BKMR model: The BKMR model was used to identify the associations of the mixture of Pb and the essential metals on the serum lipid profiles. The posterior inclusion probabilities (PIPs) for each metal and the total influence of the metal mixture on the serum lipid profiles were calculated after fitting the final model using the Markov Chain Monte Carlo (MCMC) sampler for 50,000 iterations (30). A PIP threshold of 0.5 is typically used to determine if the investigated heavy metal is important (24, 31). We also displayed the dose–response curves for each metal and the potential interactions between the metals based on predicted serum lipid profiles when holding all other metals exposure at the median, 10th, or 90th percentile using the BKMR model as previously described (13). The R package bkmr was used.

All data were analyzed using IBM SPSS Statistics (version 27) and R (version 4.2.1). A two-tailed p value <0.05 was considered statistically significant.

Results

General characteristics of participants

Table 1 describes the basic characteristics of the participants. The mean age of the overall study population was 48.6 ± 14.13 years, and 42.7% were men. The prevalence of overweight or obesity, diabetes, and hypertension in the population was 43.1, 13.3, and 32.1%, respectively. The prevalence of higher TC, higher TG, higher LDL, and lower HDL was 21.3, 13.1, 14.9, and 9.4%, respectively. The mean level of blood Mg, Mn, Ca, Fe, Cu, Zn, and Pb was 41.66 mg/L, 13.13 μg/L, 62.91 mg/L, 505.86 mg/L, 884.65 μg/L, 6.32 mg/L, and 20.00 μg/L, respectively.

The associations of blood Pb and essential metal levels with serum lipid profiles using the multivariable linear regression

Using Pearson's correlation analysis, weak-to-moderate correlations between blood metals were observed (r: 0.02 to 0.70; Supplementary Figure 2). No significant collinearity of the covariates, including the metals, were found (all VIF < 3) (Supplementary Table 1).

Figure 1 presents the associations of metals with TC, Ln TG, LDL-C, and HDL-C using the multivariable linear regression model considering multiple-metal analysis. Compared with the lowest quartile, individuals in the highest quartile of Mg had the highest β

TABLE 1 General Characteristics of participants in the study.

	Total (*n*=998)	Men (*n*=426)	Women (*n*=572)
Age, years	48.59 ± 14.13	47.36 ± 14.19	49.50 ± 14.03
Education, %			
< high school	27.0	22.5	30.2
high school	35.0	35.2	34.8
> high school	38.1	42.3	35.0
BMI, %			
Normal weight	56.9	50.2	61.9
Overweight	31.1	33.3	29.4
Obese	12.0	16.4	8.7
Smoking status, %			
No	81.1	55.9	99.8
Ever	4.7	10.8	0.2
Current	14.2	33.3	0.0
Abuse drinking, %	1.7	4.0	0.0
Hypertension, %	32.1	38.7	27.1
Diabetes, %	13.3	13.4	13.3
TC, mmol/L	5.43 ± 1.11	5.43 ± 1.13	5.42 ± 1.10
HDL-C, mmol/L	1.42 ± 0.32	1.31 ± 0.31	1.49 ± 0.31
LDL-C, mmol/L	3.23 ± 0.92	3.37 ± 0.94	3.12 ± 0.89
TG, mmol/L	1.19 ± 0.87	1.37 ± 1.10	1.10 ± 0.67
FPG, mmol/L	5.02 ± 0.73	5.04 ± 0.78	5.02 ± 0.71
HbA1c, %	5.70 ± 0.60	5.70 ± 0.60	5.60 ± 0.67
Mg, mg/L	41.66 ± 4.49	43.26 ± 4.05	40.48 ± 4.43
Mn, μg/L	13.13 ± 4.02	12.24 ± 3.49	13.79 ± 4.25
Ca, mg/L	62.91 ± 5.75	60.45 ± 5.51	64.74 ± 5.22
Fe, mg/L	505.86 ± 57.43	542.94 ± 46.15	478.25 ± 48.82
Cu, μg/L	884.65 ± 121.29	837.70 ± 107.81	919.61 ± 119.03
Zn, mg/L	6.32 ± 0.92	6.67 ± 0.84	6.06 ± 0.88
Pb, μg/L	20.00 ± 12.00	23.00 ± 11.00	18.00 ± 11.00

The baseline characteristics of participants were summarized as mean ± standard deviation for continuous variables, and frequencies for categorical variables (%).
TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoproteins cholesterol; LDL-C, low-density lipoproteins cholesterol; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; BMI, body mass index; Mg, magnesium; Mn, manganese; Ca, calcium; Fe, iron; Cu, copper; Zn, zinc; Pb, lead.

for TC [0.523 (0.284, 0.762)], Ln TG [0.149 (0.034, 0.263)], and LDL-C [0.497 (0.303, 0.692)] ($p < 0.05$, all). Compared with the lowest quartile, individuals in the highest quartile of Fe had the highest β [0.080 (0.002, 0.158)] ($p < 0.05$) for HDL. However, individuals in the highest quartile of Cu had the lowest β [−0.071 (−0.132, −0.010)] ($p < 0.05$). Compared with the lowest quartile, individuals in the highest quartile of Pb had the highest β for TC [0.451 (0.234, 0.669)], LDL-C [0.328 (0.151, 0.505)], and HDL-C [0.087 (0.027, 0.147)] (all the $p < 0.05$), while individuals in the third quartile of Pb had the highest β [0.117 (0.021, 0.213)] ($p < 0.05$) for Ln TG. The interactions between Pb and Mg on TC (p for interaction = 0.068) and LDL-C (p for interaction = 0.046) were found.

TABLE 2 Associations of the mixture of blood Pb and the essential metals with serum lipids profiles.

	Positive WQS regression	*p*	Negative WQS regression	*p*
TC	0.394 (0.265, 0.523)	<0.001	0.023 (−0.108, 0.154)	0.728
Ln TG	0.080 (0.015, 0.145)	0.016	0.019 (−0.038, 0.076)	0.510
LDL-C	0.369 (0.256, 0.482)	<0.001	0.060 (−0.044, 0.164)	0.263
HDL-C	0.071 (0.034, 0.108)	<0.001	0.013 (−0.024, 0.050)	0.503

Data are presented as the WQS regression index (95% CI), representing the overall mixture effect on serum lipids profiles. The model was adjusted for age, sex, education, smoking status, abused drink, BMI categories, hypertension and diabetes.
TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; WQS, weighted quantile sum.

The associations of the mixture of blood Pb and essential metals with serum lipid profiles using the WQS model

Table 2 presents the associations of mixed metals on lipid profiles using the WQS model. The positive WQS indices of the metal mixture were associated with all the serum lipid profiles (all the $p < 0.05$), suggesting that the metal mixture was positively associated with lipid profiles. In the positive direction, blood Pb received the highest weights for TC (49.6%), LDL-C (38.6%), and HDL-C (51.3%), and blood Mg (32.1%) received the highest weights for Ln TG (Figure 2). In the negative direction, blood Ca received the highest weights for TC (54.6%), LDL-C (60.0%), Ln TG (64.9%), and blood Cu received the highest weights for HDL-C (43.2%) (Figure 2).

The associations of the mixture of blood Pb and the essential metals with serum lipid profiles using the BKMR model

In the BKMR model, as the metal mixture approached and exceeded the 55th percentile, the TC, Ln TG, LDL-C, and HDL-C increased significantly (Figure 3). When other elements were fixed at their 25th, 50th, or 75th percentile, the associations of Mg and Pb with TC and LDL-C, the association of Mg with Ln TG, and the association of Pb with HDL-C were significant (Figure 4). Figure 5 presents the bivariate Mg and Pb exposure-response relationships. The trend of the curves of Pb on TC and LDL-C intersected when Mg was at the 10th, 50th, or 90th percentile, which suggested the interactions between Pb and Mg on TC and LDL-C. No interactions among other metals were found on the lipid profiles. In addition, an inverse U-shaped association of Pb with Ln TG was found. The non-linear relationship between Pb and Ln TG was further validated using RCS analysis (p for overall = 0.030, p for non-linear = 0.024) (Supplementary Figure 3). Supplementary Table 2 shows the important metals on lipid profiles: on TC and Ln TG, Mg was considered the most important metal (both PIPs >0.9); on LDL-C, Mg and Pb were identified as the most two important metals (both PIPs >0.9); on HDL-C, Pb was recognized as the most important metal (PIP > 0.9).

Discussion

In the current study, we examined the associations of the mixture, including blood Pb and the essential metals, with serum lipids profiles

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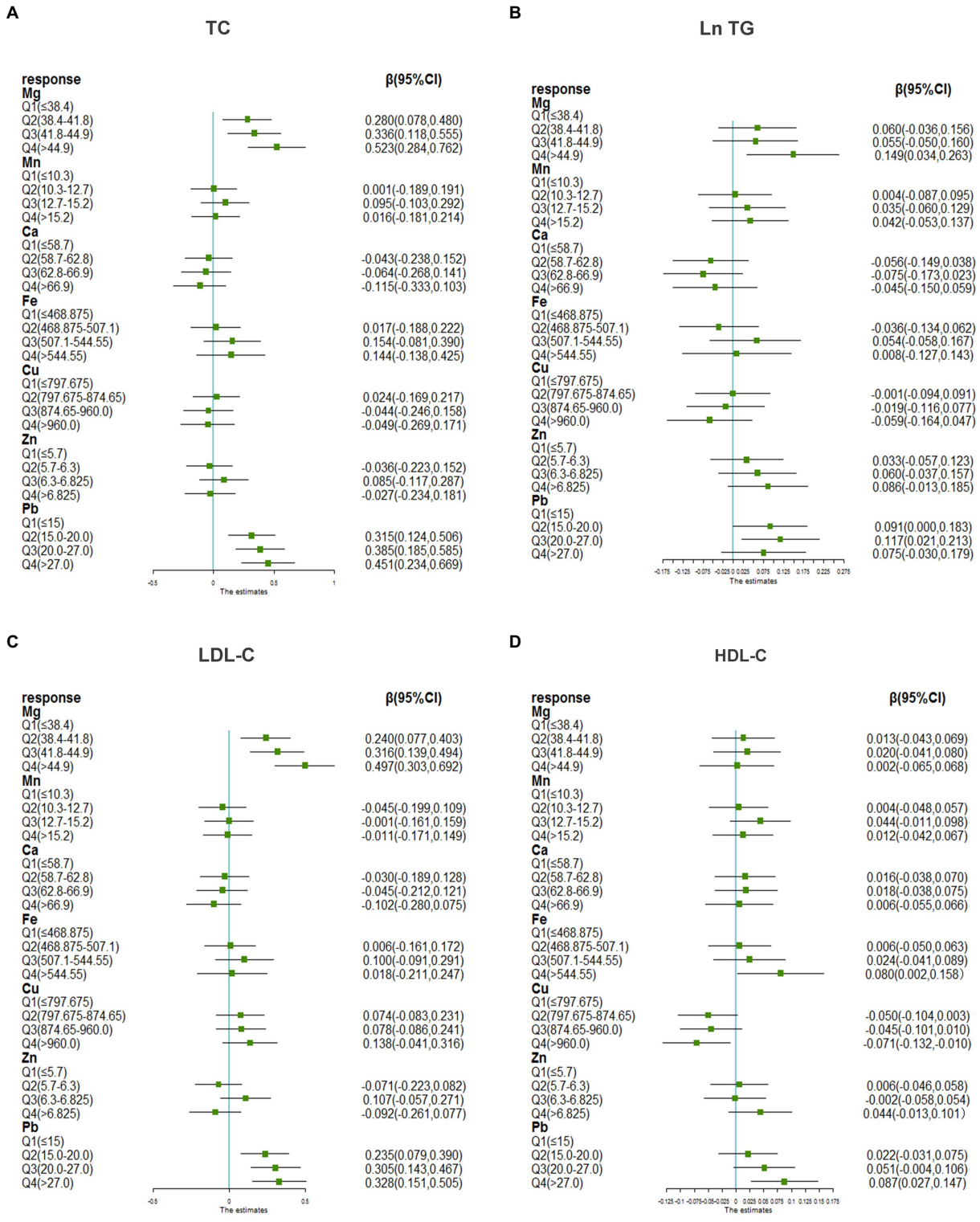

FIGURE 1
The associations of blood Pb and essential metals with serum lipid profiles using multivariable liner regression. **(A)** Metals and TC, **(B)** metals and Ln TG, **(C)** metals and LDL-C, **(D)** metals and HDL-C. TG was transformed to natural logarithm and metal concentrations were grouped into quartiles for further analysis. The model was adjusted for age, sex, educational, smoking status, abuse drinking, BMI categories, diabetes, and hypertension. TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoproteins cholesterol; HDL-C, high-density lipoproteins cholesterol; BMI, body mass index; Mg, magnesium; Mn, manganese; Ca, calcium; Fe, iron; Cu, copper; Zn, zinc; Pb, lead.

using a variety of statistical approaches. The linear regression pointed out that higher blood Pb levels were associated with higher serum TC, LDL-C, and HDL-C levels; higher blood Mg levels were associated with higher serum TC, LDL-C, and Ln TG levels. In addition, our

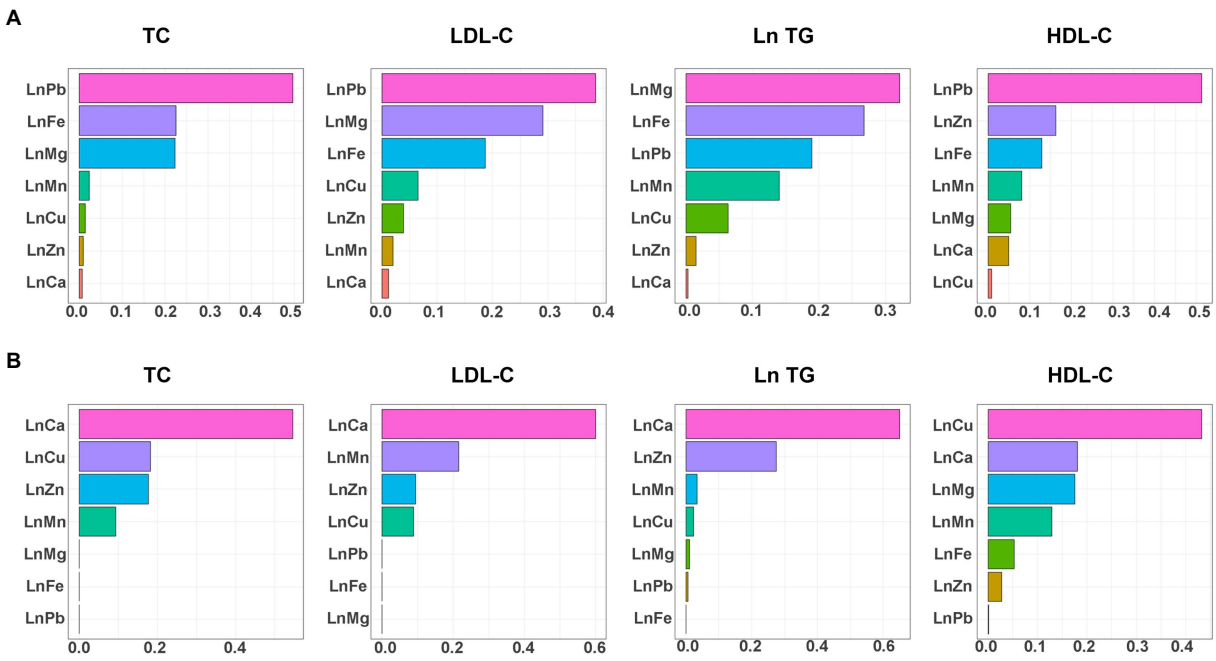

FIGURE 2
WQS regression weights of blood Pb and essential metals for serum lipid profiles. (A) The positive direction and (B) the negative direction. TG, blood Pb and essential metal concentrations were transformed to natural logarithm for further analysis. The model was adjusted for age, sex, educational, smoking status, abuse drinking, BMI categories, diabetes, and hypertension. TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoproteins cholesterol; HDL-C, high-density lipoproteins cholesterol; BMI, body mass index; Mg, magnesium; Mn, manganese; Ca, calcium; Fe, iron; Cu, copper; Zn, zinc; Pb, lead.

findings were mainly robust in BKMR and WQS regression. The metal mixture, including blood Pb and essential metals, was positively associated with the serum lipid profiles, including TC, Ln TG, LDL-C, and HDL-C. An inverse U-shaped association of Pb with Ln TG and the positive interaction between blood Pb and Mg levels on TC and LDL-C were also found. The findings implied that supplementation of multivitamin tablets should be cautious in people with low doses of Pb exposure, considering the increased risk of dyslipidemia, which could contribute to the prevention strategies for dyslipidemia.

Although most of the results in the current study seemed reliable and robust, the significantly important metals identified by these approaches are not exactly consistent. For example, blood Fe level is not significantly associated with LDL-C in the linear regression; however, WQS regression and BKMR suggested Fe was an important metal for increasing LDL-C. The inconsistency of the results can be attributed to the pros and cons of each approach. Multivariable linear regression is typically used, as its results are straightforward to interpret. However, combining metals with high correlation in the linear regression model is not recommended since it may distort the results (32). We conducted the linear regression considering multiple-metal analysis in the current study because the metals were only with weak-to-moderate correlations. By comparison, BKMR or WQS regression can include multiple metals with moderate to high correlations (30). However, mixture exposure burden and outcomes can only be examined in one direction per occasion using the WQS model (29). Although BKMR can capture the important metals in either direction or their non-linear exposure-response relationships, this method cannot determine co-exposure patterns of metals at both high and low concentrations (30). Thus, to estimate the single and combined associations of blood metals with the outcome, various statistical methods should be used, and the results interpreted together, weighing their advantages and disadvantages (30, 33).

The associations of Pb and lipid profiles determined in previous studies were inconsistent. One study (the mean of Pb is 28.07 μg/L) found that Pb levels were positively associated with the prevalence of dyslipidemia among 1,013 elders (34). Another study (the mean of Pb is 12.3 μg/L) enrolled general adults found that higher Pb was associated with elevated TC and LDL-C but not with TG (35). One recent study reported that elevated HDL-C is associated with lower blood Pb in Pb-exposed workers (the mean of Pb is 139.94 μg/L) (36). In the current study, we found that blood Pb, with relatively large weights in the metal mixture, was positively associated with TC, LDL-C, and HDL-C while non-linearly associated with Ln TG. The differences may be due to the different blood Pb levels of the participants in these studies and the non-linear relationship of Pb with TG. In animal experiments, similar conclusions were given (37, 38). Mouse exposed to Pb (5 mg/kg body weight) for 30 days orally caused higher TC, LDL-C, HDL-C, and lower TG (37). The inverse U-shaped association of Pb with TG was also observed in the Wistar rats (38).

In previous studies, conflicting results have been found regarding Mg and lipid profiles (39). One study with 492 participants reported a significantly positive association of Mg with TC and LDL-C (10), which is consistent with our results. The positive associations of Mg with TC, LDL-C, and TG were also found in two earlier studies (40, 41). However, in two other studies enrolling patients with diabetes or chronic kidney disease, negative associations of Mg with TC, LDL-C, and TG were found (3, 42). Furthermore, studies about the efficacy of Mg supplementation on decreasing serum lipids are conflicting (39). One earlier meta-analysis study suggested no significant effects of Mg supplementation on the lipid profiles among individuals with or

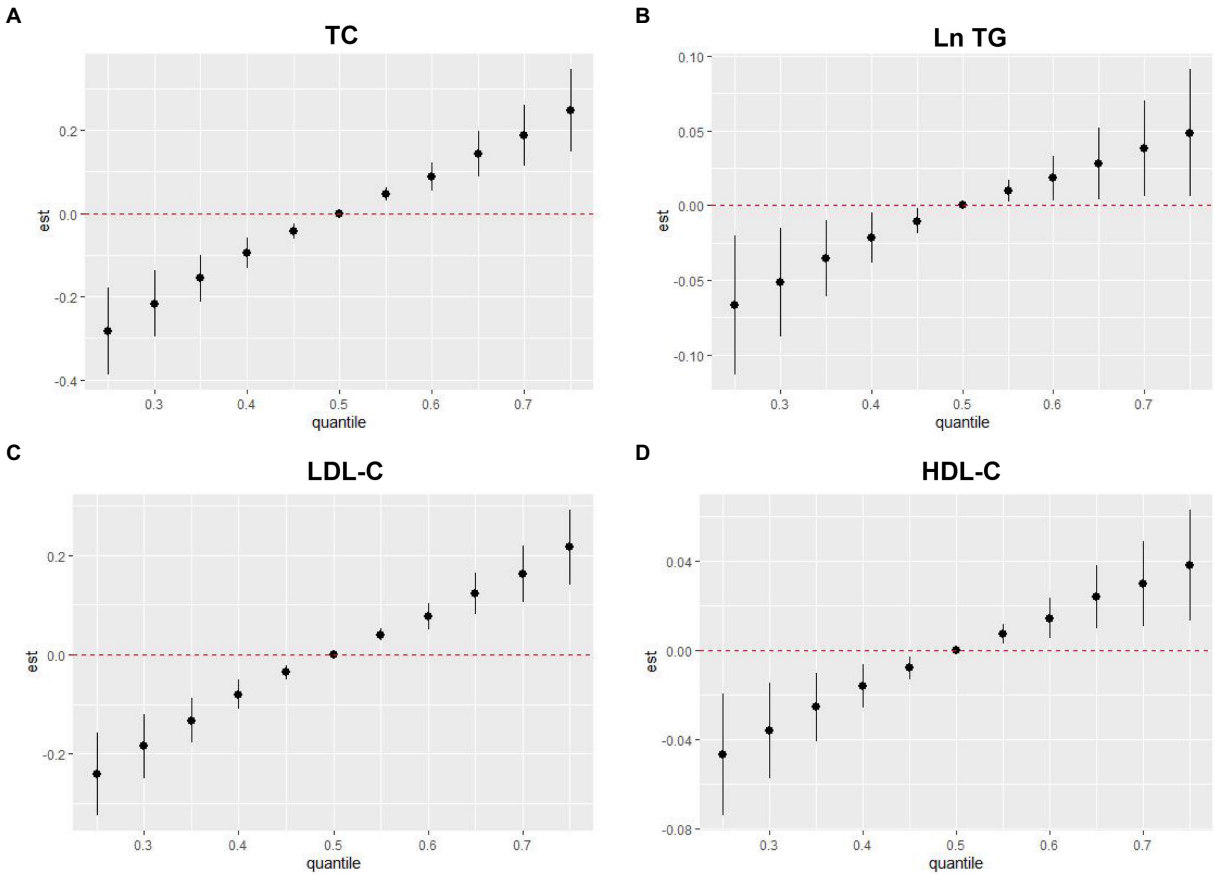

FIGURE 3
Overall associations of metal mixture including blood Pb and essential metals with serum lipid profiles in the BKMR model. **(A)** Metal mixture and TC, **(B)** metal mixture and Ln TG, **(C)** metal mixture and LDL-C, and **(D)** metal mixture and HDL-C. TG, blood Pb and essential metal concentrations were transformed to natural logarithm for further analysis. The model was adjusted for age, sex, educational, smoking status, abuse drinking, BMI categories, diabetes, and hypertension. Compared with the 50th percentile, the estimate at any percentile where the 95% confidence interval does not include 0 is considered statistically significant. TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoproteins cholesterol; HDL-C, high-density lipoproteins cholesterol; BMI, body mass index; Mg, magnesium; Mn, manganese; Ca, calcium; Fe, iron; Cu, copper; Zn, zinc; Pb, lead.

without diabetes (43). However, one recent meta-analysis study concluded that Mg supplementation could significantly lower the LDL level in patients with diabetes (44). These different findings may be accounted for the blood Mg levels of the population enrolled in our study being higher than those of patients with diabetes, chronic kidney disease, or hypomagnesemia (3, 42, 44). It indicates that overindulging or unnecessary Mg supplementation may harm lipid profiles. Another possible reason may be the simple-binding interaction between Mg and lipoprotein particles (40). The affinity of certain phospholipid head groups to Mg was given as a divalent cation, causing a positive correlation between Mg and all lipoprotein species (41).

As far as we know, no studies have investigated the relationships of a metal mixture, including Pb and essential metals, with lipid profiles, although previous studies found that the metal mixture was closely linked to diabetes (11), non-alcoholic fatty liver diseases (31, 45) and all-cause mortality (46). Using the BKMR and WQS regression models, we found that the blood metal mixture was positively associated with all of the lipid profiles. Interestingly, positive interactions between Pb and Mg on TC and LDL-C were also found. Scarce previous studies have reported the interaction between Pb and Mg on human health. It has been demonstrated that Pb can increase the production of cholesterol, leading to hypercholesterolemia by the upregulation of HMG-CoA reductase, an enzyme involved in the biosynthesis of cholesterol (47), and Mg also plays a vital role in the rate-limiting step in cholesterol synthesis at HMG-Co A reductase (40). In addition, sub-chronic lead exposure had no effects on Mg levels in any of the analyzed tissues (48), and Mg even could alleviate the adverse effects of Pb (41). Thus, we speculated that Pb might promote the binding of Mg to lipoprotein particles and further reduce the biological function of Mg. Nevertheless, future studies are needed to elucidate the interaction mechanism of Pb and Mg on lipid metabolism.

The current study still has several limitations, although it has some strengths, such as utilizing diverse statistical approaches to identify the associations of metals with lipid profiles. Firstly, causal inference cannot be drawn due to the study's cross-sectional nature. Further cohort studies and vivo experiments are needed to investigate the possible causal relationship. Secondly, despite our best efforts to adjust for the potential covariates, not all confounders or metals were measured in the current study. Thirdly, since this study included only Han Chinese participants and the number of participants was relatively small, the results may not apply to people of other ethnicities. Further studies with larger sample sizes are needed.

In conclusion, the positive associations of the metal mixture, including blood Pb and essential metals with serum lipid profiles, and the positive interactions between Pb and Mg on TC and LDL-C were observed. The levels of blood Pb, together with essential metals,

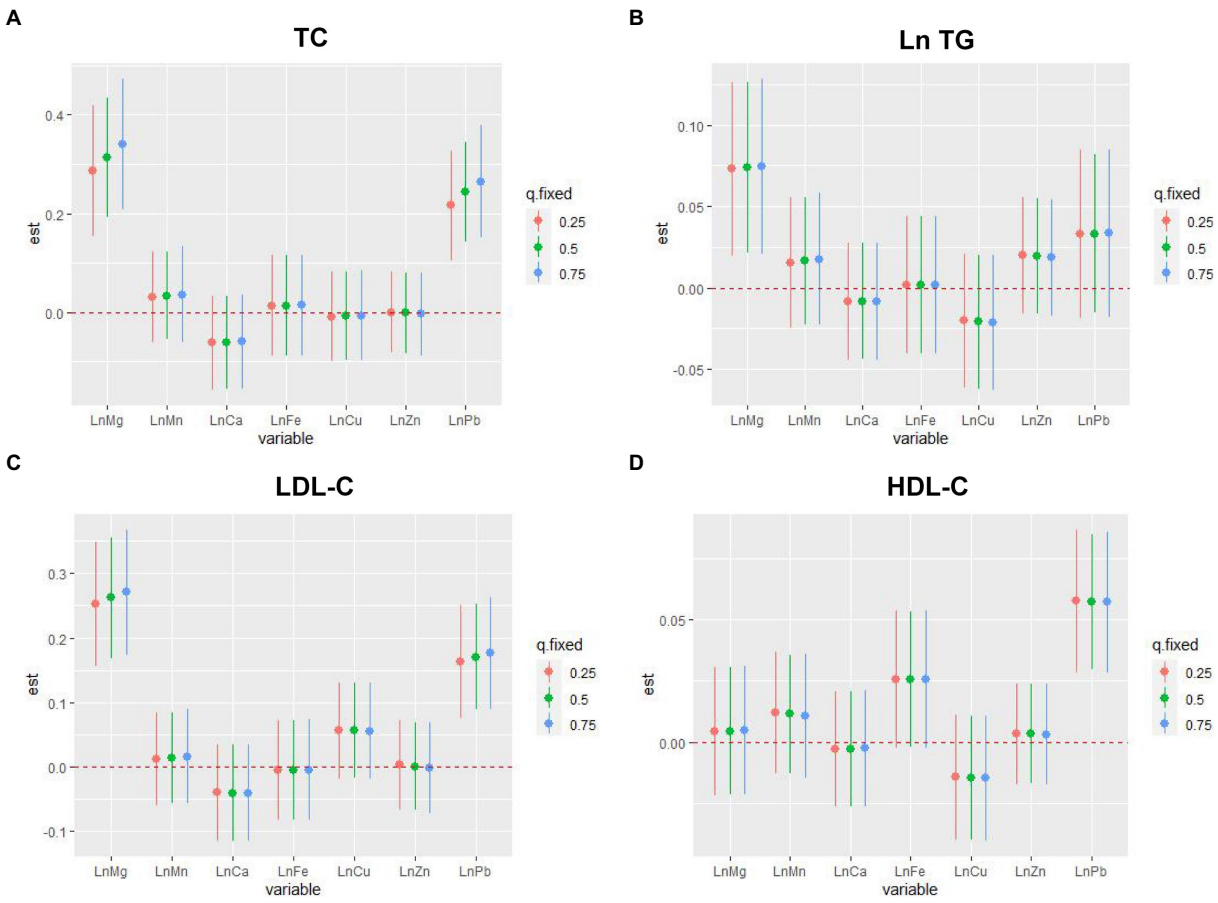

FIGURE 4
The single-exposure associations of individual metal with serum lipid profiles by the BKMR model. **(A)** Metal mixture and TC, **(B)** metal mixture and Ln TG, **(C)** metal mixture and LDL-C, and **(D)** metal mixture and HDL-C. TG, blood Pb and essential metal concentrations were transformed to natural logarithm for further analysis. The model was adjusted for age, sex, educational, smoking status, abuse drinking, BMI categories, diabetes, and hypertension. The associations were analyzed when all the other exposures were fixed to 25th, 50th, and 75th percentile. The 95% confidence interval of the estimate does not include 0 is considered statistically significant. TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoproteins cholesterol; HDL-C, high-density lipoproteins cholesterol; BMI, body mass index; Mg, magnesium; Mn, manganese; Ca, calcium; Fe, iron; Cu, copper; Zn, zinc; Pb, lead.

especially Mg levels, are suggested to be considered when assessing dyslipidemia risk. However, more evidence from prospective cohort studies with larger sample sizes and mechanism researches are still needed to validate the conclusions.

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 Ethics Committee of Shunde Hospital of Southern Medical University approved the study protocol (20211103). The patients/participants provided their written informed consent to participate in this study.

Author contributions

HW and LL performed the conceptualization. HW and DW conducted the data analysis. YaH, TL, YiH, HG, JW, ZL, YL, and XL conducted the data acquisition. HW drafted the manuscript. JS revised the manuscript and served as scientific advisors. 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.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1129169/full#supplementary-material

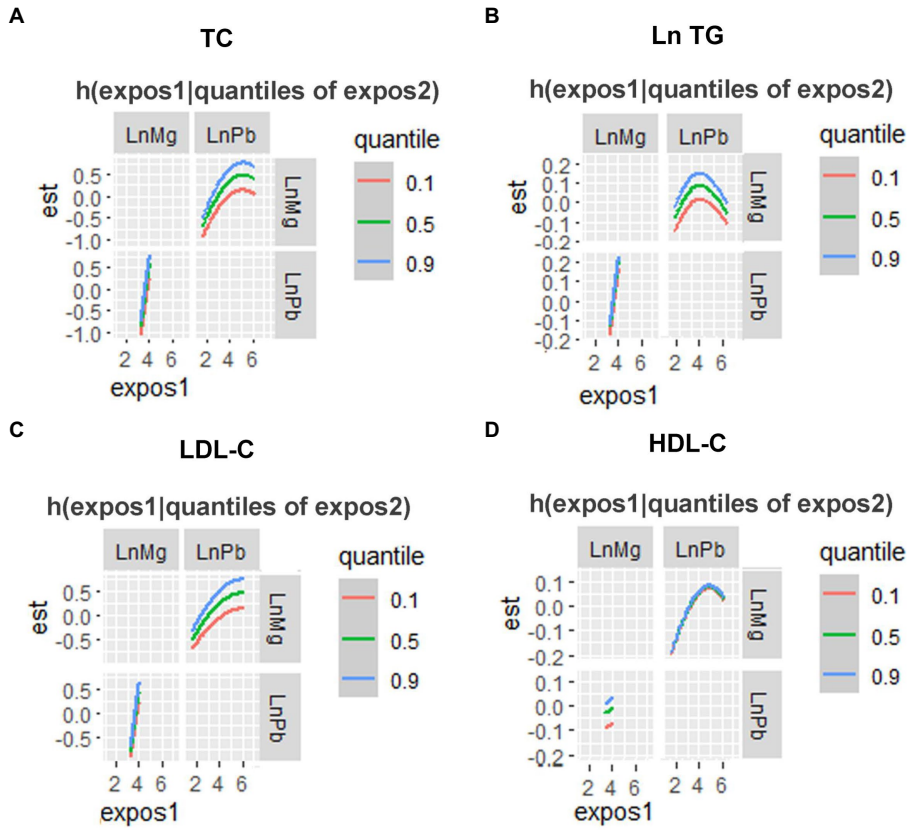

FIGURE 5
The bivariate associations of Mg and Pb exposure-response relationships of a single metal where the second element was fixed at P10, P50 and P90 analyzed by the BKMR model. **(A)** TC, **(B)** Ln TG, **(C)** LDL-C, and **(D)** HDL-C. TG, blood Pb and essential metal concentrations were transformed to natural logarithm for further analysis. The model was adjusted for age, sex, educational, smoking status, abuse drinking, BMI categories, diabetes, and hypertension. TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoproteins cholesterol; HDL-C, high-density lipoproteins cholesterol; BMI, body mass index; Mg, magnesium; Pb, lead.

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Higher dietary magnesium and potassium intake are associated with lower body fat in people with impaired glucose tolerance

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Introduction: Obesity and diabetes are public health concerns worldwide, but few studies have examined the habitual intake of minerals on body composition in people with prediabetes.

Methods: In this prospective cross-sectional study, 155 Chinese subjects with IGT [median age: 59 (53–62) years, 58% female] had an assessment of body composition including body fat percentage, oral glucose tolerance tests (OGTT), Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) and 3-day food records from nutritional programme analysis.

Results: Dietary intake of minerals was negatively correlated with body fat. People with obesity had the lowest daily consumption of iron median (IQR) 10.3 (6.9–13.3) mg, magnesium 224 (181–282) mg, and potassium 1973 (1563–2,357) mg when compared to overweight [10.5 (8.0–14.5) mg, 273 (221–335) mg, and 2,204 (1720–2,650) mg] and normal weight individuals [13.2 (10.0–18.6) mg, 313 (243–368) mg, and 2,295 (1833–3,037) mg] (p=0.008, <0.0001, and 0.013 respectively). Amongst targeted minerals, higher dietary magnesium and potassium intake remained significantly associated with lower body fat after the adjustment of age, gender, macronutrients, fibre, and physical activity.

Conclusion: Dietary magnesium and potassium intake may be associated with lower body fat in people with impaired glucose tolerance. Inadequate dietary mineral intake may play contribute to obesity and metabolic disorders independent of macronutrients and fibre consumption.

KEYWORDS

diabetes, minerals, impaired glucose tolerance, obesity, body fat, potassium, magnesium, body fat %

Introduction

Lifestyle modification is an integral component of the management of diabetes and cardiometabolic disorders. Dietary intervention improves glycaemic control and insulin resistance with 3–5% of weight loss in subjects with overweight and obese, resulting in decreased mortality and morbidity associated with type 2 diabetes (T2D) (1). Apart from prioritising

reduction in the intake of energy-dense foods, a balanced diet with the consumption of whole grains, vegetables, and fruits is also important. However, current dietary interventions predominantly focus on altered macronutrient intake, with less focus on regulating micronutrient intake.

Body fat accumulation is the single-most important risk factor for metabolic syndrome and its predisposition to diabetes (2). Lifestyle factors contributing to the former include inadequate physical activity and overnutrition, such as high intake of refined carbohydrates (3). Additionally, it can result in an inadequate intake of essential vitamins and minerals (4, 5). The relationship between mineral intake and impaired glucose tolerance (IGT) is of particular interest, given the known roles of minerals such as magnesium in improving glucose, insulin, and lipid metabolism (6). Subjects with IGT have a decompensation rate of 1–10% per year, with a cumulative incidence of T2D as high as half over time (7, 8).

A nutritional survey using 24 h dietary recall showed that higher dietary magnesium was associated with lower body mass index (BMI) in the Mexican cohort without a known diagnosis of diabetes (9), but inconsistent results were observed using food frequency questionnaires (FFQ) in patients with diabetes (10). Moreover, dietary sodium and potassium play a pivotal role in blood pressure regulation and intracellular osmolarity (11, 12). Moreover, dietary potassium was associated with obesity and metabolic syndrome (13). Few studies have investigated detailed relationships between dietary minerals and body fat composition in people with prediabetes which may also influence response to intensive dietary interventions. Furthermore, most studies used food frequency questionnaires (FFQ) or 24-h recall to capture habitual intake which may provide inaccurate data due to recall bias, retrospective and incomplete records (14). The study aimed to investigate the relationship between dietary minerals, body composition, and metabolic parameters in subjects with impaired glucose tolerance using accurate diagnosed methods (OGTT) and prospective food records. Therefore, in this study, we examined the associations between habitual minerals and anthropometric, biochemical parameters, and glycemic response in subjects with impaired glucose tolerance (IGT) using prospective 3-day food records.

Methods

Participants were recruited as part of screening for randomised controlled trial for continuous glucose monitoring (CGM) as an adjunct to lifestyle modification in IGT (NCT04588896). Participants were identified from those attending the medical outpatients at Prince of Wales Hospital, general outpatient clinics in the New Territories East Cluster, or self-referral from the community in Hong Kong SAR. We recruited participants above 18 years of age and <65 years old, BMI of 18–40 kg/m^2, not pregnant or lactating, with no history of diabetes and treatment with glucose-lowering drugs or any weight-loss treatment. Those who were participating in weight loss interventions within 3 months of the screening period were excluded. All participants underwent a 75 g OGTT was performed after an overnight fast. Glycaemic status and T2D were defined according to the ADA criteria (2009) for impaired glucose tolerance (IGT): the range of 2-h glucose level was between 7.8 mmol/L and < 11.1 mmol/L.

Dietary evaluation

This is a prospective evaluation of dietary intake using food records over a three-day period to reflect habitual consumption (records two weekdays and one weekend day). In a food record, the subject records all the food and beverages consumed, including ingredients, cooking method, and quantity of the food consumed at a given period. Upon return of the food records, a dietitian carefully screened the records according to standardised portion sizes and clarified any missing information with the patient. Food records were then analysed for daily intake of 8 targeted minerals: calcium, chromium, iron, magnesium, phosphorous, potassium, selenium, and sodium using a nutritional analysis programme (eSHA Food Processor Nutrition Analysis Software). The sodium-potassium ratio is the amount of sodium compared to potassium in the diet.

Anthropometric and biochemical measurements

Body fat percentage was assessed by an impedance biochemical analysis system [Tanita; Model: TBF-410 Body composition analyser; Nagai et al., 2008 (15); Thomas et al., 2010 (16)]. Waist and hip circumference (cm) were measured at the baseline. Height was measured with a stadiometer to the nearest 0.1 cm for the calculation of BMI. Moreover, we categorised the body weight into three groups under the World Health Organization (WHO) Asian classification for obesity (17), normal weight is between 18 and 22.9 kg/m^2, overweight is 23–26.9 kg/m^2, and obesity is higher or equal than 27 kg/m^2. Physical and activity levels were recorded using the International Physical Activity Questionnaires (IPAQ) (Chinese version) (18).

Fasting blood samples were collected following an 8-h overnight fast. Venous blood samples were collected from participants in a six-point 75 g oral glucose tolerance test (OGTT) at 0, 15, 30, 60, 90, and 120 min with C-peptide measurement. Homeostasis Model Assessment calculator (HOMA2) was used to estimate HOMA-IR and HOMA-β indices as percentages of a normal reference population developed by Oxford University (version 2.2.4 Diabetes Trials Unit, University of Oxford, Oxford, United Kingdom) (19).

Statistical analysis

Statistical analysis of all data was performed using SPSS version 26.0 (SPSS Inc., Chicago, IL, United States). To investigate the associations between the investigated minerals and markers of glucose-dependent parameters, multivariate analyses were conducted amongst the study cohort. Data were presented as a β coefficient, *p*-value, and 95% confidence interval. Values were reported as mean ± SD for parametric data or median [interquartile range] for non-parametric data. Significance was set at $p \leq 0.05$.

Results

A total of 155 individuals with IGT were in included the present analysis (Figure 1). The median age was 59 (53–62) years old with a BMI of 26 (24–29) kg/m^2 and 58% were female. Table 1 shows the

FIGURE 1
Flow chart of the study. NGT, normal glucose tolerance; IGT, Impaired glucose tolerance; DM, Diabetes mellitus.

characteristics of the study cohort. 51% of the study cohort had hypertension and 57% had dyslipidaemia. There were no significant differences in cardiovascular diseases, systolic and diastolic blood pressure, heart rate, total cholesterol, and LDL-c amongst different BMI categories (Table 1). IGT participants with obesity had higher plasma triglyceride levels and lower HDL-c when compared to overweight and normal-weight individuals ($p < 0.0001$).

Association between mineral intake, and body compositions

Individuals with obesity had the lowest consumption of iron, magnesium, and potassium (median 10.6 mg, 241 mg, and 2061 mg) compared with individuals who were overweight (11.9 mg, 282 mg, and 2,284 mg) and normal weight (14.4 mg, 314 mg, and 2,252 mg), ($p = 0.008$, <0.0001 and 0.013). There were no significant differences in other dietary minerals amongst the groups (Table 2). Body weight was positively correlated with the intake of sodium ($r = 0.16$, $p = 0.040$). Body fat was negatively correlated with calcium ($r = -0.24$, $p = 0.003$), chromium ($r = -0.27$, $p = 0.001$), iron ($r = -0.31$, $p < 0.001$), magnesium ($r = -0.40$, $p < 0.001$), phosphorus ($r = -0.39$, $p < 0.001$), potassium ($r = -0.35$, $p < 0.001$), selenium ($r = -0.27$, $p = 0.001$), and sodium ($r = -0.23$, $p = 0.004$) (Table 3). A higher intake of sodium and a ratio of sodium-potassium were correlated to an increase in body weight ($r = 0.165$, $p = 0.040$, and $r = 0.172$, $p = 0.031$).

After the exclusion of those subjects ($n = 8$) who were regularly taking minerals supplementation or Chinese medicine, dietary minerals were still significantly correlated with body fat. It was negatively correlated with calcium ($r = -0.26$, $p = 0.002$), chromium ($r = -0.29$, $p < 0.0001$), iron ($r = -0.31$, $p < 0.001$), magnesium ($r = -0.40$, $p < 0.001$), phosphorus ($r = -0.41$, $p < 0.001$), potassium ($r = -0.34$, $p < 0.001$), selenium ($r = -0.30$, $p < 0.001$), and sodium ($r = -0.22$, $p = 0.009$).

In the multivariate linear regression, there was a significant association between iron and body fat following the adjustment of age and gender (Beta coefficient = −0.277, $p = 0.006$). The association persisted following adjustment after macronutrient intake and fibre in model 1 (Beta coefficient = −0.261, $p = 0.041$) but was inconsistent after the adjustment of physical activity in model 2 (Beta coefficient = −0.179, $p = 0.167$) (Supplementary Table 1). Furthermore, dietary magnesium and potassium were significantly associated with body fat after the adjustment of age and gender (Beta coefficient = −0.020, $p = 0.001$, and −0.002, $p = 0.001$) and macronutrients, fibre (Beta coefficient = −0.024, $p = 0.003$, and −0.003, $p = 0.002$) as well as physical activity (Beta coefficient = −0.030, $p < 0.0001$, and −0.003, $p = 0.004$) (Table 4). The associations between body fat and calcium, chromium, iron, selenium, and sodium were attenuated and became insignificant after the adjustment of macronutrients and physical activity (Supplementary Table 1).

Association between mineral intake, lipid profile, insulin secretion, and insulin resistance

There were no associations between iron, magnesium, potassium, and glucose-dependent variables, such as fasting glucose, 2 h postprandial glucose, fasting C-peptide, and HOMA-IR (Table 3). Higher consumption of sodium was correlated with higher plasma triglycerides ($r = 0.168$, $p = 0.036$). A higher ratio of sodium-potassium was correlated with an increase in triglycerides ($r = 0.191$, $p = 0.017$), and HOMA-IR ($r = 0.201$, $p = 0.048$) and lower HDL ($r = -0.162$, $p = 0.044$). In the multivariate linear regression, there was no association between sodium and plasma triglycerides after the adjustment of age and gender in the base model. However, the sodium-potassium ratio was positively associated with HOMA-IR, (Beta coefficient = 0.271, $p = 0.007$), but associations attenuated after the adjustment for macronutrients and fibre (Beta coefficient = 0.130, $p = 0.227$) and physical activity (Beta coefficient = 0.031, $p = 0.821$) (Supplementary Table 2).

Discussion

In this prospective cross-sectional study, our study suggests an inverse relationship between dietary intake of minerals and body fat. Specifically, higher magnesium and potassium dietary intake was significantly associated with lower body fat, independent of age, macronutrients, physical activity, and fibre intake. The association with other minerals such as calcium, chromium, and selenium became insignificant following the adjustment of other factors.

Recent studies indicated the importance of dietary intake of minerals reduction in risk of chronic diseases, such as obesity, cardiovascular diseases, and diabetes (20, 21). In this study, we observed that magnesium and potassium intake were significantly correlated with lower body fat with persistent adjustment of macronutrients, fibre, and physical activity. Another cross-sectional study in elder adults assessed by a 3-day food record also found that dietary magnesium intake was inversely associated with BMI and metabolic syndrome (22). Magnesium is an essential cofactor for enzymes involved in glucose and insulin metabolism (23), but we did not observe any associations between magnesium and glucose-dependent parameters. We observed a low consumption of magnesium in our study cohort. The recommended daily allowance for magnesium intake is 320 mg and 420 mg for

TABLE 1 Characteristics of the study cohort.

Characteristic	Normal weight (*n*=31) >18 and <23kg/m^2	Overweight (*n*=59) 23–26.9kg/m^2	Obesity (*n*=65)≥27kg/m^2
Age, (years)*	58.2 ± 6.0	58.8 ± 6.3	54.9 ± 8.3
Female, (%)	20 ± 64.5	32 ± 54.2	39 ± 59.1
Body mass Index, (kg/m^2)**	21.5 ± 1.0	25.4 ± 1.2	30.3 ± 2.93
Body fat, (%)**	25.2 ± 6.3	29.3 ± 6.4	36.6 ± 8.8
Hypertension, number (%)	13 ± 41.9	27 ± 45.8	39 ± 59.1
Dyslipidaemia, number (%)	14 ± 45.2	35 ± 59.3	39 ± 59.1
Family history of diabetes, number (%)	18 ± 58.1	41 ± 69.5	32 ± 49.2
Systolic blood pressure, (mm Hg)	129 ± 17.8	133 ± 14.9	135 ± 17.1
Diastolic blood pressure, (mm Hg)	80.1 ± 9.0	84.6 ± 10.0	84.3 ± 11.1
Pluses (beats per min)	71.2 ± 9.5	72.4 ± 10.8	73.5 ± 10.4
Fasting glucose, (mmol/L)	5.3 ± 0.50	5.4 ± 0.52	5.4 ± 0.53
2 h plasma glucose, (mmol/L)	8.1 ± 1.3	8.5 ± 1.5	8.5 ± 1.4
Fasting C-peptide, (pmol/L)**	392 ± 155	602 ± 221	785 ± 283
2 h plasma C-peptide, (pmol/L)**	2,572 ± 757	3,134 ± 1,056	3,478 ± 932
HOMA2-IR**	0.87 ± 0.33	1.36 ± 0.51	1.76 ± 0.64
Total Cholesterol, mmol/L	5.1 ± 1.0	4.8 ± 1.0	4.9 ± 1.0
HDL-C, mmol/L**	1.5 ± 0.39	1.4 ± 0.31	1.2 ± 0.27
LDL-C, mmol/L	3.1 ± 0.85	2.8 ± 0.89	3.0 ± 0.87
Triglycerides, mmol/L**	1.0 ± 0.45	1.3 ± 0.54	1.5 ± 0.88
Light (min/week)	240 (95, 435)	210 (130, 420)	175 (77.5, 338)
Moderate (min/week)	45 (0, 142)	30 (0, 142)	0 (0, 82.5)
Vigorous (min/week)	0 (0, 67.5)	0 (0, 7.5)	0 (0, 22.5)
Sedentary (min/day)	300 (180, 390)	300 (180, 420)	360 (240, 480)

Data were shown as Mean ± SD or median (interquartile range 25th, 75th). Mean ± SD, median (interquartile range), *$p = 0.05$ and **$p \leq 0.001$.

TABLE 2 Median consumption of macronutrients and minerals by BMI categories.

	Normal (*n*=31) BMI≥18 and<23kg/m^2	Overweight (*n*=59) BMI 23–26.9kg/m^2	Obesity (*n*=65) BMI≥27kg/m^2
Energy, kcal/day	1774 (1,596, 2,113)	2032 (1802, 2,383)	1858 (1,580, 2,118)
Protein, g/day	91.8 (75.7, 110)	90.8 (82.6, 115)	87 (70.5, 107)
Fat, g/day	74.8 (56.5, 89.2)	81.1 (61.4, 97.9)	77.6 (61.7, 94.0)
Carbohydrates, g/day	195 (152, 232)	204 (159, 250)	199 (161, 243)
Sugar, g/day	38.8 (30.9, 57.3)	40.7 (28.8, 55.2)	40.2 (23.8, 66.1)
Fibre, g/day**	16.7 (13.7, 23.1)	13.5 (10.3, 17.5)	8.8 (6.5, 13.3)
Calcium (mg)	617 (497, 863)	545 (434, 737)	539 (382, 755)
Chromium (mcg)	2.7 (1.9, 5.6)	2.7 (1.9, 3.7)	19 (1.5, 3.5)
Iron (mg)*	13.2 (10.0, 18.6)	10.5 (8.0, 14.5)	10.3 (6.9, 13.3)
Magnesium (mg)**	313 (243, 368)	273 (221, 335)	224 (182, 282)
Phosphorous (mg)	1,158 (898, 1,381)	1,094 (934, 1,342)	999 (824, 1,265)
Potassium (mg)*	2,295 (1833, 3,037)	2,204 (1720, 2,650)	1980 (1,564, 2,365)
Selenium (mcg)	96.6 (73.7, 122)	91.1 (75.1, 110)	86.7 (65.2, 103)
Sodium (mg)	3,531 (2,414, 3,893)	3,754 (3,339, 4,373)	3,677 (2,881, 4,197)
Sodium-potassium ratio*	1.38 (1.07, 1.91)	1.81 (1.29, 2.18)	1.75 (1.26, 2.18)

Median (interquartile range), *$p = 0.05$ and **$p \leq 0.001$.

adult females and males, but the intake of magnesium in our cohort was lower, only 237 (192–306) mg in females and 279 (233–360) mg in males. Obesity is characterised by an increase in oxidative stress and magnesium plays an important regulator in participating as a cofactor of several enzymes, and maintenance of cell membrane stability (24, 25). It is suggested that magnesium is involved in redox homeostasis of the multifactorial antioxidant protein PARK7/DJ-1 which is in response to cellular oxidative stress that leads to fat accumulation in adipose tissue (26, 27). Moreover, magnesium affects both phosphorylase b kinase activity and glucose transporter protein activity 4 (GLUT4) by releasing glucose-1-phosphate from glycogen and regulating glucose translocation into the cell in the function of glucose metabolic pathways (28, 29). Studies indicated that magnesium supplementation reduced plasma glucose levels, and improved the glycaemic status of people with prediabetes and risk for developing diabetes (30, 31). However, data on dietary mineral data from dietary records in prediabetes are limited in current publications, patients with prediabetes or diabetes may have a largely inadequate intake of several minerals due to malnutrition and metabolic changes (32).

The gradient of potassium is responsible for maintaining cell function and is maintained in large part by the ubiquitous ion channel *via* the sodium-potassium ATPase pump. Actual dietary potassium requirements would vary with genetics, hypertensive status, and dietary sodium intake. Individuals with hypertension are more sensitive to increasing potassium intake than

TABLE 3 Spearmen correlations between minerals and metabolic parameters.

	Calcium (mg)	Chromium (mcg)	Iron (mg)	Magnesium (mg)	Phosphorus (mg)	Potassium (mg)	Selenium (mcg)	Sodium (mg)	Sodium/ potassium
Body weight, (kg)	$r = -0.02$, $p = 0.822$	$r = 0.09$, $p = 0.283$	$r = -0.09$, $p = 0.272$	$r = -0.09$, $p = 0.254$	$r = 0.08$, $p = 0.300$	$r = -0.10$, $p = 0.218$	$r = 0.02$, $p = 0.763$	**$r = 0.17$, $p = 0.040$**	**$r = 0.17$, $p = 0.031$**
BMI, (kg/m^2)	$r = -0.10$, $p = 0.213$	$r = -0.11$, $p = 0.156$	**$r = -0.23$, $p = 0.005$**	**$r = -0.28$, $p = 0.001$**	$r = -0.12$, $p = 0.128$	**$r = -0.20$, $p = 0.012$**	$r = -0.13$, $p = 0.102$	$r = 0.00$, $p = 0.994$	$r = 0.12$, $p = 0.147$
Body fat (%)	**$r = -0.24$, $p = 0.003$**	**$r = -0.27$, $p = 0.001$**	**$r = -0.31$, $p < 0.001$**	**$r = -0.40$, $p < 0.001$**	**$r = -0.39$, $p < 0.001$**	**$r = -0.35$, $p < 0.001$**	**$r = -0.27$, $p = r0.001$**	**$r = -0.23$, $p = 0.004$**	$r = 0.85$, $p = 0.298$
Systolic blood pressure, (mm Hg)	$r = -0.04$, $p = 0.590$	**$r = -0.18$, $p = 0.028$**	$r = -0.12$, $p = 0.140$	$r = -0.15$, $p = 0.054$	$r = -0.13$, $p = 0.097$	$r = -0.14$, $p = 0.092$	$r = -0.12$, $p = 0.123$	$r = 0.05$, $p = 0.560$	$r = 0.13$, $p = 0.107$
Diastolic blood pressure, (mm Hg)	$r = -0.06$, $p = 0.468$	$r = -0.05$, $p = 0.560$	$r = -0.05$, $p = 0.521$	$r = -0.11$, $p = 0.182$	$r = -0.03$, $p = 0.741$	$r = -0.13$, $p = 0.118$	$r = -0.02$, $p = 0.784$	$r = 0.09$, $p = 0.282$	$r = 0.15$, $p = 0.062$
Heart rate (beats per min)	$r = -0.12$, $p = 0.128$	$r = -0.09$, $p = 0.247$	$r = -0.10$, $p = 0.215$	$r = -0.11$, $p = 0.190$	$r = -0.14$ m $p = 0.094$	**$r = -0.19$, $p = 0.016$**	$r = -0.11$, $p = 0.158$	$r = 0.05$, $p = 0.519$	**$r = 0.18$, $p = 0.025$**
Fasting glucose, (mmol/L)	$r = 0.00$, $p = 0.999$	$r = -0.07$, $p = 0.375$	$r = 0.08$, $p = 0.305$	$r = 0.04$, $p = 0.619$	$r = 0.10$, $p = 0.196$	$r = 0.00$, $p = 0.970$	$r = 0.04$, $p = 0.598$	$r = 0.10$, $p = 0.228$	$r = 0.07$, $p = 0.059$
2 h plasma glucose, (mmol/L)	$r = -0.02$, $p = 0.796$	**$r = -0.19$, $p = 0.019$**	$r = 0.01$, $p = 0.872$	$r = -0.07$, $p = 0.401$	$r = -0.07$, $p = 0.393$	$r = -0.03$, $p = 0.687$	$r = 0.02$, $p = 0.846$	$r = 0.07$, $p = 0.410$	$r = 0.05$, $p = 0.508$
Fasting C-peptide, (pmol/L)	$r = -0.14$, $p = 0.187$	$r = 0.08$, $p = 0.440$	$r = -0.10$, $p = 0.320$	$r = -0.16$, $p = 0.114$	$r = -0.02$, $p = 0.875$	$r = -0.13$, $p = 0.213$	$r = -0.12$, $p = 0.260$	$r = 0.12$, 0.239	$r = 0.19$, $p = 0.059$
2 h plasma C-peptide, (pmol/L)	$r = -0.10$, $p = 0.333$	$r = -0.11$, $p = 0.297$	$r = -0.06$, $p = 0.541$	$r = -0.18$, $p = 0.085$	$r = -0.08$, $p = 0.457$	$r = -0.14$, $p = 0.175$	**$r = -0.22$, $p = 0.032$**	$r = 0.05$, $p = 0.595$	$r = 0.20$, $p = 0.051$
Total Cholesterol, mmol/L	$r = 0.02$, $p = 0.791$	$r = -0.08$, $p = 0.347$	$r = 0.04$, $p = 0.612$	$r = 0.04$, $p = 0.633$	$r = -0.02$, $p = 0.789$	$r = 0.07$, $p = 0.372$	$r = 0.03$, $p = 0.682$	$r = 0.08$, $p = 0.293$	$r = 0.27$, $p = 0.735$
HDL-C, mmol/L	$r = 0.05$, $p = 0.543$	$r = -0.11$, $p = 0.178$	$r = -0.07$, $p = 0.400$	$r = 0.02$, $p = 0.820$	$r = -0.09$, $p = 0.264$	$r = 0.06$, $p = 0.468$	$r = -0.11$, $p = 0.164$	$r = -0.15$, $p = 0.070$	**$r = -0.16$, $p = 0.044$**
LDL-C, mmol/L	$r = 0.02$, $p = 0.765$	$r = -0.04$, $p = 0.584$	$r = 0.07$, $p = 0.405$	$r = 0.05$, $p = 0.548$	$r = 0.02$, $p = 0.818$	$r = 0.08$, $p = 0.335$	$r = 0.10$, $p = 0.207$	$r = 0.12$, $p = 0.135$	$r = 0.04$, $p = 0.656$
Triglycerides, mmol/L	$r = -0.07$, $p = 0.391$	$r = -0.03$, $p = 0.669$	$r = 0.05$, $p = 0.522$	$r = -0.04$, $p = 0.586$	$r = 0.01$, $p = 0.861$	$r = -0.05$, $p = 0.514$	$r = 0.00$, $p = 0.977$	**$r = 0.17$, $p = 0.036$**	**$r = 0.19$, $p = 0.017$**
HOMA-IR	$r = -0.10$, $p = 0.333$	$r = 0.74$, $p = 0.470$	$r = -0.91$, $p = 0.373$	$r = -0.15$, $p = 0.373$	$r = -0.01$, $p = 0.941$	$r = -0.12$, $p = 0.228$	$r = -0.11$, $p = 0.291$	$r = 0.14$, $p = 0.184$	**$r = 0.20$, $p = 0.048$**
HOMA-B	$r = -0.15$, $p = 0.137$	$r = 0.13$, $p = 0.209$	$r = -0.13$, $p = 0.207$	$r = -0.20$, $p = 0.051$	$r = -0.08$, $p = 0.450$	$r = -0.13$, $p = 0.205$	$r = -0.12$, $p = 0.235$	$r = 0.01$, $p = 0.895$	$r = 0.12$, $p = 0.249$

BMI, body mass index; HOMA2-IR, Homeostatic Model Assessment for Insulin Resistance; Sodium-potassium ratio, the amount of sodium is divided by the amount of potassium in the diet. Bold values are p-value less than 0.05.

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TABLE 4 Multivariate analysis of associations between dietary magnesium, potassium, and body fat.

Dependent variable (Body fat)	Beta coefficient	95% CI	Adjusted R^2
Magnesium			
Base model**	−0.020	[−0.031 to −0.008]	0.485
Model 1*	−0.024	[−0.041 to −0.008]	0.489
Model 2**	−0.030	[−0.046 to −0.013]	0.545
Potassium			
Base model**	−0.002	[−0.004 to −0.001]	0.485
Model 1*	−0.003	[−0.005 to −0.001]	0.492
Model 2*	−0.003	[−0.005 to −0.001]	0.529

Body fat was included as a dependent variable with magnesium, and potassium as independent variables. Base model: adjusted for age, gender; Model 1 = base model + daily intake of total energy (carbohydrates, protein, fats, sugar, and dietary fibre); Model 2 = Model 1 + physical activities (vigorous, moderate, light exercise, and sedentary). *$p = 0.005$ and **$p \leq 0.001$

normotensive individuals and gain a greater benefit for individuals who consumed a high sodium diet (33, 34). Therefore, it is suggested that the balance of sodium and potassium is essential to reduce the risk of hypertension in a particularly high-risk population (35). Meta-analyses showed a significant reduction in blood pressure with increasing potassium supplementation (36, 37), but other studies report inconsistent results (38). High dietary sodium intake has been also correlated to high blood pressure, especially in individuals with elevated plasma triglycerides (39). In our study, the intake both dietary potassium is generally lower than that of the recommended Dietary Reference Intakes (DRIs) (2047 mg/day for females and 2,313 mg/day for males in our cohort versus 2,600 mg/day for females to 3,400 mg/day for males in the recommendation of DRI) (40). However, we observed that potassium was negatively correlated to BMI and body fat, the association between dietary potassium and body fat was robust after the adjustment of age, gender, macronutrients, and physical activity. A pooled meta-analysis indicated that adequate potassium intake and a lower urinary sodium-to-potassium ratio had a protective effect on obesity (13). A possible explanation for this could be dietary potassium maintains cell function, particularly in muscle and nerve activity, and potassium has been shown to positively correlate with an increase in muscle mass (41–43). Skeletal muscle mass plays an important role in metabolic regulation and reduces the percentage of body fat accumulation (44). Furthermore, dietary potassium may relate to Aryl hydrocarbon receptor polymorphisms which are reported to induce weight gain, glucose intolerance, and the development of obesity (45). Similar to the studies of magnesium and the risk of diabetes, studies involving potassium depletion showed that low intake of dietary potassium can lead to glucose intolerance because of the loss of intracellular potassium *via* the ATP-sensitive potassium channel. It may affect impaired insulin secretion in most excitable tissues (46–48). Therefore, habitually low intakes of dietary minerals induce changes in biochemical pathways that can increase the risk of the development of diabetes with obesity (49). Generally, a balanced diet is sufficient to supply the required balance of minerals to help support the metabolism. Recent studies indicated that dietary potassium was not associated with serum potassium or hyperkalemia in either non-dialysis-dependent chronic kidney disease (NDD-CKD) or haemodialysis (HD) patients (50, 51). However, supplement over-consumption may be negative to the metabolism, but the related study is limited.

Strength and limitations

Firstly, some important confounders were not controlled in this study, such as the use of vitamin and minerals supplements as well as traditional Chinese medicines. These could influence our results, however, 0.5% of subjects ($n = 8$) in the cohort had mineral supplementation or traditional Chinese medicine in the last 3 months prior to the screening. There was a significant correlation between body fat and minerals even after the exclusion of those subjects. Secondly, some local Chinese foods, such as dim sum, did not examine the mineral contents that affect the quantity of habitual mineral contents assessment. Thirdly, bearing in mind the limitations of BMI alone in the assessment of body composition, we did not use more sophisticated techniques such as dual-energy absorptiometry that could better investigate the relationships between dietary minerals, body fat, and muscle mass. Fourthly, we have not yet measured body mineral concentrations with the majority of work being centred on the assessment of urinary or serum minerals for a relevant indicator to evaluate the absorption rate of minerals. Furthermore, we examined the intake of habitual minerals from food records which improves the accuracy and agreement compared to other dietary assessment tools (52), such as the food frequency questionnaire (FFQ). We also assessed subjects' food records by a research dietitian (Leung K) at the dietary counselling and data were inputted by a research nutritionist (He J) and reconfirmed by a senior research nutritionist (Chu NHS) for tripartite confirmation. Finally, our sample size was relatively small and future studies should be extended to a larger multi-centred cohort.

Conclusion

A balanced diet with an adequate intake of meats, grains, vegetables, and fruits is essential to health. Apart from the quality of macronutrients, the quantity of minerals may help in different metabolism in carbohydrates and lipids that reduce the body fat, blood pressure, and glucose/ insulin response. Further confirmatory studies are needed to explore associations of dietary magnesium and potassium on the development of obesity and metabolic disorders with implications for the design of nutritional interventions.

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 Chinese University of Hong Kong (CUHK) and New Territories East Cluster (NTEC) clinical research ethics committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

NC and EC conceived the idea of the study. JH, KL, and NC were involved in data collection and supported by RM and JC. JL and NC analysed the data. NC wrote the first draft 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1169705/full#supplementary-material

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Iron metabolism and ferroptosis in diabetic bone loss: from mechanism to therapy

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Osteoporosis, one of the most serious and common complications of diabetes, has affected the quality of life of a large number of people in recent years. Although there are many studies on the mechanism of diabetic osteoporosis, the information is still limited and there is no consensus. Recently, researchers have proven that osteoporosis induced by diabetes mellitus may be connected to an abnormal iron metabolism and ferroptosis inside cells under high glucose situations. However, there are no comprehensive reviews reported. Understanding these mechanisms has important implications for the development and treatment of diabetic osteoporosis. Therefore, this review elaborates on the changes in bones under high glucose conditions, the consequences of an elevated glucose microenvironment on the associated cells, the impact of high glucose conditions on the iron metabolism of the associated cells, and the signaling pathways of the cells that may contribute to diabetic bone loss in the presence of an abnormal iron metabolism. Lastly, we also elucidate and discuss the therapeutic targets of diabetic bone loss with relevant medications which provides some inspiration for its cure.

KEYWORDS

osteoporosis, bone, diabetes, iron metabolism, ferroptosis, mechanism

1. Introduction

Osteoporosis, which is regarded as the most common bone illness worldwide, has the characteristics of low bone mass, bone tissue's microarchitectural deterioration, and declined bone strength (1). It has been determined that diabetes-specific bone characteristics, such as deficiencies in glucose/insulin metabolism, the buildup of advanced glycosylated end products (AGEs), and a lack of bone microvasculature, may constitute a novel syndrome that can be categorized as diabetic osteoporosis (DOP) (2). DOP has eclipsed other diabetes-related illnesses as the major cause of death and mutilation, substantially affecting patients' quality of life and inflicting a substantial financial burden on their families and society (3–6). Current

glucose-lowering therapeutic measures mainly consist of metformin and sulfonylureas. However, their efficacy might be enhanced. Meanwhile, a few therapies targeting diabetic mellitus were discovered to increase the possibility of fractures, such as Thiazolidinediones (TZDs) and possibly sodium-glucose cotransporter-2 (SGLT2) (2, 7, 8). Investigating the mechanisms underlying DOP can contribute to the development of new therapeutic strategies, despite the fact that researchers do not fully comprehend these mechanisms. Recent studies have demonstrated that the onset of Type 2 Diabetic Osteoporosis (T2DOP) may be correlated with the buildup of peroxides and reactive oxygen species (ROS) resulting from ferroptosis. It is also investigated that some signal molecules and signal pathways, such as NRF2/HO-1/GPX4 and SLC7A11 can ameliorate the above symptoms, providing novel possible therapeutic targets and research directions for T2DOP (9–11).

Iron metabolism is the process of iron being absorbed, transported, distributed, stored, utilized, transformed, and excreted in living organisms. The metabolism of iron is of great significance for cells. It has been discovered that iron can switch between its ferric (Fe^{3+}) and ferrous (Fe^{2+}) forms, allowing it to take and give electrons with relative ease (12). Therefore, iron metabolism is crucial to the regular functioning of several intracellular processes, and the disturbance of iron homeostasis could potentially increase the risks of many diseases. For example, iron deficiency is perceived as one of the most prevalent causes that induces anemia, while iron overload is recognized as one of the main culprits of heart diseases, bone diseases, and cognitive level-related diseases (13–17). As a significant mechanism for disease exploration, iron metabolism has gotten attention from plenty of research in exploring the relationship between iron metabolism and bone metabolism and the underlying pathways that induce osteoporosis (18–20).

Iron metabolism also impacts bone homeostasis through ferroptosis, which is a kind of iron-dependent cell death characterized by an aggregation of lipid peroxides and ROS (21). Ferroptosis has been found to be associated with the pathophysiology of diverse ailments, which includes malignant tumors, ischemic diseases, neurodegenerative diseases, as well as metabolic disorders. Substances that are induced by ferroptosis performs the ability to diminish the activated state of glutathione peroxidase 4 (GPX4) via multiple routes, resulting in a significant decrement in oxidation resistance and oxidative death in cells eventually. ROS buildup has a significant impact on the creation and survival of osteoblastic cells and their differentiation into osteocytes, thus, oxidative stress might be a major contributor to T2DOP. Wang et al. discovered ferroptosis in T2DOP rats' bone tissue, and therapies with ferroptosis inhibitors dramatically could reduce the stress of oxidation and ameliorates the symptoms of osteoporosis, although the underlying mechanisms were still far from fully understood (19).

Furthermore, findings from existed publications been proved that some medications that targeting on iron metabolism, including melatonin, Qing'e pills (22), and Artesunate (ART) (23) could relieve the systems of T2DOP to some extent, indicating the necessary to deeply invest in the research of iron and bone metabolism. Therefore, this review intends to systematically summarize the research progress of iron metabolism and diabetic bone loss, the underlying mechanisms exploration, and clinical therapies for the comprehensive evidence of further study.

2. Bone fragility in diabetes

According to the International Diabetes Federation, the alarming number of diabetics worldwide has surpassed 537 million as of 2021, and the most striking features of diabetes include chronically higher-than-standard fasting and random blood glucose, which either induce insulin deficiency [Type 1 diabetes mellitus (T1DM)] due to damage to pancreatic beta cells or progressive insulin secretion defect [Type 2 diabetes mellitus (T2DM)] from insulin resistance (24).

Diabetic complications could significantly increase the patient's risk of morbidity and mortality. Long-term diabetes is known to cause macrovascular and microvascular damage to the heart, brain, nerves, eyes, and kidneys, while significantly less attention has been given to the musculoskeletal system. The high glucose (25) environment brought on by these two factors could further potentially affect the bone metabolism, bone loss or even osteoporosis (26). Osteoporosis is defined as bone mineral density (BMD) at the femoral neck that is 2.5 standard deviations (SD) or more below the mean for young female adults (T-score less than or equal to −2.5 SD (27)), on the basis of dual-energy X-ray absorptiometry (DXA). Osteoporosis induced by diabetes mellitus, sometimes referred to as diabetic bone disease, is a chronic disease that subsequently increases bone fragility and fracture risk owing to a decrease in bone density and damage to the bone microstructure (28, 29). Research found that patients with diabetic bone disease are at a higher risk of long-term bone pain, motor dysfunction and fractures (30). More than 35% of individuals with Type 2 diabetes displayed bone loss, with 20% meeting the diagnostic criteria for osteoporosis.

Diabetic bone loss is characterized by altered bone density, altered bone turnover, reduced bone microarchitecture, and increased fracture risk. Multiple independent research demonstrate that the BMD of diabetic individuals may be decreased, constant, or even enhanced. The femur and vertebrae are the major sites of elevated BMD in patient with T2DM (31–33). Generally, having unnecessarily abundant energy and being overweight are the main causes of the rise in BMD in T2DM patients. Adaptive changes in the bone that enable the body to sustain a heavier load may also contribute to the increment of BMD (34, 35). Nonetheless, despite greater mean BMD and T-score values, there is increasing evidence that the T2DM-associated increased fracture risk is related to decreased bone quality, which may be termed "diabetic osteopathy" (36–38). The apparently contradictory finding is based on the changes in bone turnover, decreased bone microarchitecture, accumulation of AGEs, muscular weakness, anti-diabetic medication, etc., which might have the possibility to enhance the fracture risk of T2DM patients (39). Patients with T2DM usually display aberrant bone microstructure, particularly in the cancellous bone, with both a reduction in the number of trabeculae and morphological defects (40); also they also have a considerably decreased number of trabeculae and trabecular thickness in the femoral head compared to non-diabetic patients (41). Thinner cortical bones and higher porosity show a direct correlation with a decreased breaking load. Compared to the general population, individuals with T2DM had a 3% drop in radial cortical bone density and a 25% increase in cortical bone porosity (42); a smaller cross-sectional area, more cortical porosity; and a lower cortical vertebral BMD in the tibia, but not the radius with the assistance of HR-pQCT (43).

3. Bone cell biology in high glucose condition

Resorption and creation of bone are two essential components of bone remodeling. A major element in the development of osteoporosis is an imbalance in bone reconstruction. Bone remodeling, the coordinated activities of bone-resorbing osteoclasts and bone-forming osteoblasts, is required for continuous bone turnover and regeneration. Diabetes may affect all types of bone cells and promote adipose tissue formation in bone marrow. In this part, we intend to describe separately for four different cells in bone microenvironment in the context of HG: mesenchymal stem cells, osteoblasts, osteoclasts, and osteocytes (Figure 1).

3.1. Mesenchymal stem cells in HG condition

Osteoblasts are derived from multipotent mesenchymal stem cells (MSCs), which may move to the site of impairment, proliferate, and differentiate (44). MSCs may be separated from peripheral blood and nonhematopoietic tissues such as adipose tissue, trabecular bone, dermis, dental pulp, synovium and lung, despite the fact that bone marrow is assumed to be the primary source of these precursor cells (45). As the most significant MSCs obtained from bone marrow, bone marrow-derived mesenchymal stem cells (BMSCs) play crucial roles in bone tissue regeneration. Different microenvironments such as high glucose levels, inflammation, and hypoxia, would change the physiological functioning of stem cells (46). Recent study has shown that osteoporosis was associated with an increase in circulating MSCs with low osteogenic potential, highlighting the importance of BMSCs for successful bone remodeling and/or repair *in vitro* (47). A number of studies have shown that the biological activities of BMSCs were modified by chronic exposure to a diabetic pathogenic environment (48, 49).

In addition, the serine/threonine kinase glycogen synthase kinase-3, also known as GSK-3, contains two remarkably homogeneous isoforms, GSK-3a and GSK-3b, which is a broadly expressed enzyme (50). GSK-3b inhibition could increase bone density (51). In high glucose microenvironments, GSK-3b activation as well as Wnt pathway suppression impede BMSC migration and proliferation, however, lithium chloride, an inhibitor of GSK-3b, may restore the functionality of BMSCs (46), according to Zhang et al. Moreover, Yu's study demonstrated the activation of GSK3b in diabetic osteoporosis and its deleterious osteogenic affected BMSCs in a high glucose milieu through the β-catenin/Tcf7/Ccn4 signaling axis inhibition, and thus provide unprecedented perspectives into diabetes osteopathy (48).

Furthermore, as a common denominator of the numerous osteogenic signaling pathways, it's suggested to strictly mange the ROS levels for MSCs to undergo osteogenic differentiation (52). It is reported that usage of deferoxamine *in vitro*, the anti-osteogenic impact of superparamagnetic iron oxide nanoparticles was abolished, indicating that the free form of iron is significant to the inhibition of MSCs from differentiating into osteoblasts (53). Balogh et al. also approved that iron specifically prevents BMSCs from differentiating into osteoblasts without affecting adipogenic or chondrogenic differentiation (54).

In summary, high glucose condition shows an impact on mesenchymal stem cells and suppresses its differentiation process.

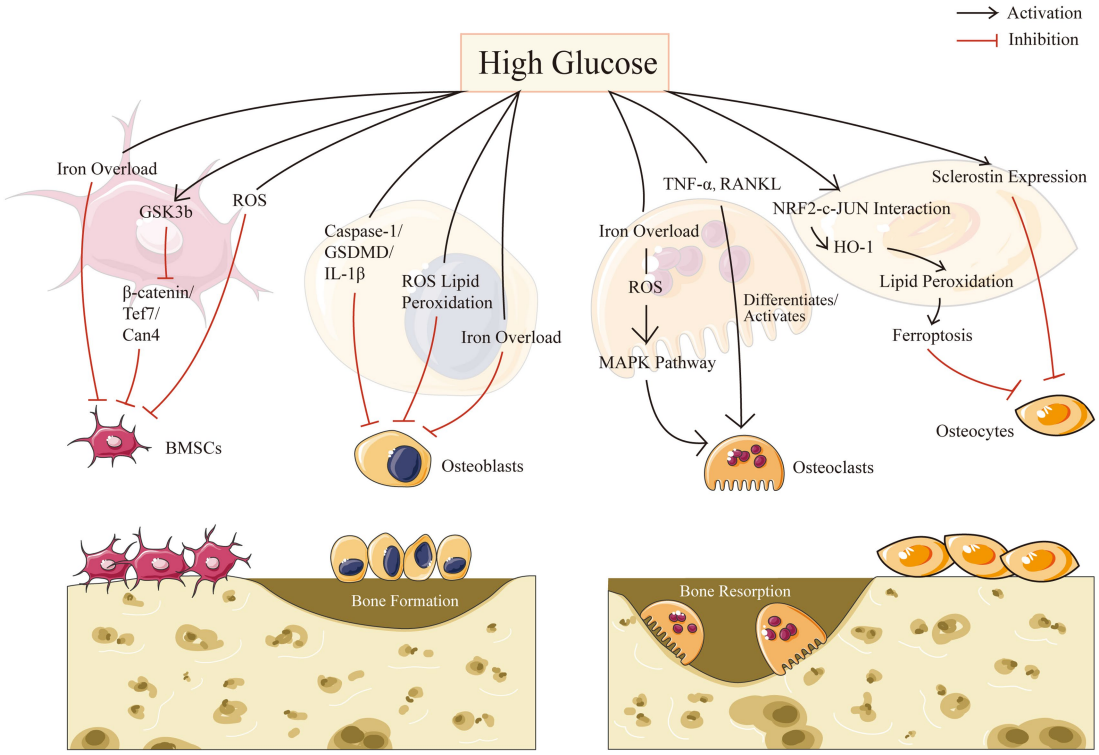

FIGURE 1
Bone cell biology in high glucose condition.

3.2. Osteoblasts in HG condition

Osteoblasts, which serve as bone-forming cells, originate from the sequential activity of transcriptional factors on mesenchymal precursors to osteoprogenitor lineages and eventually differentiate into osteocytes. Osteoblasts produce extracellular proteins such as osteocalcin, alkaline phosphatase, and type I collagen, the latter of which accounts for more than 90% of bone matrix protein. The extracellular matrix is initially secreted as unmineralized osteoid and becomes gradually mineralized when calcium phosphate concentrates as hydroxyapatite (55).

It has been demonstrated that the high glucose conditions in T2DM severely impair the biological functions of osteoblasts, resulting in an increase in the density of mitochondrial bilayers and a decrease in the number of mitochondrial cristae, and leading to the accumulation of ROS as well as lipid peroxides causing the cells to exhibit excessive oxidative stress as well as lipid peroxidation, and causing the cells to exhibit excessive oxidative stress and lipid peroxidation, accelerating apoptosis and autophagy of osteoblasts. It is reported that the proliferation and differentiation of osteoblasts could be inhibited by excessive glucose in alveolar bone through the caspase-1/GSDMD/IL-1 pathway, indicating the opposite effects from usage of caspase-1 inhibitors *in vivo* and *in vitro* (56).

HG condition could also affect osteoblasts by modulating iron metabolism as well. It was identified that iron overload reduces MC3T3 cell viability and causes apoptosis, in which they reported that an excess of iron may partially suppress osteoblast activity, and disturb the differentiation and mineralization processes of osteoblasts (57). What's more, the pathogenesis of T2DOP was significantly influenced by the osteogenic activity of osteoblasts, which was negatively influenced by iron overload caused by the increased expression of DMT1 in osteoblasts (58, 59).

In summary, HG condition not only suppress the differentiation process of osteoblasts, but also strongly affected its osteogenic function.

3.3. Osteoclasts in HG condition

Osteoclasts are end-differentiated multinucleated cells of the monocyte/macrophage lineage with unique function of resorbing bone matrix (60). Osteoclasts break down bone by secreting acids and proteolytic enzymes such cathepsin K, also known as CTSK, which break down matrix components like collagen during osteoclastogenesis (61, 62). As was known, monocytes could only differentiate into osteoclasts *in vitro* when co-cultured with cells comprising stromal cells and osteoblasts (63). Because osteoclasts and osteoblasts' respective bone resorbing and building processes are closely correlated, an adult's bone mass is generally steady. However, in many disease states such as osteoporosis, metastatic bone cancer, and inflammatory arthritis, the delicate balance is disturbed by an increase in osteoclast bone resorption activity (60).

Various studies have revealed that the high glucose condition has a certain promotion effect on the differentiation of osteoclasts, which can strengthen their bone resorption ability (64, 65). Clinical studies showed that osteoclastogenesis was more frequently accelerated by diabetes mellitus: (a) enhanced levels of tartrate-resistant acid phosphatase, a sign of increased osteoclast activity, were found in the blood of patients with T2DM (66); (b) tartrate-resistant acid phosphatase levels were higher in blood among T2DM patients (67). Studies on animals' models further approved that diabetes patients have higher osteoclast activity (68, 69): compared to normoglycemic controls, osteoclastic bone resorption was increased in T2DM rats (70). TNF-a, macrophage-colony stimulating factor, receptor activator of nuclear factor kappa-B ligand (RANKL), as well as the vascular endothelial growth factor-A were all elevated in diabetic mice, which would differentiate and activate osteoclasts (71–73).

Furthermore, osteoclasts could also be significantly influenced by iron overload, which is induced by DM. It's reported that ROS that arises from iron overload could activate the MAPK pathway, improving the differentiation capability and the bone resorption capacity of osteoclasts in bone metabolism (20). There's also evidence showed that ferritin autophagy took place when cells were iron-deficient, which makes them more susceptible to ferroptosis caused by intracellular Fe^{2+} (74, 75) Additionally, Mature osteoclasts require a greater amount of cytoplasmic free iron than other osteocytes. Hence, osteoclasts are more susceptible to ferroptosis (76, 77).

In summary, it implies that the HG condition can influence osteoclast activity, which may result in aberrant bone metabolism and osteoporosis.

3.4. Osteocytes in HG condition

Osteocytes are terminally developed osteoblasts that undergo substantial morphological changes when embedded in the mineralized bone matrix. It plays a key function throughout the homeostasis regulation of bone, with a main function to communicate with the surrounding environment (78, 79): (a) their numerous dendritic processes that protrude from the osteocyte soma in all directions and enter the 'canaliculi', which are tiny passageways by which the osteocytes could connect with other osteocytes and cells in the bone marrow or periosteum; (b) osteocytes in the interstitial tissue of the lacunar-canalicular structure come into touch with liquid, which enables these cells to function well. Consequently, the osteocyte lacunar-canalicular network provides a vast system that could detect changes in bone loading and regulate bone remodeling for the healthy skeleton, with the collaboration of other bone cells' (osteoblasts and osteoclasts) activities (80).

Osteocytes may release various signaling substances in response to loading or unloading stimuli via the SOST/DKK/Wnt or the RANKL/Osteoprotegerin (OPG) axis. It may either promote bone resorption by producing RANKL and decreasing OPG, or decrease bone resorption by flipping the RANKL/OPG ratio. Osteocytes are also the substantial producers of Dkk1(the Lrp5/6 Wnt signaling inhibitor) and sclerostin (transcription product of the SOST genes) in connection to bone formation (78, 81, 82). It's interesting to note that patients with T1DM and T2DM had higher serum levels of sclerostin (83, 84), indicating variations in glucose concentration may have impact on the cells most crucial for maintaining bone health as sclerostin is largely produced by osteocytes. Moreover, Blood glucose levels significantly above and below the normal range of 80–140 mg/dl may have detrimental effects on osteocytes (85). Another study showed that diabetes caused osteocytes to alter over time and upregulate the sclerostin gene, that might be mediated by local glucose concentrations and could have a significant effect on the deterioration of bone quality (85–87).

Furthermore, it's suggested that inhibiting the ferroptosis pathway in diabetic mice prevented DOP and osteocyte death (10). Traditional cell death inhibitors such as Z-VAD-FMK and Nec-1 had no impact in rescuing osteocytes from the death induced by high glucose and high fat (HGHF) circumstances. Furthermore, they concluded excessive lipid peroxidation may be the primary source of cell damage in the diabetic milieu and that ferroptosis may be strongly associated with the underlying molecular process of cell osteocyte death. Altogether, high glucose level could induce longstanding changes in osteocytes via upgrading sclerostin expression and inducing ferroptosis, resulting in the imbalance of bone metabolism eventually (10).

In summary, the high glucose level in the blood caused by T2DM alters the dynamic equilibrium between bone formation and bone resorption in a normal organism, resulting to a variety of complications such as T2DOP.

4. Iron-related protein and bone formation in HG

Studies have approved that proteins involved in iron metabolism have a very clear connection to bone metabolism. Here we give some summaries. Table 1 summarizes iron-related proteins and bone metabolism in high glucose condition.

4.1. Ferritin

Zarjou et al. found that the ferroxidase activity of ferritin was responsible for the suppression of osteoblasts' activities (75). By observing the effects of ceruloplasmin (a protein with ferroxidase activity but no iron sequestration ability) and examining the osteoblast-specific genes expression, they discovered that ferritin ferroxidase activity might inhibit the production and subsequent activity of alkaline phosphatase (ALP). Thus, the ferritin ferroxidase activity could not only inhibit the exclusive osteoblast product osteocalcin which in turn affect calcification, but also downregulate the osteoblast-specific genes such as core binding factor α-1, alkaline phosphatase and osteocalcin (75).

Additionally, it has been demonstrated that mitochondrial ferritin (FtMt) reduces oxidative stress and maintains intracellular iron homeostasis (25). If FtMt expresses excessively, it will lessen ferroptosis that happens in osteoblasts under HG environments, whereas if FtMt becomes silent, it can stimulate autophagy in mitochondrial via the ROS/PINK1/Parkin pathway, leading to an increase in osteoblasts ferroptosis (74). In T2DOP, FtMt was showed to prevent ferroptosis in osteoblasts by decreasing oxidative stress produced by excess ferrous ions, while FtMt deficiency increased mitophagy in the pathogenesis of T2DOP (74, 89).

4.2. HEP

Hepcidin (HEP), which is produced and secreted by liver cells, regulates iron homeostasis. It can connect to the ferroportin (FPN) receptor, which is a type of transmembrane protein, to prevent cellular iron from entering the bloodstream (11, 123). The sole iron output protein in vertebrates up to this point is FPN (90–92). If FPN activation induced by HEP is inadequate or inefficient, the organism may experience iron overload and perhaps iron deposition in the skeletons. Causing numerous ROS production, mitochondrial biogenesis, peroxisome proliferator-activated receptor gamma coactivator-1beta (PGC-1β) expression in osteoclasts and ultimately resulting in osteoporosis (93). In addition, there's also a study concluding that BMP/SMAD signaling pathway was discovered to possess the ability to regulate the expression level of HEP (94). Xu et al. not only found that HEP stimulated osteoblast intracellular Ca2+ in a dose-dependent manner, but also revealed that the process mention above is facilitated by voltage-dependent L-type calcium channels, which indicated an unignorable effect that HEP had on bone metabolism (95).

4.3. Tfr2

In mammalian cells, there are two distinct transferrin receptors (Tfrs) (96). Transferrin receptor 1 (Tfr1) is predominantly expressed and binds to Fe^{3+}-loaded holo-Tf with great affinity. Plasma iron flows attached to the iron transporter protein transferrin and is absorbed by endocytosis that mediated by Tfr1 under physiological circumstances. Tfr1 is regulated post-transcriptionally by intracellular iron status through the iron-regulatory protein system (97), resulting in elevated Tfr1 under low iron circumstances and diminished Tfr1 under high iron conditions (98). Bhaba's reported that Tfr1absence resulted in a >50% drop in osteoclast lineage cells in the total osteoblasts intracellular iron concentration (99). However, Tfr1-deficiency had no impact on the iron levels in monocytes and pre-osteoclasts. It has been determined that mature osteoclasts procured extracellular iron mostly via using Tf and heme (99). This study found that iron uptake regulated by Tfr1 is a key iron acquisition route in osteoclast lineage cells, which significantly regulates bone remodeling of trabecular in the perpendicular and axial bones via female and male mice models (99). Also, the increased cytoplasmic iron generated by Tfr1 was approved to be especially essential for mitochondrial energy consumption and cytoskeletal structure in osteoclasts, however, it still showed slight impact on the differentiation of osteoclasts (99).

Transferrin receptor 2 (Tfr2) is another crucial regulator of hepcidin, which is proposed to control iron homeostasis. Tfr2 is known for controlling systemic iron levels, but it also promotes healthy erythropoiesis (100–103). Tfr2 has recently been identified a novel extrahepatic function, controlling bone mass directly by osteoblasts in the research from Martina Rauner's team (104). They reported that Tfr2, which is predominantly located in osteoblasts, governed bone production but had little effect on the systemic iron homeostasis. Furthermore, Tfr2 could also activate p38 MAPK signaling in osteoblasts, which leads to the induction of the Wnt inhibitor sclerostin and limits bone formation, hence, Tfr2 functions as a unique regulator of bone mass via modifying the BMP-p38 MAPK-Wnt signaling axis (104).

4.4. IRP

Iron regulatory protein 1 (IRP1) and iron regulatory protein 2 (IRP2) post-transcriptionally control the metabolism of iron in

TABLE 1 Iron-related proteins and bone metabolism in high glucose condition.

Protein	Mechanism	Effect in Ferroptosis	Basic foundation	Origin and distribution *in vivo*	Biochemistry and molecular structure	References
Ferritin	ROS/PINK/Parkin	Ferroptosis in OB, osteocalcin and CBF-a1 inhibition	Primary iron storage proteins of most living organisms, members of a broad superfamily of ferritin-like diiron-carboxylate proteins	Almost all body tissues especially liver cells and reticuloendothelial cells	Iron-free (apoferritin) molecule is a protein shell composed of 24 protein chains arranged in 432 symmetry. 2 types of chains (subunits): H or M (fast) and L (88), which differ in rates of iron uptake and mineralization.	(74, 75, 89)
HEP	BMP/SMAD	Increased ROS production, mitochondrial biogenesis, and PGC-1β expression in osteoclasts	An antibacterial and antifungal protein	Expressed in the liver	Cysteine-rich, forms a distorted beta-sheet with an unusual disulphide bond found at the turn of the hairpin.	(90–95)
Tfr	BMP/p38MAPK/ Wnt	Tfr1 is a key player in the uptake of iron-loaded transferrin into cells, Tfr2 binds transferrin but with a significantly lower affinity than Tfr1	TfR1 may also participate in cell growth and proliferation	Tfr1: widely expressed Tfr2: hepatocytes, hematopoietic cells, and duodenal crypt cells	Tfrs are homodimeric type II transmembrane proteins containing three distinct domains: protease-like, apical or protease-associated, and helical domains.	(96–104)
IRP	Through post-transcriptional regulation of iron metabolism-related proteins to maintain cellular iron homeostasis	Decreased expression of bone formation markers such as TFRC and ferritin	Sustaining normal mitochondrial function			(105–107)
METTL3	Upregulating the ASK1/p38 signaling pathway to induce ferroptosis	Induction of ferroptosis in OB	Regulating various processes such as the circadian clock, differentiation of embryonic and hematopoietic stem cells, cortical neurogenesis, response to DNA damage, differentiation of T-cells and primary miRNA processing, playing an important role in various kinds of tumors	Almost all body tissues		(108–111)
DMT1		Suppression of the OB osteogenic function	Having a role in gastrointestinal uptake of metals and in transferrin dependent trafficking of iron and manganese, Cu2+, Cd2+	Widely expressed	DMT1 is a 12-transmembrane-domain protein, having at least four isoforms: two are derived from N-terminal alternatives and two are from C-terminal alternatives	(112–115)
HO-1	NRF2 and c-Jun/ HO-1	Catalyzing heme oxidation to produce a significant amount of free labile iron, inducing ferroptosis in osteocytes	Catalyzing heme degradationholds antioxidant, anti-inflammatory, cytoprotective, proliferative, and angiogenic properties	Expressed in low quantities under normal conditions except in tissues that involve the degradation of senescent red blood cells, such as the spleen, liver, and bone marrow		(89, 116–120)
GSH	XC-system/GSH/ GPX4 axi	Reduced osteoblast ferroptosis and enhanced osteogenic activity.	Converting peroxide (R-OOH) into alcohol (R-OH) and decreasing the toxicity of lipid peroxides	Widely expressed		(19, 121, 122)

HEP, hepcidin; Tfr2, transferrin receptor 2; IRP2, iron regulatory protein 2; METTL3, methyltransferase-like 3; DMT1, divalent metal transporter 1; HO-1, heme oxygenase-1; GSH, glutathione; OB, osteoblast.

vertebrate cells (105). Zhang et al. demonstrated that iron drove the transcription of NADPH oxidase 4 (NOX4) by dissociating IRP1 and thereby depressed osteogenesis in bone metabolism. Mechanically, they revealed that the NOX4 locus includes iron-response element-like sequences, which are bound by IRP1. Upon iron binding, IRP1 dissociates from the IRE-like sequences, resulting in the activation of NOX4 transcription. Osteoblasts with increased NOX4 accumulate lipid peroxide and show obvious alterations in mitochondrial morphology and function (106). In mouse bone tissue after the deletion of IRP2, investigation has discovered the expression of the genes for the proteins that served as iron transporter (FLT, FPN1, and TFR1). This is a disease characterized by scant trabecular bone, which could induce the reduction of iron concentration and the downregulated expression of bone formation markers (107). Therefore, a lack of IRP2 may prevent the iron transporter from transferring, which results in a lack of iron and affects bone metabolism. However, additional research will be required in the future to understand this conclusion because the underlying process is currently elusive.

4.5. METTL3

Methyltransferase-like 3 (METTL3), one of the m^6A writers, is approved to play a role in the pathophysiology and growth of bone-related disorders including osteoporosis, arthritis, and osteosarcoma (108). Nonetheless, there is controversy regarding the link between osteoporosis and METTL3 expression. For instance, one study found that overexpression of METTL3 in bone marrow monocytes protected mice against osteoporosis induced by estrogen deprivation, while disruption of METTL3 in mice destroyed bone formation, decreased osteogenic differentiation, and improved marrow obesity (109). Another study demonstrated a negatively regulatory role of METTL3 in osteogenesis process by activating NF-κB pathway, which was considered as a significant osteogenic differentiation inhibitor. And METTL3 was found to induce the expression of MYD88, an upstream regulator of NF-κB pathway, through control m6A methylation status of MYD88-RNA (110).

Furthermore, researchers have discovered that METTL3 may be involved in high glucose and palmitic acid (HGPA)-induced osteoporosis via activating the ASK1/p38 signaling pathway, in which they noticed that METTL3 knockdown prevented HGPA-induced activation of ASK1/p38 signaling (111). The fact that the expression of the ferroptosis-inhibitory proteins GPX4 and SLC7A11 was markedly repressed further provided evidence that activating ASK1/p38 pathway was responsible for the induction of ferroptosis (111).

4.6. DMT1

Divalent metal transporter 1 (DMT1) is a 12-transmembrane-domain protein that is present in various tissues, such as bone, kidney, and duodenum. DMT1 transports lots of divalent cations (112). It is the main apical transporter in charge of absorbing intestinal Fe^{2+} and it is found to be widely expressed in endosomal compartments, in a place where it is in responsibility of exporting Fe^{2+} throughout the transferrin cycle (112, 113). As a result, iron overload and DMT1 expression are closely connected. DMT1 plays a role in the absorption of other metals in addition to its role in the metabolism of iron and manganese, and it also involves in the transfer of Cu^{2+} and Cd^{2+} (114, 115).

Further studies proved that the overexpression of DMT1 could lead to iron overload in osteoblasts, thus suppressing the osteogenic function of osteoblasts. Liu et al. discovered that human hFOB1.19 osteoblasts treated with ferric ammonium citrate (FAC) expressed more DMT1 compared with those untreated cells (58). Zhang et al. found that there were less of the autophagosome accumulation that was caused by FAC in DMT1-shRNA hFOB1.19 cells, which suggested that DMT1 controls the levels of Fe2+ in osteoblasts, which has an impact on the cellular accumulation of autophagosomes (59). In summary, DMT1 expression could enhance in the bone tissue of type 2 diabetic condition, then DMT1 induces iron overload in osteoblasts, and ultimately affects the osteogenic function of osteoblasts.

4.7. HO-1

Heme oxygenase-1 (HO-1) is a cellular inducible oxidative stress regulator that oxidizes heme to produce biliverdin, carbon monoxide, and free ferrous iron (116). The role HO-1 plays in ferroptosis is still up for dispute at this time. Numerous studies showed that elevated HO-1 expression prevented oxidative stress in cells and prevented ferroptosis (37, 117, 118). For instance, Adedoyin et al. discovered that $HO\text{-}1^{-/-}$ cells demonstrated higher erastin-induced cell death when compared to $HO\text{-}1^{+/+}$ renal proximal tubule cells (119). Other researchers, however, identified that excessive HO-1 caused organ failure and exacerbated ferroptosis (89, 120). According to Fang et al., inhibiting HO-1 expression reduced ferroptosis in cardiomyopathy in models *in vivo* and *in vitro* (89). Tang et al. noted that blocking HO-1 activity should be a reliable way to prevent ferroptosis in the retinal pigment epithelium (120). It therefore demonstrated that HO-1 was a double-edged sword that functions differently in distinct tissues and disease models.

HO-1 plays important roles in bone metabolism. Yang's team approved that the group with DOP had much more lipid peroxidation occurred *in vivo* via DOP mouse model, indicating that the high-glucose microenvironment could induce osteocyte ferroptosis. Then they went further demonstrated the concrete mechanism of how high-glucose microenvironment induced intracellular iron overload. In diabetic microenvironment, HO-1 transcription was activated upstream by the heterodimer of NRF2 and c-JUN and activation of HO-1 catalyzes heme oxidation produced a significant amount of free labile iron (10). What's more, Ma's finding also supports the theory that HO-1 might mediate HGHF-induced osteocyte ferroptosis (9). HO-1 activation and ferroptosis are both mutually causal and can lead to an endless loop of mutual promotion (10, 121).

4.8. GSH

Ferroptosis can also be induced by the depletion of glutathione (GSH) and the reduction in GPX4 activity (121). GSH is a protective substance in cells and the main substrate of GPX4, which can combine with lipid peroxide to reduce ROS, so as to play an important role in antioxidant. The body's lipid antioxidant system is regulated by GPX4 as its principal regulator. To protect biofilm

systems against ferroptosis damage, GSH is employed as a cofactor to convert peroxide (R-OOH) into alcohol (R-OH) and decrease the toxicity of lipid peroxides. However, the body's decreased GSH levels displays impacts on GPX4 activity, which is required for ferroptosis to occur. Numerous synthesis routes, such as glutathione synthetase (GSS) and nicotinamide adenine dinucleotide phosphate, are the source of GSH (121). A disulfide bond connecting the heavy chain SLC3A2 and the light chain SLC7A11 creates the cystine-glutamate reverse transporter protein known as the XC-system. It mediates the 1:1 exchange of glutamate and cystine inside and outside the cell. The extracellular glutamate concentration influences the transport rate of the XC-system, and an elevated glutamate concentration inhibits cystine uptake and GSH production, which results in altering the GPX4 activity alteration and ferroptosis (11, 122).

This XC-system/GSH/GPX4 axis is one of the main pathways that HG induces ferroptosis. According to Zhao et al., system XC-mediated suppression of ATF3 activity resulted in the induction of osteoblast ferroptosis in high glucose conditions, and these occurrences aided in the pathogenesis of T2DOP (19). They found that ATF3 was upregulated by HG *in vivo* and *in vitro*, which reduced the expression of SLC7A11 and the amounts of intracellular GSH and extracellular glutamate (19). ATF3 inhibition then boosted GPX4 levels and decreased the buildup of ROS and lipid peroxides and these modifications reduced osteoblast ferroptosis and enhanced osteogenic activity. According to Ma et al., osteoblasts from osteoporotic individuals with T2DM developed a lot of ferroptosis lipid peroxides. The down-regulated expression of GPX4 and SLC7A11 in osteoblasts mitochondria and the XC-system were correlated with these lipid peroxides (9).

In summary, high glucose condition induces the imbalance of iron metabolism (ferroptosis and iron overload) via abundant pathways like Nrf2/HO-1, METTL3, XC- system/GSH/GPX4. Some proteins, such as METTL3 and DMT1, also contribute dramatically to the regulation of iron metabolism. It indicated the necessary to explore deeply on the association between iron and bone metabolism and underlying pathways.

5. Iron-related signaling pathways and bone formation

5.1. NRF2/HO-1/GPX4

Figure 2 showed the association between iron overload and osteoporosis in osteoblast and osteoclast. Activating the NRF2/HO-1 channel considerably lowers ferritin levels while reducing oxidative stress and it prevents ferroptosis and promotes bone production (124). The nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway is directly downstream of ROS and controls the transcription of antioxidant response element-dependent genes to sustain cellular redox homeostasis and regulate oxidative mediators (125). Recent studies demonstrated that melatonin activated the Nrf2/HO-1 pathway and increased levels of the antioxidant enzymes HO-1 and NAD(P)H dehydrogenase [quinone] 1 to prevent kidney damage caused by diabetes and exert neuroprotective effects (126, 127). Additionally, it has been noted that Nrf2 guarded cancer cells from ferroptosis brought on by erastin or RSL3 (128).

Researchers have found the NRF2/HO-1/GPX4 pathway had an impact on osteoblast. Ma et al. reported that activation the NRF2/HO-1 channel considerably lowers ferritin levels while reducing oxidative stress. NRF2 initiates the cellular peroxidation and defense process by activating the downstream enzymes glutathione peroxidase and superoxide dismutase (SOD). Additionally, it eliminates hazardous elements like ROS, and further reduce the toxic effects to osteoblasts (9, 129).

Furthermore, in ferroptosis, the antioxidant system Nrf-2/HO-1 could be suppressed. In the absence of Nrf-2, the activity and expression of the GPX4 protein is reduced and the severity of iron death is enhanced. It indicates that both the Nrf-2/HO-1 antioxidant system and iron death may be regulated under inflammatory settings (130). Additionally, researchers found the Nrf2/GPX4 pathway played an important role in age-related osteoporosis. Using 18 female wild type and 16 Nrf2-knockout (KO) mice as experimental subjects, Kubo et al. found that old Nrf2-specific KO mice showed reduced bone mass, which significantly implied that chronic Nrf2 deficiency made a great contribution to the progression of osteoporosis specifically in aging females (131). Yang et al. determined that 1,25(OH)2D3 can delay age-related osteoporosis via activating Nrf2 antioxidant signaling pathway and inhibition of oxidative stress, which provided support for the significant impact Nrf2 signaling pathway had on age-related osteoporosis (132, 133). Moreover, by evaluating the effect of 1,25(OH)2D3 on the Nrf2/GPX4 signaling pathway in MC3T3-E1 cells, other researchers also concluded that VDR activation inhibited osteoblast ferroptosis by activating the Nrf2/GPX4 signaling pathway, which indicates that there is a broad and profound link between the association of iron death and osteoblast (134).

5.2. NF-κB signaling pathways

To limit osteogenic development, nuclear factor κB (NF- κB) produces inflammatory molecules, suppresses Wnt signaling, and stimulates Smad and MAPK signaling pathways in osteoblasts. These changes caused by NF- κB mentioned above will ultimately activate ferroptosis (135, 136). Through its control over the production of a network of inducers and effectors that characterize responses to pathogens, NF-κB plays a crucial part in the cellular stress response as well as in inflammation (137). Inflammatory cytokines are released as a result of host defense mechanisms in reaction to inflammation, which activates the NF-κB pathway (138). Postnatal bone development requires BMPs, which also promote the expression of the matrix proteins osteocalcin and bone sialoprotein. Osteopenia, bone fragility, and spontaneous fracture are caused by a decrease in BMP activity (139, 140). The Wnt signaling system also promotes bone growth. When Wnt signaling is activated, β-nuclear catenin's expression rises, which in turn causes osteocalcin and bone sialoprotein to express more strongly. Inflammation inhibits Wnt signaling by increasing the expression of Wnt antagonists such as Dkk1 or sclerostin (82).

NF-κB regulates transcription positively in practically every conditions. The latest research has shown that NF-κB may interfere with the transcription of gene and chemokines were suppressed when noncanonical NF-κB subunits bound to the κB sites (141, 142). Interferon-b expression at the degree of promoter is directly suppressed by the activation of noncanonical NF-κB (143). Thus, RelB-p52 heterodimers were formed because of the noncanonical

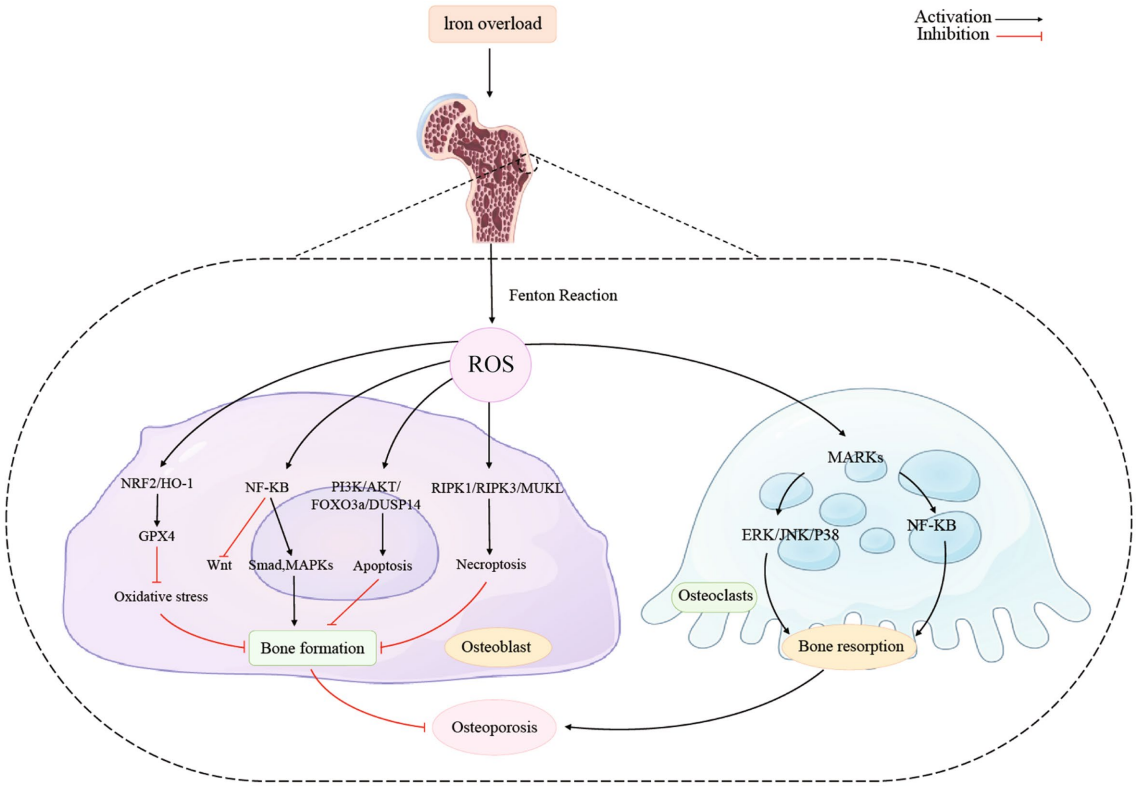

FIGURE 2
Iron overload and osteoporosis in osteoblast and osteoclast.

pathway activation, which caused NF-κB to have a detrimental impact. In Tarapore's study, the researchers discovered that NF-κB was crucial for the decreased production of matrix proteins brought on by inflammatory reactions, which eventually affected bone formation. Activation of NF-κB inhibits the production of matrix proteins both Wnt- and BMP-stimulated. This suppression entailed b-catenin and Runx2 inhibition by binding to neighboring consensus sites and NF-κB, to directly interacted with the involvement of response elements in the promoter regions of bone matrix proteins (144). Furthermore, Other studies also found that significant impacts of NF-κB on bone formation, by approving that it stimulates inflammatory factors and stimulates Smad and MAPK signaling pathways in osteoblasts to prevent osteogenic differentiation (107, 144, 145).

5.3. PI3K/AKT/FOXO3a/DUSP14

Iron overload significantly suppresses osteoblast proliferation and induces apoptosis through the PI3K/AKT/FOXO3a/DUSP14 channel, thus inhibiting bone formation in HG. It has been discovered that the PI3K/AKT signaling pathway contributes to signal transmission that is connected to cell proliferation, differentiation, invasion, and apoptosis (146). Specifically, researchers have reported that the proliferation and development of rat osteoblasts required activation of the PI3K/AKT signaling pathway (147).

The FOXO3a gene belonging to the FOXO subfamily. The transcription of FOXO3a is suppressed by pAKT, which regulates the phosphorylated process of FOXO3a. Members of the DUSP family are intimately connected to cellular proliferation as well. According to a prior study, DUSP4 promotes the growth and invasion of colorectal cancer cells. Xia et al. discovered that iron overload reduced osteoblasts growth and promoted apoptosis greatly through the PI3K/AKT/FOXO3a/DUSP14 channel (148). By noticing that the impact of iron overload in osteoblasts was greatly reduced by overexpressing DUSP14, their team demonstrated that through the inhibition of DUSP14 expression, iron overload may endanger the proliferation of osteoblasts. Additionally, iron overload enhanced p-AKT and p-FOXO3a expression in osteoblasts. FOXO3a could directly attach to the DUSP14 promoter and DUSP14 may therefore represent a unique element in the PI3K/AKT/FOXO3a pathway (149). In summary, PI3K/AKT/FOXO3a/DUSP14 signaling pathway is potentially in charge of cell defense in the presence of iron overload stress.

5.4. RIPK1/RIPK3/MLKL

In the iron overload-induced osteoblast apoptosis process, ROS could promote phosphorylation of RIPK1 and RIPK3 and create a positive vicious circle involving RIPK1/RIPK3/MLKL. Sufficient evidences suggest oxidative stress induced by iron overload is the primary factor in the pathophysiology of osteoporosis (150–152). It also appears that iron toxicity is intimately linked to cell death in illnesses from iron overload (153). Apoptosis and necrosis have been historically considered to be the two primary fundamental processes of cell death (154). ROS, as was already established, were crucial for

the apoptosis that was induced by iron overload in the osteoblasts. Nevertheless, Tian's research revealed that necrosis may also be strongly related to the characteristics of osteoblasts death from iron overload (155). Similar occurrences have been observed in earlier research, which indicated that necrosis may be the principal mechanism of cell death for osteoblastic cells in iron overload-associated bone disorders (156).

The precise mechanisms through which iron overload induces osteoblastic cells to necrotize remains not fully understood. An example of planned necrosis is necroptosis, which is distinguished by morphological variations of necrosis and is greatly reliant on regulating RIPK1, RIPK3, and MLKL. The phosphorylated MLKL eventually goes to the plasma membrane via oligomerization and penetrates, and then triggers necroptotic cell death (157, 158).

Tian's team firstly demonstrated how ROS were crucially regulated in iron overload-induced necroptosis and found that ROS brought on by iron overload encourage necroptosis by creating a positive feedback loop with the involvement of RIPK1/RIPK3. The results of Tian's study showed a dose-dependent rise in RIPK1 and RIPK3 phosphorylation as well as total protein expression in the osteoblastic cells following exposure to FAC. Nonetheless, following FAC treatment, the osteoblasts' protein expression of MLKL showed no appreciable change. The addition of Nec-1, GSK872, or NSA inhibited iron overload-induced necrotic cell death in osteoblasts. Their findings illustrated iron overload induced necroptosis in osteoblasts cells, at least partially through the RIPK1/RIPK3/MLKL pathway, and finally inhibited bone formation (155).

In summary, iron absorption, storage, and excretion abnormalities, together with the aberrant expression of iron-related proteins IRP2, FtMt, TFR1, TFR2, HEP, and ferritin ferroxidase, may result in alterations in iron content. Multiple signaling pathways like NRF2/HO-1, PI3K/AKT/FOXO3a/DUSP14, RIPK1/RIPK3/MLKL, and NF-κB, are warranted be explored more for the targeted interventions of the imbalanced bone remodeling process.

6. Iron-related signaling pathways and bone resorption

Osteoclasts are multinucleated large cells that are differentiated from bone marrow monocytes and come from the hematopoietic cell lineage (64). Two essential cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-B ligand (RANKL), affects the development of monocytes into osteoblasts. The cytokine M-CSF regulates the process by which BMMSCs differentiate into preosteoblasts and their proliferation, whereas RANKL controls the process by which preosteoblasts differentiate into osteoblasts and the activity of mature osteoblasts (159). Furthermore, cytokines such as tumor necrosis factor (TNF) and interleukin (IL) (160) could regulate the formation of osteoblasts (161). It was shown that RANKL was also linked to the recruitment of the non-receptor tyrosine kinase and tumor necrosis factor-associated receptor (TNFR) (162). c-Src acts to activate signaling pathways involved in osteoclast differentiation and maturation, such as NF-κB signaling pathway (163), and MAPK signaling pathway while TNFR acts to activate the Akt signaling pathway, which in turn induces the expression of nuclear factor of activated T-cell (NFATc). NFATc is the core transcription factor of osteoclasts, which ultimately mediates osteoclast differentiation, fusion and degradation of inorganic and organic bone matrix (164). The common signaling pathways for osteoblasts include OPG/RANKL/RANK, NF-κB, c-src-PIK3-AKT, MAPK, and CN-NFAT, all of which were approved crucial for controlling osteoclast development (165). However, the latest research revealed that the NF-κB signaling pathway and the MAPK signaling pathway were mostly responsible for T2DOP in the case of ferroptosis caused by HG conditions (20).

6.1. NF-κB signaling pathway

The intrinsic immune system's NOD, LRR, and pyrin domain-containing protein 3 (NLRP3) inflammatory vesicles recognize pathogens like viruses and bacteria, and activate inflammatory factors to mediate inflammation. It has been discovered that in osteoclasts, however, NLRP3 played a critical role in promoting osteoclast maturation and increasing bone resorption (166). A recent study showed that mice osteoclasts that expressed NLRP3 in particular did not undergo systemic inflammation. The amount of osteoclasts stayed the same, but the bone mass decreased by around 50% (165). The NLRP3 inflammasome performs a variety of tasks in both young and old persons. Bone loss in old mice lacking NLRP3 is increased through bone resorption rather than bone formation. Similarly, MCC950 inhibited osteoclast development by reducing caspase-1 activation, but not observed in young mice. Moreover, the transcription factor NF-κB, could encourage the production of molecules that control the development of inflammatory vesicles with the NLRP3 gene (163). And it's demonstrated that the ROS generated in the high glucose state led to the phosphorylation of MAPK-related proteins, which in turn activated the MAPKs pathway and subsequently the NF-κB pathway. This increased the expression of NLRP3 in the internal environment, which in turn promoted the maturation of osteoclasts and increased osteoclastic bone resorption (163).

6.2. ERK/JNK/p38 pathway

Three different signaling pathways of MAPK, MAPK kinase (MEK or MKK), and kinase of MAPK kinase (MEKK or MKKKK), make up the MAPK signaling system. Together, these three kinases that can be activated in any order, regulates a range of significant physiological and pathological reactions, including cellular development, differentiation, stress, and inflammatory responses (167). ERK, JNK, p38/MAPK, and ERK5 are the four primary branching points of the MAPK pathway. JNK and p38 have comparable roles in inflammation, apoptosis, and cell growth; and the ERK pathway primarily controls cell growth and differentiation; and Ras/Raf protein serves as its upstream signal. These kinases used in the branching route are all different and can be used as biomarkers in the pathway.

As a downstream branching pathway of the MAPK pathway, ERK/JNK/p38 pathway is another signaling pathway that might contribute to osteoporosis. Related studies have shown that the ERK/JNK/p38 pathway plays an important role in promoting the differentiation of preosteoclasts, promoting the survival of osteoclasts and inhibiting osteoclast apoptosis (164). In contrast, iron deficiency with moral hyperglycemia enhances the expression of ROS increases, which in turn increases the expression of RANKL,

thus promoting the ERK/JNK/p38 pathway for greater differentiation of pro-osteoclasts (164). This increases the bone resorption effect of osteoclasts, causing a disruption in the homeostasis of bone resorption and bone formation, which in turn leads to osteoporosis.

7. Therapeutic targets and drugs targeting iron metabolism for DOP

7.1. Preclinical monitoring: evaluating diabetes-specific risk factors for osteoporosis

In addition to the age-related risk factors and other established fracture causes, a comprehensive investigation of risk variables is required for the clinical examination of bone fragility in diabetes patients. Bone fragility is a distinct risk factor for fractures in both T1DM (168) and T2DM (169), and is substantially linked with the length of the condition. Individuals with T1DM are more likely to fracture more frequently and experience bone loss even when they are young (131, 170). Due to the fact that osteoporosis is a frequent complication of T1DM, DXA testing, and laboratory checks to identify additional risk factors, such as hypogonadism would be generally recommended by physicians. Hofbauer et al. advised testing for blood 25-hydroxyvitamin D (25[OH]D) in diabetics who were institutionalized (i.e., Living in a care facility such as a nursing home) or at risk of falls and fractures in order to identify a rapidly curable cause of falls and fractures. The initial bone assessment for determining fracture risk would also strongly be taken into consideration in the testing.

Poor glycaemia control was identified strongly associated with increased bone fragility, with a HbA1c threshold of more than 9% (75 mmol/mol) in individuals with T2DM and more than 9% (63 mmol/mol) in individuals with T1DM (171). Moreover, routine assessments should be made of hypoglycemic episodes, which can result in cardiovascular events, falls, and fractures in both type 1 (172) and type 2 (173) of diabetes. Consequently, it is advised to maintain stringent glycemic control in individuals who are younger and have the condition earlier. Strict glycaemia control's skeletal benefits in patients with long-term disease, diabetic comorbidities, and a history of falls must be weighed against the elevated risk of falls and cardiovascular events brought on by hypoglycemia. Currently, sulfonylureas and thiazolidinediones are used cautiously in patients at risk of fractures, metformin, glucagon-like peptide-1 (GLP-1) receptor agonists, SGLT2 inhibitors, and DPP-4 inhibitors exhibits a safe bone profile for type 2 diabetes (174, 175). Moreover, Metformin was found to lower the incidence of fractures in T2D patients and increased bone mass and bone quality in ovariectomized (OVX) rats. The underlying mechanism contained decreased RANKL expression and osteoclast inhibition (176, 177). Another study demonstrated that metformin limits bone marrow stromal stem cells' ability to produce succinate and lessens the stimulatory effects of succinate in promoting osteoclast development, and bone resorption (178); while a recent study reported that metformin usage did not increase BMD (179), and similar osteo-protective effect was also seen in non-diabetic OVX (180). Further studies are needed for the inconsistent findings in clinical practice.

7.2. General interventions and classic anti-osteoporosis drugs

This is a consensus that unless their serum 25(OH)D concentration is at least 20 ng/ml, all diabetics are recommended to take vitamin D supplements. In obese patients, calorie restriction to lower body weight is frequently used to halt the onset of diabetes mellitus; nevertheless, weight loss is considered to be linked to decreased bone mass. Thus, it is strongly advised that people with T2DM and obesity control their weight by carefully supervised exercise (181), which could strengthen bones aid patients with diabetes mellitus in preventing bone loss. According to a meta-analysis, people with T2DM who follow a Mediterranean diet rich in fresh fruits, vegetables, and fish have a lower incidence of fractures and microvascular sequelae (182). The unhealthy eating habits of high sugar and fat should be quitted. Also, bad lifestyle choices like drinking too much alcohol and smoking need to be carefully avoided.

Some clinical trials illustrated that alendronate (183) and teriparatide (184) displayed some therapeutic effects in diabetes mellitus through post-hoc analyses. According to Langdahl's study, teriparatide showed similar effects in lowering fracture risk for diabetic patients as general patients (185). Alendronate has been shown to reduce postmenopausal osteoporosis patients' fasting glucose and insulin resistance in preclinical diabetes mellitus (186). Dagdelen et al. found that alendronate had a more muted effect on increasing forearm BMD in postmenopausal osteoporosis patients with diabetes mellitus than in postmenopausal osteoporosis patients without diabetes mellitus, but it had no appreciable difference in effect on BMD in the hip and vertebrae between the two patient groups (187). Some studies proved that the mechanism of postmenopausal osteoporosis is also related to iron metabolism. For example, the postmenopausal spine may be protected against bone loss by dietary iron (188). Ni et al. suggested and tried to testify that an alternative method of treating postmenopausal osteoporosis might be to induce ferroptosis in osteoclasts by inhibiting Hypoxia-Inducible Factors (HIF-1) and ferritin (189). A recently created anti-osteoporosis medication called Romosozumab, the first sclerostin inhibitor licensed by the U.S. FDA, targets sclerostin, has demonstrated remarkable effectiveness in treating postmenopausal osteoporosis (190). Now that Picicca et al. demonstrated that diabetes caused osteocytes to alter over time and upregulate the sclerostin gene, we assumed that Romosozumab may be a very effective drug in treating DOP reasonably, which is also a potential research direction (85).

7.3. Pharmacological regulating iron metabolism and anti-ferroptosis therapies for DOP

To the best of our knowledge, there remain no randomized controlled trials examining the effectiveness and security of anti-ferroptosis medications in individuals with diabetes osteoporosis. And there are no specific medications to treat DOP currently, and many studies merely explored the potential treatment impact in animal tests, with many studies focusing on simply the prospective therapeutic targets. We know that the distance from animal studies to clinical trials is long and this would be a potential research direction, people are all looking forward to a potent drug for DOP. Figure 3 showed the

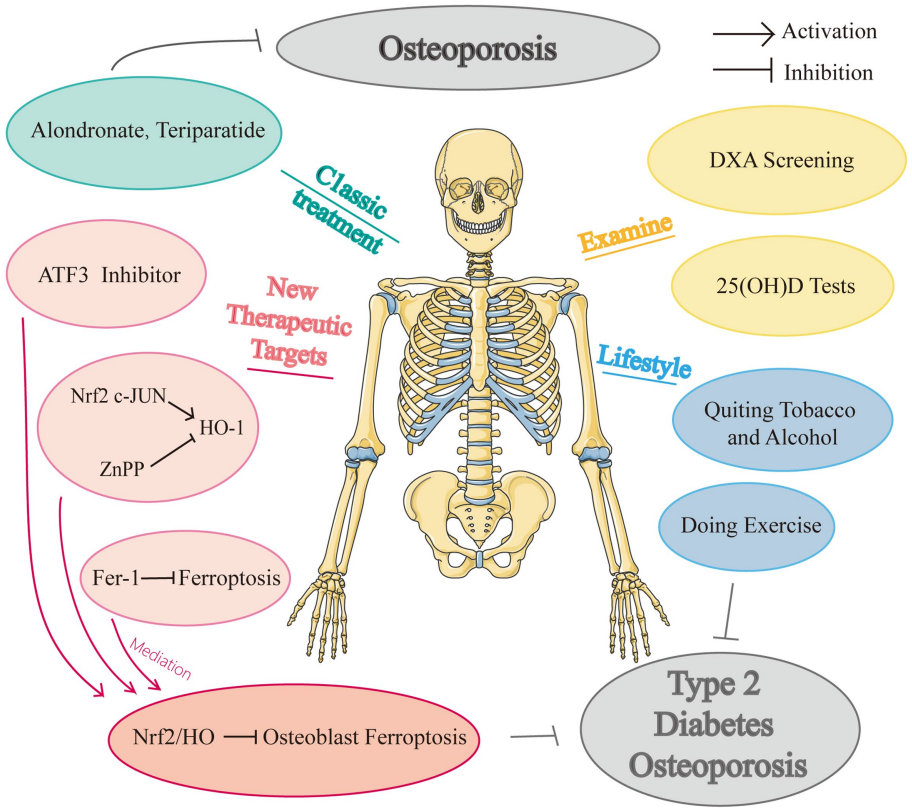

FIGURE 3
Drugs and potential therapeutic targets about iron metabolism in high glucose condition.

drugs and potential therapeutic targets about iron metabolism in high glucose condition.

Iron affects several phosphate and bone illnesses, as was previously indicated (191). Iron homeostasis should always be maintained for healthy cellular activity. Many studies have revealed that the major feature of ferroptosis is iron excess-induced aberrant iron metabolism. Increased iron intake decreased stable iron, and iron outflow would together induce ferroptosis. The six-transmembrane prostate epithelial antigen 3 (STEAP3) transforms ferric iron to ferrous iron when the Tfr1 on the cell membrane binds to circulating iron. DMT1 then releases divalent iron into the cytoplasm's labile iron pool (LIP). Notably, because of their significant LIP storage, lysosomes are considered to be the major organelles responsible for cellular ferroptosis, which shows potentially desirable potential disease targets (192).

Moreover, iron overload-induced liver ferroptosis in transferrin knockout mice is greatly reduced by both treatments with Fer-1 and hepatocyte-specific Slc39a14 deletion (193). Deferoxamine, an iron chelator, inhibits ferroptosis, has been demonstrated its clinical potential. Bordbar et al. found that in comparison to other regimens, combination therapy with Deferasirox and Deferoxamine had the greatest effect on lowering blood ferritin, despite its negligible value, and decreasing bone loss in the lumbar spine and femoral neck (194). Accordingly, Fer-1, was found to be an effective ferroptosis inhibitor because of its ability to scavenge lipid (195). Emerging studies have indicated that ferroptosis is involved in metabolic disease, cardiomyopathy, neurodegeneration, ischemia–reperfusion injury, and the effects of cancer (89, 196). Targeting ferroptosis may be an effective strategy for treating DOP.

Yang et al. applied a mouse model of DOP and established the critical involvement of ferroptosis in DOP-induced osteocyte death both *in vivo* and *in vitro* (10). The increased expression of HO-1 caused intracellular iron overload and heme breakdown, which subsequently triggered the oxidation of lipids. For this mechanism to work, nuclear factor-like 2 and c-direct JUN's binding were required. Furthermore, inhibiting ferroptosis greatly reversed trabecular degeneration and osteoclast death. Iron atrophylinkage and HO-1 activation are causally connected and may result in a self-feeding vicious cycle. These all offered prospective therapeutic targets for upcoming DOP therapy plans: ZnPP (an HO-1 inhibitor) and Fer-1. Intriguingly, treatment with Fer-1 consistently had a higher therapeutic outcome than that with ZnPP, indicating that using Fer-1 to scavenge intracellular lipid peroxides may be a more effective treatment plan for DOP. Furthermore, their study showed ZPP and Fer-1 therapy in diabetic mice also prevented lacunar emptying and osteocyte death in addition to restoring trabecular balance. In conclusion, stopping the ferroptosis pathway could prevent DOP and osteocyte mortality in diabetic mice.

In addition, System Xc, an amino acid antiporter that is made up of two subunits of the xCT light chain (catalytic subunit, encoded by the SLC7A11 gene), and the heavy chain (chaperone subunit, encoded by the SLC3A2 (197, 198)), mediates the exchange of extracellular cystine and intracellular glutamate on the cell membrane. The expression level of SLC7A11 is typically positively correlated with the

activity of the antiporter, playing a critical role in preventing ferroptosis caused by lipids. Because the light chain encoded by SLC7A11 is responsible for the primary transport activity, and the heavy chain subunit SLC3A2 primarily serves as a chaperone protein (19). Several research have demonstrated the therapeutic benefits of melatonin, which is a strong endogenous antioxidant. Thus, if melatonin may neutralize ROS, could this be a possible method by which melatonin treats DOP? Ma's study might provide the solution (9). It has been demonstrated that melatonin can enhance bone microstructure both *in vivo* and *in vitro* by inhibiting osteoblasts' ability to ferroptosis in which melatonin lowered ROS levels, elevated SLC7A11 levels, and boosted GPX4 activity by opening the NRF-2/HO-1 antioxidant channel Also, it reduced the toxicity of lipid peroxides to shield the biofilm system from ferroptosis, enhancing osteoblast 's capacity for osteogenesis and bone microstructure (9). Another study discovered that melatonin can inhibit the ERK signaling pathway and lower osteoblast autophagy levels, delaying the pathological development of DOP (199).

In other disease-related osteoporosis, such as postmenopausal osteoporosis, there are some specific medicines or therapeutic schedules. For example, some researchers suggested that some special types of osteoporosis, anti-resorptive medication should be used after anabolic therapy, similar to the therapeutic sequence used to treat common osteoporosis (200). And the best BMD improvements were seen in postmenopausal women with osteoporosis who received these sequential medications in this order in clinical studies (201). However, this has not yet been established for those with diabetes, and there is no clinical trial to prove this therapy (190). But this can be a potentially effective treatment option. According to Zhang et al. (93), postmenopausal osteoporosis is prevented by hepcidin-induced reductions in iron concentration and PGC-1 expression, which adversely affect osteoclast differentiation, so maybe hepcidin can play a role in treating DOP in the future?

8. Conclusion and outlooks

Long-term, poorly controlled diabetes commonly culminates in diabetic bone disease with fragility fractures, which has a considerable impact on socioeconomic and public health burdens. The advent of knowledge of the biological mechanisms and implicated pathways, coupled with improved multiscale imaging of bone, has made it feasible to gain new insights into the increased bone fragility in diabetes at many levels. In this review, we systematically summarize the diverse mechanism and pathways of ferroptosis in osteoblasts, osteoclasts, and other key cells, and attempt to comprehend the regulatory targets of interventions and treatments in clinical practice, applying the identified biomarkers as guides, aiming to highlight the near-term opportunities to elaborate the execution mechanisms and targeted therapeutics of iron metabolism and ferroptosis to T2DOP. For further research, it is necessary to clarify the diagnostic criteria for DOP in patients of varying ages and disease trajectories, and to reach a consensus. Although there have been some studies exploring the mechanism of iron metabolism and ferroptosis in diabetic bone loss, the mutual effect among these key proteins and pathways remains unclear, and the relative importance of each mechanism in the development of diabetic osteoporosis has not been explored, which is meaningful to find key therapeutic targets. Finally, there is no specific medicine to treat diabetic patient with osteoporosis, therefore developing new treatment strategy for patients with DOP is promising and significant. Advances in iron metabolism and ferroptosis are particularly noteworthy.

Author contributions

HY, ZZhao, and JB contributed to conception and design of the review. JB, YY, DZ, ZZhuo, TS, HL, and ZH performed the literature search, drafted the manuscript, and constructed the figures and tables. HY and ZZhao reviewed 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.

Publisher's note

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Are (poly)phenols contained in 100% fruit juices mediating their effects on cardiometabolic risk factors? A meta-regression analysis

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Background: The consumption of 100% fruit juices has not been associated with substantial detrimental outcomes in population studies and may even contribute to improving the cardiometabolic profile if included in a healthy balanced diet. The main contributors to such potential beneficial effects include vitamins, minerals, and likely the (poly)phenol content. This study aimed to investigate whether the (poly)phenols contained in 100% fruit juices may mediate their effects on cardiometabolic risk factors based on published randomized controlled trials (RCT).

Methods: A systematic search in PubMed/MEDLINE and Embase, updated till the end of October 2022, was carried out to identify RCT providing quantitative data on (poly)phenol content in 100% fruit juices and used as an intervention to improve cardiometabolic parameters such as blood lipids, glucose, and blood pressure. Meta-regression analysis was performed to calculate the effect of the intervention [expressed as standardized mean difference and 95% confidence intervals (CI)] using the (poly)phenol content as moderator.

Results: A total of 39 articles on RCT investigating the effects of 100% fruit juices on cardiometabolic risk factors reporting data on total (poly)phenol and anthocyanin content were included in the analysis. Total (poly)phenol content was substantially unrelated to any outcome investigated. In contrast, each 100mg per day increase in anthocyanins was related to 1.53mg/dL decrease in total cholesterol (95% CI, −2.83, −0.22, p=0.022) and 1.94mg/dL decrease in LDL cholesterol (95% CI, −3.46, −0.42, p=0.012). No other potential mediating effects of anthocyanins on blood triglycerides, glucose, systolic and diastolic pressure were found, while a lowering effect on HDL cholesterol after excluding one outlier study was observed.

Discussion: In conclusion, the present study showed that anthocyanins may mediate the potential beneficial effects of some 100% fruit juices on some blood lipids. Increasing the content of anthocyanins through specific fruit varieties or plant breeding could enhance the health benefits of 100% fruit juices.

KEYWORDS

polyphenols, anthocyanins, fruit juice, blood preasure, blood lipids, blood glucose, metabolic

1. Introduction

Over the last decades, dietary (poly)phenols have been a focus of major interest due to their potential health benefits. This heterogeneous group of molecules is widely spread in the plant kingdom and is commonly found in fruits and vegetables. Depending on their chemical structure, they can be classified as flavonoids and non-flavonoids as the two major groups, but there is a great variety of molecules with many diverse properties and functions within each family (1). Regarding research on humans, there is growing evidence from observational studies showing a lower risk of incidence and mortality from cardiovascular diseases (CVD) associated with higher intakes of total and major classes of flavonoids, such as flavonols, flavones, flavanones, anthocyanins, and flavan-3-ols (2, 3). Epidemiological evidence suggests that higher dietary intakes of flavonoids, particularly from fruits, may have beneficial effects on the risk of CVD incidence and mortality (2, 3), type 2 diabetes incidence (4), and hypertension (5). One of the key dietary sources of flavonoids is fruit, and a recent meta-analysis reports a 10% lower risk of CVD with each 100 g/day increased fruit intake, peaking at 300 g/day for ischemic heart disease risk (6). Moreover, studies on specific fruits, such as citrus and berries, reported stronger positive associations for cardiovascular prevention (7). The observed evidence may provide a rationale for including (poly)phenol-rich foods and beverages in the recommended diet as a potential strategy to prevent CVD.

The consumption of 100% fruit juices is generally considered a secondary choice compared with whole fruits. One reason for this view is the loss of fiber when juice is extracted from the fruit (8). Country-specific dietary guidelines vary considerably regarding the place of 100% fruit juices in a healthy balanced diet, ranging from advice to avoid them to counting one daily serving of juice as a serving of fruit (9). However, since compliance with whole fruit consumption in the general population is relatively low (10), the consumption of 100% fruit juices could still be considered an appealing and cost-effective alternative when whole fruit consumption is not possible. Moreover, previous studies showed that overall fruit and vegetable consumption contributes up to one third of daily fiber intake in low whole-grain consumers, but reaches around one fifth in adequate whole-grain consumers (11–13). This suggests that the major contribution to dietary fiber is not fruit but other dietary sources, such as whole grains. Hence, it is possible that components of fruits other than dietary fiber may play a role in preventing CVD.

Another reason why 100% fruit juice is viewed as an inferior choice to whole fruit is associated with the classification of the natural sugars in fruit juices, but not whole fruits, as free sugars (14). Dietary recommendations suggest limiting free sugars regardless of their source in the diet. While there is undisputed evidence that high consumption of sugar-sweetened beverages, a major source of free sugars, is detrimental to metabolic health and body weight control (15), a meta-analysis of randomized controlled trials (RCT) revealed that higher consumption of 100% fruit juices does not increase the risk of cardiometabolic risk factors. On the contrary, the study reported null effects on body weight, blood lipids, and glucose metabolism, while a beneficial effect toward blood pressure and arterial compliance was found (16). However, no data have been reported on potential components of 100% fruit juices that may exert possible beneficial effects, or at least counterbalance the presence of free sugars. With this hypothesis, we aimed to explore whether the content of (poly)phenols may mediate or modify the effects of 100% fruit juices on major cardiometabolic risk factors in dietary intervention trials.

2. Methods

2.1. Systematic search and study selection

A systematic search for all studies examining interventions with (poly)phenol-containing 100% fruit juices and their effects on cardiometabolic biomarkers was performed using PubMed/MEDLINE and Embase from their inception until the end of August 2022 and updated till the end of October 2022. The search strategy was based on combining the relevant keywords related to 100% fruit juices and cardiometabolic risk factors used in combination as MeSH terms and text words (Supplementary Table 1). Reference lists of eligible studies were also examined for any additional studies not previously identified. If more than one study reporting results from the same trial was retrieved, only the study including the most comprehensive data was included in the meta-regression analysis. Studies that provided insufficient statistical data were excluded. The systematic search and study selection were performed by two independent authors (A.M. and J.G.). The design, analysis, and reporting of this study followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Studies were eligible if they met the following inclusion criteria: (i) randomized controlled trials with the independent-group pre-test–post-test design reporting on the changes in cardiometabolic risk factors (blood pressure, blood lipid profile, and blood glucose levels); (ii) studies evaluating the effect of the intervention with 100% fruit juice; (iii) studies reporting the (poly)phenolic content of the 100% fruit juice; (iv) studies with control drinks not containing (poly)phenols; (v) studies exploring long-term effects of the intervention (at least 1 week); and (vi) studies reporting on adult populations and not reporting on patients with end-stage degenerative diseases, or on pregnant women. The study protocol was registered in the PROSPERO International Prospective Register of Systematic Reviews database (ID number: CRD42022339493).

2.2. Data extraction and quality assessment

Data from all included studies were extracted using a standardized electronic form. The following information was collected: first author name, publication year, study design and location, population age and gender, sample size and intervention duration, type of intervention and its main characteristics (including phenolic content), type of comparator, details on the outcome of interest, and measures needed to calculate size effects for each intervention at the beginning and at the end of the trial. The Cochrane risk of bias tool was used to evaluate the quality of included studies (17). Two investigators assessed the methodological quality independently, and any incongruity was resolved by consensus (A.M. and J.G.).

2.3. Statistical analyses

Analyses were conducted separately on studies with different cardiometabolic risk factor measurements and for different (poly) phenol types. Within each measurement, all results were converted into standard units, and mean differences (MD) of pre-post changes between the two intervention groups (juice vs. control) were calculated. In reports which fail to provide sufficient data for computing effect size estimates properly accounting for the paired nature of the design, the correlation between measurements before and after each intervention was imputed at level 0.5, whereas the correlation of change-from-baseline measures between active and placebo treatment periods was set at 0 (18). Finally, effect sizes were harmonized using a random-effects model with DerSimonian and Laird estimator of between-study variance. Heterogeneity was assessed by the I^2 statistic and formally complemented by the Cochran Q-test under a level of significance set at 0.1. Publication bias was verified by visual inspection of funnel plots for asymmetry and by quantitative method, Egger's regression test. Pooled results were reported as MD with 95% confidence intervals (CI) with two-sided p values, with values of p less than 0.05 considered statistically significant. To then verify whether the retrieved effects were associated with the (poly) phenol content in 100% fruit juices, the daily amount of (poly)phenol [meaning (poly)phenol content in the intervention arm] was treated as a moderator of the association between intervention and cardiometabolic risk factors measurements. Therefore, meta-regression analyses were conducted with the total and specific (poly) phenol content in the intervention arm additionally incorporated into the models. The significance and sign of the slope coefficient of the meta-regression line were tested to show the direction and strength of the dose–response relationship. A sensitivity analysis was conducted to assess the stability of results by testing alternative models excluding one study each time and pooling estimates for the rest of the studies. Subgroup analyses by health status of participants were performed. All analyses were performed with R software (Development Core Team, Vienna, Austria, version 4.0.4).

3. Results

3.1. Main characteristics of the included studies

Figure 1 shows the process of the literature search and selection. The initial database search identified 6,779 potential articles, of which 6,616 were excluded based on title and abstract assessment leaving 163 articles. After full-text examination, 124 articles were identified as ineligible based on one or more of the following reasons: (i) inadequate intervention, (ii) inadequate or lack of comparator, (iii) reporting on acute effects, (iv) providing insufficient statistics, (v) not providing phenolic content of the intervention, and (vi) inadequate study design. Finally, 39 articles met the inclusion criteria (19–57).

The main characteristics of the 25 parallel (19–24, 26, 29, 30, 32, 35, 36, 38, 39, 41, 43–45, 48, 50, 51, 54–57) and 14 crossover (25, 27, 28, 31, 33, 34, 37, 40, 42, 46, 47, 49, 52, 53) RCT on 100% fruit juices and cardiometabolic outcomes are provided in Table 1. Eligible studies reported on the following juices: pomegranate, cranberry, tart cherry, Concord grape, blueberry, blood orange, chokeberry, bayberry, strawberry, blackcurrant, *Aronia melanocarpa*, plum, and mixed berry juices. Studies conducted on other 100% fruit juices (i.e., orange juices) did not provide enough data to actually perform analyses on specific (poly)phenol contained (i.e., flavanones and flavan-3-ols) while available data was retrieved for anthocyanins. Included studies involved adult participants, being at low and high cardiovascular risk. The intervention duration varied from 1 to 16 weeks. Most of the trials provided measures on more than one of the investigated outcomes, including blood pressure ($n = 32$), blood lipids ($n = 32$), and blood glucose levels ($n = 27$). The risk of bias assessment showed that when considering overall risk of bias, the majority of the studies were subjected unclear risk of bias (Supplementary Figures 1, 2).

3.2. Effect of total (poly)phenol content in 100% fruit juices on cardiometabolic biomarkers

A total of 24–27 comparisons (7–9 from crossover design and 15–20 from parallel design studies, depending on the outcome) were included in the analysis (Table 2). No significant effects of 100% fruit juice intervention on cardiometabolic biomarkers were detected after the meta-analysis of included studies, nor any potential mediating effect of total (poly)phenol content in the intervention groups (Figure 2; Supplementary Figure 3). In some subgroup analyses, a borderline protective activity of juices was observed toward lowering the concentration of LDL cholesterol (LDL-C) in high CVD risk individuals (MD = −3.93, 95% CI: −8.30, 0.43, $p = 0.077$) and significant effect on triglycerides (TG) in individuals with high CVD risk (MD = −9.52, 95% CI: −16.28, −2.76, $p = 0.006$), in trials lasting at least 6 weeks (MD = −6.80, 95% CI: −12.31, −1.28, $p = 0.016$): however, these effects were not related to the total (poly)phenol content (Supplementary Table 2).

Overall, none of the cardiometabolic biomarkers showed an asymmetrical pattern in the funnel plot that might be indicative of publication bias (Supplementary Figure 4).

3.3. Effect of anthocyanin content in 100% fruit juices on cardiometabolic biomarkers

Eighteen comparisons from randomized clinical trials with repeated measures and parallel design (19–21, 23, 24, 26, 32, 35, 36, 39, 41, 45, 48, 55, 57) and 10 from crossover design (27, 34, 37, 40, 42, 46, 47, 49, 52, 53) depicted the effect of anthocyanins contained in 100% fruit juices on total cholesterol concentration. An overall influence of juice intervention on lipid measurement, regardless of dose anthocyanins consumed with juice, was significant (MD = −4.62, 95% CI: −8.51, −0.72, $p = 0.020$; Table 3; Supplementary Figure 5). The relationship was dose-dependent with the stronger effect for juices containing larger amounts of anthocyanins: each 100 mg/day increase in anthocyanin content was accompanied with 1.53 mg/dL decrease in total cholesterol (95% CI: −2.83, −0.22, $p = 0.022$ per Δanth = +0.1 g/day; Table 3; Figure 3). Introducing the moderator to analysis reduced heterogeneity from 22.6 to 10.4%. Subgroup analysis revealed a beneficial effect of consumption of anthocyanin-rich juice compared to control drink in crossover design studies (MD = −6.67, 95% CI: −11.43, −1.92, $p = 0.006$) with no further effect of

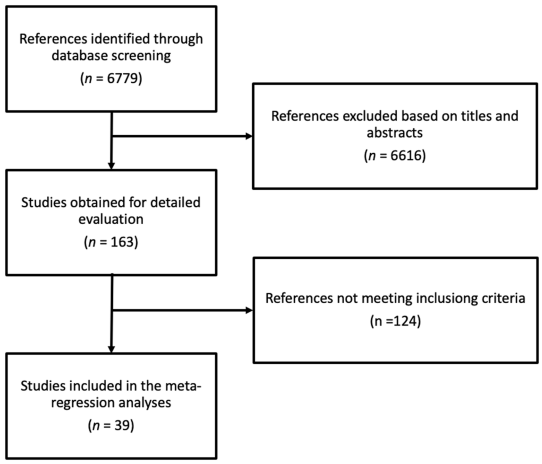

FIGURE 1
Flow chart of study selection process.

anthocyanin content (Supplementary Table 3). On the contrary, in parallel studies, anthocyanin content mediated the juice activity, enhancing the decline of lipid marker levels (ΔMD = −3.35; 95% CI: −5.41, −1.29, $p = 0.001$ per Δanth = +0.1 g/day). Both the overall effects of juice consumption and the effects of anthocyanin content were significant for trials lasting less than 6 weeks (Supplementary Table 3). Moreover, the exclusion of one trial (40), which was identified as an influential point in meta-regression, resulted in a significant slope (ΔMD = −3.55; 95% CI: −6.50, −0.61, $p = 0.018$) in the analysis of crossover studies, confirming additional benefit from the consumption of juices richer in anthocyanins (Supplementary Table 4).

There was no evidence of an effect of 100% fruit juices rich in anthocyanins on HDL cholesterol (HDL-C) concentration (Table 3; Figure 3) based on 28 comparisons from RCT [nine from crossover (27, 34, 40, 42, 46, 47, 49, 52, 53) and 19 from parallel design studies (19–21, 23, 24, 26, 32, 36, 39, 41, 45, 48, 51, 55, 57)] for total (MD = 0.97, 95% CI: −0.96, 2.90, $p = 0.324$; $I^2 = 76.9\%$; Supplementary Figure 5) and subgroup analyses (Supplementary Table 3). In the subgroup analysis by study design, the exclusion of one influential study (40) reduced unexplained heterogeneity between crossover trials to a low level ($I^2 = 26.9\%$) and showed the significant impact of anthocyanins content toward raising the concentration of HDL cholesterol (Supplementary Table 4): the larger amount of anthocyanins in 100% fruit juice was associated with an additional increase in MD between the intervention and the placebo of 1.59 (MD = 1.81, 95% CI: −1.02, 4.64, $p = 0.210$ overall effect and ΔMD = 1.59, 95% CI: 0.34, 2.84, $p = 0.013$ per Δanth = +0.1 g/day).

Twenty-four comparisons [nine from crossover (27, 34, 37, 42, 46, 47, 49, 52, 53) and 15 from parallel design studies (19–21, 23, 24, 26, 39, 41, 45, 48, 51, 55, 57)] were included in the analysis verifying the influence of 100% fruit juice interventions on LDL-C levels. A marginally significant protective activity of 100% fruit juice consumption was observed (MD = −3.97, 95% CI: −7.97, 0.03, $p = 0.052$) with moderate heterogeneity between trials ($I^2 = 34.2\%$; Table 3; Supplementary Figure 5). The higher amounts of anthocyanins enhanced the LDL cholesterol-lowering effect, which was manifested by a further decline of MD between 100% fruit juice and comparator of −1.94 mg/dL (95% CI: −3.46, −0.42, $p = 0.012$) of LDL cholesterol with each 0.1 g/day increase in the dose of anthocyanins (Figure 3). Simultaneously, a reduction of heterogeneity to 16.5% after introducing the moderator to a model was noted. Subgroups analysis showed a significant dose-dependent impact of juices rich in anthocyanins on LDL cholesterol in studies examining subjects with low CVD risk (ΔMD = −2.72, 95% CI: −4.59, −0.85, $p = 0.004$ per Δanth = +0.1 g/day), trials with follow up shorter than 6 weeks (ΔMD = −2.63, 95% CI: −4.44, −0.83, $p = 0.004$ per Δanth = +0.1 g/day), and the marginally significant result was detected in both crossover trials (ΔMD = −3.44, 95% CI: −6.99, 0.11, $p = 0.058$ per Δanth = +0.1 g/day) and parallel trials (ΔMD = −1.69, 95% CI: −3.39, 0.01, $p = 0.051$ per Δanth = +0.1 g/day; Supplementary Table 3).

Twenty-six comparisons (10 from crossover (27, 34, 37, 40, 42, 46, 47, 49, 52, 53) and 16 from parallel design studies (19–21, 23, 24, 26, 32, 39, 41, 45, 48, 51, 55, 57)) were included in the analysis verifying the influence of 100% fruit juice interventions on TG concentration. A significant impact of juice consumption on TG measurement favoring intervention against the control drink was found, as evidenced by a 9.55 mg/dL larger decrease (MD = −9.55, 95% CI: −13.52, −5.57, $p < 0.001$) in TG during follow-up (Table 3; Supplementary Figure 5), however, with no further effect of anthocyanin content in the beverages (ΔMD = −0.60, 95% CI: −2.07, 0.88, $p = 0.429$ per Δanth = +0.1 g/day; Figure 3). Subgroup analysis showed a significant dose-dependent impact of juices rich in anthocyanins on TG in trials with a follow up <6 weeks (ΔMD = −3.68, 95% CI: −6.82, −0.54, $p = 0.022$ per Δanth = +0.1 g/day). The overall effect of juice was protective toward lowering TG for studies lasting longer than 6 weeks, in both crossover and parallel studies, however, with no further impact of anthocyanin content (Supplementary Table 3). Moreover, after the exclusion of one study (40), meta-regression resulted in a marginally significant slope (ΔMD = −2.24; 95% CI: −4.56, 0.07, $p = 0.058$ per Δanth = +0.1 g/day) showing a tendency toward a stronger impact of juices higher in anthocyanins (Supplementary Table 4).

Twenty-four comparisons [10 from crossover (27, 28, 34, 37, 40, 42, 46, 47, 49, 53) and 14 from parallel design studies (19, 21, 23, 24, 26, 29, 39, 41, 48, 51, 55, 57)] tested the effect of juice interventions on glucose concentration. No evidence of the impact of 100% fruit juices rich in anthocyanins on glucose measurement was detected in the total sample of studies (Table 3; Supplementary Figure 5) nor in subgroup analyses (Supplementary Table 3). In the sensitivity analysis, after the exclusion of the influential study of (40), higher anthocyanin content was associated with a decrease in blood glucose in crossover design trials (ΔMD = −1.89, 95% CI: −3.60, −0.18, $p = 0.030$ per Δanth = +0.1 g/day; Supplementary Table 4).

Finally, there was no evidence of the effect of 100% fruit juices rich in anthocyanins on blood pressure based on 29 comparisons from RCT [10 crossover (27, 33, 37, 40, 42, 46, 47, 49, 52, 53) and 19 parallel design (23, 24, 26, 29, 32, 35, 36, 39, 41, 48, 50, 51, 55–57); Table 3;

Supplementary Figure 5] nor in subgroup analyses (Supplementary Table 3).

In the case of cholesterol, HDL and triglycerides signs of an asymmetrical pattern in the funnel plot that might be indicative of publication bias was detected (Supplementary Figure 6).

4. Discussion

In this study, we attempted to investigate the role of (poly)phenol content in relation to 100% fruit juice consumption and cardiometabolic risk factors through a meta-regression analysis of RCT. The results showed no significant role of total (poly)phenols in any outcomes investigated. However, a higher content of anthocyanins in 100% fruit juices significantly increased the lowering of total cholesterol and LDL cholesterol; the mediating effects seemed to be stronger in studies that included individuals at high CVD risk (i.e., with metabolic syndrome or multiple cardiovascular risk factors), with a potential additional significant effect also on HDL cholesterol when excluding an outlier study. No further effects were detected on TG, blood glucose or blood pressure. This study adds another dimension to the scientific literature and suggests that (poly)phenols should be taken into account in future dietary intervention trials of 100% fruit juice consumption.

Numerous observational and intervention studies have been conducted to identify the potential impact of 100% fruit juice consumption on such biomarkers, often reporting contrasting results (16). No substantial harm concerning blood glucose and obesity risk has been observed, while a potential protective effect (or an inverse association) was found for blood pressure and the risk of CVD (8). Compared to previous meta-analyses (16) an effect of 100% fruit juice consumption and blood pressure could not be found, probably due to the smaller number of studies included with available data on (poly) phenol content. It has been suggested that the beneficial effects on such cardiometabolic outcomes are related to the potassium content of 100% fruit juices, as this mineral may affect blood pressure and lower the risk of stroke (58, 59). However, none of the research conducted up to date explored the potential mediating effect of other bioactive components in 100% fruit juices, such as (poly)phenols.

Although, in this study, we were not able to demonstrate the role of (poly)phenols in the association between 100% fruit juices and blood pressure or any other outcome, we found that anthocyanins may be potential mediators of improvements in blood lipids in RCT administering 100% fruit juices. Other meta-analyses showed that purified anthocyanin and anthocyanin-rich berry supplementation could significantly reduce blood LDL cholesterol and increase HDL cholesterol (60–63). Moreover, a recent umbrella review concluded that anthocyanins improved plasmatic lipids, glucose metabolism, and endothelial function, without affecting blood pressure in RCT (64). Hence, current evidence is consistent with our findings, suggesting a substantial role of anthocyanins in the observed effects related to 100% fruit juice consumption.

The rationale behind the potential positive effects of (poly)phenols and, specifically, anthocyanins in 100% fruit juices, is supported by the extensive share of scientific literature providing a variety of potential mechanisms. Several preclinical studies conducted *in vitro* or on animals show that (poly)phenols (such as anthocyanins cyanidin-3-glucoside and peonidin-3-glucoside and their metabolites) may affect cellular antioxidant status and inflammation by increasing endogenous antioxidant defenses through activation of genes encoding antioxidant enzymes and modulating various inflammatory pathways (i.e., nuclear factor, erythroid 2–like 2, NF-kB, etc.) (65, 66). Moreover, clinical studies suggest that anthocyanins may improve blood lipid profile by increasing reverse cholesterol transport, regulating HDL functionality, increasing HDL antioxidant capacity, and HDL cholesterol efflux capacity, whereas reducing HDL lipid hydroperoxides (67). Finally, an emerging and growing body of literature is further investigating the role of (poly)phenols and their metabolites on gut microbiota and its potential mediating role on inflammation and prevention of non-communicable diseases (68, 69).

Concerning the comparison between whole fruits and 100% fruit juice, the lack of fiber in the latter is generally considered a limitation from a nutritional point of view. However, the health benefits of fruit appear to go beyond its fiber content, and may instead depend on its overall mineral, vitamin, and possibly (poly) phenol content (70). Only recently, increased attention has been given to the (poly)phenol content of 100% fruit juices as a potential mediator of their health effects (71). A direct comparison of the bioavailability of phenolic compounds in whole fruit and 100% fruit juice suggests that the liquid matrix and lower pectin content of 100% fruit juices could allow for higher intestinal (poly)phenol absorption compared with the solid matrix and higher pectin content of whole fruit (72, 73). Indeed, (poly)phenols are released after a series of mechanical and chemical processes to break down food structure. The ingested molecules in the small intestine are only a small fraction, while the vast majority reach the colon and follow a substantial transformation by the gut microbiota into small-molecular-weight phenolic metabolites, which are ultimately absorbed and further conjugated (74, 75) The whole process seems to be influenced by the food matrix, since the bioavailability of (poly)phenols in whole fruit can be affected by interaction with complex structures (i.e., cell wall or biopolymer interactions), while those in 100% fruit juices might be more easily absorbed even in the small intestine (76). However, it is still unclear what happens to the non-digestible fraction of (poly)phenols reaching the colon and how that affects the gut microbiota and the production of metabolites further absorbed, which could potentially mediate the effects on human health.

There are limitations of the present study that should be considered. First and foremost, data on the (poly)phenol content of 100% fruit juice were available only in a minority of studies. Thus, the overall size effects estimated in the present study may not reflect the entirety of published RCT. However, the aim of this study was not to establish the effects of 100% fruit juice consumption and cardiometabolic risk factors, which have been considered elsewhere (16), but rather to test whether their (poly) phenol content could be considered a mediator for the retrieved effects (in available studies). Second, while the content of a specific (poly)phenol class (i.e., anthocyanins) is more straightforward to compare, the total (poly)phenol content may include a different proportion of the various (poly)phenol classes; given the large variety in chemical composition, pharmacokinetic properties, and mechanisms of action characterizing the different (poly)phenol classes, this approach may not be optimal to determine which bioactive components of 100% fruit juice may be mediating the observed effects on health. Third, related to the

TABLE 1 Main characteristics of the randomized clinical trials on 100% fruit juices and cardiometabolic outcomes.

Author, year, country	Participant characteristics	Sex; mean age	Design	Duration of intervention	Intervention	Comparison	Intervention juice phenolic content (concentration)	Outcomes of interest
Cerdá (19), Spain	30 patients with chronic obstructive pulmonary disease	M; I: 60 years, C: 63.4 years	Parallel	5 weeks	Pomegranate juice (400 mL/day)	Placebo beverage (400 mL/day)	Polyphenols: 6,650 mg/L Anthocyanins: 475 mg/L	LP, GP
Duthie (20), Scotland	20 healthy volunteers	F; I: 27.3 years, C: 28.3 years	Parallel	2 weeks	Cranberry juice (750 mL/day)	Placebo beverage (750 mL/day)	Polyphenols: 1,136 mg/L Anthocyanins: 2.80 mg/L	LP
Hollis (21), United States	51 overweight subjects	MF; I: 22 years, C: 26 years	Parallel	12 weeks	Concord grape juice (480 mL/day)	Placebo beverage (480 mL/day)	Polyphenols: 1,945 mg/L Anthocyanins: 398 mg/L	LP, GP
Park (22), Korea	40 subjects with borderline hypertension	M; I: 43 years, C: 46 years	Parallel	8 weeks	Concord grape juice (5.5 mL/kg/day)	Placebo beverage (5.5 mL/kg/day)	Polyphenols: 2,108 mg/L	LP, BP
Basu (23), United States	48 obese individuals with metabolic syndrome	MF; I: 51.5 years, C: 48 years	Parallel	8 weeks	Blueberry juice (960 mL/day)	Water (960 mL/day)	Polyphenols: 1,692 mg/L Anthocyanins: 773 mg/L	LP, GP, and BP
Basu (24), United States	27 subjects with metabolic syndrome	MF; I: 48 years, C: 45 years	Parallel	8 weeks	Strawberry juice (960 mL/day)	Water (960 mL/day)	Polyphenols: 2,089 mg/L Anthocyanins: 160 mg/L	LP, GP, and BP
Dohadwala (25), United States	64 patients with prehypertension and stage 1 hypertension	MF; 43 years	Crossover	8 weeks	Concord grape juice (7 mL/kg/day)	Placebo beverage (7 mL/kg/day)	Polyphenols: 1,970 mg/L	LP, GP, and BP
Basu (26), United States	31 subjects with metabolic syndrome	F; 52 years	Parallel	8 weeks	Cranberry juice (480 mL/day)	Placebo beverage (480 mL/day)	Polyphenols: 954 mg/L Anthocyanins: 52 mg/L	LP, GP, and BP
Dohadwala (27), United States	44 patients with coronary artery heart disease	MF; 62 years	Crossover	4 weeks	Cranberry juice (480 mL/day)	Placebo beverage (480 mL/day)	Polyphenols: 1,740 mg/L Anthocyanins: 196 mg/L	LP, GP, and BP
Buscemi (28), Italy	19 subjects with increased cardiovascular risk	MF; I: 48 years, C: 35 years	Crossover	7 days	Red orange juice (500 mL/day)	Placebo beverage (500 mL/day)	Polyphenols: 419 mg/L Anthocyanins: 71.3 mg/L	GP
Krikorian (29), United States	21 older subjects with mild, age-related memory decline	MF; I: 78 years, C: 75 years	Parallel	16 weeks	Concord grape juice (6.3–7.8 mL/kg/day)	Placebo beverage (6.3–7.8 mL/kg/day)	Polyphenols: 2,091 mg/L Anthocyanins: 425 mg/L	GP, BP
Lynn (30), United Kingdom	48 healthy participants	MF; I: 39 years, C: 36.1 years	Parallel	4 weeks	Pomegranate juice (330 mL/day)	Placebo beverage (330 mL/day)	Polyphenols: 18.6 mmol/L	BP
Tsang (31), United Kingdom	28 overweight or obese volunteers	MF; 50.4 years	Crossover	4 weeks	Pomegranate juice (500 mL/day)	Placebo beverage (500 mL/day)	Polyphenols: 1,685 mg/L	LP, GP, and BP
Flammer (32), United States	69 subjects with peripheral endothelial dysfunction and cardiovascular risk factors	MF; I: 44.8 years, C: 51.4 years	Parallel	4 m	Cranberry juice (460 mL/day)	Placebo beverage (460 mL/day)	Polyphenols: 1,740 mg/L Anthocyanins: 151 mg/L	LP, BP

(Continued)

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TABLE 1 (Continued)

Author, year, country	Participant characteristics	Sex; mean age	Design	Duration of intervention	Intervention	Comparison	Intervention juice phenolic content (concentration)	Outcomes of interest
Ruel (33), Canada	35 healthy overweight participants	M; 45 years	Crossover	4 weeks	Cranberry juice (500 mL/day)	Placebo beverage (500 mL/day)	Polyphenols: 800 mg/L Anthocyanins: 42 mg/L	BP
Guo (34), China	44 participants with features non-alcoholic fatty liver disease	MF; 21.2 years	Crossover	4 weeks	Bayberry juice (500 mL/day)	Placebo beverage (500 mL/day)	Polyphenols: 2,702 mg/L Anthocyanins: 835 mg/L	LP, GP
Khan (35), United Kingdom	64 healthy subjects	MF; I (low): 55 years, I (high): 51 years, C: 51 years	Parallel	6 weeks	Low-polyphenol blackcurrant juice (1,000 mL/day) High-polyphenol blackcurrant juice (1,000 mL/day)	Placebo beverage (1,000 mL/day)	Low-polyphenol blackcurrant juice Polyphenols: 273 mg/L Anthocyanins: 40 mg/L High-polyphenol blackcurrant juice Polyphenols: 815 mg/L Anthocyanins: 143 mg/L	LP, BP
Lynn (36), United Kingdom	46 healthy subjects	MF; I: 38.3 years, C: 37.2 years	Parallel	6 weeks	Tart cherry juice (250 mL/day)	Placebo beverage (250 mL/day)	Anthocyanins: 1,094 mg/L	LP, BP
Siasos (37), Greece	26 healthy smokers	MF; 26.3 years	Crossover	2 weeks	Concord grape juice (7 mL/kg/day)	Placebo beverage (7 mL/kg/day)	Polyphenols: 1,970 mg/L Anthocyanins: 296 mcmol/L	LP, GP, and BP
Sohrab (38), Iran	44 patients with type 2 diabetes	MF; I: 55 years, C: 56.9 years	Parallel	12 weeks	Pomegranate juice (250 mL/day)	Placebo beverage (250 mL/day)	Polyphenols: 1,946 mg/L	GP
Novotny (39), United States	56 healthy volunteers	MF; I: 49.8 years, C: 51.3 years	Parallel	8 weeks	Cranberry juice (480 mL/day)	Placebo beverage (480 mL/day)	Polyphenols: 720.8 mg/L Anthocyanins: 42.9 mg/L	LP, GP, and BP
Loo (40), Finland	37 patients with mildly elevated blood pressure	MF; 55.8 years	Crossover	8 weeks	Chokeberry juice (300 mL/day)	Placebo beverage (300 mL/day)	Polyphenols: 7,313 mg/L Anthocyanins: 3,413 mg/L	LP, GP, and BP
Kojadinovic (41), Serbia	23 subjects with metabolic syndrome	F; 40–60 years	Parallel	6 weeks	Pomegranate juice (300 mL/day)	Water (not reported)	Polyphenols: 2,938 mg/L Anthocyanins: 21 mg/L	LP, GP, and BP
Moazzen (42), Iran	30 volunteers with metabolic syndrome	MF; 51.6 years	Crossover	1 weeks	Pomegranate juice (500 mL/day)	Placebo beverage (500 mL/day)	Anthocyanins: 100.46 mg/L	LP, GP, and BP
Paquette (43), Canada	41 overweight or obese subjects with insulin resistance	MF; I: 57 years, C: 60 years	Parallel	6 weeks	Strawberry and cranberry juice (120 mL/day)	Placebo beverage (120 mL/day)	Polyphenols: 2,775 mg/L	LP, BP

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TABLE 1 (Continued)

Author, year, country	Participant characteristics	Sex; mean age	Design	Duration of intervention	Intervention	Comparison	Intervention juice phenolic content (concentration)	Outcomes of interest
Chai (44), United States	34 older subjects	MF; I: 70.0 years, C: 69.5 years	Parallel	12 weeks	Tart cherry juice (480 mL/day)	Placebo beverage (480 mL/day)	Polyphenols: 938.8 mg/L	LP, GP, and BP
Bakuradze (45), Germany	57 healthy volunteers	M; I: 23 years, C: 24 years	Parallel	8 weeks	Fruit (red grape, lingonberry, apple, blueberry, strawberry, aronia, and acerola) juice (750 mL/day)	Placebo beverage (750 mL/day)	Polyphenols: 3,600 mg/L Anthocyanins: 274.5 mg/L	LP
Hollands (46), United Kingdom	41 overweight participants	MF; 52.2 years	Crossover	28 days	Blood orange juice (500 mL/day)	Blonde orange juice (500 mL/day)	Anthocyanins: 100 mg/L	LP, GP, and BP
Martin (47), United States	26 overweight or obese participants	MF; 41 years	Crossover	4 weeks	Tart cherry juice (240 mL/day)	Placebo beverage (240 mL/day)	Polyphenols: 4,140 mg/L Anthocyanins: 65 mg/L	LP, GP, and BP
Pokimica (48), Serbia	84 individuals at cardiovascular risk	MF; I (low): 40.8 years, I (high): 42.3 years, C: 39 years	Parallel	4 weeks	Low-polyphenol chokeberry juice (100 mL/day) High-polyphenol chokeberry juice (100 mL/day)	Placebo beverage (100 mL/day)	Low-polyphenol chokeberry juice Polyphenols: 2942.8 mg/L Anthocyanins: 283 mg/L High-polyphenol chokeberry juice Polyphenols: 11771.1 mg/L Anthocyanins: 1,133 mg/L	LP, GP, and BP
Desai (49), United Kingdom	12 participants with metabolic syndrome	MF; 50 y	Crossover	7 days	Montmorency tart cherry juice (130 mL/day)	Placebo beverage (130 mL/day)	Anthocyanins: 2076.9 mg/L	LP, GP, and BP

(Continued)

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TABLE 1 (Continued)

Author, year, country	Participant characteristics	Sex; mean age	Design	Duration of intervention	Intervention	Comparison	Intervention juice phenolic content (concentration)	Outcomes of interest
do Rosario (50), Australia	31 older adults with cognitive impairment	MF; I (low): 76.1 years, I (high): 75.1 years, C: 74.9 years	Parallel	8 weeks	Low-anthocyanin Queen Garnet Plum juice (250 mL/day) High-anthocyanin Queen Garnet Plum juice (250 mL/day)	Apricot juice (250 mL/day)	Low-anthocyanin Queen Garnet Plum juice Anthocyanins:188 mg/L High-anthocyanin Queen Garnet Plum juice Anthocyanins: 804 mg/L	BP
Johnson (51), United States	19 individuals with metabolic syndrome	MF; I: 29.3 years, C: 44.2 years	Parallel	12 weeks	Tart cherry juice (480 mL/day)	Placebo beverage (480 mL/day)	Polyphenols: 4458.3 mg/L Anthocyanins: 366.7 mg/L	LP, GP, and BP
Li (52), United Kingdom	15 healthy overweight or obese participants	MF; 28.7 years	Crossover	2 weeks	Blood orange juice (400 mL/day)	Placebo beverage (400 mL/day)	Anthocyanins: 24 mg/L	LP, BP
Richter (53), United States	40 adults with elevated blood pressure	MF; 47 years	Crossover	8 weeks	Cranberry juice (500 mL/day)	Placebo beverage (500 mL/day)	Polyphenols: 674.7 mg/L Anthocyanins: 9.6 mg/L	LP, GP, and BP
Stojković (54), Serbia	54 dyslipidemic individuals	MF; I: 41.1 years, C: 38.5 years	Parallel	4 weeks	*Aronia melanocarpa* juice (100 mL/day)	Placebo beverage (100 mL/day)	Polyphenols: 11771.1 mg/L	LP, GP, and BP
Heiss (55), Germany	44 healthy adults	M; I: 25 years, C: 25 years	Parallel	1 m	Cranberry juice (500 mL/day)	Placebo beverage (500 mL/day)	Polyphenols: 1,050 mg/L Anthocyanins: 108 mg/L	LP, GP, and BP
Hillman (56), United States	20 healthy adults	MF; I: 28 years, C: 27 years	Parallel	30 days	Montmorency tart cherry juice (480 mL/day)	Placebo beverage (480 mL/day)	Polyphenols: 3,304.2 mg/L Anthocyanins: 945.8 mg/L	BP
Sinclair (57), United Kingdom	44 healthy adults	MF; I (cherry): 32.8 years, I (blueberry): 34.1 years, C: 35.1 years	Parallel	20 days	Montmorency tart cherry juice (260 mL/day) Blueberry juice (260 mL/day)	Placebo beverage (260 mL/day)	Montmorency tart cherry juice Anthocyanins: 2,462 mg/L Blueberry juice Anthocyanins: 2,977 mg/L	LP, GP, and BP

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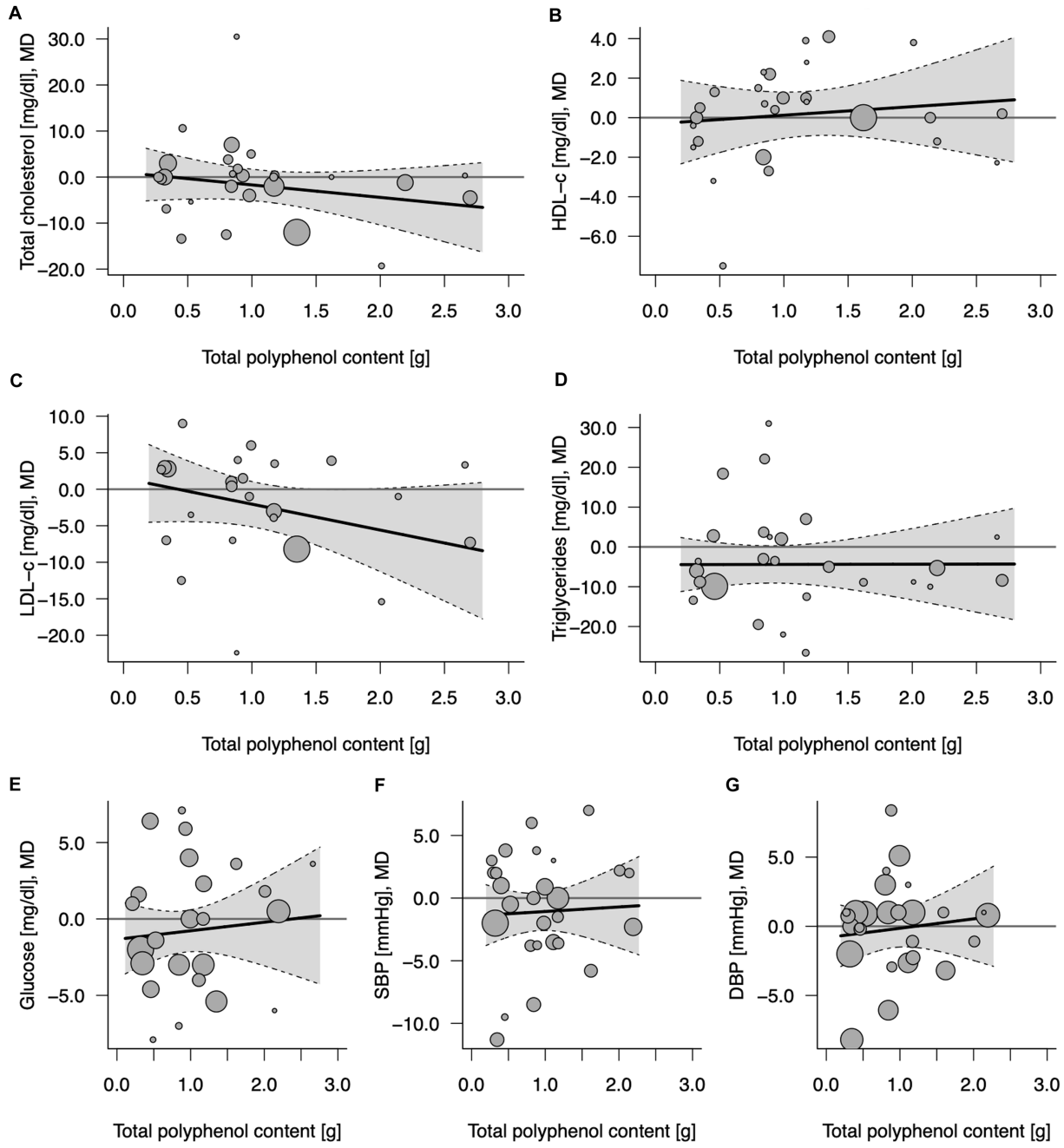

FIGURE 2
Potential mediating effect of total (poly)phenol content in 100% fruit juice in randomized controlled trials on cardiovascular risk factors: **(A)** Total cholesterol (mg/dL), **(B)** HDL-C (mg/dL), high-density lipoprotein-cholesterol, **(C)** LDL-C (mg/dL), low-density lipoprotein-cholesterol; **(D)** TG (mg/dL), **(E)** Glucose (mg/dL), **(F)** DBP (mmHg), diastolic blood pressure; and **(G)** SBP (mmHg), systolic blood pressure. Solid lines depict regression slopes and reflect how the mean differences in measurement of each specific cardiometabolic biomarker between juice and control change across the (poly) phenol content. Gray shadows represent confidence interval regions for regression slopes. Bubbles reflect observed study-specific mean differences in biomarkers between juice and control and the point sizes are a function of the model weights.

above limitation, we could not include other (poly)phenol classes or produce significant analyses due to a lack of data from existing RCT. Fourth, the studies included participants with different health status (i.e., healthy and unhealthy), thus, the effects of the intervention may differ across studies; in fact, we observed stronger size effects when analyzing studies conducted on patients with metabolic syndrome, but residual confounding should be still considered. Fifth, overall diets are generally controlled in both intervention and control groups, but, given the wide variety of foods containing (poly)phenols, there cannot be an absolute exclusion of confounding effects from the external intake of phenolic compounds.

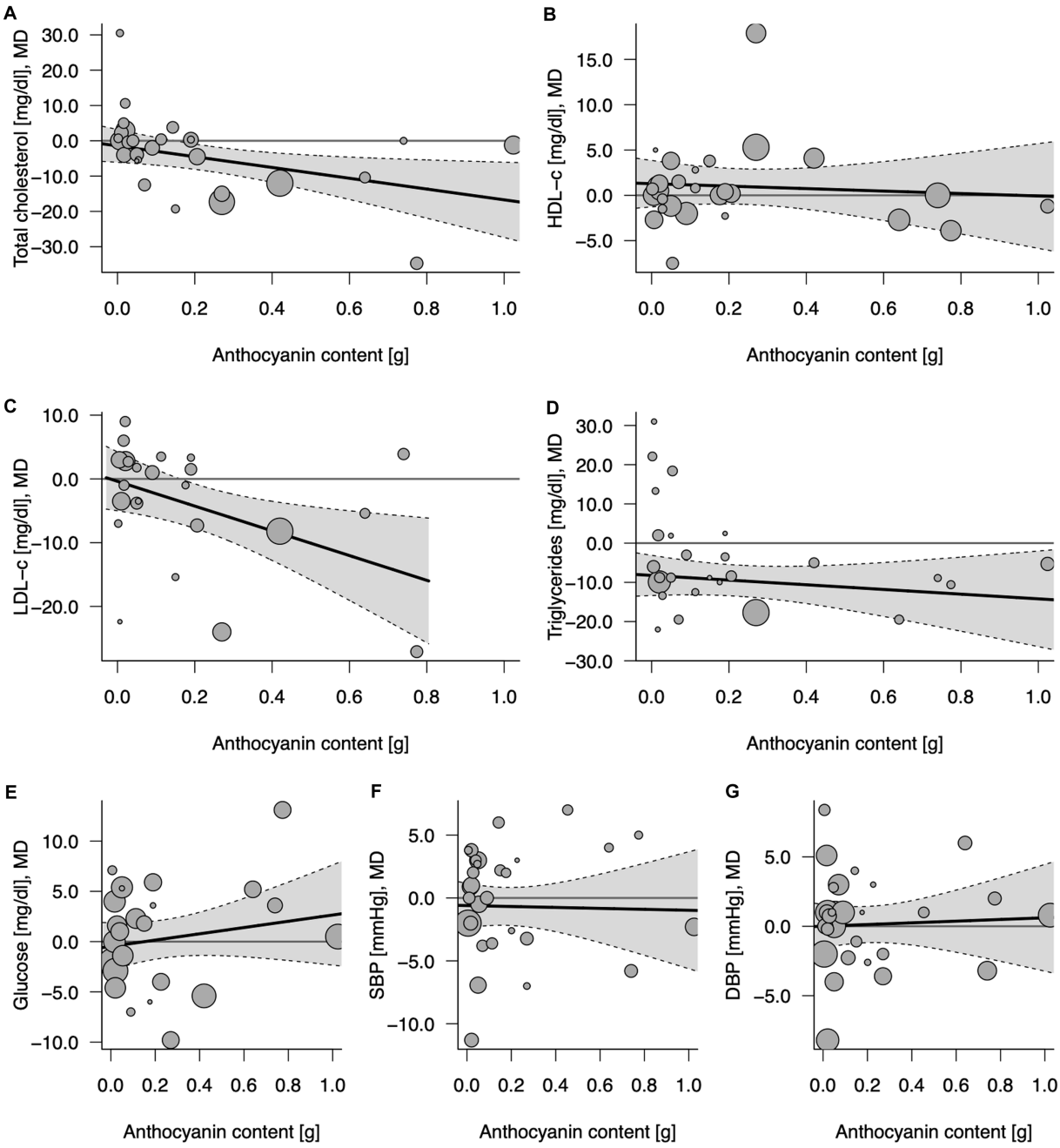

FIGURE 3
Potential mediating effect of total anthocyanin content in 100% fruit juice in randomized controlled trials on cardiovascular risk factors: **(A)** Total cholesterol (mg/dL), **(B)** HDL-C (mg/dL), high-density lipoprotein-cholesterol, **(C)** LDL-C (mg/dL), low-density lipoprotein-cholesterol; **(D)** TG (mg/dL), **(E)** Glucose (mg/dL), **(F)** DBP (mmHg), diastolic blood pressure; and **(G)** SBP (mmHg), systolic blood pressure. Solid lines depict regression slopes and reflect how the mean differences in measurement of each specific cardiometabolic biomarker between juice and control change across the anthocyanin content. Gray shadows represent confidence interval regions for regression slopes. Bubbles reflect observed study-specific mean differences in biomarkers between juice and control and the point sizes are a function of the model weights.

5. Conclusion

In conclusion, the present study found that anthocyanins may mediate some of the potential beneficial effects of 100% fruit juices on specific blood lipids. Considering the relevance of this for CVD prevention, it is strongly encouraged that future RCT on 100% fruit juices measure and report the total and specific (poly)phenol content to provide further data to be considered in additional meta-analyses. If these findings are confirmed in future studies, there could be a human health advantage to increasing the (poly)phenol content of 100% fruit juices through the use of specific fruit varieties or targeted plant breeding.

TABLE 2 Effect of 100% fruit juice vs. control in randomized controlled trials on cardiovascular risk factors and potential mediating effect of total (poly) phenol content.

		Overall effect of juice vs. control drink				Total polyphenol content effect (per 1g/day)			
Cardiometabolic biomarker	*n* comparisons	MD (95% CI)	*p*	I^2 [%]	p_{heter}	ΔMD^6 (95% CI)	*p*	I^2 [%]	p_{heter}
Total cholesterol	27	−1.85 (−5.25; 1.55)	0.286	0	0.853	−2.72 (−7.94; 2.51)	0.308	0	0.861
HDL-C	27	0.20 (−0.93; 1.33)	0.727	0	0.996	0.43 (−1.39; 2.26)	0.640	0	0.995
LDL-C	24	−2.24 (−5.33; 0.85)	0.156	0	0.921	−3.55 (−8.61; 1.51)	0.169	0	0.951
Triglycerides	26	−4.40 (−9.09; 0.28)	0.066	0	0.944	0.05 (−6.92; 7.02)	0.989	0	0.923
Glucose	24	−0.86 (−2.18; 0.46)	0.202	15.6	0.245	0.56 (−1.77; 2.89)	0.636	17.9	0.219
SBP	27	−1.11 (−2.57; 0.34)	0.133	16.5	0.223	0.36 (−2.31; 3.03)	0.791	19.4	0.188
DBP	27	−0.21 (−1.53; 1.11)	0.757	45.4	0.006	0.68 (−1.80; 3.16)	0.590	45.9	0.006

DBP, diastolic blood pressure; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MD, mean difference; SBP, systolic blood pressure.

TABLE 3 Effect of 100% fruit juice vs. control in randomized controlled trials on cardiovascular risk factors and potential mediating effect of anthocyanin content.

		Overall effect of 100% fruit juice vs. control				Anthocyanin content effect (per 0.1g/day)			
Cardiometabolic biomarker	*n* comparisons	MD (95% CI)	*p*	I^2 [%]	p_{heter}	ΔMD (95% CI)	*p*	I^2 [%]	p_{heter}
Total cholesterol	28	−4.62 (−8.51; −0.72)	0.020	22.6	0.141	−1.53 (−2.83; −0.22)	0.022	10.4	0.311
HDL-C	28	0.97 (−0.96; 2.90)	0.324	76.9	<0.001	−0.14 (−0.85; 0.57)	0.708	76.8	<0.001
LDL-C	24	−3.97 (−7.97; 0.03)	0.052	34.2	0.052	−1.94 (−3.46; −0.42)	0.012	16.5	0.237
Triglycerides	26	−9.55 (−13.52; −5.57)	<0.001	0	0.669	−0.60 (−2.07; 0.88)	0.429	0	0.651
Glucose	24	0.22 (−1.63; 2.08)	0.814	45.1	0.009	0.31 (−0.29; 0.91)	0.308	45.5	0.010
SBP	29	−0.70 (−2.16; 0.77)	0.353	14.9	0.239	−0.04 (−0.57; 0.49)	0.891	17.9	0.201
DBP	29	0.11 (−1.17; 1.39)	0.865	41.4	0.011	0.06 (−0.39; 0.51)	0.792	43.2	0.009

DBP, diastolic blood pressure; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MD, mean difference; SBP, systolic blood pressure.

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

AM, PM, and JG contributed to conception and design of the study. AM and JG organized the database. AM performed the statistical analysis. AM, WC, and JG wrote the first draft of the manuscript. AM, WC, CM, AR, IB, AA, DR, PM, and JG wrote the sections 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1175022/full#supplementary-material

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The contradictory role of branched-chain amino acids in lifespan and insulin resistance

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Branched-chain amino acids (BCAAs; a mixture of leucine, valine and isoleucine) have important regulatory effects on glucose and lipid metabolism, protein synthesis and longevity. Many studies have reported that circulating BCAA levels or dietary intake of BCAAs is associated with longevity, sarcopenia, obesity, and diabetes. Among them, the influence of BCAAs on aging and insulin resistance often present different benefits or harmful effects in the elderly and in animals. Considering the nonobvious correlation between circulating BCAA levels and BCAA uptake, as well as the influence of diseases, diet and aging on the body, some of the contradictory conclusions have been drawn. The regulatory mechanism of the remaining contradictory role may be related to endogenous branched-chain amino acid levels, branched-chain amino acid metabolism and mTOR-related autophagy. Furthermore, the recent discovery that insulin resistance may be independent of longevity has expanded the research thinking related to the regulatory mechanism among the three. However, the negative effects of BCAAs on longevity and insulin resistance were mostly observed in high-fat diet-fed subjects or obese individuals, while the effects in other diseases still need to be studied further. In conclusion, there is still no definite conclusion on the specific conditions under which BCAAs and insulin resistance extend life, shorten life, or do not change lifespan, and there is still no credible and comprehensive explanation for the different effects of BCAAs and insulin resistance on lifespan.

KEYWORDS

branched-chain amino acids, aging, insulin resistance, lifespan, longevity

1. Introduction

Branched-chain amino acids (BCAAs), a general term for leucine, isoleucine and valine, are a common protein supplement (1). BCAAs play an important physiological role in regulating protein synthesis, metabolism, food intake and aging (2). Among them, BCAA circulatory levels are strictly regulated by catabolism, and BCAA abnormalities are associated with obesity, insulin resistance, type 2 diabetes, heart disease and cancer. All of the amino acids classified as BCAAs are catalyzed by branched-chain amino acid transaminase 1/2 (BCAT1/2) to form branched-chain ketoacid (BCKA), which is then oxidized by the branched-chain ketoacid dehydrogenase (BCKDH) complex to form the final metabolites. These metabolites enter the tricarboxylic acid cycle to generate energy. To maintain the catabolic cycle, BCAT2 reversibly catalyzes the initial steps of BCAA catabolism to regenerate BCAAs and α-KG (α-Ketoglutarate) (3, 4). Hence, we speculate that the increase in circulating BCAA levels caused by various diseases may be the

result of BCAA metabolic disorder, which occurs by BCKA accumulation, changes in BCKDH activity, etc. (5) (Figure 1).

BCAAs are closely related to abnormal glucose and lipid metabolism, and studies on animal models have shown that dietary BCAAs are important inducers of insulin resistance (6), and limiting dietary BCAAs can help prevent insulin resistance (7). BCAAs can induce insulin resistance by promoting fat accumulation in muscles and transporting 3-hydroxyisobutyric acid (a catabolic intermediate of valine) across the vascular endothelium (8). Insulin resistance refers to a decrease in the efficiency of insulin in promoting glucose uptake and utilization and is associated with metabolic syndrome and type 2 diabetes mellitus (T2DM). Previous studies have linked aging to high blood pressure, high glucose levels and low insulin levels (9). Insulin resistance is an important cause and symptom of cardiovascular disease, T2DM and other diseases, and its occurrence causes great damage to organs (10). Insulin resistance in the aging process also causes sarcopenia (11). However, low levels of insulin resistance tend to contribute to longevity (12). It has been reported that low levels of insulin/IGF-1 signaling can prolong life. In *C. elegans* (*Caenorhabditis elegans*), mutations in the insulin/IGF-1 receptor gene DAF-2 or other genes in this pathway extend lifespan (13). In addition, in some insulin-related knockout mouse models, in which insulin resistance is induced in one or more tissues, a significant increase in the lifespan of mice can be observed (14, 15). All of these findings suggest that inhibition of insulin-related pathways promotes longevity. However, these contradictory reports raise a more important question: Is insulin resistance harmful or beneficial to humans?

To explore the above questions, scientists began to take life expectancy as an indicator and explored the relationship between BCAA intake and life expectancy. The results show that BCAAs are associated with longevity/aging and metabolic regulation through a variety of mechanisms. However, some studies suggest that BCAA restriction may be beneficial to longevity/metabolic health. High intake of red meat containing high levels of BCAAs may be associated with the promotion of age-related diseases (16). Other studies suggest that BCAA supplements may improve aging and prolong the life of middle-aged mice in multiple ways (17). This paradoxical finding is consistent with the contradictory relationship between insulin resistance and longevity. This information also suggests that BCAAs, insulin resistance and longevity may be closely correlated.

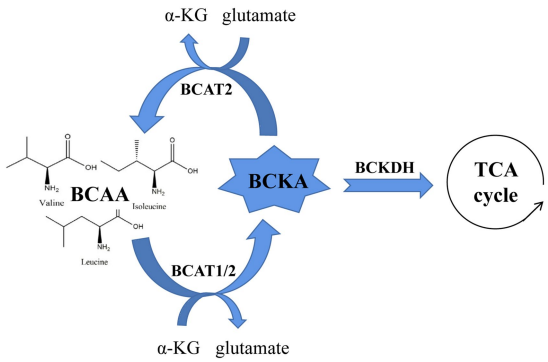

FIGURE 1
A brief schematic of BCAA catabolism and the enzymes that regulate this process.

Therefore, this review summarizes and discusses the results of pairwise studies on BCAAs, insulin resistance and longevity and raises some questions. First, in terms of BCAAs and longevity, are BCAA-restricted and BCAA-rich diets good or bad for humans? Second, is insulin resistance induced by BCAAs beneficial or harmful? Third, under what circumstances does BCAA-induced insulin resistance occur? Fourth, does insulin resistance increase or decrease lifespan? This review provides an overview of recent studies and possible regulatory mechanisms from the above aspects. In addition, a new theory that insulin resistance is unrelated to longevity will be discussed in the following sections.

2. BCAAs and lifespan

The elderly population is increasing as a result of increased life expectancy, improved health status and improved medical services. The number of people aged 65 and over reached 617 million (8.5%) in 2015 and is expected to rise to 1.6 billion by 2050. Globally, from 1990 to 2017, life expectancy increased by 7.4 years, from 65.6 years in 1990 to 73.0 years in 2017 (18). While the average lifespan has increased, the corresponding healthy lifespan has not (19). Recent studies have shown that the average age of health is declining yearly, and nutrition appears to be an important factor in increasing healthy life expectancy. Disorder of protein metabolism due to aging is often accompanied by sarcopenia, which greatly reduces the quality of life of elderly individuals (20). With increasing age, human mitochondrial function gradually decreases through a decrease in mitochondrial DNA copy number and the ATP production rate (21). Dietary supplementation fortified with BCAAs may help to slow mitochondrial decay, improve the clinical situation of malnourished elderly patients (22), and increase healthy life expectancy, and this approach is more effective than simple dietary recommendations (19). Its mechanism may be related to increases in mitochondrial biogenesis and antioxidant capacity in the muscle of aged rats induced by BCAA-rich foods. Studies have shown that BCAA supplementation in middle-aged mice significantly increased the occurrence of mitochondrial biogenesis in the heart and skeletal muscle and significantly improved the oxidative stress state in the heart and skeletal muscle by increasing the activity of mTORC1. Following these alterations, the lifespan of middle-aged mice was extended by 12% (17).

On the other hand, supplementation with BCAAs can also improve aging by promoting muscle protein synthesis and iron content (23), and BCAA supplementation significantly inhibits the decrease in serum albumin in elderly patients compared with that in young patients (24). In addition, BCAA supplementation can promote wound healing, reduce the occurrence of kidney and liver diseases with age (25), and reduce inflammatory markers in patients with chronic heart failure (26).

Furthermore, after the addition of BCAAs, the structure of the intestinal flora in mice was changed, and the rate of changes in the intestinal flora in mice due to age was slowed. This finding suggests that BCAAs affect the intestinal flora and intestinal metabolism. The concentration of lipopolysaccharide binding protein in the serum of BCAA-group mice was low. These changes indicate reduced antigen load in the host intestinal tract. It has been suggested that adding BCAAs to the diet can improve the intestinal flora and promote healthy aging (27).

Endogenous BCAA accumulation also has a positive effect on longevity. In *C. elegans*, BCAT-1 gene silencing in wild-type adults increases endogenous BCAA accumulation and prolongs lifespan (28). In addition, in a population study, taking the long-lived village in East Asia as the latest research object shows that endogenous BCAAs have a potential protective effect on leukocyte telomere length and weakness and suggests a potential synergistic effect between BCAAs and leukocyte telomere length during healthy aging (29) (Figure 2).

High levels of BCAAs are often detected in the plasma of patients with T2DM and obese people (28, 30). However, considering the health benefits of BCAAs, the correlations between high levels of BCAAs and insulin resistance, obesity and T2DM seem contradictory. Studies have shown that an increase in circulating BCAAs was associated with an increasing risk of tissue damage both in mice, and maybe eventually lead to the mortality in mice, while BCAA restriction ranging from 50% (0.5 x BCAAs) to 85% can result in an additional increase in lifespan of Drosophila, and the amplitude of increase could reach about 10%. The possible mechanism by which BCAAs negatively regulate lifespan is by reducing TORC1 activity, reducing the phosphorylation level of S6K (S6 kinase), downregulating S6K mRNA translation, and inducing autophagy (31). BCAAs are effective activators of the TOR signal, and S6K is a direct downstream target of this signal. Research results show that overexpression of ARGK-1 can extend the life of *C. elegans* by activating the regulation of the energy sensor AAK-2/AMPK to RSK-1/S6K (32). Life expectancy was also increased by 9% in S6K knockout mice, suggesting that downregulation of S6K can prolong the lifespan of lower animals and mammals (33).

Studies on rapamycin-induced Drosophila autophagy (34) and dietary restriction-triggered *C. elegans* autophagy (35) have demonstrated that initiation of autophagy can lead to prolonged lifespan. In addition, adverse effects of leucine, the main component of branched-chain amino acids, on longevity have been reported. Leucine can induce senescence of MC3T3-E1 cells through DNA damage, which has a negative effect on the proliferation of bone cells. These results may provide new insights into previous studies on the supplementation of amino acids to promote bone health (36). Depletion of leucine prolongs the life of leucine-auxotrophic cells, leading to cell senescence and cell cycle stagnation in the G1 phase, which are dependent on genes in the Ecl1 family (37). These studies suggest that leucine, as one of the main components of BCAAs, may play an important role in the negative effects of aging.

In recent years, an increasing number of studies have begun to seek the best way to regulate lifespan through the study of branched-chain amino acid metabolism. Among them, BCAA catabolism regulated by miR-277 is the best way to study the regulation of BCAAs on lifespan. Overexpression and inhibition of miR-277 can shorten the lifespan, especially when consuming foods containing excessive protein. Expression of miR-277 reduced the metabolic capacity of BCAAs. The resulting increase in BCAA concentration may stimulate TOR kinase (38, 39), thereby shortening lifespan. Transgenic

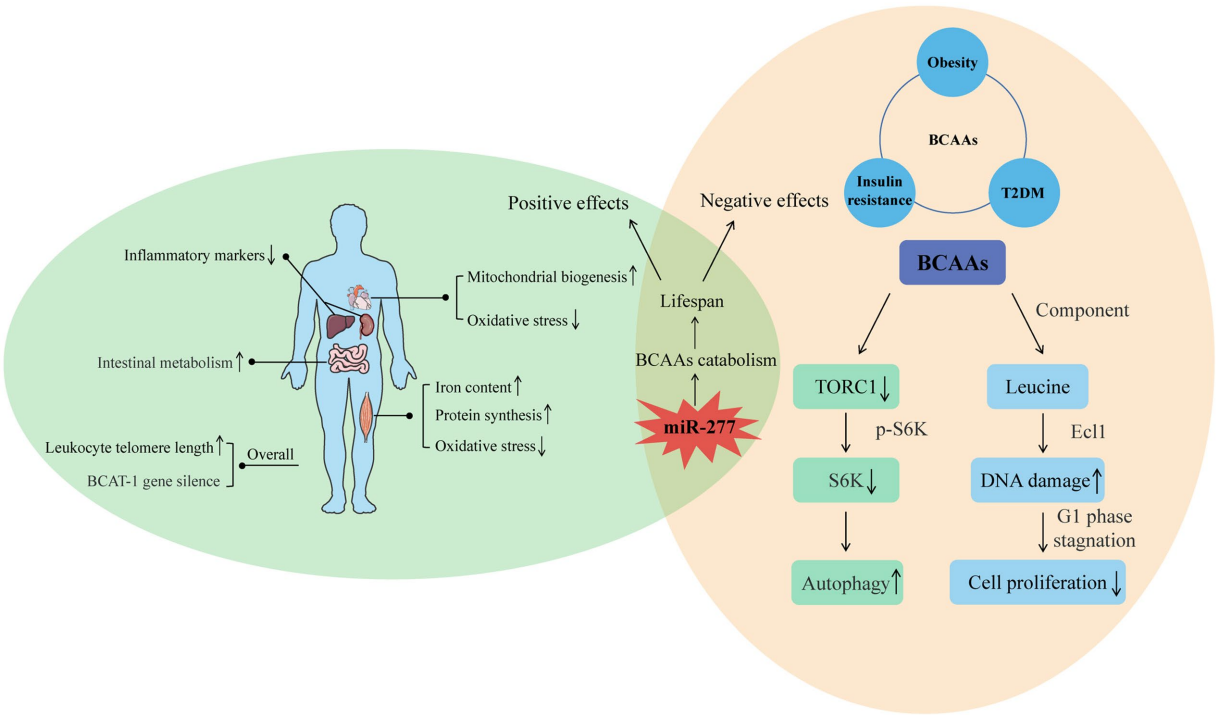

FIGURE 2
Positive and negative effects of BCAAs on the body. For the positive, the intake of BCAAs can improve the function of muscle and heart, reduce the inflammation of liver and kidney, and enhance intestinal metabolism. Moreover, increased endogenous BCAAs caused by BCAT-1 gene silence and increased leukocyte telomere length can also have a positive effect on longevity. For the negative, the effects of BCAAs on insulin resistance, obesity, and T2DM indicate their negative influence on lifespan. Furthermore, the molecular mechanism of BCAAs on shortened life is related to the autophagy induced by decreased TORC1, and the decreased cell proliferation induced by DNA damage. From the two sides, miR-277 seems a potential regulatory target whose expression level is associated with lifespan through adjusting BCAAs catabolism.

inhibition with the use of miRNA sponges also shortens the lifespan, especially on a protein-rich diet (40). Changes in miRNA expression in adult Drosophila showed that miR-277 was significantly downregulated in adult Drosophila, which was consistent with its effect on lifespan. Therefore, optimal metabolic adaptation and optimal longevity effects seem to be achieved by regulating BCAA catabolism by miR-277 (Figure 2).

In summary, the effects of BCAAs on longevity have many controversial consequences, and the possible mechanisms are complex. It is certain that the positive and negative regulation of BCAAs on longevity has a great relationship with the external environment, diseases and intake. For example, supplementing BCAAs under specific dietary restrictions can improve metabolic health, improve glucose tolerance, and reduce fat accumulation (41), while BCAAs in combination with a high-fat diet tend to lead to the development of obesity-related insulin resistance (42). The regulatory mechanism of BCAAs on longevity may be related to the regulation by miR-277 of BCAA catabolism.

3. BCAAs and insulin resistance

Many studies have confirmed that BCAAs may play a causal role in the pathogenesis of type 2 diabetes (43–45), and BCAAs contribute to the development of obesity-related insulin resistance in humans and rodents on a high-fat diet (HFD) (46). Studies of male rats and humans have shown that high-level BCAA intake is associated with high fat intake, leading to insulin resistance and glucose intolerance, which ultimately result in metabolic syndrome (42, 47). In obese mice induced by a high-fat diet, BCAA supplementation exacerbated obesity-related hepatic glucose and lipid metabolism disorders by weakening Akt2 signaling, thereby causing severe hepatic metabolic disorders and hepatic insulin resistance (48). Moreover, supplementation with BCAAs combined with a high-fat diet was associated with increased plasma BCAA concentrations in adult rats (42). Moreover, reducing BCAAs in the diet in this situation moderately improves glucose tolerance and reduces fat mass increase (49). Similarly, high BCAA levels in breast milk are associated with a high risk of insulin resistance in the children of obese women (50). A maternal high-fat diet alone or supplemented with BCAAs can impair the glycemic control of these children in adulthood. Male and female offspring fed high-fat diets containing BCAAs showed serious insulin intolerance and elevated fasting blood glucose levels, which might be related to the influence of the hypothalamic ER-α pathway (51). In addition, daily intake of BCAAs, namely, leucine, isoleucine, and valine, by mothers with gestational diabetes may also increase the risk of overweight and insulin resistance in their children (7). Other studies have not found an association between dietary BCAA intake and adult plasma BCAA concentrations (52, 53). In contrast, supplementing the maternal diet with BCAAs prevents age-related and diet-related insulin intolerance, thereby protecting offspring.

In healthy individuals, BCAAs appear to play beneficial roles, including reducing the risk of obesity, increasing muscle mass, potentially improving glucose sensitivity, and possibly exerting therapeutic effects in patients with cirrhosis and encephalopathy (54–57). However, high levels of circulating BCAAs in serum or plasma are thought to be associated with obesity and insulin resistance (58). Studies have shown that long-term exposure to high levels of BCAAs stimulates hyperlipidemia and obesity, which has a positive association with fasting glucose levels, LDL and triglyceride levels and a negative correlation with HDL-C and inhibits the TCA (tricarboxylic acid cycle) directly through BCKA accumulation (54, 59–61). When BCAA metabolic disorders occur, such as in metabolic syndrome, the catabolites of valine, isoleucine, and leucine may accumulate and produce negative metabolic effects. Furthermore, the risk of developing T2DM increases because of the genetic predisposition to BCAA metabolism impairment (62). Several studies have reported that plasma or serum BCAA levels can predict the development of T2DM (63).

BCAA metabolites are associated with the risk of obesity and insulin resistance. However, it is unknown which BCAAs, that is, leucine, valine or isoleucine, influence insulin resistance. Studies using diet deprivation—the complete removal of an amino acid from the diet—have found that eliminating leucine from the diet for 1 week significantly improved blood glucose control in mice (64, 65). Moreover, the glucose tolerance and pyruvate tolerance of mice fed a low-leucine diet were significantly increased, indicating that leucine restriction could enhance gluconeogenesis (41). Leucine can also regulate glucose uptake by L6 myotubes independent of the mTORc1 and AKT signaling pathways, and the concentration of leucine in the culture medium has a dose-dependent relationship with non-insulin-stimulated glucose uptake in cells (66). Nevertheless, low concentrations of leucine did not change insulin signaling and were not associated with insulin resistance but increased the lipid content in myotubes (67). A cohort study of women without prediabetes also showed that leucine supplementation had no effect on insulin sensitivity (68).

Recent studies have shown that valine supplementation alone has no effect on pAkt and Akt in myotubes and was not associated with insulin stimulation or different levels of insulin resistance (69). However, valine in combination with leucine, isoleucine or protein can affect insulin signaling and related metabolic pathology. Moreover, excessive intake of valine can independently induce insulin resistance. 3-Hydroxyisobutyric acid (3-HIB) is a catabolic intermediate of valine (70). In animals, 3-HIB is secreted by muscle, which activates the transport of endothelial fatty acids, stimulates the uptake of muscle fatty acids, and promotes muscle lipid accumulation and insulin resistance (8). The addition of 3-HIB to white and brown adipose cell cultures increased fatty acid uptake and regulated insulin-stimulated glucose uptake in a time-dependent manner (70). In humans, 3-HIB has been shown to be associated with insulin resistance in subjects with diabetes and with insulin resistance in overweight and obese individuals (71). It was shown that in a cohort of 4,942 men and women, cyclic 3-HIB increased with elevated levels of hyperglycemia and T2DM. In addition, after weight loss, cyclic 3-HIB concentrations increase briefly and then decrease significantly.

Studies of isoleucine have shown that it can significantly increase muscle and fat mass and cause insulin resistance. Furthermore, it can also upregulate the levels of key lipogenic proteins and myogenic proteins. More importantly, through mitochondrial function lesions, isoleucine can harm the gastrocnemius and tibialis anterior and lead to cavitation, swelling, cristae fractures, etc. In addition, isoleucine promotes myogenesis and increases lipid droplet accumulation in myotubes. In general, isoleucine can increase muscle mass and induce insulin resistance through myogenesis and intracellular lipid deposition (72).

Although most of the recent studies have confirmed that an appropriate supply of BCAAs to the normal body has no or a positive effect on insulin sensitivity, in the diabetic or obese body or in the case of an excessive supply of BCAAs to the normal body, BCAAs have negative effects on insulin sensitivity and sometimes can lead to insulin resistance. At present, there are several speculations about the mechanism of this phenomenon: one is the catabolite of BCAAs, that is, branched-chain ketone acids (BCKAs). The expression of BCAA catabolic enzymes in the hearts of fasting mice was decreased, and circulating BCKAs were increased, while BCAAs were not increased. Similar increases in circulating BCKAs were associated with changes in BCAA catabolic enzyme expression in diet-induced obesity (DIO) mice. Exposure of muscle cells to high levels of BCKAs inhibits insulin-induced AKT phosphorylation and reduces glucose uptake and mitochondrial oxygen consumption. Changes in the intracellular BCKA clearance rate by gene-regulated expression of BCKDK and BCKDHA have similar effects on AKT phosphorylation. Therefore, excessive amounts of BCKAs are the real cause of insulin resistance (73). Second, it has been hypothesized that mammalian target of rapamycin complex 1 (mTORC1) is overactivated in the presence of amino acid overload, leading to a reduction in insulin-stimulated glucose uptake, which is caused by insulin receptor substrate (IRS) degradation and reduced Akt-AS160 activity (56). However, this hypothesis can only explain the negative effects of excessive intake of BCAAs but cannot explain the regulatory mechanism of insulin sensitivity by the addition of BCAAs in disease models. Furthermore, the results still do not explain the findings on HMB (the metabolite of leucine – hydroxy-p-methylbutyric acid), which is thought to reduce insulin resistance (74). HMB may reduce insulin resistance and hepatic steatosis by inhibiting GLUT-2 in the liver of high-fat diet-fed rats (75). For humans, acute HMB treatment improves insulin resistance after glucose loading in young men but has no effect on insulin sensitivity in older men (76). The above phenomena are not consistent with the effect of leucine on insulin resistance, and there is no recent research on the mechanism of this contradiction, so the regulation of these phenomena requires further investigation.

4. Lifespan and insulin resistance

The improvement in insulin resistance is associated with longevity. The metabolic traits in centenarians are maintaining insulin sensitivity and a lower incidence of diabetes (77), which suggests that glucose homeostasis may play a crucial role in health and longevity. At present, much of the increase in longevity and health is achieved by improving insulin sensitivity. New insights into certain features of vegetarianism suggest that vegetarianism can improve insulin resistance and dyslipidemia and related abnormalities by limiting proteins or certain amino acids (leucine or methionine) and has the potential to extend life (78). Aging PASK-deletion mice exhibited overexpression of the longevity gene FoxO3a and a normal HOMA-IR index, which simultaneously confirmed that mice lacking PASK had better insulin sensitivity and glucose tolerance (79). *Drosophila melanogaster* fed a high-sugar diet showed signs of insulin resistance and a reduced lifespan (80). Moreover, in mice and humans, an increase in circulating BCAAs is associated with a high risk of insulin resistance and diabetes, as well as an increased mortality in mice (31). Studies of calorie restriction (CR) have shown that eating high-calorie foods can impair metabolism and accelerate aging; in contrast, CR can prevent age-related metabolic diseases and extend life (81), and the main mechanism by which calorie restriction prolongs life is to improve aging by enhancing insulin sensitivity (82, 83), which also suggests a positive correlation between increased longevity and insulin sensitivity. In a study of mice fed a medium-fat or high-fat diet, it was observed that low insulin levels significantly increased lifespan, high insulin levels contributed to age-dependent insulin resistance, and decreased basal insulin levels could extend lifespan (84). These results suggest that increased insulin levels and insulin sensitivity have a positive effect on longevity.

Although several studies have shown that improved insulin sensitivity promotes health and longevity, there is also clear evidence that insulin sensitivity is not necessarily related to healthy aging and may even be counterproductive. In some insulin-related gene knockout mouse models, insulin resistance is induced in one or more tissues, and a significant increase in the lifespan of mice can be observed. The average lifespan of mammalian insulin-like growth factor type 1 receptor (IGF-1R) knockout mice was 26% longer than that of wild-type mice, and the knockout mice showed stronger antioxidant stress resistance (85). Fat-specific insulin receptor knockout (FIRKO) in both male and female mice increased the average lifespan by approximately 134 days (18%) (14). Female insulin receptor substrate (IRS) 1 knockout mice also showed signs of longevity (15). Furthermore, rapamycin extended the lifespan of mice by inhibiting mTORC1 and impaired glucose homeostasis to induce insulin resistance by interfering with mTORC2 signaling (86). S6K1 knockout female mice showed an extended lifespan and induced loss of insulin sensitivity (33).

Previous studies have focused on the positive and negative correlations between insulin resistance and lifespan, but recent findings suggest the more interesting possibility that insulin resistance or metabolic defects may be independent of longevity, which also explains the above two opposite results. As insulin resistance is independent of lifespan, the opposite results of lifespan may be led by other different factors, and insulin resistance is not the main reason for lifespan-changing. From this study we could see that a high-sugar diet can shorten the lifespan of Drosophila, which could be saved by water supplementation. In contrast, metabolic defects, which are widely thought to lead to reduced survival, have been shown to be unrelated to water. Drosophila that had been watered on a high-sugar diet still showed all the metabolic defects similar to diabetes and had the same survival rates as healthy controls, suggesting that obesity and insulin resistance by themselves did not shorten fly life. The mechanism by which a high-sugar diet regulates lifespan in Drosophila is thought to be a water-dependent way of regulating uric acid production: the high-sugar diet promotes the accumulation of uric acid (a final product of purine catabolism) and enhances this process. It is obviously shown that renal calculi occur on a 20% sucrose diet, with impaired renal tubule secretion and impaired purine metabolism leading to uric acid accumulation in the urinary cavity. Furthermore, this phenomenon is completely restored by water or allopurinol treatment (87). Mice deficient in fatty acid binding protein (FABP) showed extended metabolic health cycles, which were protective against insulin resistance, glucose intolerance, inflammation, deterioration of adipose tissue integrity, and fatty liver disease. However, the mice lacking FABP showed no signs of longevity. These data suggest that metabolic health in mice can be detached from

longevity in the absence of caloric restriction, suggesting that these pathways may act independently (88) (Figure 3).

5. Conclusion

This review summarizes the contradictory role of branched-chain amino acids in lifespan and insulin resistance. In this review, we attempt to explain these conflicting findings from a physiological and pathological perspective and to draw conclusions about the possible regulatory mechanisms between BCAAs and aging, BCAAs and insulin resistance, and aging and insulin resistance. When explaining the above arguments, it should be noted that (1) metabolic regulation of the body is very complex, and the existence of aging and insulin resistance may also have an impact on metabolism, including some compensatory effects; (2) the effects of BCAA supplementation on aging and insulin resistance should be comprehensively analyzed in combination with sex, age, dietary conditions and basic diseases; (3) at present, exogenous BCAAs are not well correlated with endogenous BCAA levels in plasma and serum; and (4) there are some great differences in diseases, aging, food intake and water intake, especially in animal models. Therefore, BCAA-based research must refer to the basic survival data of the research subject for comprehensive analysis. Due to the complexity of metabolism and body responses, it is difficult to draw convincing conclusions about the effects of BCAAs on aging and insulin resistance.

From the screening of the literature, the following possible regulatory mechanisms of BCAAs, aging and insulin resistance can be concluded: (1) Autophagy can significantly extend the lifespan of yeast, *C. elegans* and mice. However, autophagy alone is neither sufficient to extend the lifespan nor necessary to extend the lifespan. In the absence of autophagy, the inhibition of protein synthesis in animals that were fed adequate food could extend life. In summary, autophagy may have a specific regulatory mechanism to prolong life, and its specificity may be related to the environment of the food supply and the regulation and triggering of longevity pathways. To some extent, this also explains the mechanism by which BCAAs and insulin resistance influence lifespan. However, there is currently no reliable study on the triggering mechanism by which insulin resistance affects autophagy, and the study examining the trigger of autophagy by BCAAs also shows contradictory results. This suggests that a certain limit of autophagy may have a positive effect on the regulation of lifespan, while excessive autophagy may have a negative effect, but there is no clear conclusion as to what the limit is. (2) Endogenous BCAAs have a significant influence on lifespan. Although there is no exact correlation between endogenous and exogenous BCAA levels at present, the conclusion that endogenous BCAA accumulation caused by diseases such as obesity and T2DM leads to aging and damage to the body has been very clear. Therefore, the negative effects of BCAAs on the body based on insulin resistance and various diseases can be explained. However, elderly individuals have low concentrations of BCAAs in the plasma, but this was not the case in young individuals and children (89, 90). Therefore, the positive effects of BCAA supplementation on aging can also be explained. However, theories based on endogenous BCAAs cannot explain the positive effects of insulin resistance on longevity. To date, many hypotheses have been proposed based on these contradictions. However, there are various problems, such as the lack of credible evidence and the inability to

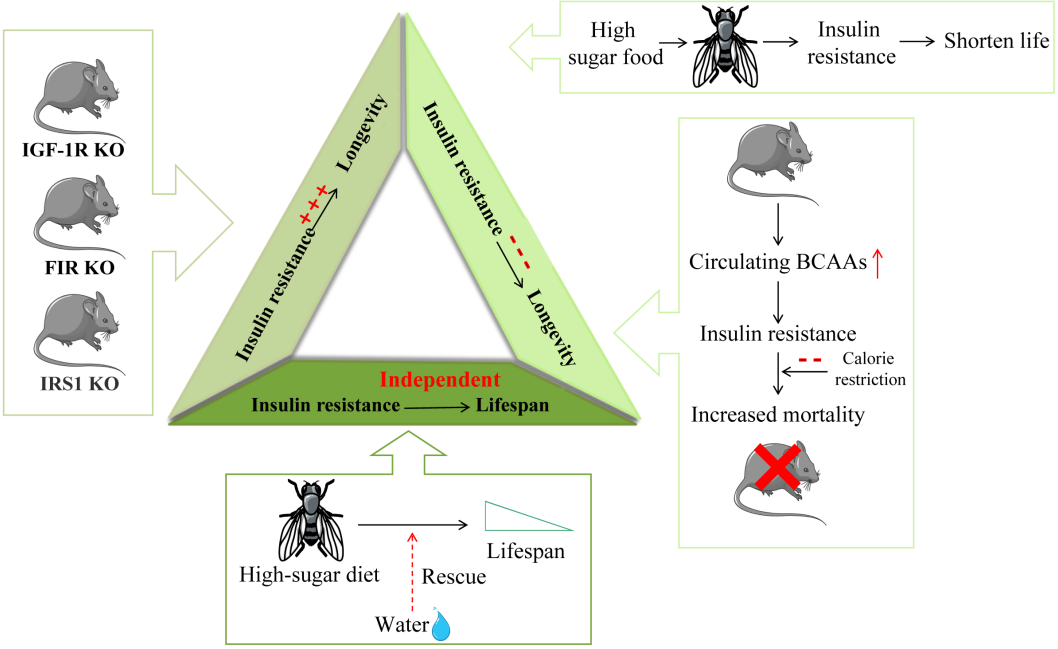

FIGURE 3
The different relationship between insulin resistance and longevity. Insulin resistance in insulin-related gene IGF-1R, FIR, and IRS1 knockout mice is induced in one or more tissues and is accompanied by a significant extension of mouse lifespan. On the other hand, Drosophila fed a high-sugar diet are characterized by insulin resistance and a shortened lifespan. Increased circulating BCAAs in mice lead to insulin resistance development and increased mortality in mice. Furthermore, calorie restriction (CR) can extend life by improving insulin sensitivity. Moreover, the shortened lifespan caused by high sugar feeding in Drosophila can be saved by water, suggesting that insulin resistance may be independent of lifespan regulation.

explain some of the results. Therefore, there are no credible theories about the regulatory mechanisms of BCAAs, aging and insulin. However, two conclusions are convincing: one is that BCAAs remain an effective supplement for aging and related metabolic changes, and the other is that maintaining endogenous BCAAs within a reasonable range is indeed important for health. Therefore, there is still a long way to go to further explore the relationship between BCAAs, longevity and insulin resistance based on existing research.

Besides, the metabolites of BCAAs have been the focus of attention in recent years. Studies were no longer limited to BCAAs study on lifespan and insulin resistance. Studying the separate amino acids of BCAAs and the metabolites of BCAAs is on the rise. This can further research the mechanism of BCAA-regulated metabolic disorders in the body. In this review, we mainly talked about the metabolite of valine – 3-HIB. This metabolite could lead to the activation of fatty acid transportation in some organs, which is related to insulin resistance. After that, the insulin resistance was induced in the body. In summary, the 3-HIB is a promising marker for detecting insulin resistance. However, as the essential factor of BCAAs, leucine has fewer reports about its metabolites that have an effect on insulin resistance. So we have reason to believe that with the discovery of more metabolites of BCAAs that are in association with insulin resistance, the mechanism of BCAAs and insulin resistance will be more promising. And these metabolites will be the potential targets to treat insulin resistance in T2DM.

In conclusion, recent studies suggest that endogenous BCAAs, BCAA metabolism and mTOR-related autophagy play important roles in the relationships among BCAAs, longevity, and insulin resistance. The recent discovery that insulin resistance may be independent of longevity has expanded our understanding of the regulatory mechanisms among the three. However, there is still no definite conclusion on the specific conditions under which BCAAs and insulin resistance extend life, shorten life, or do not change lifespan, and there is still no credible and comprehensive explanation for the different effects of BCAAs and insulin resistance on lifespan. In addition, similar confusion occurs between BCAAs and insulin resistance. These problems are attracting increasing research interest, and the study of these problems is conducive to the elucidating the rational use of BCAAs, identifying a treatment for T2DM and the study of longevity.

Author contributions

YaL and YiL supervised the entire project. HY drafted the manuscript and prepared the figures. KL and JW revised the manuscript. All authors have read and agreed to the published version of 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.

Publisher's note

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Protective effects and molecular mechanisms of tea polyphenols on cardiovascular diseases

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Aging is the most important factor contributing to cardiovascular diseases (CVDs), and the incidence and severity of cardiovascular events tend to increase with age. Currently, CVD is the leading cause of death in the global population. In-depth analysis of the mechanisms and interventions of cardiovascular aging and related diseases is an important basis for achieving healthy aging. Tea polyphenols (TPs) are the general term for the polyhydroxy compounds contained in tea leaves, whose main components are catechins, flavonoids, flavonols, anthocyanins, phenolic acids, condensed phenolic acids and polymeric phenols. Among them, catechins are the main components of TPs. In this article, we provide a detailed review of the classification and composition of teas, as well as an overview of the causes of aging-related CVDs. Then, we focus on ten aspects of the effects of TPs, including anti-hypertension, lipid-lowering effects, anti-oxidation, anti-inflammation, anti-proliferation, anti-angiogenesis, anti-atherosclerosis, recovery of endothelial function, anti-thrombosis, myocardial protective effect, to improve CVDs and the detailed molecular mechanisms.

KEYWORDS

aging, cardiovascular diseases, tea polyphenols, prevention, treatment

1. Introduction

With the advent of an aging society, aging-related issues are becoming a growing concern (1). Aging-related diseases have also become the most common diseases among middle-aged and elderly people (1, 2). Cardiovascular disease (CVD) is closely related to aging and is a serious threat to the lives and health of middle-aged and elderly people (3). CVD is a complex disease that involves multiple environmental and genetic factors, particularly atherosclerosis (AS), which mainly affects the large and middle arteries (3). This disease is characterized by lesions in the affected arteries starting from the intima, followed by a combination of lesions, including lipid accumulation, fibrous tissue proliferation and calcification, along with degenerative changes in the middle layers of the arteries (4). The secondary lesions of AS include lipid deposition, intimal thickening, thrombosis, inflammatory cell infiltration, subintimal inflammation, vessel wall remodeling, neovascularization, plaque rupture, intraplaque hemorrhage, plaque rupture and local thrombosis, which eventually cause narrowing or blockage of the vascular lesion, resulting in ischemic injury to the affected organs (4). AS and related diseases, such as

coronary heart disease (CHD) and myocardial infarction, have become serious threats to human health and have become the leading causes of high morbidity, disability and mortality worldwide (5). Hence, there is a growing interest in exploring new ways to prevent and treat aging-related CVD.

With aging, cardiomyocytes gradually develop physiological changes such as hypertrophy, senescence, lipofuscin aggregation, fibrosis, and apoptosis, which lead to cardiac hypertrophy and heart failure (6). Vascular endothelial cells, smooth muscle cells and extracellular matrix gradually change, resulting in reduced endothelial function, a thickened intima, vascular sclerosis, increased arterial pressure, reduced number of capillaries and decreased permeability, which further cause tissue ischemia and hypoxia, oxidative stress damage and necrosis (7, 8). Eventually, this causes AS, CHD, and atherosclerotic occlusive disease (8, 9). In addition, during aging, physiological changes in glucose and lipid metabolism also occur, resulting in diabetes, hyperlipidemia, and metabolic syndrome, which in turn damage cardiovascular function and can lead to diseases such as diabetic heart disease (10, 11). Therefore, blocking these mechanisms may be a therapeutic strategy to resist aging-related CVD.

Tea is the second most widely consumed beverage after water and has been consumed for thousands of years in China (12). Tea is the dried young leaves or leaf buds of *Camelliasinenis o. Ktze.*, a plant in the *Camelliaceae* family, and is used as a drink with great nutritional, health and medicinal value (13, 14). Depending on the degree of fermentation, tea is divided into six main categories: white (not fermented), green (not fermented), yellow (slightly fermented), oolong (deeply fermented), black (deeply fermented), and dark (deeply fermented) (15, 16). Tea is rich in many biologically active components, such as polyphenols, pigments, polysaccharides, alkaloids, free amino acids and saponins (15–17). Although tea contains several chemicals, tea polyphenols (TPs) play a major role in promoting health, and green tea contains far more polyphenols than other teas (12, 18–20). A large amount of evidence confirms that TPs are effective antioxidants with anti-inflammatory, antiradiation, and antiaging properties that can prevent CVD (20, 21).

The treatment methods for CVDs are mainly divided into two categories: (1) drug conservative treatment; (2) active surgical treatment. No matter which treatment method is used, it needs to be carried out on the basis of improving lifestyle, such as a light diet, rest, physical exercise, controlling weight, blood pressure, blood sugar, blood lipids, quitting smoking, and limiting alcohol. These are important lifestyle improvement measures to reduce the risk of CVDs recurrence. Compared to these traditional treatment methods, tea is widely recognized as a healthy beverage, and multiple studies have confirmed that drinking tea regularly can reduce the risk of CVDs (22–24). This may be related to various components in tea, and TPs have the effects of lowering blood lipids, antioxidation, and inhibiting thrombosis. There have been many reports describing the role of TPs in the prevention and treatment of CVDs (25–27). These articles focus on a particular component of tea or emphasize a particular CVD. In this article, we provide a detailed review of the classification and composition of teas and an overview of the causes of aging-related CVDs; then, we focus on ten aspects of the effects of TPs to improve CVD and the detailed molecular mechanisms.

2. Physicochemical properties and composition of TPs

Many bioactive TPs have been identified in dry tea leaves, including flavonols, flavonoids, anthocyanins, and phenolic acids (23, 24, 28, 29). Flavanols are the main components of TPs, and flavanols are dominated by catechins and their derivatives (15, 16). According to their chemical structure, catechins can be divided into four main types: (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin (EC) (18). EGCG is the most physiologically active substance among catechols, accounting for approximately 50%–70%, and the physiological effects of green tea are mainly exerted by EGCG (18). Unlike green tea, oolong and black teas are fermented, and their catechins are oxidized to theaflavins (including four isomers: theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3,3′-gallate) (Table 1) (18). These theophyllins exert cardiovascular protective effects, but their antioxidant activity may be lower than that of catechins (18).

3. Traditional risk factors for CVD

3.1. Abnormal blood pressure

Hypertension is a chronic CVD (32). A study showed that for every 10 mmHg reduction in systolic blood pressure, the risk of major cardiovascular events (e.g., coronary heart disease, stroke, heart failure) is significantly reduced (33). Therefore, effective control of blood pressure can reduce the incidence of cardiovascular-related

TABLE 1 Tea polyphenol composition of different tea leaves.

Tea	Components	References
White tea	EC, GC, EGC, CG, ECG, GCG, EGCG	(15, 16)
Green tea	EC, GC, EGC, CG, ECG, GCG, EGCG	(15, 16, 18)
Yellow tea	EC, GC, EGC, CG, ECG, GCG, EGCG	(15, 16)
Oolong tea	Catechins, gallic acid, TF, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3,3′-gallate, thearubigins	(18, 30, 31)
Black tea	Catechins, gallic acid, TF, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3,3′-gallate, thearubigins	(18, 30, 31)
Dark tea	Catechins, gallic acid, TF, theaflavin-3-gallate, theaflavin-3′-gallate, theaflavin-3,3′-gallate, thearubigins	(18, 30, 31)

CG, catechin gallate; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-gallate; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin-3-gallate; GC, gallocatechin; GCG, gallocatechin gallate; TF, theaflavin.

diseases, morbidity and mortality (34). In addition, prehypertension is already prevalent and accompanied by increased aortic stiffness, impaired elasticity, decreased cardiac function, and diminished insulin resistance (34). Hence, hypertension is not only a chronic form of CVD but also worsens the morbidity and mortality of major CVD (35).

3.2. Abnormal metabolic indices

3.2.1. Abnormal glucose metabolism

Abnormal fasting plasma glucose (FPG) increases the risk of CVD (36). Abnormal glucose metabolism, especially hyperglycemia, leads to oxidative stress, microvascular damage, vascular tone and endothelial damage, as well as platelet aggregation and embolism (37, 38). In addition, hyperglycemia induces certain inflammatory factors [tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP), etc.] and inflammatory reactions, all of which cause varying degrees of damage to cardiomyocytes, blood vessels, and even the heart (39).

3.2.2. Dyslipidemia

Lipids are the general term for neutral fats [triacylglycerols (TG) and total cholesterol (TC)] and lipids in plasma (phospholipids, glycolipids, sterols, and steroids), which are essential for the basic metabolism of living cells (40). Among them, cholesterol [low density lipoprotein (LDL) and high density lipoprotein (HDL)] and TG are closely related to the development of atherosclerosis (AS) (40, 41). Studies have shown that for every 1 mmol/L reduction in LDL-C, the risk of CVD is reduced by 21% to 24% (41, 42).

3.3. Poor lifestyle habits

3.3.1. Smoking

It is estimated that tobacco use causes approximately 10% of CVD worldwide (43–45). Tobacco contains approximately 4,000 chemicals, of which nicotine, carbon monoxide and other components stimulate blood pressure, lead to coronary AS, increase blood and platelet viscosity, reduce the ability to dissolve blood clots and oxygen-carrying capacity of hemoglobin, and even induce ventricular fibrillation, increasing the incidence of cardiovascular events (46, 47).

3.3.2. Alcohol consumption

Many studies now indicate that small amounts of alcohol consumption can moderately reduce the risk of myocardial infarction (48, 49). However, the effects of heavy alcohol use on exacerbating CVD cannot be ignored. Both long-term heavy drinking and occasional heavy drinking can, to varying degrees, decrease HDL-C, increase plasma viscosity and fibrinogen concentration, cause platelet aggregation, impair endothelial function, increase inflammatory responses, increase heart rate, and inhibit cardiac contractile function, thereby increasing the incidence of CVD, morbidity and mortality (50, 51).

3.3.3. Diet

The structure, quantity, and type of diet can also influence the occurrence of cardiovascular events (52, 53). For example, a high-salt diet can exacerbate vasoconstriction, leading to elevated blood pressure and plasma cholesterol and contributing to the development of AS (52–55). Sugar can increase blood viscosity and slow blood flow, which, combined with damage to the vascular endothelium, causes the generation of a large number of atherosclerotic plaques that block blood vessels and trigger the occurrence of acute cardiovascular events (56, 57). A high-fat diet can cause obesity or overweight, leading to metabolic disorders such as hyperlipidemia, hypertension, and other CVDs (58, 59).

3.3.4. Sleep and mental factors

It is reported that in patients with insomnia, serum HDL is low, while TG level is high (60, 61). In addition, CVDs are closely related to psychological conditions such as depression, chronic psychological stress, post traumatic stress disorder (PTSD), and anxiety (62).

3.4. Others

Numerous epidemiological studies have shown that sex, age, and family history influence the incidence and mortality rates of CVD (63, 64). With increasing age, the onset of various metabolic diseases, and the reduction in the body's immune system, CVD increases each year (65). Moreover, the prevalence and mortality rates are higher in men than in women, especially in premenopausal women (66). Postmenopausal women lack the protective mechanisms of a specific physiological period, and with the decrease in estrogen levels, the metabolism of the body changes, leading to an increase in the incidence of CVD (65, 67).

4. Molecular mechanism of the cardioprotective effect of TPs

As a natural polyphenol complex, TPs are characterized by their simple availability and wide range of biological effects (68, 69). In recent years, TPs have been shown to have good preventive and curative effects against AS, thrombosis, myocarditis, coronary artery disease, antiarrhythmia and myocardial ischemia/reperfusion (I/R) injury (70). Studies have shown that the cardioprotective effects of TPs are closely related to their antioxidant, anti-inflammatory, and blood viscosity-altering characteristics (68, 70). Here, we have reviewed the relevant literature and summarized ten mechanisms of TPs associated with protection against CVD (anti-hypertension, lipid-lowering effects, anti-oxidation, anti-inflammation, anti-proliferation, anti-angiogenesis, anti-AS, recovery of endothelial function, anti-thrombosis, myocardial protective effect),. Undoubtedly, TPs can significantly reduce the risk of CVDs by reducing the factors related to CVDs.

4.1. Hypotensive effects

Hypertension is a major risk factor for CVD and a common disease with a high incidence worldwide that is characterized by elevated arterial pressure (71). At present, there are many drugs that treat hypertension and can effectively lower blood pressure but have large side effects and fluctuate greatly while lowering blood pressure

(72, 73). Therefore, the screening of functional food factors with antihypertensive effects is critical for the prevention and treatment of hypertension. One of the pathogeneses of hypertension is elevated levels of renin, angiotensin, and aldosterone, and so patients with hypertension will experience high renin in their bodies (74). Aqueous extracts of fermented oolong and black teas strongly inhibit renin (74). In addition, supplementation with white, black and green teas in obese mice prevented the development of hypertension (75). Further analysis revealed that this antihypertensive effect was mainly associated with increased expression of antioxidant enzymes induced by TPs such as gallic acid, xanthine and flavan-3-ol (75). In a randomized, double-blind, controlled crossover study, black tea intake increased functionally active circulating angiogenic cells compared to placebo, thereby greatly offsetting the reduction in blood flow-mediated dilation due to fat intake (76). In two epidemiological studies [ATTICA and MEDiterranean ISlands (MEDIS)], green tea is rich in high levels of catechins (e.g., EGCG) compared to black tea and, therefore, significantly reduces the likelihood of hypertension in adults aged 50 years and older (77). In addition, tannins in tea have been shown to have a hypotensive effect on rats (78). Gao et al. (52, 53) found that green tea had an antihypertensive effect on hypertension induced by a high salt diet in aged male rats, and its main mechanism of action included inhibiting the activity of the renin-angiotensin II-aldosterone system, altering the expression of sodium-potassium pumps in heart, kidney and aortic tissues and increasing the synthesis of nitric oxide in endothelial cells.

4.2. Lipid-lowering effects

Hyperlipidemia is an important factor that induces CVD. An increase in LDL-C and a decrease in HDL-C in serum can cause arterial endothelial cell damage, increase permeability and accelerate LDL-C deposition in the subendothelium of blood vessels (79). In recent years, a large number of studies have shown that TPs can significantly reduce serum TC, TG, and LDL-C levels and increase HDL-C levels in patients with hyperlipidemia, which can protect vascular endothelial function (79). For example, serum levels of cholesterol, LDL and TG were reduced and HDL was significantly increased in experimental rats fed a high-cholesterol diet after the administration of beverages containing theaflavin and theaflavin (80). Results from a clinical trial of tea drinking habits and HDL in Chinese adults found that in people aged 60 years or older, serum HDL concentrations decreased more slowly in tea drinkers compared to non-tea drinkers, suggesting a significant association between tea consumption and HDL-C (81). In a randomized, controlled trial, ingestion of GTC for 4 consecutive weeks significantly reduced fasting serum TG levels (82). TPs have been widely demonstrated to improve lipid metabolism abnormalities by modulating gut microbial species and functions. Ma et al. (83) found that different doses of TPs could regulate intestinal redox status and the intestinal microbiota through different patterns, thus improving the disorders of lipid metabolism induced by a high-fat diet (HFD). Wang et al. (84) found that green tea leaf powder could reshape the intestinal microbiota in the cecum of mice and increase satiety hormone secretion, there by reducing lipid metabolism disorders in mice fed a HFD. Conversely, excessive intake of TPs reduced their beneficial effects on intestinal health (83). Moreover, TPs were effective in reducing leptin in rat serum and inhibiting fatty acid uptake, thereby improving lipid and antioxidant levels (85). It is worth noting that the lipid-lowering effect of black teas (such as Liubao and Pu′er teas) is increased significantly after fermentation compared to that of the raw material, probably due to the significant increase in browning and gallic acid in the tea leaves after fermentation (86).

4.3. Inhibiting oxidation

Oxidative stress is present throughout the pathology of AS, and another important effect of TPs is their antioxidant properties (87). Due to the number and structure of phenolic hydroxyl groups, catecholates and theaflavins are excellent electron donors and effective free radical scavengers (87). *In vitro*, the antioxidant effects of catechols and theaflavins against human LDL oxidation were similar, and the antioxidant capacity of polyphenols was in the following order: TF3 > ECG ≥ TF2B ≥ TF2A ≥ TF1 ≥ EC > EGC (88). In addition, after drinking 600 mL of green tea daily for 4 weeks, plasma levels of oxidized LDL (ox-LDL) were reduced in smokers (89). The inhibition of ROS-producing enzymes by TPs may also enhance their antioxidant effects. Both catechols and TFs inhibit the expression of inducible NO synthase (iNOS). Another physiological source of ROS occurs during the oxidation of hypoxanthine and xanthine to uric acid (87, 90). This reaction is catalyzed by xanthine oxidase, which has now been shown to be inhibited by catechol and theaflavin. Several studies have shown that catechol induces a variety of enzymes involved in cellular antioxidant defense mechanisms (87, 90, 91). Negishi et al. (91) found that oral administration of TPs for 2 weeks induced peroxidase in the aorta in spontaneously hypertensive rats. In endothelial cells, EGCG significantly induced subtilisin oxygenase-1 through activation of AKT and Nrf2, resulting in significant protection against hydroperoxide-regulated oxidative stress (87, 90). *In vitro*, TPs ameliorated heat stress injury in cardiomyocytes by upregulating Keap1-Nrf2-ARE signaling to enhance its antioxidant capacity and inducing the expression of heat shock proteins (69). Moreover, in Wistar rats, TPs attenuated the HFD-induced increase in intima-media thickness and significantly inhibited vascular oxidative damage (92). In addition, TPs can inhibit the oxidation of lipoproteins *in vivo*. In a clinical study, urinary levels of 4-O-methylglutamic acid were significantly increased after subjects took green and black tea, suggesting that intake of TPs could inhibit LDL oxidation *in vivo* (93). Besides, in a randomized, placebo-controlled, double-blind, crossover trial, green tea extract was ingested, with EGCG and EGC as the main components. Both of them rapidly bind LDL particles and reduce the degree of oxidation of LDL, thereby reducing the risk of AS associated with oxidative stress (94).

4.4. Inhibiting proliferation

The proliferation and migration of vascular smooth muscle cells (VSMCs) play key roles in the formation and development of AS, postvalvular restenosis and graft vascular lesions (95). *In vivo* and *in vitro* experiments showed that catechols inhibited VSMC proliferation and migration (95). Among catechols, EGC, ECG and EGCG were significantly more effective than catechins and epicatechins in preventing proliferation (95). Kim et al. (96) found that EGCG

blocked the transition of VSMCs from G1 to S phase by initiating the expression of p21/WAF1, which in turn inhibited NF-kappaB and AP-1-mediated VSMC proliferation. Additionally, the antiproliferative effects of TPs include interactions with growth factors involved in the proliferation and migration of VSMCs, such as fibroblast growth factor (bFGF) (97, 98). EGCG also significantly inhibits c-Jun nuclear translocation and AP-1 binding activity and reduces iNOS expression (99). Moreover, TPs can interact with the matrix metalloprotein (MMP) system, which contributes to the migration, proliferation, and neointima formation of VSMCs after vascular injury (100). In a rat model of carotid artery injury, catechins reduced MMP-2 activity by upregulating matrix metalloproteinase (MMP)-2 and TIMP-2, thereby inhibiting neointimal proliferation and improving vascular remodeling (100). Furthermore, in a carotid artery injury model, EGCG reduced VSMC proliferation by inhibiting extracellular signal-regulated kinase (ERK), but c-jun and p38 signaling was not affected (101). Moreover, EGCG was shown to inhibit the expression of apoptosis-related proteins and attenuate apoptosis in VSMCs induced by H_2O_2 (102).

4.5. Anti-inflammation

Acute and chronic inflammation plays a key role in the development of CVD (103, 104). TPs can modulate immune responses and have potential anti-inflammatory activity. For example, in rats fed an atherosclerotic diet, the administration of 0.2% green tea extract (Polyphenon®) resulted in a significant reduction in serum inflammatory markers (CRP) (103). A clinical study showed that consistent use of green tea or green tea extract significantly reduced serum amyloid alpha, which is an important CVD risk factor, in obese individuals with metabolic syndrome (105). In another randomized, double-blind trial, long-term black tea consumption reduced platelet activation and lowered plasma CRP levels in healthy men, leading to long-term cardiovascular health maintenance (106). Moreover, in female rats with chronic inflammation, supplementation with TPs suppressed the innate immune response to chronic inflammation, thereby alleviating the development of myocardial fibrosis (107). In the early stages of atherosclerosis, leukocytes adhere to vascular endothelial cells and gradually migrate to the vessel wall. EGCG significantly reduced the migration of neutrophils to the endothelial cell monolayer by inhibiting chemokine production (108). *In vitro* experiments revealed that EGCG treatment inhibited TNF-α-induced adhesion of THP-1 cells to human umbilical vein endothelial cells (109). Moreover, EGCG reduced the expression of intracellular adhesion molecule 1, which affected the adhesion and migration of peripheral blood monocytes and CD8+ T cells (110). In RAW264.7 macrophages, EGCG inhibited NF-κB activation and reduced lipopolysaccharide (LPS)-induced TNFα production in a dose-dependent manner (111). In obese mice fed a HFD, TPs reduced the serum levels of TNFα, IL-1β and IL-6 by inhibiting the activation of NF-κB (28). Lu′an GuaPian tea, which is a green tea, is rich in kaempferol-3-O-rutinoside (KR), which can protect against cardiovascular disease by inhibiting TLR4/MyD88/NF-κB signaling and protect against myocardial injury (112). In addition, endothelial cells control vascular tone and permeability and are important for maintaining vascular homeostasis (113). Reddy et al. (113) found that EGCG reduced inflammation and decreased vasodilation by inhibiting the NF-κB pathway, thereby protecting against endothelial dysfunction and delaying the onset of CVD. In addition to the NF-κB signaling pathway, TPs improved the species abundance of the intestinal microbiota in the cecum, thereby improving the intestinal inflammatory response (114). Additionally, TPs could increase the expression of intestinal tight junction proteins to maintain the integrity of the intestinal barrier, thereby improving intestinal flora dysbiosis and reducing systemic inflammatory responses in obese mice (28, 115).

4.6. Improving the vascular endothelium function

The pathophysiological features of the cardiovascular system are characterized by a decrease in protective vasoactive substances in the endothelium, which is called endothelial dysfunction (43, 44). Numerous studies have shown that TPs improve endothelial cell function, lower blood pressure and have vasodilatory effects (116–118). For example, in obese prehypertensive women, short-term daily intake of GTE could improve endothelial function (119). Excessive accumulation of ROS is one of the important causal factors leading to endothelial cell dysfunction and hypertension (120). In bovine carotid artery endothelial cells (BCAECs), TPs could inhibit ROS production by reducing nicotinamide adenine dinucleotide phosphate (NADPH) expression, thereby alleviating angiotensin (Ang) II-induced endothelial cell hyperpermeability and possibly preventing the development of CVD (120). Moreover, in endothelial cells, TPs can bind endothelial extracellular superoxide dismutase (eEC-SOD) to inhibit LDL oxidation and thus counteract atherosclerosis (121). Endothelial nitric oxide synthase (eNOS) is a source of nitric oxide in endothelial cells and plays an important role in maintaining the function of endothelial cells (122). Caveolin-1 (Cav-1) is a negative regulator of eNOS that can affect cardiovascular function in multiple ways (123). Liu et al. (123) found that in BCAECs, TPs activated ERK1/2 and inhibited p38MAPK signaling in a dose-dependent manner, downregulating Cav-1 expression and thereby protecting endothelial cells. In addition, TPs can reduce the expression and secretion of plasminogen activator inhibitor-1 (PAI-1), a regulator that plays a key role in AS and hypertensive disease, in endothelial cells in a time-and dose-dependent manner, contributing to cardiovascular protection (123). In isolated rat mesenteric arteries, (-)-epicatechin increased NO concentrations in the vasculature and promoted vasodilation by activating iberiotoxin-sensitive K^+ channels (116). A clinical study showed that acute black tea intake could activate NO production in endothelial cells, thereby reducing the risk of CVD (124). Kim et al. (125) found that EGCG increased LC3-II production and autophagosome formation in primary bovine aortic endothelial cells (BAECs), thereby reducing lipid accumulation and improving the development of CVD.

4.7. Inhibiting angiogenesis

Angiogenesis is an important pathological cause of the development of CVD (126). For instance, myocardial infarction (MI) is mainly associated with partial or complete occlusion of

microvessels at the site of the lesion (126). Myocardial ischemia–reperfusion mainly refers to the production of necrotic material by ischemic cells when a patient has a myocardial infarction (127). After revascularization, blood passes through the necrotic myocardium in a short time to create reperfusion damage and increase cellular necrosis, which aggravates the symptoms of infarction and leads to malignant arrhythmias (127). To combat these conditions, restoring blood supply to the infarcted area can reduce cardiac remodeling and improve myocardial function (126). Vascular endothelial growth factor (VEGF), a homodimeric vasoactive glycoprotein, is a key regulator of angiogenesis. VEGF levels are significantly elevated in the serum of patients with different CVDs and are often associated with a poor prognosis (126). A growing number of studies have shown that TPs can protect against CVD by suppressing VEGF-mediated angiogenesis. In HUVECs, EGCG blocks the formation of the vascular endothelial growth factor receptor 2 complex, which in turn inhibits VEGF-mediated angiogenesis (128, 129). In a high-cholesterol diet male New Zealand White rabbit atherosclerosis model, green tea consumption significantly reduced VEGF expression in foam cells and smooth muscle cells, and it is hypothesized that green tea may slow the progression of atherosclerosis by reducing VEGF-induced angiogenesis (128). EGCG also inhibits angiogenesis by reducing the expression of the angiogenic factor bFGF (basic fibroblast growth factor) (130). After EGCG pretreatment, endothelial cells could induce the expression of membrane-type-1 matrix metalloproteinase (MT1-MMP), which promoted endothelial cell migration, and Cav-1, which caused tube formation, was significantly decreased, suggesting that EGCG inhibits angiogenesis (131).

4.8. Antiatherosclerosis

AS is the underlying cause of CVD (132). The development of AS has been associated with multiple molecular mechanisms, including endothelial dysfunction, inflammation, oxidative stress, and dysfunctional lipid metabolism (132). The protective effect of TPs on AS has been widely reported (133, 134). For example, a clinical study from Japan showed that patients who consumed >3 cups of green tea/day had a lower prevalence of coronary artery disease (CAD) than those who consumed <1 cup/day, suggesting that green tea intake may help improve coronary artery atherosclerosis in the Japanese population (135). TPs inhibit oxLDL production and thus IKB kinase (IKK)-mediated NF-κB activation in a dose-dependent manner and reduce the production of the proinflammatory cytokine TNF-α (134). In a mouse model of AS, EGCG reduced proinflammatory genes and increased antioxidant protein expression in the mouse aorta, and serum C-reactive protein, monocyte chelator protein-1 and ox-LDL were significantly decreased after EGCG treatment (133). Theaflavins in tea not only reduced the concentrations of *F*(2)-isoprostane, vascular superoxide, vascular leukotriene B(4) and plasma-SP-selectin in the aorta but also enhanced eNOS activity, thereby improving NO bioavailability to alleviate the development of AS in apolipoprotein E-deficient (ApoE−/−) mice (136). Changes in the gut microbiota are also closely associated with the development of AS (137). Liao et al. (137) found that TPs promoted the proliferation of intestinal *bifidobacteria* in ApoE−/− mice, thereby reducing total cholesterol and LDL cholesterol levels and reducing HFD-induced AS plaques. In addition, TPs increased the expression of autophagic markers (such as LC3, Beclin1 and p62) in the vascular wall of mice, ameliorated lipid metabolism disorders and inhibited AS plaque formation (138).

4.9. Inhibiting thrombosis

Platelet activation and subsequent thromboembolism are important pathophysiological mechanisms of ischemic CVD (139). The antithrombotic effect of green tea catechins is achieved mainly through the inhibition of platelet aggregation (140). EGCG has been reported to exert its inhibitory effect on platelet viability through several mechanisms: the inhibition of collagen-mediated phospholipase (PL) Cgamma2, blockade of protein tyrosine phosphorylation, and the enhancement of $Ca^{2(+)}$-ATPase activity, thereby reducing platelet aggregation and alleviating atherothrombosis (140). In addition, GTC did not alter anticoagulant activity but mainly altered antiplatelet activity to exert antithrombotic effects in human platelet aggregation assays induced by ADP, collagen, epinephrine, and the calcium ion polymer A23187 *in vitro* (141). EGCG has also been shown to stimulate tyrosine phosphorylation of platelet-associated proteins (e.g., Syk and SLP-76) and reduce the phosphorylation levels of focal adhesion kinases, thereby improving platelet aggregation (142). Moreover, Kang et al. (143) found that catechol modulates the reduction in intracellular calcium levels in platelets, which led to Ca^{2+}-ATPase activation and the inhibition of IP3 production, thereby inhibiting fibrinogen-GPIIb/IIIb binding and reducing platelet aggregation. Inflammatory and oxidative responses caused by endothelial cell injury play equally important roles in thrombosis (144). A recent study showed that EGCG combined with warfarin significantly reduced thrombus weight in a rat model of deep vein thrombosis (144). Further *in vitro* studies showed that the combination of EGCG and warfarin protected HUVECs from oxidative stress and prevented apoptosis, and the specific mechanism involved the inhibition of HIF-1α-mediated activation of PI3K/AKT and ERK1/2 signaling (144).

4.10. Myocardial protective effects

Ischemia is an extremely common pathological process in myocardial lesions (145). The protective effect of TPs against myocardial injury may be due to their ability to inhibit oxidative stress associated with ischemic injury (145). For example, in a cardiac hypertrophy model in rats established by abdominal aortic constriction (AC), myocardial tissue had increased malondialdehyde (MDA) levels and decreased superoxide dismutase (SOD) activity (146). In contrast, after EGCG treatment, the MDA levels in myocardial tissue decreased, and SOD activity increased. These

results suggest that EGCG ameliorates myocardial injury in rats by inhibiting oxidative stress (146). In a rat model of diabetic cardiomyopathy, TPs significantly improved myocardial function in rats, and cardiomyocyte disorders and hypertrophy were significantly improved (147). An in-depth study revealed that TPs significantly upregulated LC3-II/I and Beclin-1 expression and reduced SQSTM1/p62 expression in rat myocardial tissue (147). In addition, ingestion of TPs significantly alleviated heat stress injury in hen cardiomyocytes at 38°C, as evidenced by the downregulation of myocardial injury-related indicators (LDH, CK, CK-MB and TNF-α), and the mechanism mainly involved Keap1-Nrf2-ARE and heat shock protein (Hsp)-related heat stress responses (69). Interestingly, a recent study showed that despite the low plasma concentration of polyphenols, polyphenols were transported to the arterial intima at pH 7.4 in the form of bound lipoproteins, and polyphenol levels were significantly elevated in endothelial cells and macrophages (148). Thereafter, such high local concentrations of polyphenols protect the heart through direct antioxidant effects (148). In addition, TPs alleviate myocardial fibrosis in female rats by attenuating chronic inflammation and suppressing innate immune responses (149).

Overall, TPs improve aging-related CVDs in the following five ways (Figure 1, Table 2): (1) TPs cause activation of autophagic flux; (2) TPs inhibit ox-LDL-mediated NF-κB, ERK1/2, p38MAPK, and JNK-induced inflammatory responses; (3) TPs activate the NRF2-mediated antioxidant signaling pathway; 4; (4) TPs improve vascular endothelial cell function via PI3K/AKT/eNOS pathway; (5) TPs inhibit VEGF-mediated angiogenesis. By modulating these molecular mechanisms, TPs can improve aging-related CVDs.

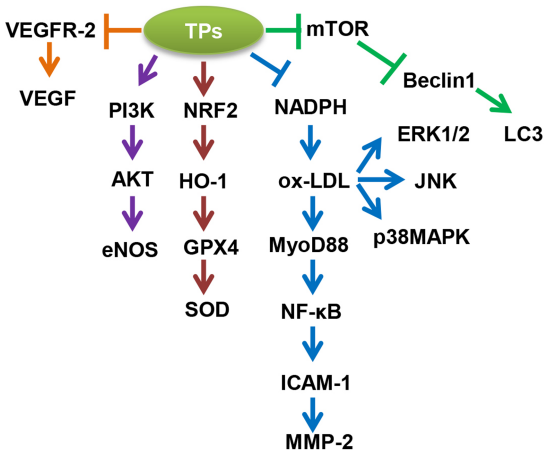

FIGURE 1
Molecular mechanism of tea polyphenols (TPs) to improve aging-related cardiovascular diseases (CVDs). AKT, AKT serine/threonine kinase 1; eNOS, nitric oxide synthase 3; ERK1/2, mitogen-activated protein kinase 1; GPX4, glutathione peroxidase 4; HO-1, heme oxygenase 1; ICAM-1, intercellular adhesion molecule 1; JNK, mitogen-activated protein kinase 8; LC3, microtubule associated protein 1 light chain 3; MMP-2, matrix metallopeptidase 2; mTOR, mechanistic target of rapamycin kinase; MyD88, MYD88 innate immune signal transduction adaptor; NADPH, 2,4-dienoyl-CoA reductase 1; NF-κB, nuclear factor kappa B subunit 1; NRF2, NFE2 like bZIP transcription factor 2; ox-LDL, oxidized low density lipoprotein; p38MAPK, mitogen-activated protein kinase 14; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta; SOD, superoxide dismutase 1; VEGF, vascular endothelial growth factor A; VEGFR-2, kinase insert domain receptor.

TABLE 2 References related to molecular mechanism diagrams.

Title	References
Tea polyphenols enhanced the antioxidant capacity and induced Hsps to relieve heat stress injury	(69)
(-)-Epicatechin gallate blocks the development of atherosclerosis by regulating oxidative stress *in vivo* and *in vitro*	(87)
The inhibitory effect of (-)-epicatechin gallate on the proliferation and migration of vascular smooth muscle cells weakens and stabilizes atherosclerosis	(150)
Green tea polyphenols inhibit human vascular smooth muscle cell proliferation stimulated by native low-density lipoprotein	(151)
EGCG protects vascular endothelial cells from oxidative stress-induced damage by targeting the autophagy-dependent PI3K-AKT–mTOR pathway	(152)
(-)-Epigallocatechin-3-gallate inhibits eNOS uncoupling and alleviates high glucose-induced dysfunction and apoptosis of human umbilical vein endothelial cells by PI3K/AKT/eNOS pathway	(153)
EGCG protects against homocysteine-induced human umbilical vein endothelial cells apoptosis by modulating mitochondrial-dependent apoptotic signaling and PI3K/Akt/eNOS signaling pathways	(154)
Potent inhibition of VEGFR-2 activation by tight binding of green tea epigallocatechin gallate and apple procyanidins to VEGF: relevance to angiogenesis	(155)

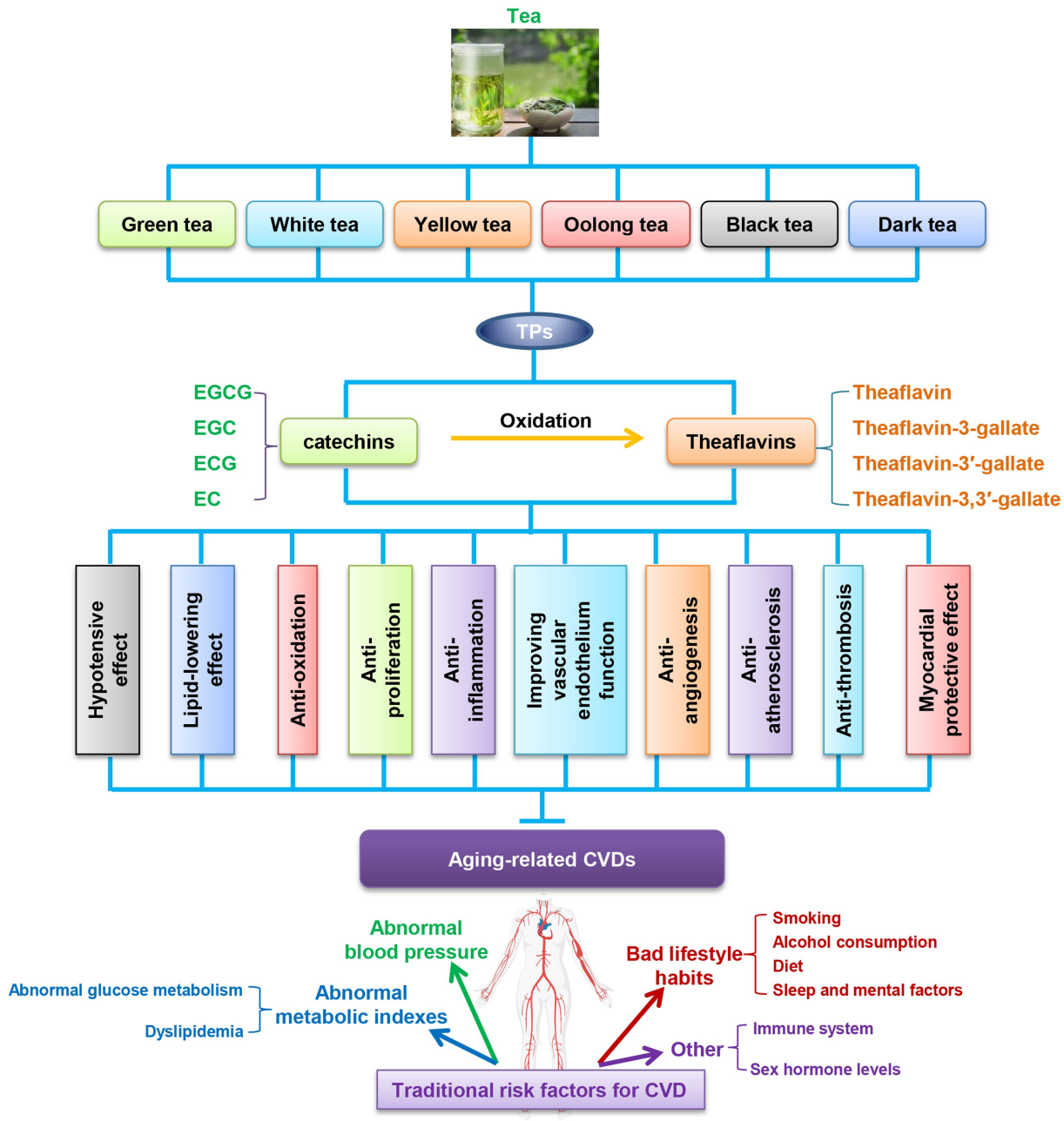

FIGURE 2
TPs improve aging related-CVDs in ten ways. CVDs, cardiovascular diseases; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-gallate; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin-3-gallate; TPs, tea polyphenols.

5. Conclusion

Natural substances originating from natural food and plants are of great interest due to their low toxicity, low cost and easy availability. However, the underlying physiological mechanisms of these substances are not fully understood, especially with respect to the cardiovascular system.

The pathophysiological process of CVD is multifactorial and can be affected by tea components in several processes: anti-hypertension, lipid-lowering effects, anti-oxidation, anti-inflammation, anti-proliferation, anti-angiogenesis, anti-AS, recovery of endothelial function, anti-thrombosis, myocardial protective effect (Figure 2). However, a large number of unresolved issues exist that limit the clinical use of TPs. The debated issues are mainly related to dose, specificity, potency, feasibility and short-or long-term side effects in humans. Although naturally occurring polyphenols are generally considered pharmacologically safe, it is also important to note the presence of deleterious effects of these compounds in the body, which are largely dependent on their distribution in the body and the type of cells on which they act. In addition, the bioavailability of TPs is relatively low when administered orally, and the effective transport of TPs to target organs is an important issue (156). Moreover, some

components of tea polyphenols can also interact with nutrients in the body as well as conventional drugs, which are also potential safety issues (156). To address these issues, animal experiments, large cohort studies and human intervention trials are very necessary in the future.

In conclusion, a growing body of data suggests that TPs have an important role in the prevention and treatment of CVD by interfering with multiple signal transduction pathways. However, the specific molecular roles of TPs in various cells need to be studied in great depth.

Author contributions

JG, YaL, and YiL wrote the paper. KL collected the references. 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 micronutrient content of the diet is correlated with serum glucose biomarkers and lipid profile and is associated with the odds of being overweight/obese—a case-control study

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Background: A low micronutrient intake has been reported to contribute to the double-burden of obesity, increasing the risk for chronic diseases such as cardiovascular disease, diabetes, cancer, and mental disorders. This case-control study compared micronutrient intake profiles in overweight/obese vs. normal-weight individuals. We hypothesized that a low intake of certain micronutrients would increase the odds of being overweight/obese.

Methods: The case group (n=812 adults) consisted of individuals with a BMI of ≥25 kg/m^2, and the control group (n=793) had BMIs of 17.9–24.9 kg/m^2. A validated 124-item food frequency questionnaire was used to determine micronutrient-related dietary-quality, using the index of nutritional quality (INQ), calculated as the fraction of a micronutrient consumed vs. its dietary requirement. In addition, body surface area (BSA) was calculated according to the Mosteller formula.

Results: The control group had significantly higher INQ-scores of vitamin A, vitamin C, calcium, magnesium, and selenium compared to the case group. Furthermore, individuals with normal BSA (≤1.91 m^2 for men; ≤1.71 m^2 for women) had significantly higher INQ scores of vitamin C, calcium, magnesium, and zinc compared to participants with high BSA. In multivariable adjustment regression models, INQs of vitamin C (OR_{BMI}=0.79, 95%CI: 0.64–0.97; OR_{BSA}=0.81, 95%CI, 0.68–0.97) and magnesium (OR_{BMI}=0.69, 95%CI: 0.47–0.99; OR_{BSA}=0.71, 95%CI: 0.52–0.97) were significantly associated with the odds of obesity/overweight (in both BMI and BSA categories).

Conclusion: The significant association between micronutrient levels of the diet, especially of vitamin C and magnesium, with both obesity criteria, emphasized the importance of certain micronutrients in the obesity/overweight causal network.

KEYWORDS

antioxidants, minerals, vitamins, body surface area (BSA), dietary assessment, insulin, public health, weight control

1. Background

The prevalence of obesity and overweight in most countries is increasing rapidly, becoming a non-communicable pandemic (1, 2). Various factors, including genetics, gender, physical activity, economic/social/cultural/religious factors, as well as dietary factors, are intertwined with this pandemic (2, 3). The etiology of obesity is so complex and controversial that it is almost impossible to control or limit all of the contributing factors. On the other hand, obesity is strongly entangled with society's health both directly and indirectly (2). Persons with obesity/overweight are at high risk for certain chronic diseases, including cardiovascular disease (CVD), type 2 diabetes (T2D), hypertension, various cancers, as well as mental disorders (4). In addition to the personal impact for concerned individuals, obesity/overweight imposes high costs on the countries' health systems, both directly and by increasing the prevalence of co-morbidities such as non-communicable diseases (5). It should also be noted that even in communicable diseases, obese/overweight patients tend to be hospitalized longer, take more time to recover, respond later to treatment, or require more medication to recover, and they are also more likely to be re-infected (6–8). These factors drastically increase the treatment costs of obese/overweight people. On average, the annual treatment costs of a person with obesity are almost 30% higher than those of an individual with normal weight (9). Other additional costs, such as treating diseases for which obesity is a risk factor, are indirect and are not readily computable (9). However, dealing with persons with obesity in different countries and societies can be discriminatory, and these people often also suffer also psychologically (10).

Therefore, studying the various factors affecting the incidence and prevalence of obesity/overweight can be part of an effective strategy toward reducing the associated complications and consequences. From a nutritional point of view, various factors are influential regarding the incidence and prevalence of obesity/overweight. For example, several studies have examined the association between chrono-nutrition/meal timing (11, 12), macronutrient intake and their distribution ratio (13), calorie intake, as well as dietary patterns/behaviors (14), and micronutrient intake and the prevalence of obesity/overweight (3, 15).

Many studies and health associations, such as the World Health Organization (WHO), have highlighted the double burden of malnutrition of persons having obesity and micronutrient deficiency (16). Despite having a diet rich in energy and providing enough macronutrients—or even too many—many persons with overweight/obesity were reported to have low vitamin and mineral status due to marginal intake. For instance, obesity has often been associated with a low status of vitamin A (17), iron (18), zinc (19), and vitamin D status (20). Vitamins and minerals are involved in a huge array of metabolic processes, and reduced intake or status increases the risk of bodily dysfunctions (21). For example, low zinc status may compromise immune function as well as the efficiency of antioxidant enzymes such as catalase or superoxide dismutase (22) and has been related to decreased leptin levels (23) and, thus, satiety. Low intake of vitamin A has likewise been linked to increased obesity, perhaps due to its role in modulating RAR/RXR receptors that set the stage for many cellular developments and immune functions (24).

Thus, while many mechanisms and associations between micronutrients and overweight/obesity have been reported and documented, contradictions remain and require further investigation and documentation (1, 25–27). Our present study aimed at comparing the quality of the diet regarding micronutrient intake in individuals with obesity/overweight vs. normal-weight individuals. We hypothesized that a diet of lower nutrient density regarding micronutrients and decreased intake of vitamins and minerals would be related to increased odds of obesity/overweight.

2. Materials and methods

2.1. Participants

In this population-based case-control study, the case group [$n = 812$; overweight/obese individuals based on body mass index (BMI)] and the control group ($n = 793$; individuals with normal BMI) were selected from the patient's caregivers attending Arak Medical Centers, Arak, Iran. First, we briefly explained the study's objectives to the patient's caregivers (who had come to visit the patients), and if persons were interested in joining the study, their height and weight were measured. Based on the calculated BMIs, participants were allocated into case groups (overweight/obese) or controls (normal weight). However, we additionally categorized them in statistical analyses based on the body surface area (BSA) after considering inclusion and exclusion criteria.

Following the completion of the questionnaires, participants were referred to the specified accredited laboratories for biological tests. The case group consisted of individuals with a higher or equal BMI of 25 kg/m^2, and the control group had a BMI of 17.9–24.9 kg/m^2. The cases and controls were frequently matched in terms of age and gender. In this regard, 50.4% ($n = 409$) of the case group and 50.2% ($n = 398$) of the control group were women. The mean age was 47.6 ± 13.0 years in the case group and 48.4 ± 12.7 years in the control group. Written informed consent was received from all participants, and the Arak University of Medical Science Ethics Committee, Arak, Iran, approved the study protocol (Ethics Committee No. IR.ARAKMU.REC.1398.094). The study protocol and method have been published elsewhere (28, 29).

2.1.1. Inclusion and exclusion criteria

The inclusion were: (a) Lack of active diseases such as CVD and cancer; (b) Not following a special diet such as vegetarian; (c) No major change in diet in the past year; (d) No pregnancy or lactation; (e) No medication or dietary supplements in the past 6 months; (f) Willingness to participate in the study; (g) Absence of drug addiction; (k) Living in Arak for the past 5 years, I. Being in the age range of 18–81 years. An exclusion criteria was reporting less than 80% of the questions in the questionnaire.

2.1.2. Assessment of dietary intake

A validated and reliable 124-item food frequency questionnaire (FFQ), including commonly consumed Iranian food items, was used to assess dietary intake (30). Individuals were asked to report the amount and frequency of food and beverage consumption in the previous year. Frequency of use was reported as "never use," "daily," "weekly," "monthly" or "annually." The "daily" or "weekly" intake in

the season in question was asked for seasonal food items. Food intake was evaluated by means of the Nutritionist IV software (First Databank, Hearst Corp., San Bruno, CA, United States), and the daily average of macro- and micronutrients was extracted. The total energy was obtained as the sum of 9 kcal/g for fat, 4 kcal/g for protein, 4 kcal/g for carbohydrates, and 2 kcal/g for dietary fiber. Daily intake was aggregated as a total of food in 1 year and divided by 365 to obtain the average daily intake.

During several sessions, a nutritionist provided the training to complete the questionnaires accurately to medical staff. All questionnaires were completed in person by the trained medical staff.

2.1.3. Assessment of blood tests

After 8–12 h of fasting, 5 mL of intravenous blood was obtained from all participants. Serum was then collected following centrifugation (2,000 g for 10 min) and stored at −80°C until further analyses. Blood glucose biomarkers and lipid profiles were measured by approved laboratories using the following methods.

According to the kit instructions, fasting blood glucose (FBG, in mg/dL), insulin (mIU/mL), glycated hemoglobin (HbA1C, in mmol/mol), low-density lipoprotein cholesterol (LDL-C, in mg/dL), triglycerides (TG, in mg/dL), high-density lipoprotein cholesterol (HDL-C, in mg/dL), total cholesterol (in mg/dL) were assessed using relevant commercial ELISA kits (all provided by Pishtazteb Co.; Tehran, Iran), following the manufacturer's guidelines.

2.1.4. Assessment of anthropometric and socioeconomic variables

For all participants, BMI was obtained by dividing squared height (meters) by weight (kg). Seca scales (Seca; Hamburg, Germany) measured the individuals' weight with an accuracy of 50 g, and the height was assessed using a tape measure attached to the wall with an accuracy of 1 cm. The BMI was considered "normal" if the value was between 17.9 and 24.9 kg/m^2, and obese/overweight if the value was equal to or higher than 25 kg/m^2. Body surface area (BSA) was calculated according to the Mosteller formula: BSA = (height (cm) * weight (kg)/3,600)$^{1/2}$ (31). The BSA was considered 'normal' if the mean value was ≤1.91 m^2 for men and ≤1.71 m^2 for women.

A general information questionnaire collected information on age (year), gender (female/male), marital status (single/married/widow, divorced, separated, not willing to mention, living with a partner), education (lower than high school diploma/higher than diploma), regular physical activity (yes/no), smoking (yes/no), alcohol (yes/no), history of CVD (yes/no), T2D (yes/no), and hypertension (yes/no).

2.1.5. Assessment of dietary quality regarding micronutrient intake

We estimated micronutrient quality using each micronutrient's index of nutritional quality (INQ) for vitamins A, C, D, E, K, thiamin, riboflavin, niacin, biotin, folate, pantothenic acid, B12, and B6 and calcium, iron, magnesium, zinc, copper, selenium, and manganese. For calculating INQs of micronutrients in which existed a defined recommended dietary allowance (RDA) or adequate intake (AI) in dietary reference intake (DRI) tables, we used the following formula: INQ = (consumed amount of a micronutrient per 1,000 kcal)/(RDA or AI of that micronutrient per 1,000 kcal) (19, 20, 23). FFQ-derived dietary data were used to calculate INQ scores.

2.2. Statistical analysis

Original power calculations were based on an assumed p-value of 0.05 (alpha), a targeted power of 0.80, and an assumed P_0 (probability of exposure in controls), and P_1 (probability of exposure in case subjects) or odds ratio, based on literature findings.

Q-Q normality plots and the Kolmogorov–Smirnov (KS) test were employed to verify the normality of the variables' distribution. For non-normal distributed variables, a log-transformation was performed (log-transferred data were used in all analyses). The INQs were investigated across the following characteristics of participants (both BMI and BSA groups): age, gender, education, smoking, alcohol, marital status, regular physical activity, history of CVD, T2D, and hypertension analyzed by independent t-tests or chi-squared (χ^2) tests for continuous and categorical variables, respectively. Odds ratio (OR) and 95% confidence intervals (CI) for obesity/overweight using BMI and also BSA as outcomes were estimated using logistic regression in three models. In model A, crude ORs and 95% CIs were reported. In model B, we adjusted for age and gender. Model C was model B+ with additional adjustments for education, smoking, alcohol, marital status, regular physical activity, history of CVD, T2D, and hypertension. Bivariate and partial correlation were used to investigate the correlation between INQs and blood glucose handling markers and lipid profile. Partial correlation was done by controlling for age, gender, education, smoking, alcohol, marital status, regular physical activity, history of CVD, T2D, and hypertension. All statistical analyses were performed using SAS® 9.3 (SAS Institute Inc., Cary, NC); all p-values were based on two-sided tests. A p-value below 0.05 (2-sided) was considered statistically significant. Data reported in the descriptive analyses represent mean and standard deviation (SD) or number (percentage).

3. Results

The distribution of participants' characteristics across BMI groups is represented in Table 1. There was a significant difference in the history of the disease. Participants with obesity/overweight reported a higher prevalence of a history of CVD, T2D, and hypertension compared to normal-weight individuals (all p-values < 0.01). In addition, there was a significant difference between blood glucose handling biomarkers and lipid profile between cases and controls. Individuals with obesity/overweight had a significantly higher level of FBG, insulin, HbA1C, LDL-C, TG, and total cholesterol compared to normal-weight participants. On the contrary, the control group exhibited a significantly higher level of HDL-C compared to the case group.

A comparison of the INQs of the participants based on BMI groups is represented in Table 2. Controls (normal BMI) had a significantly higher INQ for vitamin A, vitamin C, calcium, magnesium, and selenium compared to the case group.

Table 3 represents a comparison of the INQs of the participants based on BSA groups. Individuals with normal BSA (different

TABLE 1 Distribution of characteristics across BMI groups of participants of the case-control study[f].

Characteristics	Mean±SD or number (%)			*p*-value[a]
	Cases (*n*=812)	Controls (*n*=793)	Total (*n*=1,605)	
Age (years)	47.6 ± 13.0	48.4 ± 12.7	48.0 ± 12.9	0.26
BMI (kg/m^2)	27.8 ± 2.5	22.2 ± 1.7	25.0 ± 3.5	**<0.01**
FBG (mg/dL)	94.2 ± 21.0	92.1 ± 18.1	93.2 ± 19.6	**0.03**
Insulin (mIU/mL)	8.1 ± 11.9	7.2 ± 4.6	7.7 ± 9.0	**0.04**
HbA1C (mmol/mol)	18.8 ± 15.5	15.1 ± 12.2	17.0 ± 14.1	**<0.01**
LDL-C (mg/dL)	129.9 ± 34.6	126.5 ± 33.4	128.2 ± 34.1	**0.05**
TG (mg/dL)	128.5 ± 77.8	117.0 ± 88.1	122.8 ± 83.2	**<0.01**
HDL-C (mg/dL)	52.1 ± 14.0	56.0 ± 14.2	54.0 ± 14.2	**<0.01**
Cholesterol (mg/dL)	202.4 ± 38.3	198.1 ± 36.2	200.3 ± 37.4	**0.02**
Gender:				0.96
Women	409 (50.3%)	398 (50.2%)		
Men	403 (49.7%)	395 (49.8%)		
Education:				0.71
Diploma and low literate	545 (67.1%)	525 (66.2%)		
Higher than diploma	267 (32.8%)	268 (33.8%)		
Smoking status:				0.99
Non-smokers	680 (83.7%)	664 (83.7%)		
Alcohol consumption				0.75
No	723 (89.0%)	702 (88.5%)		
Marital status:				0.91
Married	615 (75.7%)	600 (75.6%)		
Single	136 (16.7%)	137 (17.2%)		
Other	61 (7.6%)	56 (7.2%)		
Regular physical activity:				0.06
No	628 (77.4%)	580 (73.2%)		
T2D:				**<0.01**
Yes	246 (30.3%)	190 (24.0%)		
CVD:				**<0.01**
Yes	204 (25.1%)	109 (13.7%)		
Hypertension:				**<0.01**
Yes	495 (61.0%)	332 (41.8%)		

[f]A BMI equal to or higher than 25 kg/m^2 was considered obese/overweight and between 17.9 and 24.9 kg/m^2 as normal weight.
[a]Independent Student's *t*-test was used for comparing continuous variables, and a Chi-square test was used for categorical variables; significant *p*-values are shown in bold.SD, Standard deviation; BMI, Body mass index; HbA1C, Glycated hemoglobin; LDL-C, Low-density lipoprotein cholesterol; TG, Triglycerides; HDL-C, High-density lipoprotein cholesterol; T2D, Type 2 diabetes; CVD, Cardiovascular diseases.

categories were considered for gender) had significantly higher INQ of vitamin C, calcium, magnesium, and zinc compared to participants with high BSA. Comparing the results of Tables 2, 3 revealed that only INQ of vitamin C, calcium, and magnesium differed significantly between the controls (normal BMI or normal BSA) and cases in both categories (BMI and BSA categories).

Table 4 represents ORs and CIs for the association between INQs and BMI groups. The INQ of vitamin A, C, calcium, magnesium, and selenium as continuous variables in all three models (crude model, age and gender-adjusted model, and multivariable-adjusted model) was significantly associated with BMI. The INQ of iron, however, was only significantly associated with BMI in the age and gender (model 2) as well as the fully adjusted model (model 3).

Table 5 shows ORs and CIs for the association between INQ and BSA groups. The INQ of vitamin C, magnesium, and zinc as continuous variables in all three models (crude model, age and gender-adjusted model, and multivariable-adjusted model) was significantly associated with BSA. The INQ of calcium was only significantly associated with BSA in the crude model. Comparing the results of Tables 4, 5 revealed that only the INQ of vitamin C and magnesium were significantly associated with both

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TABLE 2 Comparison of the participants' index of nutritional quality (INQ) regarding micronutrient intake patterns based on BMI groups[f].

INQs	Mean±SD			RDA (or AI) Women-Men	*p*-value[a]
	Cases (*n*=812)	Controls (*n*=793)	Total (*n*=1,605)		
Vitamin A	0.56 ± 0.29	0.60 ± 0.31	0.58 ± 0.30	700–900 μg/d (RAE/d)	**0.02**
Vitamin D	0.10 ± 0.07	0.10 ± 0.08	0.10 ± 0.08	15–15 μg/d	0.66
Vitamin E	0.89 ± 0.41	0.91 ± 0.43	0.90 ± 0.42	15–15 mg/d	0.32
Vitamin K	1.88 ± 1.00	1.93 ± 1.05	1.91 ± 1.03	90–120 μg/d	0.37
Vitamin C	1.29 ± 0.58	1.38 ± 0.64	1.33 ± 0.61	75–90 mg/d	**<0.01**
Thiamin	1.36 ± 0.62	1.39 ± 0.64	1.38 ± 0.63	1.1–1.2 mg/d	0.43
Riboflavin	1.27 ± 0.54	1.31 ± 0.56	1.29 ± 0.55	1.1–1.3 mg/d	0.16
Niacin	1.39 ± 0.59	1.42 ± 0.61	1.40 ± 0.60	14–16 mg/d	0.26
Vitamin B6	1.14 ± 0.45	1.16 ± 0.48	1.15 ± 0.47	1.3–1.7 mg/d	0.32
Folate	1.21 ± 0.51	1.25 ± 0.51	1.23 ± 0.51	400–400 μg/d	0.12
Vitamin B12	1.62 ± 1.00	1.68 ± 1.04	1.65 ± 1.02	2.4–2.4 μg/d	0.25
Biotin	0.89 ± 0.34	0.91 ± 0.35	0.90 ± 0.35	30–30 μg/d	0.27
Pantothenic acid	1.07 ± 0.45	1.09 ± 0.45	1.08 ± 0.45	5–5 mg/d	0.32
Calcium	0.75 ± 0.31	0.80 ± 0.32	0.77 ± 0.31	1,000–1,000 mg/d	**<0.01**
Iron	1.67 ± 0.62	1.71 ± 0.66	1.70 ± 0.64	18–8 mg/d	0.14
Magnesium	0.99 ± 0.32	1.03 ± 0.35	1.01 ± 0.34	320–420 mg/d	**0.03**
Zinc	1.10 ± 0.42	1.14 ± 0.45	1.12 ± 0.43	8–11 mg/d	0.09
Copper	2.08 ± 1.05	2.12 ± 1.08	2.10 ± 1.07	900–900 μg/d	0.44
Manganese	3.09 ± 1.34	3.18 ± 1.38	3.13 ± 1.36	1.8–2.3 mg/d	0.18
Selenium	1.61 ± 0.63	1.72 ± 0.77	1.66 ± 0.70	55–55 μg/d	**<0.01**

[f]A BMI equal to or higher than 25 kg/m^2 was considered obese/overweight and between 17.9 and 24.9 kg/m^2 as normal.
[a]Independent sample *t*-test was used for comparing INQs; significant *p*-values are shown in bold. RAE: retionoic acid equivelents.

categories of obesity (BMI and BSA categories) in the fully adjusted model.

In addition, we performed sensitivity analyses based on gender groups for the fully adjusted model considering BMI and BSA. The sensitivity analysis results were in line with the main results, although the number of significant associations was higher in men than in women (Supplementary Table S1).

Finally, Figure 1 represents the partial correlation controlling for age, gender, education, smoking, alcohol, marital status, regular physical activity, history of CVD, T2D, and hypertension between INQs and blood glucose handling markers and lipid profile. In the partial model, there was a significant correlation between the INQ of vitamin A and HDL-C and LDL-C; calcium and FBS, HDL-C, LDL-C; iron and HDL-C; vitamin D and insulin, riboflavin and HDL-C; vitamin B6 and FBS; folate and HDL-C; vitamin K and insulin, HbA1C; magnesium and HbA1C, LDL-C, total cholesterol, and triglycerides; zinc and HDL-C; manganese and HDL-C. Similar results were obtained in bivariate correlation models (Supplementary Table S2).

4. Discussion

In studying the dietary patterns regarding micronutrient adequacy based on BMI grouping, a significantly lower score of the INQ was found for vitamins A and C, calcium, magnesium, and selenium of cases vs. controls. Similarly, based on the BSA grouping, a significant difference was observed between the INQ of vitamin C, calcium, magnesium, and zinc between cases and controls; and vitamin C, calcium, and magnesium were found to differ based on both categories. Our study, based on multivariate-adjusted models, showed significantly higher INQs of vitamin A, C, calcium, magnesium, and selenium, which were inversely related to overweight/obesity based on BMI, and also a lower INQ for vitamin C, magnesium, and zinc in the overweight/obesity group based on and BSA, with only vitamin C and magnesium being significantly inversely associated with both classifications.

The association between INQ and obesity/overweight (based on BMI) has been studied in two previous studies (3, 32). Gholamalizadeh et al. (3) examined the association between the risk of obesity and INQ in adolescent boys. They reported a significant inverse association between the INQ of vitamin C, iron, vitamin B6, pantothenic acid, selenium, and magnesium and the risk of obesity in adolescent boys (3). They also reported that after adding different confounders to the model, a significant inverse relationship, in addition to the above, was observed between the INQ of zinc and obesity risk (3).

Mehrdad et al. (32) investigated the relationship between INQ and obesity in adults. Similar to the present study, their results emphasized an inverse association between the INQ of vitamin D and manganese and obesity. The association between vitamin D intake and obesity has been shown in other studies (33–35) and may be explained by the importance of this vitamin for the immune system and systemic

TABLE 3 Comparison of the participants' index of nutritional quality (INQ) regarding micronutrient intake patterns based on BSA groups[f].

INQs	Mean±SD		*p*-value[a]
	Cases (*n*=818)	Controls (*n*=787)	
Vitamin A	0.58 ± 0.30	0.59 ± 0.30	0.65
Vitamin D	0.10 ± 0.07	0.11 ± 0.08	0.06
Vitamin E	0.89 ± 0.42	0.91 ± 0.43	0.25
Vitamin K	1.87 ± 1.02	1.95 ± 1.04	0.14
Vitamin C	1.30 ± 0.57	1.37 ± 0.64	**0.02**
Thiamin	1.38 ± 0.60	1.38 ± 0.66	0.94
Riboflavin	1.28 ± 0.54	1.30 ± 0.55	0.30
Niacin	1.39 ± 0.60	1.41 ± 0.61	0.53
Vitamin B6	1.13 ± 0.47	1.17 ± 0.46	0.13
Folate	1.21 ± 0.50	1.24 ± 0.52	0.16
Vitamin B12	1.65 ± 1.01	1.65 ± 1.03	0.96
Biotin	0.89 ± 0.35	0.90 ± 0.35	0.60
Pantothenic acid	1.07 ± 0.44	1.08 ± 0.47	0.53
Calcium	0.76 ± 0.31	0.79 ± 0.32	**0.04**
Iron	1.70 ± 0.63	1.68 ± 0.65	0.44
Magnesium	0.99 ± 0.32	1.03 ± 0.35	**<0.01**
Zinc	1.09 ± 0.40	1.15 ± 0.46	**<0.01**
Copper	2.09 ± 1.04	2.12 ± 1.10	0.48
Manganese	3.08 ± 1.35	3.19 ± 1.37	0.09
Selenium	1.66 ± 0.67	1.67 ± 0.74	0.85

[f]The BSA was considered "normal" if the mean value was ≤1.91 m^2 for men and ≤1.71 m^2 for women.
[a]Independent sample *t*-test was used for comparing INQs; significant *p*-values are shown in bold.

inflammation and oxidative stress that are aggravating factors in obesity (36, 37). In the present study, vitamin D intake was similar, though rather quite low in both groups, a fact that had been previously reported for the region (3). However, none of the two studies (3, 32) have investigated the relationship between the quality of micronutrients and "obesity" based on the BSA, so it is impossible to compare results in this regard.

The finding that vitamin C was significantly associated with obesity in our study (based on BMI and BSA) and also by Gholamalizadeh et al. (3) maybe is due to its antioxidant and, thus, indirect anti-inflammatory effect (38–40). In fact, studies have shown that people exposed to a high amount of reactive oxygen species (ROS) are more prone to being obese or overweight (41). However, it should also be noted that obesity itself can result in increased production of ROS (41). In addition, vitamin C is a good indicator of fruit and vegetable intake in general (42), and thus its consumption may reflect an altogether more healthy dietary pattern that also contains more dietary fiber and also secondary plant metabolites such as polyphenols and carotenoids that also appear to play some protective roles against obesity (43–45).

Although our study was not able to examine the possible mechanisms underlying the role of vitamin C and obesity, its antioxidant properties have been reported to protect to a certain degree from inflammation and oxidative stress (38–40). On the other hand, persons in this study, including the cases, consumed 25–30% more vitamin C than the RDA and can thus be judged as having an adequate vitamin C status. Nevertheless, the evidence remains controversial, and taking vitamin C supplements does not appear to prevent obesity or being overweight (46), especially if persons are not deficient in this vitamin. However, recommending foods rich in vitamin C can be an effective strategy to improve inflammatory and oxidative stress status and decrease CVD risk in people with obesity/overweight (47, 48).

In addition, the role of magnesium—having a significant association with overweight/obesity with both BMI and BSA—in preventing atherosclerosis, and stroke, lowering blood pressure, cholesterol, and triglycerides, correcting irregular heartbeat, and reducing insulin requirements in diabetic individuals has been well demonstrated (49, 50). Although the mechanisms of its direct effects in preventing or controlling obesity are controversial, long-term low magnesium intake has been associated with increased insulin resistance, leading to a cascading network of factors that eventually might lead to obesity or overweight (51, 52). Magnesium participates in a large number of energy-related enzymatic reactions (53) and has been shown to potentially reduce blood pressure, hypertriglyceridemia, and hyperglycemia (54). It also has been emphasized that its intake has rather globally declined during the last decades (55). Although the mentioned mechanisms may partially explain the relationship between low magnesium intake and obesity, they cannot justify the whole causal network (56). Especially as in the present study, most persons appeared to reach the RDA, and a deficiency is therefore not likely.

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TABLE 4 Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between INQ and BMI groups[f].

INQs[l]	Crude model		Adjusted model 1		Adjusted model 2	
	ORs and CI 95%[a]	*p*-value	ORs and CI 95%[b]	*p*-value	ORs and CI 95%[c]	*p*-value
Vitamin A	**0.68 (0.49–0.94)**	**0.02**	**0.58 (0.39–0.87)**	**<0.01**	**0.59 (0.39–0.89)**	**0.01**
Vitamin D	0.76 (0.22–2.64)	0.66	1.10 (0.24–5.07)	0.89	1.19 (0.24–5.73)	0.82
Vitamin E	0.89 (0.70–1.12)	0.32	0.87 (0.65–1.16)	0.34	0.85 (0.63–1.15)	0.30
Vitamin K	0.95 (0.87–1.05)	0.37	0.95 (0.84–1.07)	0.38	0.96 (0.85–1.08)	0.49
Vitamin C	**0.78 (0.66–0.92)**	**<0.01**	**0.77 (0.63–0.94)**	**0.01**	**0.79 (0.64–0.97)**	**0.03**
Thiamin	0.94 (0.80–1.09)	0.43	0.91 (0.75–1.11)	0.38	0.89 (0.73–1.09)	0.28
Riboflavin	0.88 (0.73–1.05)	0.16	0.91 (0.73–1.14)	0.43	0.90 (0.72–1.14)	0.41
Niacin	0.91 (0.77–1.07)	0.26	0.84 (0.69–1.03)	0.09	0.85 (0.69–1.04)	0.11
Vitamin B6	0.90 (0.73–1.11)	0.32	0.95 (0.74–1.22)	0.71	1.01 (0.77–1.31)	0.93
Folate	0.86 (0.71–1.04)	0.12	0.89 (0.70–1.13)	0.36	0.90 (0.70–1.15)	0.41
Vitamin B12	0.94 (0.86–1.04)	0.25	0.94 (0.84–1.06)	0.32	0.93 (0.83–1.05)	0.30
Biotin	0.85 (0.65–1.12)	0.27	0.93 (0.66–1.31)	0.68	0.92 (0.64–1.30)	0.64
Pantothenic acid	0.89 (0.72–1.11)	0.32	0.86 (0.66–1.11)	0.26	0.84 (0.64–1.10)	0.21
Calcium	**0.62 (0.45–0.84)**	**<0.01**	**0.58 (0.40–0.85)**	**<0.01**	**0.59 (0.40–0.87)**	**<0.01**
Iron	0.89 (0.76–1.04)	0.14	**0.78 (0.64–0.95)**	**0.01**	**0.79 (0.65–0.96)**	**0.02**
Magnesium	**0.72 (0.54–0.97)**	**0.03**	**0.69 (0.48–0.98)**	**0.04**	**0.69 (0.47–0.99)**	**0.05**
Zinc	0.82 (0.66–1.03)	0.09	0.90 (0.68–1.19)	0.48	0.94 (0.70–1.25)	0.66
Copper	0.96 (0.88–1.05)	0.44	0.98 (0.88–1.10)	0.80	0.99 (0.88–1.11)	0.88
Selenium	**0.79 (0.69–0.91)**	**<0.01**	**0.71 (0.60–0.85)**	**<0.01**	**0.70 (0.58–0.84)**	**<0.01**
Manganese	0.95 (0.88–1.02)	0.18	0.97 (0.89–1.06)	0.50	0.96 (0.88–1.05)	0.43

[a]Crude model.
[b]Model A: Age and sex-adjusted.
[c]Model B: Model A+ Education, smoking, alcohol, marital status, regular physical activity, history of CVD, type 2 diabetes, and hypertension adjusted.
[f]The BMI equal to or higher than 25 kg/m^2 was considered obese/overweight and between 17.9 and 24.9 kg/m^2 as normal weight.
[l]All values are based on log-transformed data; significant *p*-values are shown in bold.

The advantages of using INQs are that it considers nutrient intake based on total calorie intake and compares them with the RDA (57–59). As a result, it is expected that the obtained results will be more realistic than the study of merely the amount of micronutrient intake. However, it should be emphasized that studies with longitudinal designs with appropriate sample sizes are needed to confirm the sensitivity and specificity of the INQ as well as its applicability in nutritional studies.

One of the important strengths of our study is investigating the correlation between INQ and blood-based biomarkers. While remarkably, vitamin C was not associated with any measured biomarker, magnesium was related to decreased HbA1C, total cholesterol, and LDL-C. Earlier studies had already emphasized that persons with a better magnesium status tended to show improvements in glucose control and blood lipids (60, 61). We can only speculate on the absence of a relation of vitamin C and any measurable effects. It is possible that the action of vitamin C rather influenced markers of inflammation and oxidative stress, which were not assessed in the present study, and that differences in vitamin C were not pronounced enough to produce changes in the observed biomarkers of blood lipids and glucose control.

Therefore, this investigation allowed us to measure the predictive power of INQ. Furthermore, one of the limitations of nutritional studies is the lack of attention to discussing the calibration of the studied methods, i.e., that the results obtained may be significant but, in fact, have little or no clinical importance or significance. Therefore, the results of our study, in addition to being significant, could also point toward clinical importance. Another strength of the study was using a valid and reliable FFQ. This allowed us to have a comprehensive and complete overview of micronutrient intake. In addition, a population-based case-control study design allowed us to control for a wide range of variables and confounders. However, due to budget limitations, we had to disregard some potential confounding factors, such as genetic differences.

Thus, disregarding genetic differences was one of the major limitations of our study. However, as the study was conducted with a relatively appropriate sample size in a population-based design, following age and gender-matched design, it can be concluded that the results can be generalized to the majority of the population living under similar conditions. Another limitation of our study was using FFQ, which is prone to recall bias. However, trained personnel completed the FFQs, and also, because the questionnaire had already been validated in previous studies, it seems that this bias would not

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TABLE 5 Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between INQ and BSA groups[f].

	Crude model		Adjusted model 1		Adjusted model 2	
INQs	ORs and CI 95%[a]	*p*-value	ORs and CI 95%[b]	*p*-value	ORs and CI 95%[c]	*p*-value
Vitamin A	0.93 (0.67–1.28)	0.65	1.01 (0.71–1.43)	0.95	1.06 (0.75–1.50)	0.72
Vitamin D	0.30 (0.08–1.04)	0.06	0.41 (0.10–1.56)	0.19	0.46 (0.12–1.80)	0.26
Vitamin E	0.87 (0.69–1.10)	0.25	0.98 (0.77–1.26)	0.92	1.02 (0.79–1.31)	0.87
Vitamin K	0.93 (0.84–1.02)	0.14	0.92 (0.83–1.01)	0.10	0.91 (0.82–1.02)	0.11
Vitamin C	**0.83 (0.70–0.97)**	**0.02**	**0.80 (0.67–0.95)**	**0.01**	**0.81 (0.68–0.97)**	**0.02**
Thiamin	0.99 (0.85–1.16)	0.94	1.02 (0.87–1.21)	0.76	1.02 (0.87–1.21)	0.75
Riboflavin	0.91 (0.76–1.08)	0.30	0.93 (0.77–1.12)	0.46	0.95 (0.78–1.15)	0.63
Niacin	0.95 (0.80–1.11)	0.53	0.94 (0.79–1.12)	0.53	0.96 (0.81–1.15)	0.70
Vitamin B6	0.85 (0.69–1.05)	0.14	0.91 (0.73–1.14)	0.42	0.96 (0.77–1.21)	0.76
Folate	0.87 (0.72–1.06)	0.16	0.90 (0.73–1.10)	0.31	0.91 (0.74–1.12)	0.40
Vitamin B12	0.99 (0.90–1.01)	0.96	1.00 (0.90–1.11)	0.99	1.00 (0.91–1.11)	0.90
Biotin	0.93 (0.70–1.22)	0.60	0.91 (0.68–1.23)	0.57	0.93 (0.69–1.26)	0.66
Pantothenic acid	0.93 (0.75–1.15)	0.53	0.94 (0.75–1.19)	0.64	0.97 (0.77–1.23)	0.81
Calcium	**0.73 (0.54–0.99)**	**0.05**	0.75 (0.54–1.05)	0.09	0.79 (0.57–1.11)	0.17
Iron	1.06 (0.91–1.23)	0.44	1.06 (0.90–1.25)	0.43	1.08 (0.91–1.27)	0.36
Magnesium	**0.67 (0.51–0.92)**	**0.01**	**0.70 (0.51–0.95)**	**0.02**	**0.71 (0.52–0.97)**	**0.04**
Zinc	**0.75 (0.60–0.94)**	**0.01**	**0.68 (0.53–0.87)**	**<0.01**	**0.70 (0.55–0.89)**	**<0.01**
Copper	0.97 (0.88–1.06)	0.48	0.96 (0.87–1.05)	0.40	0.96 (0.87–1.06)	0.46
Selenium	0.98 (0.86–1.13)	0.85	0.97 (0.83–1.12)	0.68	0.99 (0.85–1.15)	0.91
Manganese	0.94 (0.87–1.01)	0.09	0.93 (0.86–1.01)	0.08	0.94 (0.87–1.01)	0.12

[a]Crude model.
[b]Model A: Age and sex-adjusted.
[c]Model B: Model A+ education, smoking, alcohol, marital status, regular physical activity, history of CVD, type 2 diabetes, and hypertension adjusted.
[f]The BSA was considered "normal" if the mean value was ≤1.91 m^2 for men and ≤1.71 m^2 for women.Significant *p*-values are shown in bold.
INQ, Index of nutritional quality; BSA, Body surface area.

seriously harm the results. Another limitation of our study was the lack of body composition indicators such as body fat or fat-free tissue percentage. Budget restrictions and lack of access to more sophisticated devices such as DEXA were the main reasons for this limitation. However, as one of our important strengths, we used two different definitions of obesity (BMI and BSA) to minimize this limitation, although we recommend that future studies consider body composition for more accurate results.

After considering strengths and limitations, potentials of the present study include revealing how the intake of specific micronutrients affects glucose biomarkers and lipid profile levels in individuals. Understanding these associations can provide valuable insights into the role of micronutrients in metabolic health. By comparing the micronutrient intake of overweight/obese individuals with that of non-overweight individuals (controls), this study can establish a potential link between micronutrient intake and weight status. This can contribute to developing targeted interventions for obesity prevention and management. Thus, the ultimate goal of this study was to provide evidence-based recommendations for improving metabolic health and reducing the risk of overweight/obesity.

Finally, the predominant limitation of this study was that as a case-control study, the design can identify associations but cannot determine causality. Various confounding factors, such as overall diet quality, physical activity levels, and genetic predispositions, may influence the observed relationships. Addressing these confounding factors and considering potential biases are crucial to strengthen the study's findings. Additionally, accurately assessing dietary intake can be challenging due to reliance on self-reported data, recall bias, and variations in portion sizes and food composition. Overcoming these challenges requires meticulous study design, robust data collection methods, and appropriate statistical analyses.

5. Conclusion

The results of our study add to the evidence of the role of dietary micronutrient adequacy in people with overweight and obesity, in line with the double burden of malnutrition. The association observed between INQ of vitamin C and magnesium with respect to both obesity criteria (BMI and BSA) highlights the importance of these two micronutrients. Possible mechanisms need to be further explored and should be followed in the following cohort-design studies. In conclusion, our study highlighted the role of dietary adequacy regarding micronutrient intake in association with overweight/obesity

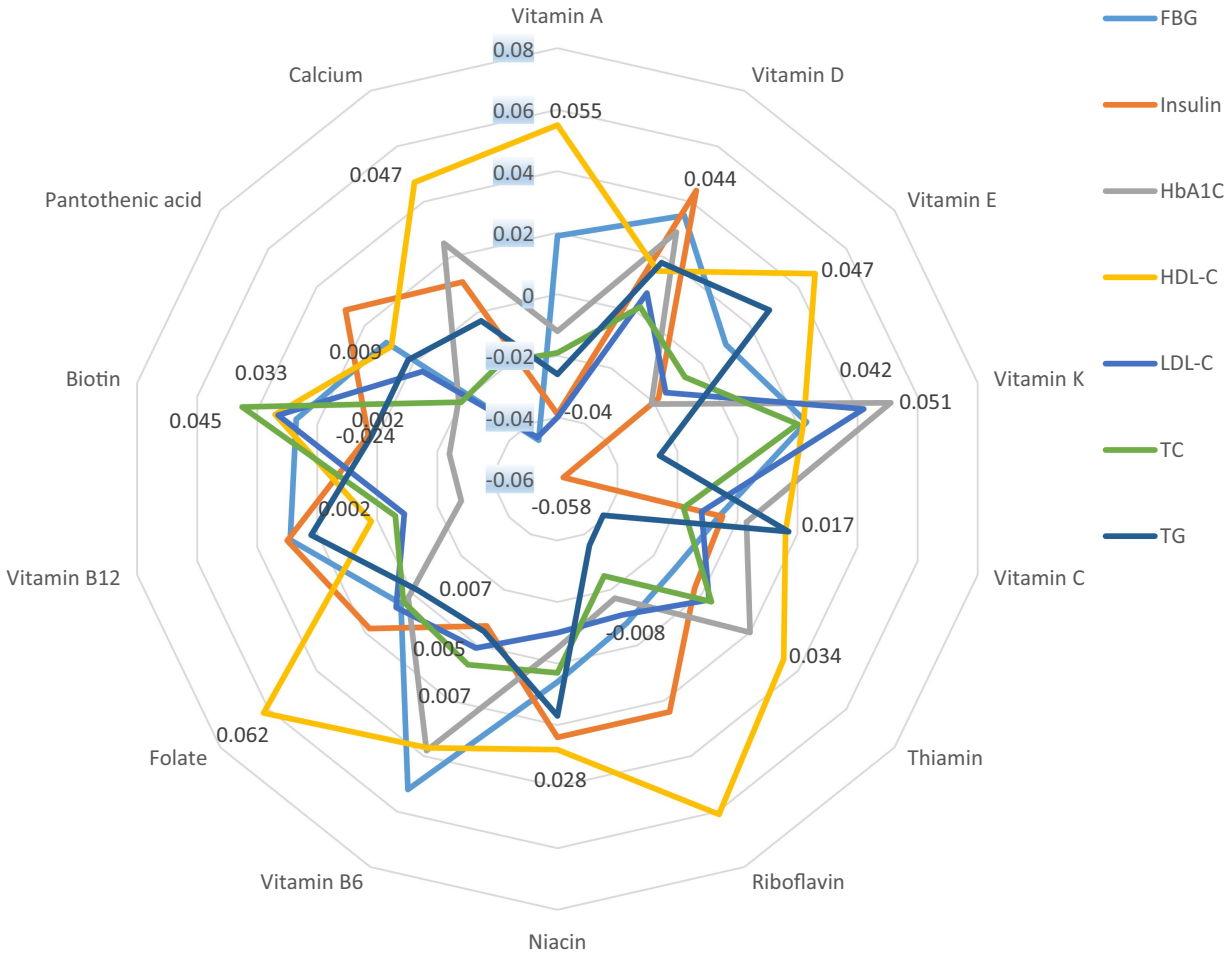

FIGURE 1
Partial correlation* between INQs and blood glucose handling markers and lipid profile controlling for age, gender, education, smoking, alcohol, marital status, regular physical activity, history of CVD, type 2 diabetes, and hypertension. INQ, Index of nutritional quality; FBG, Fasting blood glucose; HbA1C, Glycated hemoglobin; LDL-C, Low-density lipoprotein cholesterol; TC, Total cholesterol; TG, Triglycerides; HDL-C, High-density lipoprotein cholesterol. * Only significant values are shown. Results regarding micronutrients that had no significant correlation with any of the indicators and bivariate correlation models are shown in Supplementary Table S1.

and considers the use of the INQ to be preferable to the use of micronutrient intake alone to assess nutritional status. In addition, considering different definitions (classifications) of obesity may affect the results; therefore, considering the most suitable methods of defining obesity, including body composition analysis along with the more traditional BMI or BSA, would be necessary.

Data availability statement

The data presented in this study are available on request from the corresponding author. Due to our institute's rules and laws, the data are not publicly available.

Ethics statement

The studies involving human participants were reviewed and approved by Arak University of Medical Science Ethics Committee, Arak, Iran, approved the study protocol (Ethics Committee No. IR.ARAKMU.REC.1398.094). The patients/participants provided their written informed consent to participate in this study.

Author contributions

FV designed the study, performed the statistical analyses, and interpreted the data. WR was involved in the data collection. FV and SHD drafted the manuscript. TB provided expertise and oversight on the 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/fnut.2023.1148183/full#supplementary-material

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Glossary

CVD	Cardiovascular Disease
T2D	Type 2 Diabetes
WHO	World Health Organization
BMI	Body Mass Index
BSA	Body Surface Are
FFQ	Food Frequency Questionnaire
FBG	Fasting Blood Glucose
HbA1C	Glycated Hemoglobin
LDL-C	Low-Density Lipoprotein Cholesterol
TG	Triglycerides
HDL-C	High-Density Lipoprotein Cholesterol
INQ	Index of Nutritional Quality
RDA	Recommended Dietary Allowance
AI	Adequate Intake
DRI	Dietary Reference Intake
OR	Odds Ratio
CI	Confidence Interval
SD	Standard Deviation
ROS	Reactive Oxygen Species

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The protective effect of serum carotenoids on cardiovascular disease: a cross-sectional study from the general US adult population

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Background: Cardiovascular disease (CVD) has become a key global health issue. Serum carotenoids are associated with CVD, while their effects on different diseases remain unclear. Herein, the relationship between the concentration of serum carotenoid and the CVD risk was investigated using nationwide adult samples obtained from the USA.

Materials and methods: Data of National Health and Nutrition Examination Survey (NHANES) in 2001–2006 were employed. The association of serum carotenoids (total, lycopene, β-carotene, α-carotene, lutein/zeaxanthin, and β-cryptoxanthin) with CVD was explored by using multivariate logistic, linear and weighted quantile sum (WQS) regression analyses. Eventually, data from 12,424 volunteers were analyzed for this study.

Results: Multivariate model data showed that lutein/zeaxanthin, α-carotene, lycopene, and β-cryptoxanthin were negatively associated with the prevalence of CVD (p <0.05). In comparison with the first quartile, the fourth quartile was associated with α-carotene ([OR]=0.61 [0.47–0.79]), β-cryptoxanthin (OR=0.67 [0.50–0.89]), lutein (OR=0.69 [0.54–0.86]), and lycopene (OR=0.53 [0.41–0.67]). WQS analysis revealed that the combination of serum carotenoids had negative correlation with the prevalence of total CVD (OR=0.88, 95% CI: 0.85–0.92, p <0.001). Additionally, dose–response analysis demonstrated a negative linear association of hypertension with all the carotenoids involved (p >0.05 for non-linearity).

Conclusion: The concentration of serum carotenoids had negative correlation with the prevalence of CVD, with a more significant negative effect against heart attack and stroke.

KEYWORDS

serum carotenoids, cardiovascular disease, coronary heart disease, lycopene, NHANES, WQS

1. Introduction

CVD involves the blood vessels or heart and include congestive heart failure (CHF), coronary heart disease (CHD), rheumatic heart disease, heart attack, peripheral artery disease, angina, and stroke. Indeed, the CVD-caused death cases worldwide reached 17.8 million in 2019 and may increase to 23 million by 2030 (1, 2). Epidemiological evidence has suggested that the CVD risk is negatively correlated with the diet quality (3).

In terms of nutrition and health, some carotenoids can be used as vitamin A precursors, where α-carotene, β-carotene and β-cryptoxanthin can be converted to vitamin A. In daily diet, carotenoids can be obtained from lettuce, carrots, tomatoes and oranges (4). Carotenoids can be divided into hydrocarbon carotenoids and oxygen-containing derivatives of hydrocarbon carotenoids according to the chemical structure. Hydrocarbon carotenoids include lycopene, β-carotene, and α-carotene, and oxygenated derivatives of hydrocarbon carotenoids (e.g., xanthophylls, neoxanthin, violet xanthin, lutein, and β-cryptoxanthin) (5, 6). Carotenoids have antioxidant activity, which can prevent and treat CVD. In addition, carotenoids may be involved in cellular signaling pathways correlated with inflammation and oxidative stress (OS), thereby inhibiting OS and inflammation (7). It has been demonstrated that the total concentration of carotenoid in blood lower than 1,000 nmol/L is related to a high risk of chronic diseases (8). Epidemiological studies have shown that 2–20 mg of lycopene intake per day can effectively prevent CVD (9). The effects on atherosclerosis and hypertension are even more pronounced (9, 10). Nevertheless, some studies have delivered different conclusions. Specifically, the correlation of increased carotenoid intake and reduced CVD risk remains controversial. A non-linear dose–response meta-analysis showed that the risk of cardiovascular death did not change with increasing dietary β-carotene intake (11). However, β-carotene was associated with increased all-cause mortality in another study of beta carotene supplements, and one-time beta carotene use was positively associated with cardiovascular events. Hence, treatment by β-carotene alone is not recommended for CVD (12). Indeed, the use of carotenoids or vitamin E supplements to counteract CVD or carcinoma has been opposed (13, 14).

This study aims to investigate the correlation of the serum level of carotenoids with the CVD risk by using nationwide adult samples obtained from the USA. Specifically, data of National Health and Nutrition Examination Survey (NHANES) in 2001–2006 were utilized to assess the effect of serum carotenoids on CVD.

2. Materials and methods

2.1. Experimental design

The NHANES is a nationwide survey aiming to the evaluate nutritional status and health of the population. It was executed by the Centers for Disease Control and Prevention (CDC) of the USA. This study combined interview and physical examination (15). The interviews covered various fields, including health, diet demographic, and socioeconomic information. Informed consent was obtained from each participant and approval of the NHANES protocol was obtained from the NCHS Research Ethics Review Committee. From 2001 to 2006, a total of 31,509 individuals participated in NHANES. 9,331 of the 31,509 participants were excluded as they had no data on serum carotenoids. Meanwhile, participants aged below 18 years old were excluded. Additionally, pregnant participants were excluded. Eventually, data from 12,424 adults from NHEANES were included in this analysis (Supplementary Figure S1).

2.2. Measurement of carotenoid concentrations

Serum levels of β-carotene, lycopene, lutein/zeaxanthin, α-carotene, and β-cryptoxanthin were measured by using high performance liquid chromatography (HPLC) for NHANES 2001–2002 and 2005–2006, while the serum levels of six carotenoids were determined by utilizing a comparable HPLC method for NHANES 2002–2003. This method was performed based on multiwavelength photodiode absorbance (absorbance = 450 nm). NHANES 2003–2004 data were converted to equivalent carotenoid measurements by HPLC using a regression method. Total carotenoid concentrations in serum were determined based on the serum levels of the five carotenoids mentioned above. Protocols and quality control were designed accordingly (16, 17).

2.3. Determination of covariates

In the NHANES study, household interviews (using standardized questionnaire) and medical assessments were employed to collect data. Age, gender, education level, household income race, smoking and alcohol drinking history, physical activity, BMI, energy intake level, hyperlipidemia, diabetes and hypertension of the participants were obtained based on previous studies with confounding covariates for CVD.

In terms of race and ethnicity, the participants were grouped as "Mexican American," "Other Hispanic," "Non-Hispanic White," "Black "and "other." In terms of education levels, the participants were grouped as "less than high school," "high school," and "high school and above." Poverty was determined based on the household income-to-poverty ratio (household income-to-poverty ratio < 1 indicates poverty). In terms of smoking history, participants who smoked less than 100 cigarettes during lifetime were categorized as never smokers, participants who smoked over 100 cigarettes during lifetime were categorized as current smokers, and participants who smoked more than 100 cigarettes but had quit were categorized as former smokers. In terms of drinking history, the participants were grouped as 'no drinking', 'low to moderate drinking' (less than two drinks and one drink daily for the male and the female, respectively), and 'heavy drinking' (more than two drinks and one drink daily for the male and the female, respectively). The energy intake was defined as the average of dietary intake in 2 days. In terms of physical activity, the participants were grouped as 'inactive', 'insufficiently active' and 'active'. Hypertension and diabetes were identified based on self-reported histories of physician-diagnosed hypertension (yes or no), physician-diagnosed diabetes (yes or no), anti-hypertensive medications (yes or no), and anti-hyperglycemic medications (yes or no).

2.4. Statistical analysis

National estimates were effectively generated using weighted analyses, and CDC guidance was followed with adjustment for

TABLE 1 Survey-weighted features of participants with available data on serum levels of all carotenoids involved in NHANES 2001–2006 (n =12,424).

Characteristics	*N* (%)
Age, %	
18–39 years old	4,023 (32.38)
40–59 years old	3,972 (31.97)
≥60 years old	4,429 (35.65)
Male, %	6,308 (50.77)
Race/ethnicity, %	
Mexican American	2,510 (20.20)
Other Hispanic	431 (3.47)
Non-Hispanic White	6,534 (52.59)
Non-Hispanic Black	2,485 (20.00)
Others	464 (3.73)
Education level, %	
Below high school	3,622 (29.15)
High school	3,018 (24.29)
Above high school	5,784 (46.56)
Family income-to-poverty ratio, %	
≤1.0	2,156 (17.83)
1.1–3.0	4,935 (40.82)
>3.0	5,000 (41.35)
Smoking history, %	
Never smoker	6,238 (50.21)
Former smoker	3,322 (26.74)
Current smoker	2,864 (23.05)
Drinking history, %	
Nondrinker	2,921 (23.51)
Low-to-moderate drinker	8,460 (68.09)
Heavy drinker	1,043 (8.40)
Body mass index, kg/m^2	
<25.0	3,839 (30.90)
25.0–29.9	4,460 (35.90)
>29.9	4,125 (33.20)
Physical activity, %	
Inactive	3,476 (27.98)
Insufficiently active	6,090 (49.02)
Active	2,858 (23)
Energy intake, kcal/day	
Low	5,240 (42.18)
Adequate	4,539 (36.53)
High	2,645 (21.29)
Hypercholesterolemia, %	4,539 (34.35)
Hypertension, %	4,141 (33.33)
Diabetes, %	1,302 (10.48)
Carotenoids supplement use, %	3,512 (28.27)
Congestive heart failure, %	428 (3.44)
Coronary heart disease, %	587 (4.72)
Angina, %	467 (3.76)
Heart attack, %	603 (4.85)
Stroke, %	475 (3.82)
Cardiovascular disease, %	1,527 (12.29)

Data are expressed in the form of n (percentage); sampling weights are used to calculate demographic data; n and percentages denote the sample number and the survey-weighted value, respectively.

over-sampling of minority subgroups. Continuous and categorical variables were expressed as median (interquartile spacing) and absolute values (percentages), respectively. NHANES 2001–2006 correlation coefficients of serum and dietary levels of carotenoids in adults were determined by utilizing the Spearman correlation method. The interquartile spacing of serum levels of carotenoids was determined according to the distribution of the target population. The data were log-transformed and divided into quartiles, wherein the lowest quartile was regarded as the benchmark. The inter-group differences of categorical variables, non-normally distributed continuous variables, and normally distributed continuous variables were assessed by utilizing one-way ANOVA test, Kruskal-Wallis test and $\chi 2$ test, respectively.

Two statistical models were developed in this study. Specifically, Model 1 was adjusted for gender (male or female), age (18–39, 40–59, or ≥60), and race; Model 2 was adjusted for Model 1 and education level, household income (income-to-poverty ratio ≤ 1.0, 1.1–3.0, or >3.0), smoking history, drinking history, BMI (<25.0 kg/m^2, 25.0–29.9 kg/m^2, or > 29.9 kg/m^2), energy intake level (low, adequate, or high), physical activity, hypertension, diabetes, hypercholesterolemia, and supplement use (yes or no).

The correlation of the total carotenoid levels with CVD-related outcomes was assessed by using weighted quantile sum (WQS) regression. Herein, a weight in an index, which indicates the contribution to the overall protective association, was assigned to each carotenoid. In the model, 40% of the data was assigned to the training set and 60% to the validation set, meanwhile the training set was bootstrapped 1,000 times to maximize the likelihood function of the linear model. The proposed WQS regression model was exposed to adjustment for the factors mentioned above. Serum level of one single carotenoid ≥0.1 was regarded as a significant contributor. The dose–response correlation of carotenoids in serum and the prevalence of CVD was explored by using a restricted cubic spline (RCS) regression model with different percentiles (10th, 50th, and 90th). ANOVA was used to clarify the nonlinearity. R software was employed for all statistical analyses. $p < 0.05$ (two-sided) denoted statistical significance.

3. Results

3.1. Baseline features of the participants

50.77% of the 12,424 participants enrolled were male and the overall CVD-weighted prevalence was 12.9%. Table 1 shows the survey-weighted health and sociodemographic features of the respondents.

TABLE 2 ORs (95% CIs) of CVD prevalence among adults in NHANES 2001–2006.

	Carotenoids in serum (μg/dL)				
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}
α-Carotene					
Crude	1 [xx]	0.90 (0.75–1.09)	0.82 (0.67–1.00)	0.61 (0.49–0.75)	<0.001
Model 1	1 [xx]	0.72 (0.59–0.87)	0.57 (0.46–0.69)	0.40 (0.32–0.49)	<0.001
Model 2	1 [xx]	0.75 (0.60–0.93)	0.72 (0.58–0.88)	0.61 (0.47–0.79)	0.001
β-Carotene					
Crude	1 [xx]	0.96 (0.80–1.16)	1.03 (0.89–1.18)	0.94 (0.79–1.12)	0.616
Model 1	1 [xx]	0.80 (0.65–0.97)	0.65 (0.55–0.76)	0.46 (0.39–0.55)	<0.001
Model 2	1 [xx]	0.87 (0.70–1.08)	0.84 (0.70–1.02)	0.73 (0.59–0.91)	0.008
β-Cryptoxanthin					
Crude	1 [xx]	0.70 (0.60–0.80)	0.60 (0.53–0.69)	0.49 (0.40–0.61)	<0.001
Model 1	1 [xx]	0.72 (0.61–0.85)	0.59 (0.51–0.68)	0.43 (0.34–0.54)	<0.001
Model 2	1 [xx]	0.84 (0.68–1.03)	0.74 (0.62–0.88)	0.67 (0.50–0.89)	0.002
Lutein/zeaxanthin					
Crude	1 [xx]	0.79 (0.67–0.93)	0.72 (0.61–0.84)	0.76 (0.65–0.89)	<0.001
Model 1	1 [xx]	0.69 (0.56–0.85)	0.55 (0.46–0.65)	0.48 (0.39–0.58)	<0.001
Model 2	1 [xx]	0.77 (0.61–0.98)	0.66 (0.52–0.82)	0.69 (0.54–0.86)	<0.001
Lycopene					
Crude	1 [xx]	0.46 (0.38–0.56)	0.34 (0.29–0.40)	0.23 (0.18–0.29)	<0.001
Model 1	1 [xx]	0.66 (0.54–0.79)	0.57 (0.47–0.68)	0.41 (0.33–0.52)	<0.001
Model 2	1 [Reference]	0.76 (0.64–0.91)	0.68 (0.56–0.82)	0.53 (0.41–0.67)	<0.001
Total carotenoids					
Crude	1 [xx]	0.63 (0.53–0.75)	0.58 (0.48–0.69)	0.46 (0.38–0.54)	<0.001
Model 1	1 [xx]	0.65 (0.54–0.78)	0.58 (0.48–0.71)	0.36 (0.30–0.43)	<0.001
Model 2	1 [xx]	0.73 (0.61–0.87)	0.75 (0.60–0.93)	0.55 (0.45–0.68)	<0.001

3.2. Distribution and concentration of serum levels and dietary intake of carotenoids

The baseline distribution and serum levels of the carotenoids involved are summarized in Supplementary Table S1. As observed, the serum levels of β-carotene, lutein/zeaxanthin, lycopene, β-cryptoxanthin, and α-carotene were 23.56, 19.62, 15.75, 9.38, and 4.41 μg/dl, respectively. The total concentration of serum carotenoids was 72.71 μg/dl. The mean concentrations of dietary lycopene, β-carotene, lutein and zeaxanthin, α-carotene, and β-cryptoxanthin were 6,216.10, 1,931.16, 1,386.18, 372.00, and 137.90 μg/dl, respectively. The Spearman correlation coefficients of carotenoids in serum were 0.19–0.76, indicating a moderate to strong correlation. Among them, α-carotene and β-carotene showed the strongest correlation (r = 0.76; see Supplementary Figure S2).

3.3. Association of carotenoids in serum with prevalence of CVD

The serum levels of carotenoids involved were categorized into quartiles. Herein, the minimum quartile was regarded as the benchmark, and its associations with CVD prevalence were assessed. According to Table 2, carotenoids except for β-carotene were negatively related to CVD prevalence. Model 1 was generated after adjusting for age, sex, and race, and all carotenoids showed a negative association with the prevalence of CVD compared to the benchmark. Model 2 was adjusted for all other factors mentioned above on the basis of Model 1. In the Model 2, lutein/zeaxanthin (OR = 0.69, 95% CI: 0.54–0.86, $p < 0.001$) and lycopene (OR = 0.53, 95% CI: 0.41 ~ 0.67, $p < 0.001$) were significantly and negatively related to CVD prevalence, compared to the benchmark. However, the negative associations of β-cryptoxanthin, β-carotene, and α-carotene with CVD were attenuated in Model 2.

Model 1 was adjusted for age (18–39, 40–59, or ≥ 60), sex (male or female), and race (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black or Other);

Model 2 was adjusted for Model 1 plus education level (below high school, high school, or above high school), family income-to-poverty ratio (≤1.0, 1.1–3.0, or > 3.0), smoking status (never smoker, former smoker, or current smoker), drinking status (nondrinker, low-to-moderate drinker, or heavy drinker), BMI (<25.0 kg/m^2, 25.0–29.9 kg/m^2, or >29.9 kg/m^2), energy intake levels (low, adequate, or high), physical activity (inactive, insufficiently active, or active), hypercholesterolemia (yes or no), diabetes (yes or no), hypertension (yes or no), and supplement use (yes or no).

3.4. WQS regression analysis of the negative correlation of the total serum carotenoids with total and specific CVD

The association of total concentrations of serum carotenoids with various CVD-related outcomes was assessed by using WQS regression (Table 3). As observed, the combination of serum carotenoids was negatively related to the prevalence of total CVD (OR = 0.88, 95% CI: 0.85–0.92, p <0.001). In addition, the combination of serum carotenoids was also associated with the prevalence of specific CVD, with multivariable-adjusted ORs of 0.91 (95% CI: 0.85–0.98, p = 0.010) for CHF; 0.91 (95% CI, 0.86–0.96, p = 0.001) for CHD; 0.93 (95% CI, 0.87–0.99, p = 0.034) for angina; 0.88 (95% CI, 0.83–0.94, p <0.001) for heart attack; and 0.89 (95% CI, 0.84–0.95, p <0.001) for stroke. Figure 1 shows the weights of each serum carotenoid in the overall protective effects on different CVDs.

TABLE 3 WQS regression model used for assessment of the correlation of the serum carotenoids with total and specific CVD.

	OR	95% CI	p value
Total CVD	0.88	0.85–0.92	<0.001
Specific CVD			
Congestive heart failure	0.91	0.85–0.98	0.010
Coronary heart disease	0.91	0.86–0.96	0.001
Angina	0.93	0.87–0.99	0.034
Heart attack	0.88	0.83–0.94	<0.001
Stroke	0.89	0.84–0.95	<0.001

WQS, weighted quantile sum; CI, confidence interval; and OR, odds ratio. WQS regression model was exposed to adjustment for gender, race, age, household income, education level, drinking history, smoking history, BMI, physical activity, energy intake levels, hypercholesterolemia, hypertension, and diabetes.

3.5. RCS analysis of the correlation of CVD with serum carotenoids

The dose–response correlation of the five carotenoids and total carotenoids with CVD prevalence was visualized by utilizing RCS regression with multivariate adjustment (Figure 2). All carotenoids and total carotenoids were negatively and linearly related to the prevalence of CVD, respectively (α-carotene: p was 0.410, Figure 2A; β-carotene: p was 0.816, Figure 2B; β-cryptoxanthin: p was 0.733, Figure 2C; lycopene: p was 0.387, Figure 2D; lutein/zeaxanthin: p was 0.285, Figure 2E; total carotenoids: p was 0.781, Figure 2F).

4. Discussion

The correlation of serum levels of carotenoids and CVD prevalence was explored. The results exhibited that the serum level of total carotenoids was negatively related to CVD. Among the five carotenoids, β-cryptoxanthin and lycopene were negatively related to

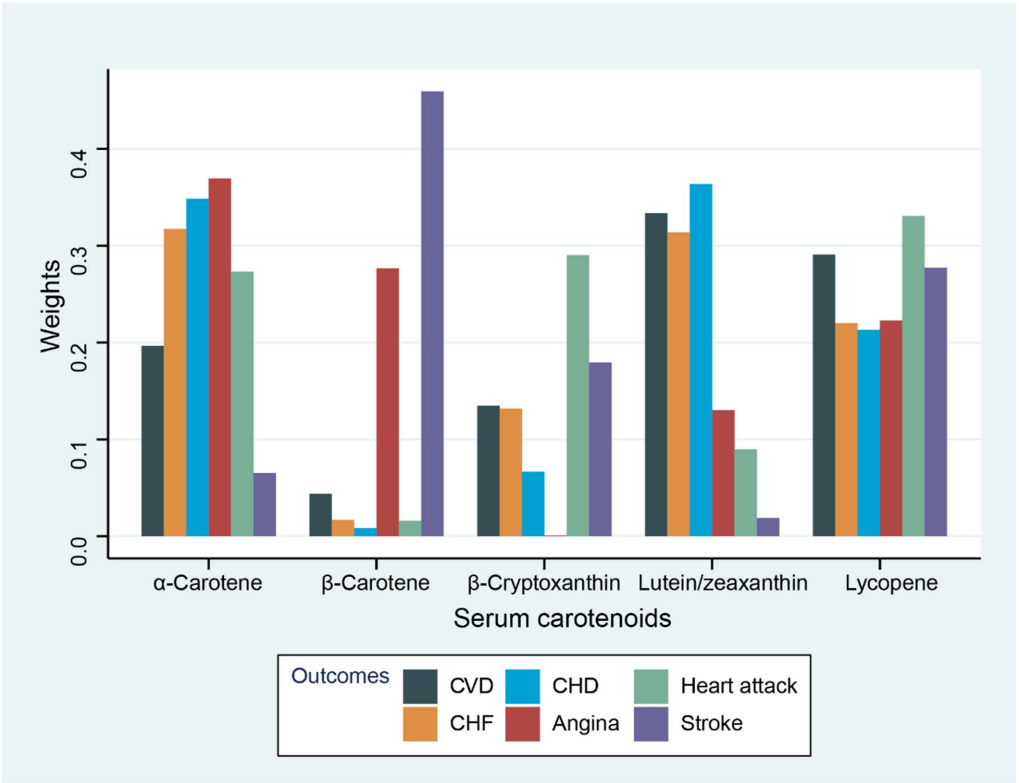

FIGURE 1
Weights from WQS regression for the serum carotenoids and CVD-related outcomes with adjustment for gender, race, age, household income, drinking history, education level, BMI, energy intake level, smoking history, physical activity, hyperlipidemia, diabetes, and hypertension.

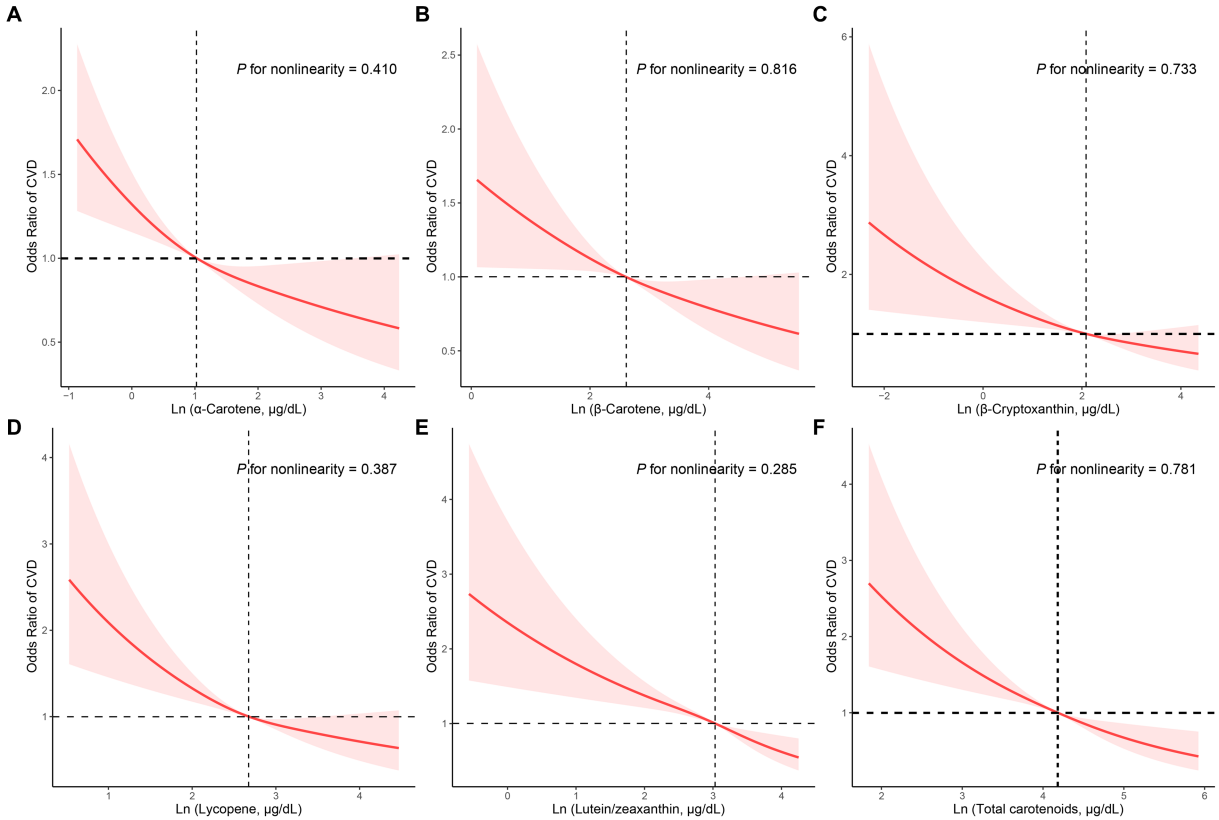

FIGURE 2
RCS analysis with multivariate adjustment of the correlation of the carotenoids and CVD prevalence. **(A)** α-Carotene, **(B)** β-Carotene, **(C)** β-Cryptoxanthin, **(D)** Lycopene, **(E)** Lutein/zeaxanthin, **(F)** Total carotenoids.

the prevalence of CVD. In addition, WQS analysis showed that serum levels of total carotenoids were related to myocardial infarction and stroke in CVD. Additionally, RCS analysis showed a linear negative correlation of serum carotenoids with CVD.

Higher dietary intake or blood carotenoid concentrations were associated with a reduced risk of cardiovascular disease (18). Previous studies have shown that the cardiovascular risk can be reduced by increasing the intake of β-carotene or serum/plasma levels of β-carotene (19). A population-based study involving middle-aged male participants shows that the risk of CHF is negatively correlated with the serum level of β-carotene (20). For α-carotene, a high serum level of α-carotene can relieve CVD (21). A stratified analysis revealed that an elevated serum level of α-carotene can trigger beneficial changes in the variability of heart rate of adults (22). As an antioxidant (23), β-Cryptoxanthin may have anti-cancer effects (24) and reduce the risk of osteoporosis (25). However, few studies discussing the correlation of β-Cryptoxanthin with CVD have been reported. A cross-sectional analysis from Mikkabi concluded that the serum level of b-cryptoxanthin is inversely proportional to the risk of atherosclerosis (26).

However, the effect of carotenoid supplements on cardiovascular disease has shown different results. A recent meta-analysis suggests that the incidence of major CVD is not related to β-carotene supplementation (27). Meanwhile, the incidence of coronary atherosclerotic heart disease has no significant association with β-carotene (12). For lycopene, most of the research is concentrated on lycopene supplements. It has been demonstrated that lycopene supplementation can deliver significant reductions in LDL cholesterol (28). In a human dietary intervention study, reductions in lipid and LDL oxidation were observed after 1 week of lycopene supplementation (10, 29). Additionally, lycopene improves the function of high-density lipoprotein (HDL) (30, 31). Lycopene in serum has beneficial effects on CHD, which is consistent with the present study, demonstrating that lycopene also exhibited beneficial effects on CHF, angina, heart attack, and stroke (32).

The present study also clarifies the role of β-Cryptoxanthin in CVD. In addition to CHD, β-Cryptoxanthin has a great effect on heart attack. However, the protection of β-Cryptoxanthin on CVD was not enhanced in Model 2 adjusted for household income, education level, and smoking history. Meanwhile, lutein/zeaxanthin may have beneficial effects on CVD by alleviating chronic inflammation in CVD patients (33). Indeed, the risks of coronary heart disease and stroke are inversely proportional to the intake or concentration of lutein/zeaxanthin (34) as lutein/zeaxanthin can improve vascular tone and endothelial function (35). Additionally, in a cohort study based on older adults, serum lutein/zeaxanthin levels were significantly associated with telomere length, which is associated with increasing age and age-related diseases such as stroke, diabetes, cardiovascular disease and cancer (36). In the present study, however, stroke had no significant correlation with lutein/zeaxanthin.

Oxidative stress (OS) plays a key role in CVD. It is also related to various abnormalities, including systemic inflammation, immune cell activation, sympathetic nervous system excitation, renal dysfunction, cardiovascular remodeling, vascular dysfunction, and endothelial

damage, by triggering redox signaling of reactive oxygen species (ROS) (37, 38). As an antioxidant, carotenoids can inhibit peroxidation, eliminate free radicals, scavenge lipid peroxyl radicals, and reduce ROS-induced damage (39). Additionally, high serum levels of carotenoids are related to low thickness of carotid intima-media, which has preventive implications for CHD (40).

Despite that the protective effect of carotenoids against CVD has been demonstrated, the specific contribution of each carotenoid to this effect remains unclear. This study revealed that serum carotenoids protect human body from CVD, with the protective effects of lutein and lycopene being well established. However, the protective effects of β-cryptoxanthin, β-carotene and α-carotene on CVD were negligible after model adjustment. Indeed, the intakes of beta-carotene and lycopene are negatively related to death, stroke, and coronary heart disease, while total carotenoid, lycopene, β-cryptoxanthin, β-carotene, and α-carotene are negatively related to all-cause mortality from CVD (18). This study indicated that the five carotenoids and total serum carotenoids have a negative linear correlation with the prevalence of CVD. WQS regression analysis indicated negative correlations of total serum carotenoids with specific CVDs, especially stroke and heart attack. However, the findings remain to be confirmed by future studies.

This study presents several advantages. First, it serves as a preliminary assessment of the overall protective effect of different carotenoids against CVDs. The protective effects of carotene, lycopene, and lutein on CVD were confirmed, while the protective effects of β-carotene, α-carotene and β- cryptoxanthin were negligible after as adjustment for education level and poverty. Second, this study was based on a large yet representative sample and the results were statistically significant. Third, WQS regression was employed to preserve statistical effects and avoid unstable regression coefficients. In addition, this approach reveals correlations between exposures and exposure-outcomes.

Nevertheless, several limitations are also noted. Due to the observational study design, no causal relationships could be determined. In other words, the causal relationship between serum levels of carotenoids and CVD prevalence has not been fully understood. Additionally, other dietary or environmental factors (e.g., artificial sweeteners, vitamin D, dietary fiber, and heavy metals) may still have influences on the conclusions even after model adjustment as carotenoids are mainly obtained from fruits and vegetables (41–44). The limitations of the WQS regression analysis may also affect the conclusions as the all-positive and all-negative dependent variables have the same effects on exposure.

5. Conclusion

With adjustment for age, race, gender, poverty, education level, smoking history, alcohol history, BMI, energy intake level, physical activity, hyperlipidemia, diabetes, and hypertension supplement, serum levels of carotenoids (total, lycopene, α-carotene, lutein/zeaxanthin, β-carotene, and β-cryptoxanthin) were negatively associated with the prevalence of CVD. However, the effect of β-carotene on CVD remains unclear. Additionally, the five serum carotenoids had inverse linear correlations with CVD prevalence. There was a strong negative correlation between serum concentrations of lycopene and the prevalence of CVDs (e.g., CHF, CHD, angina, heart attack, and stroke). Nevertheless, future studies clarifying the complex interactions between different carotenoids in serum, as well as their effects on CVDs, are of great significance.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/participants or patients/participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

MW designed the present study and performed the data analysis. RT and DD contributed equally to the writing of this article. RZ and YQ critically revised and edited the manuscript 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1154239/full#supplementary-material

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Evaluation of the safety and potential lipid-lowering effects of oral hydrogen-rich coral calcium (HRCC) capsules in patients with metabolic syndrome: a prospective case series study

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Background: Metabolic syndrome is characterized by a cluster-like occurrence of conditions such as hypertension, hyperglycaemia, elevated low-density lipoprotein (LDL) cholesterol or triglycerides (TG) and high visceral fat. Metabolic syndrome is linked to the build-up of plaque within the artery, which leads to disorders of the circulatory, nervous and immune systems. A variety of treatments target the regulation of these conditions; nevertheless, they remain dominant risk factors for the development of type 2 diabetes (T2DM) and cardiovascular disease (CVD), which affect 26.9% of the US population. Management and intervention strategies for improving cholesterol and/or TG are worthwhile, and recent studies on hydrogen treatment are promising, particularly as molecular hydrogen is easily ingested. This study aimed to investigate the lipid-lowering effects and quality of life (QOL) improvement of hydrogen-rich coral calcium (HRCC) in patients with metabolic syndrome.

Methods: The patients, all Taiwanese, were randomly assigned to 3 different doses (low, medium, and high) of HRCC capsules. The primary outcome was the adverse effects/symptoms during this 4-week use of HRCC capsules. The secondary outcome was lipid profile changes. Complete blood count, inflammatory biomarkers, and QOL were also measured before and after the course of HRCC.

Results: Sixteen patients with metabolic syndrome completed this study (7 males, 9 females; mean age: 62years; range: 32–80). No obvious adverse effects were recorded. Only changes in blood TG reached significance. The baseline TG value was 193.19μL (SD=107.44), which decreased to 151.75μL (SD=45.27) after 4weeks of HRCC (p=0.04). QOL showed no significant changes.

Conclusion: This study is the first human clinical trial evaluating HRCC capsules in patients with metabolic syndrome. Based on the safety and potential TG-lowering effects of short-term HRCC, further long-term investigations of HRCC are warranted.

Clinical trial registration: [ClinicalTrials.gov], identifier [NCT05196295].

KEYWORDS

metabolic syndrome, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, molecular hydrogen, hydrogen-rich coral (HRCC)

1. Introduction

Metabolic syndrome is a group of conditions that includes risk factors such as obesity, elevations in blood pressure, blood triglyceride (TG), and fasting glucose levels, and low high-density lipoprotein cholesterol (HDL-C) (1). Together, these conditions raise our risk of coronary heart disease, type 2 diabetes (T2DM), stroke, and other serious health problems (2). The first-line treatment for metabolic syndrome includes weight reduction and increased exercise. Nevertheless, it can be difficult to implement exercise in daily life. Genetic predisposition also plays a role in the development of these risk factors (1). Diseases such as T2DM and cardiovascular disease (CVD) promote a pro-inflammatory state and require therapeutic attention. Drug targeting metabolic syndrome focus on reducing blood pressure and blood glucose levels, and include medications such as angiotensin-converting enzyme (ACE) inhibitors (such as Capoten and Vasotec) for blood pressure, cholesterol medications (3) such as statins, niacin, and Zetia, medications for treating diabetes, such as metformin (Glucophage), pioglitazone (Actos), and rosiglitazone (Avandia) (4), and low-dose aspirin, which can reduce the risk of heart attack and stroke. Although niacin and fibrates are observed to raise HDL-C and reduce TG and non-HDL-C concentrations, they modestly (if at all) reduce low-density lipoprotein cholesterol (LDL-C) (5).

The development of metabolic syndrome is primarily related to insulin resistance (6), in which insulin is inefficiently used to lower glucose and triglyceride levels. The conditions that characterize Metabolic syndrome result from an increase in hormones induced by free fatty acids (7), which are stored in adipose cells, and can influence how the body controls blood glucose levels (7). Inflammation is a risk factor for insulin resistance, high blood pressure, and diseases of the heart and blood vessels (8). In the presence of excess adipose tissue, immune cells interact with fat cells to produce pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), which promote an inflammatory state. These cytokines can cause plaque, a waxy substance, to build up inside blood vessels and obstruct blood flow. Individuals with Metabolic syndrome present with a pro-inflammatory state, which is recognized by elevated concentrations of C-reactive protein (CRP) levels (9, 10). CRP is observed to rise as the number of conditions associated with metabolic syndrome increases, and is elevated in obese patients who experience a corresponding decrease in CRP with weight loss (9). CRP has also been associated with future cardiovascular events (10). A higher erythrocyte sedimentation rate (ESR) is also associated with the development of metabolic syndrome and obesity (11), and a higher eosinophil count is associated with metabolic, cardiac, and pulmonary outcomes (12). Therefore, targeting the inflammatory response and lipid metabolism is an important strategy for treating metabolic diseases (13). The white blood cells (WBCs) are observed to increase significantly in patients with metabolic syndrome. A high WBC count positively correlates with insulin resistance and TG levels and is negatively associated with HDL-C (14). Thus, comprehensive plasma profiling can improve understanding on the overall risk factors for developing metabolic syndrome.

Molecular hydrogen is able to regulate inflammatory processes involved in cytokine and lipid metabolism (13). Kamimura et al. (15) demonstrated that molecular hydrogen reduces hepatic oxidative stress and alleviates fatty liver in db/db mice and fatty liver induced in wild-type mice by a high-fat diet. In patients with T2DM or impaired glucose tolerance (IGT), hydrogen-rich water (HRW) intake was associated with a trend toward decreased serum concentrations of oxidized LDL-C and free fatty acids and increased adiponectin and extracellular-superoxide dismutase (16). The levels of modified LDL-C (i.e., modifications that increase the net negative charge of LDL-C), small dense LDL, and urinary 8-isoprostane significantly decreased by 15.5% (16). In another report, the use of HRW decreased serum LDL-C and improved HDL function in patients with potential metabolic syndrome (17). Furthermore, LeBaron et al. (18) demonstrated that high-concentration HRW improves several biomarkers of cardiometabolic health in middle-aged men and women with metabolic syndrome, including body mass index, waist-hip ratio, resting heart rate, blood lipids, blood glucose, inflammation, and redox homeostasis. Recent research found reduced lipogenesis and enhanced lipolysis in the liver of rats exposed 2 h daily to hydrogen gas (H_2) either by drinking HRW or by inhaling 4% H_2 gas, a finding that was associated with a loss in visceral fat and brown adipose tissue and a reduction in serum lipids (19). Beyond regulating inflammation and lipid metabolism, hydrogen treatments are also shown to affect hypertension (20) and regulate the homeostasis in the cardiovascular system and related metabolic activities. Sugai et al. (20) showed in an animal model that H_2 significantly suppresses increases in blood pressure after 5/6 nephrectomy. Molecular hydrogen has also been used to improve pulmonary hypertension (PAH) in a rat model by suppressing macrophage accumulation, reducing oxidative stress, and modulating the STAT3/NFAT axis, the latter which are important transcription factors associated with the immune response (21). Hydrogen supplements are available in three forms: encapsulated molecular hydrogen, H_2 inhalation, and HRW. As H_2 inhalation and HRW are well-established resources for ameliorating conditions induced by dysregulations in lipid and glucose metabolism, attention has been shifted to the potential of molecular hydrogen in managing

diabetes mellitus, inflammation and cardiovascular health (22). Recent research has shown improvements in T2DM through the inhibition of oxidative stress which involves mechanisms that are associated with the toll-like receptor 4 (TLR4), pathogen recognition, the myeloid differentiation primary response 88 (MyD88), signaling within immune cells, and the NF-κB signaling pathway, a regulator of innate immunity (23). It has also been reported that molecular hydrogen treatment decreases fasting blood glucose levels, increases hepatic glycogen synthesis, and improves insulin sensitivity (23). Given these findings, we are interested in the effects of molecular hydrogen on TG and cholesterol regulation, which are implicated in the development of insulin resistance. Hydrogen-rich coral calcium (HRCC) capsules were certified by the Taiwan Food and Drug Administration (FDA) in 2020.[1] We then investigated the safety and potential lipid-lowering effects of HRCC.

2. Materials and methods

2.1. Study design

The patients with metabolic syndrome were recruited from the outpatient department (OPD) in Min-Sheng General Hospital, Taiwan. Participants were screened to determine eligibility by doctors and underwent a series of tests involving questionnaires and examinations. In this study, the patients who met 3 or more of the following criteria were designated to have metabolic syndrome: (1) waist circumference: men ≥90 cm/women ≥80 cm, (2) systolic pressure ≥ 130 mmHg and/or diastolic pressure ≥ 85 mmHg or the use of antihypertensive drug, (3) fasting blood sugar level of 100 mg/dL or higher or use of antidiabetic medication, (4) fasting TG level ≥ 150 mg/dL or the use of lipid-lowering agent and (5) low HDL-C level: men <40 mg/dL/women <50 mg/dL (24). Consenting participants were allocated into 3 groups defined by their doses of HRCC capsules (low, $n = 5$; medium, $n = 5$; high, $n = 6$). The patients, who <18 years or unwilling to participate in this study were excluded. The patients received 1 (low), 3 (medium), or 6 (high) capsules of HRCC to take alongside conventional treatment daily for 4-week. The primary outcome was to measure the adverse effects/symptoms during this 4-week use of HRCC. The secondary outcome was to compare lipid profiles and other biomarkers changes including complete blood count (CBC), inflammatory biomarkers (erythrocyte sedimentation rate, ESR60'; high-sensitivity-CRP, hs-CRP), and haemoglobin A1c (HbA1C). The QOL was also checked before and after the use of HRCC. Patients were excluded from this study if serious adverse side effects/symptoms occurred. At the week 0, participants were instructed on the experimental procedure, and their consents were obtained. They received blood drawn and completed the pre-test including Brief Fatigue Inventory-Taiwan (BFI-T) and control status scale for diabetes (CSSD70) surveys. The participants also checked the changes of body weight during the 28 days. Finally, the participants had blood drawn and completed the post-test BFI-T and CSSD70 surveys. Research assistants provided the assistance and closely monitor the adverse effects/symptoms.

1 https://consumer.fda.gov.tw/Food/CapsuleAuditQueryDetail.aspx?nodeID=165&id=1096000144

2.2. Materials

Hydrogen capsules (PURE HYDROGEN) were purchased from HoHo Biotech Co., Ltd. (Taipei, Taiwan). Each capsule contained 170 mg HRCC which contained 1.7*10^21 molecules of hydrogen (approximately 24 cups of 1,200 ppb/0.6 mM, 200 mL of hydrogen water). Therefore, the low dose contained 170 mg, the medium dose contained 510 mg, and the high dose contained 1,020 mg HRCC.

2.3. Measurements of adverse effects/symptoms

We measured any adverse effect/symptom up to 28 days, which were codified according to the Common Terminology Criteria for Adverse Events (NCI CTCAE v5.0). The measures included changes from baseline (at day 28) for the following: (1) routine blood parameters; (2) routine urine parameters; (3) responses captured by the BFI-T scale (6 questions), which is a 9-item, 11-point rating scale developed to assess subjective fatigue. The first three questions measure fatigue severity with 0 indicating "no fatigue" (total range: 0 to 100); and (4) responses captured by the CSSD70 questionnaire, in which the minimum score was 0 (most severe) and the maximum score was 2 (healthiest), total range: 0 to 22.

2.4. Statistical analysis

The patients with metabolic syndrome were enrolled in the study and provided data on body weight, TG, LDL-C, HDL-C, HbA1C, complete blood count, hs-CRP, ESR60', BFI-T, and CSSD70. Data were expressed as mean and standard deviation (STD). Differences for which $p < 0.05$ were considered statistically significant. The analysis was conducted using paired-sample t tests and the statistical software Prism was used for statistical analyses.

3. Results

3.1. Demographics

A total of 16 patients who were confirmed to have metabolic syndrome completed this study (7 males, 9 females; mean age: 62 years; range: 32–80). The demographics were described in Table 1. None of the patients that were administered HRCC reported experiencing adverse effects or toxicity responses. A total 4 out of 16

TABLE 1 The demographic and baseline characteristics of the patients (n=16).

	Male	Female	Total
Patients	7	9	16
Age, mean ± SD (years)	59.17	65.25	62.64
TG (mg/dL)	203	185.55	193.19
HDL-C (mg/dL)	42.86	49.55	46.63
LDL-C (mg/dL)	105.28	109.88	107.88
HbA1C (%)	7.26	6.64	6.91

patients reported improvements in sleep, and 4 out of 16 patients presented improvements in energy (Table 2).

3.2. Body weight

The average weight of all patients before and after HRCC treatment was 73.91 kg (SD = 20.73) and 73.71 kg (SD = 20.53), respectively. No significant differences in body weight were observed [Figure 1A; $t(16) = 0.57$, $p = 0.57$]. The average weight of patients that received the high dose was 86.11 kg at baseline, which decreased to 86 kg after treatment (difference: −0.12 kg). The average weight of patients that received the medium dose was 69.4 kg at baseline, which decreased to 68.9 kg (difference: −0.5 kg). The average weight of patients that received the low dose was 63.76 kg at baseline, which decreased to 63.8 kg (difference: 0.04 kg) (Figure 1B).

TABLE 2 Qualitative responses reported by the patients (n=16).

Number	Patient	Sex	Age	Dosage (capsules/day)	Qualitative response before treatment	Qualitative response after treatment	Weight change (kg)
1	HM012	F	32	6	Look to lose weight	Felt no specific changes	0
2	HM013	F	70	6	Have low platelets, high blood pressure, I cannot squat down. Knee joint surgery is needed, but due to low platelets, doctors do not recommend surgery.	Dizziness, nothing else	0
3	HM014	M	38	6	Diabetes, feet have very obvious pigmentation, the calf also throbbing. Taking medicine helps.	Felt no specific changes	−0.7
4	HM015	F	46	6	Breast cancer, low energy	Defecation is much improved and sleep is much better	0
5	HM017	F	71	6	Great improvement in mental and sleep quality	Sleep improved	0
6	HM018	M	58	6	Diabetes	My hands are still numb, and I do not feel anything else	0
7	HM006	M	71	3	Diabetes	Energy improved; wanted to continue treatment	−1
8	HM007	F	72	3	Eating will cause nausea. May need kidney dialysis, and have control over eating. Urine protein drugs. Tiredness every day.	Energy improved, and bowel movements were significantly smoother	−0.5
9	HM008	F	80	3	Often chest tightness, the heart is not comfortable	No show	0
10	HM009	M	N/A	3	Hard of hearing. The heart has 2–3 stents, calf pain occasionally.	Severe sleep disturbance and panic disorder. Taking capsules in the afternoon greatly improves sleep. The panic disorder no longer occurs, and the mood is much brighter.	0
11	HM010	M	53	3	It is not particularly uncomfortable, it is purely a matter of endocrine	No specific changes	2
12	HM001	F	75	1	Showed interest in HRCC	Felt but the condition was preexisted	1
13	HM003	F	80	1	Taking bone-guiding health products, diabetes medicine, very talkative, menopause	CSSD70 improved	0.8
14	HM004	M	63	1	A little hard to hear, hands are shaking	Energy improved in the first week after the treatment but did not feel specific changes after	0
15	HM005	M	68	1	Occasionally throbbing on the soles of the feet, tiredness, moods fluctuating	Energy improved; could not sleep well at night	0
16	HM016	F	68	1	Have diabetes, high blood pressure, cholesterol	Sleep is much better, can fall asleep deeply	0

3.3. Serum lipid profile (TG, HDL-C, LDL-C) and HbA1C

The TG value was 193.19 mg/dL (SD = 107.44) at baseline, which decreased to 151.75 mg/dL (SD = 45.27) after 4 weeks [Figure 2A; $t(16) = 4.19$, $p = 0.04$]. The HDL-C value was 46.63 mg/dL (SD = 10.71) at baseline, which increased to 47.31 mg/dL (SD = 12.36) after 4 weeks [Figure 2B; $t(16) = 0.62$, $p = 0.54$]. The LDL-C value was 107.88 mg/dL (SD = 31.49) at baseline, which decreased to 101.13 mg/dL (SD = 31.13) after 4 weeks [Figure 2C; $t(16) = 1.20$, $p = 0.25$]. The baseline HbA1c value was 6.91% (SD = 0.68) at baseline, which decreased to 6.88% (SD = 0.71) after 4 weeks [Figure 2D; $t(16) = 0.34$, $p = 0.74$].

3.4. Complete blood count (CBC)

The WBC value was 6,957.5 μL (SD = 2,396.52) at baseline, and was 7,154.38 μL (SD = 2,361.75) after 4-week [Figure 3A; $t(16) = 0.97$, $p = 0.35$]. The HGB value at baseline was 13.18 g/dL (SD = 2.14), and was 13.05 g/dL (SD = 2.17) after 4-week [Figure 3B; $t(16) = 0.92$, $p = 0.37$]. The RBC value was 432.81*10^4 μL (SD = 66.49) at baseline, and was 431.56*10^4 μL (SD = 68.85) after 4-week [Figure 3C; $t(16) = 0.21$, $p = 0.83$].

3.5. Inflammatory biomarkers (ESR 60′ and hs-CRP)

The ESR 60′ value was 44.4 mm/dL (SD = 33.94) at baseline, which decreased to 43.2 mm/dL (SD = 40.79) after 4-week. A decreasing trend was observed; however, a one-sample t test showed no significant difference with treatment [Figure 4A; $t(16) = 0.28$, $p = 0.79$]. The hs-CRP value was 0.94 mg/dL (SD = 2.19) at baseline, which also decreased to 0.74 mg/dL (SD = 1.71) after 4-week. A decreasing trend was observed; however, a one-sample t test showed no significant difference with treatment [Figure 4B; $t(16) = 1.45$, $p = 0.14$].

3.6. Quality of life (QOL)

Both the results of BFI-T scale score and CSSD70 score showed no obvious changes before and after the 4-week use of HRCC (Figure 5).

4. Discussion

This study evaluated the potential TG-lowering effect of oral HRCC capsules in patients with metabolic syndrome. While the detailed mechanism is not well known, the significant decrease in TG could help explain the findings regarding the role of hydrogen in mediating TG by reducing excess oxidative stress. Future investigations should focus on the long-term efficacy of HRCC in patients with metabolic syndrome, especially the effects on HDL-C, LDL-C, and HbA1C. Larger sample sizes and longer experimental periods are needed to establish clinical efficacy and to determine the optimal dose.

The short-term safety endpoint of this study, which was used to test the safety of oral HRCC capsules at different doses, was met. After 1 month of treatment, HRCC was not associated with any patient-reported acute adverse effects. Most of the biomarkers measured in this study, including plasma or urinary biomarkers, WBC, HGB, RBC, HDL-C, LDL-C, HbA1C, inflammatory indicators such as CRP, eosinophils, and ESR, and body weight did not change significantly after treatment, except for TG, which

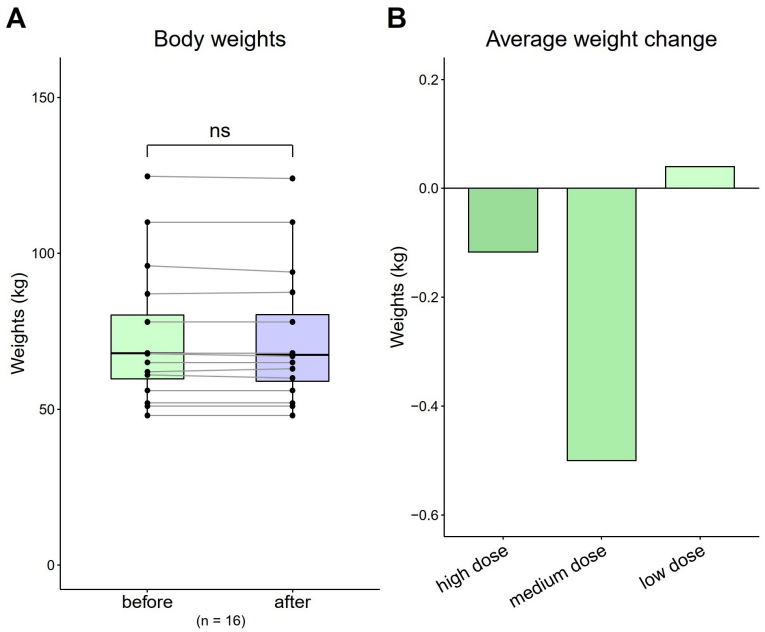

FIGURE 1
Total and average weight changes (n=16). **(A)** Total body weight of all patients. **(B)** Average weight change by dose. Data are represented by a line plot and a bar graph (*p<0.05; **p<0.01; ***p<0.001) and were analyzed using the paired t test.

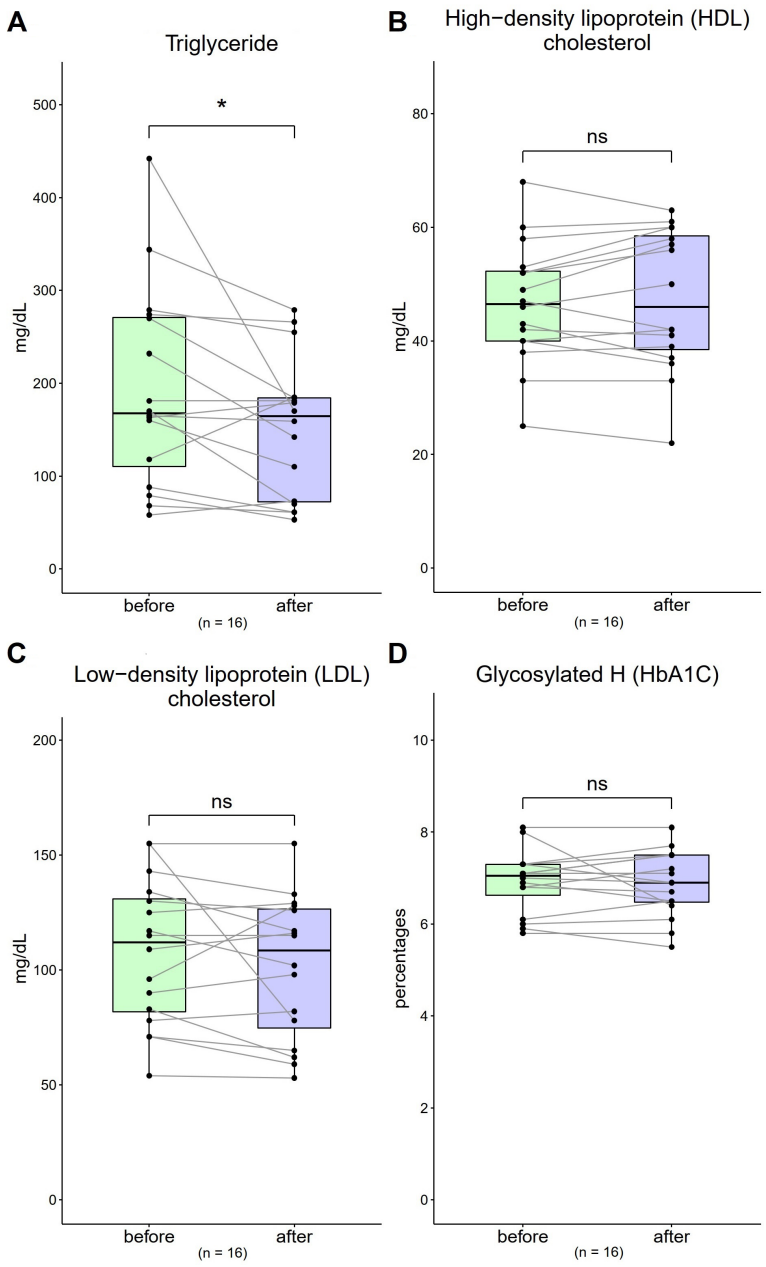

FIGURE 2
Changes in triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and haemoglobin A1c (HbA1c) levels from 0 to 4 weeks (n = 16). **(A,C)** The normal ranges are <150mg/dL and <130mg/dL for TG and LDL-C, respectively. **(B)** The normal range for HDL-C is >40mg/dL. **(D)** The normal range of HbA1c is 4.0–6.0%. Data are represented by line plots (*p < 0.05; **p < 0.01; ***p < 0.001) and were analyzed using the paired t test.

decrease significantly after treatment. In this study, BFI-T and health in type 2 diabetic patients (CSSD70) did not significantly change after the use of HRCC. Notably, the baseline CSSD70 score was 16.36 (out of the maximum of 22), which is considered high and an indication of positive health status in diabetic patients. The baseline BFI-T was 29 (out of the maximum of 100), which is considered mild. Diabetes is often associated with fatigue and dizziness (25). Signs such as increased thirst, frequent urination, urinary incontinence, numbness of the hands and feet, calf and foot pain are commonly associated with diabetes (26). Future research should be done in a larger sample over a longer time to understand the effects of HRCC capsules on the BFI-T and CSSD70. Qualitative indicated that 4 out of 16 patients experienced improvements in sleep and 4 out of 16 patients experienced improved energy, findings that require further investigation. H_2 gas has been shown to have the potential to regulate mitochondrial energy metabolism (27). Kamimura et al. (15) reported that prolonged consumption of HRW significantly altered fat and body weight in db/db obese mice by stimulating energy metabolism. Hydrogen treatment has also been documented to have anti-fatigue effects, and boost running performance and torso strength in healthy adults given hydrogen inhalation therapy (28).

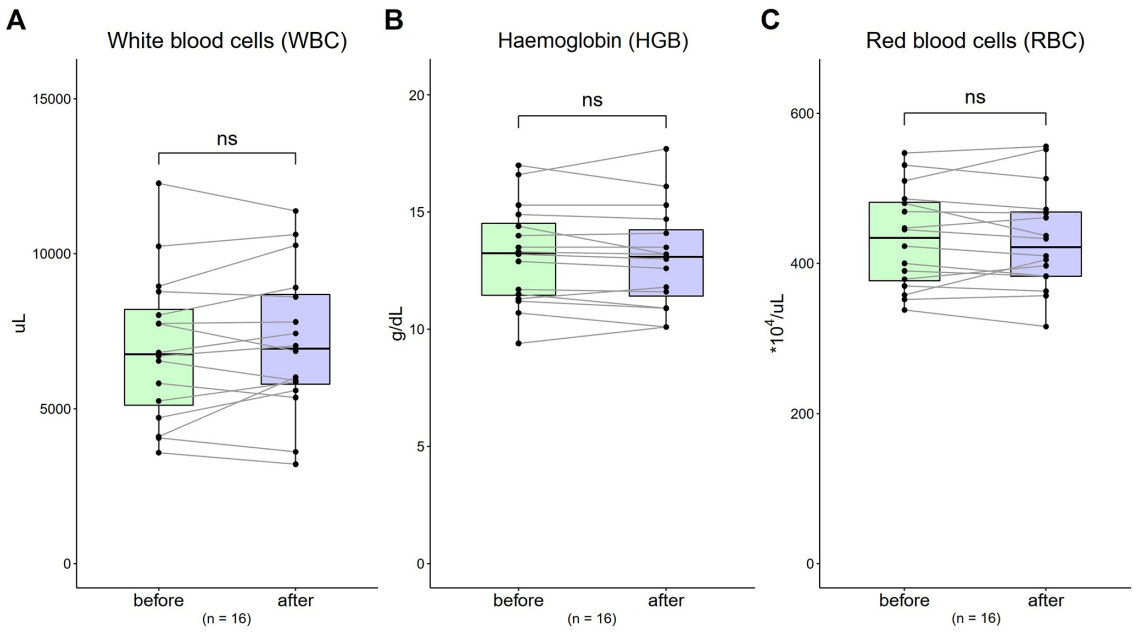

FIGURE 3
Changes in complete blood count (CBC) from 0 to 4weeks (n=16). **(A)** WBC=white blood cells (normal range: 3250~9,160/μL); **(B)** HGB=haemoglobin (normal range, males: 13.1~17.2; normal range, females: 11.0~15.2g/dL); **(C)** RBC=red blood cells (normal range, males: 421~590; normal range, females: 378~525 *10^4/μL). Data are represented by line plots (*p<0.05; **p<0.01; ***p<0.001) and were analyzed using the paired t test.

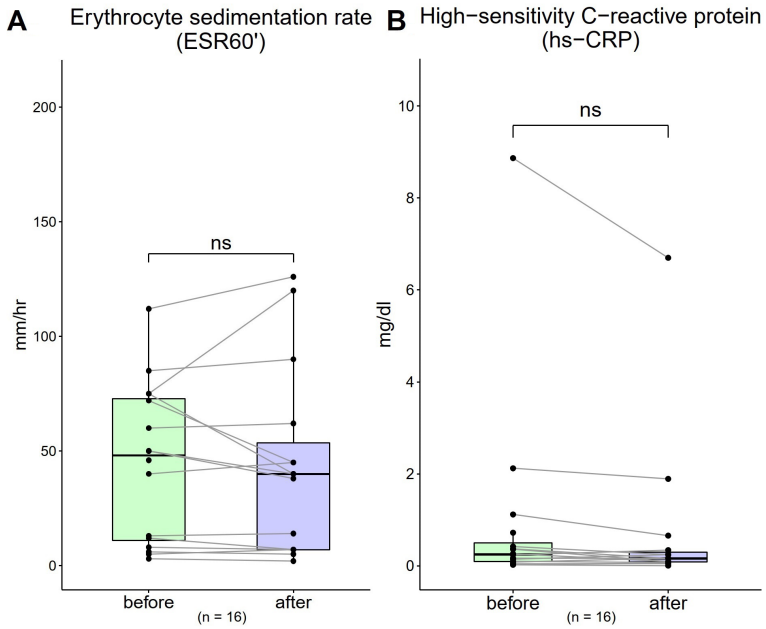

FIGURE 4
Changes in inflammatory biomarkers from 0 to 4weeks (n=16). **(A)** Erythrocyte sedimentation rate (ESR)-60 (normal range: males, <10; females, <15mm/1h) **(B)** high-sensitivity C-reactive protein (hs-CRP) (normal range: < 1mg/dL). Data are represented as line plots (*p<0.05; **p<0.01; ***p<0.001) and were analyzed using the paired t test.

Inhalation of hydrogen gas is the most straightforward therapeutic method and has been used worldwide. Inhalation of H_2 ensures a long retention time and high dose in the body. Inhaled hydrogen diffuses through the alveoli into the plasma and is transported throughout the body through the blood. Nevertheless, the different administration methods of hydrogen may have different effects (29). A pharmacokinetic study of a single inhalation of hydrogen gas in pigs showed that the hydrogen level in the carotid artery peaked immediately after breath holding, and it dropped to 1/40 of the peak value 3 min later. The peak hydrogen

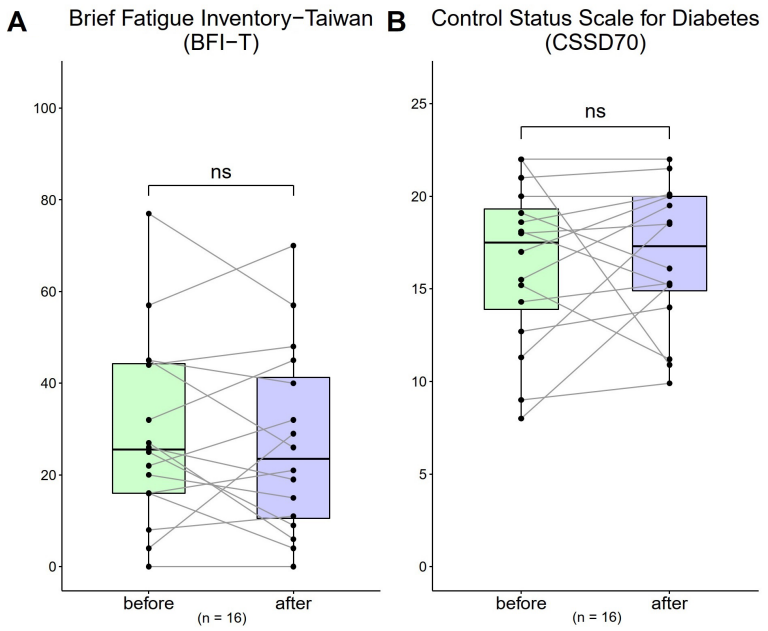

FIGURE 5
Changes in Brief Fatigue Inventory-Taiwan (BFI-T) and the control status scale for diabetes (CSSD70) scores from before to after treatment (n=16). **(A)** The BFI-T consisted of 10 questions, each scored from 0 to 10, with 0=most healthy and 10=most severe (total range: 0 to 100); **(B)** The CSSD70 questionnaire consisted of 11 questions, each scored from 0 to 2 (total range: 0 to 22). Data are represented by line plots (*p<0.05; **p<0.01; ***p<0.001) and were analyzed using the paired t test.

concentration in venous blood was much lower than that in arterial blood, which indicated that hydrogen is not simply diffused but diffuses while being carried through the blood stream (29). Molecular hydrogen/saline injection is a method that can rapidly supply a large amount of hydrogen to the body and allows direct application of hydrogen to the affected area. However, this method is invasive and difficult for patients to accept, and it has the potential risk of cross-infection. In addition, it could be dangerous if hydrogen is injected directly into the skin or vein. Thus, the different administration routes of hydrogen need to be considered according to the needs of the patient to guarantee the most benefit. A previous study found that preinhalation of hydrogen gas could protect against caerulein-induced acute pancreatitis in mice by inhibiting inflammation and oxidative stress in the early stage (30). Some clinical trials have also demonstrated that hydrogen gas inhalation treatment is safe and effective in patients with chronic obstructive pulmonary disease (COPD) and asthma (31, 32). Therapeutic effects in patients with oxidative stress owing to cardiac arrest and inflammation have also been shown (33, 34). Recently, to confront the coronavirus disease-2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), starting with the Chinese Clinical Guidance (7th edition) for COVID-19 Pneumonia Diagnosis and Treatment issued by the China National Health Commission, the inhalation of oxygen mixed with hydrogen gas (66.6% hydrogen gas and 33.3% oxygen) has been recommended given the significant role of hydrogen in ameliorating lung function decline, emphysema, and inflammation among patients with pulmonary diseases (35). In summary, the therapeutic effects of hydrogen have been highlighted in various human diseases or functional disorders. Future trials should assess self-reported lifestyle changes, provide a historical control, and compare a drug treatment group with a control group to clarify the effects of oral HRCC capsules.

To the best of our knowledge, this is the first human study investigating the safety and therapeutic efficacy of oral molecular hydrogen capsules in patients with metabolic syndrome. One strength of this study is the comprehensive evaluation of serum biomarkers in response to different doses. Unfortunately, the sample size, sample size per dose group, and short-term use of the current cohort were limiting factors. In addition, there was a large age difference between the groups. It will be important to compare the results between the different sexes. The long-term therapeutic effects of HRCC capsules and the influence of the dose in large populations should be assessed in future studies.

5. Conclusion

This first human clinical trial evaluating HRCC capsules in patients with metabolic syndrome demonstrated their safety and a potential therapeutic effect from lowering TG. No adverse effects were observed. Findings regarding CBC, HbA1C, hs-CRP and ESR-60, a fatigue indicator (BFI-T), and the CSSD70 questionnaire all indicate that HRCC did not cause obvious adverse effects. Future studies with a larger sample size are required to perform a long-term safety evaluation and assess HRCC efficacy in metabolic syndrome, such as high blood pressure, elevated fasting glucose levels, and low HDL-C. After further study, the therapeutic effects of HRCC capsules

in combination with medications such as statins and niacin could help to prevent and treat metabolic syndrome or other metabolic diseases.

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 research protocol was approved by the Institution Review Board (IRB) of Min-Sheng General Hospital (MSIRB). The approval number is 2021007-A. The patients/participants provided their written informed consent to participate in this study.

Author contributions

K-YW, H-YC, J-RC, T-YW, P-JH, and W-WC performed the material preparation, data collection, and analysis. S-HC, J-RC, P-JH, W-CH, K-YW, C-FC, and M-CS wrote the first draft of the manuscript. F-CL, P-JH, and FD supervised the manuscript. All authors contributed to the study conception and design, commented on all versions of the manuscript, and read and approved the final manuscript.

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Conflict of interest

FD, J-RC, K-YW, C-FC, H-YC, and W-CH were employed by the HoHo Biotech Co., Ltd. T-YW was employed by HoGo Force Co., Ltd.

The authors declare that this study received funding from HoHo Biotech. The funder had the following involvement in the study: experiment design, data collection, and the decision to publish.

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Glossary

HRCC	Hydrogen-rich coral calcium
TG	Triglyceride. A TG test is a blood test that measures the amount of fat in the blood called TG. High TG may increase the risk of a heart attack or stroke
T2DM	Type 2 diabetes
Cardiovascular diseases (CVD)	Cardiovascular diseases
QOL	Quality of life
HDL-C	High-density lipoprotein cholesterol, often referred to as "good" cholesterol
LDL-C	Low-density lipoprotein cholesterol, often called the "bad" cholesterol because it adheres to the walls of blood vessels, increasing the probability of developing health problems such as heart attack or stroke
TNFα	Tumor necrosis factor α (TNFα). An inflammatory cytokine that is produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signaling events within cells, leading to necrosis or apoptosis
IL-6	Interleukin-6, a cytokine that plays a central role in host defence due to its wide range of immune and haematopoietic activities
ESR	Erythrocyte sedimentation rate is the rate at which red blood cells in anticoagulated whole blood descend in a standardized tube over a period of 1 h
CRP	C-reactive protein. A wide variety of inflammatory conditions can cause elevated CRP levels
WBC	White blood cells
BFI-T	Brief Fatigue Inventory-Taiwan (BFI-T)
CSSD70	Control status scale for diabetes questionnaire. Each question is scored with a minimum value of 0 (the most severe), and a maximum value of 2 (the most healthy). Total range from 0 to 22
RBC	Red blood cells
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
MCHC	Mean corpuscular haemoglobin concentration
HbA1C	The haemoglobin A1c test measures the amount of blood sugar (glucose) attached to haemoglobin
CHOL	A blood test that can measure the amount of cholesterol and triglycerides in the blood
hs-CRP	High-sensitivity CRP
HGB	The haemoglobin test measures the quantity of haemoglobin contained in the red blood cells

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The effect of maternal vitamin D deficiency during pregnancy on glycolipid metabolism of offspring rats and the improvement of vitamin D intervention after weaning

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Background: Vitamin D deficiency during pregnancy is common, but whether maternal vitamin D status affects glycolipid metabolism of offspring remains unclear.

Objective: To evaluate the effect of maternal vitamin D deficiency during pregnancy on the glycolipid metabolism of offspring at different life-cycles (from birth to adulthood) and to explore the improvement of different dosages of vitamin D supplementation.

Methods: Sprague–Dawley rats were fed vitamin D-deprived (VDD group) or standard vitamin D diets (SC group) during pregnancy, and their diets were changed to standard vitamin D diets during lactation (the offspring were sorted into $VDD_{offspring}$ and $SC_{offspring}$ groups). After weaning, rats in the $VDD_{offspring}$ group were randomly assigned to the $VDD_{offspring}$, $VDD_{offspring}$-S_{3300} and $VDD_{offspring}$-S_{10000} groups with diets containing standard, medium and high dosages of vitamin D for 12 wk. Serum was collected for biochemical analyses at postnatal Day 21, postnatal Day 56 and postnatal Day 84. Oral glucose tolerance test (OGTT) was performed at postnatal Day 70.

Results: Compared to $SC_{offspring}$, rats in the $VDD_{offspring}$ group had significantly lower birth weight with faster weight gain and higher levels of lipid metabolism in early life. After near adulthood, the differences in weight and lipid metabolism between the two groups disappeared. OGTT showed significantly higher blood glucose levels in the $VDD_{offspring}$ group at 30 min, 60 min, and 90 min. The continuation of vitamin D supplementation at medium and high dosages after weaning did not cause any obvious changes in weight or glycolipid metabolism (except for postprandial hyperglycemia). OGTT demonstrated that the glucose levels in the $VDD_{offspring}$-S_{3300} group were lowest at all the time points and that those in the $VDD_{offspring}$-S_{10000} group were the highest at 30 min, 60 min, and 90 min among the three groups.

Conclusion: The adverse effects of vitamin D deficiency during pregnancy on glycolipid metabolism in offspring vary in different stages. Over a long time period, adequate vitamin D supplementation is beneficial to glycolipid metabolism for the offspring of subjects with vitamin D deficiency during pregnancy; however, further improvement is required.

KEYWORDS

vitamin D deficiency, body weight, lipid metabolism, glucose metabolism, offspring, pregnancy, rats

Introduction

Vitamin D, which is a prohormone, not only plays an important role in calcium and phosphorus homeostasis and bone health (1), but also exerts pleiotropic actions regarding cellular differentiation and proliferation, immune regulation and metabolism (2, 3). Sufficient vitamin D is generally considered a protective factor for maintaining good health and for preventing diseases. Notably, maternal vitamin D deficiency during pregnancy (25-hydroxyvitamin D [25(OH)D] < 30 nmol/L) is very common, even in areas with high ultraviolet exposure, which has been observed as a worldwide public health problem (4, 5). Thus, more attention has been focused on the role of vitamin D during pregnancy.

Recently, the adverse effects of vitamin D deficiency on glycolipid metabolism during pregnancy has been reported by numerous studies, including elevated risks for gestational diabetes mellitus (GDM), higher blood glucose and dyslipidemia in pregnancy (6–10). Furthermore, fetal and neonatal vitamin D status completely relies on the conditions of the mother (11), and there is a question as to whether vitamin D deficiency during pregnancy has an effect on glycolipid metabolism in the offspring. However, related studies have been poorly investigated. A maternal and infant study found that vitamin D-deficient women had higher concentrations of triglycerides and lower concentrations of high-density lipoprotein cholesterol (HDL-C) and Homeostasis model assessment- β (HOMA-β) in infant umbilical arterial blood in comparison with pregnant women with adequate vitamin D. Additionally, the triglyceride level in the umbilical artery was negatively correlated with maternal serum 25(OH)D concentration (12). Another cohort study with 1882 mother–child pairs showed that a 10 nmol/L increase in maternal 25(OH)D was associated with a 0.02 (99% CI: −0.03, −0.004) decrease in Homeostatic model assessment of insulin resistance (HOMA-IR), and a 0.13% (99% CI: −0.3, −0.003) decrease in body fat percentage at the approximate age of 5-6-years-old (13). Nevertheless, a study from India indicated that in addition to higher fasting insulin resistance at the age of 9.5 years, maternal vitamin D deficiency during pregnancy did not affect blood glucose and fasting lipid levels in the offspring at age 5 or 9.5 (14). An observational cohort study followed 149 healthy pregnant women pointed maternal vitamin D levels did not correlate with neonatal serum glucose or insulin levels (15). To date, all of the results of relevant studies are inconsistent and conflicting (5, 16, 17). These differences may be explained by several confounding factors including sample size, geographical location, gestational age at sampling, end time of observation indicator and the rate of vitamin D deficiency (16, 17).

Moreover, most research has mainly emphasized the effects at the early stages of offspring (neonatal or infant stages). According to the Developmental Origins of Health and Disease (DOHaD) theory, the effects of adverse nutritional environment *in utero* on fetal heath are long-term and even irreversible (5, 18). Additionally, some other studies concerning the association between vitamin D deficiency during pregnancy and lipid metabolism of their offspring were performed based on obesity diet models, which face difficulties in elucidating the independent effects of vitamin D on lipid metabolism (19–21). To better control the confounding factors, we constructed a prenatal vitamin D deficient rat model based on a normally structured diet (only vitamin D deficiency or not) to assess the independent effect of maternal vitamin D deficiency during pregnancy on the glycolipid metabolism of their offspring at different life cycles (from birth to adulthood). Moreover, the offspring with vitamin D deficiency during pregnancy were given different dosages of vitamin D supplementation (standard, medium and high dosages) after weaning, in an attempt to explore whether vitamin D supplementation in offspring could promote the adverse effects of vitamin D deficiency during pregnancy.

Methods

Animal study

Sprague–Dawley rats (7-weeks-old, Shanghai SLAC Laboratory Animal Co., Ltd) were housed in incandescent light devoid of ultraviolet B radiation on a 12 h light/12 h dark cycle at 22 ± 2°C and 60% relative humidity. They were randomly divided into a standard group and a vitamin D-deficient group according to initial weights. The sufficient group (SC; n = 4) was fed a standard vitamin D diet containing 1,000 IU vitamin D3/kg (AIN-93G with 1,000 IU vitamin D_3/kg, Trophic Animal Feed High-tech Co., Ltd., China), and the vitamin D-deficient group (VDD; n = 8) was fed a vitamin D-deprived diet containing 25 IU vitamin D_3/kg (AIN-93G with 25 IU vitamin D_3/kg, Trophic Animal Feed High-tech Co., Ltd., China). After a 4-week deficiency induction period, serum 25(OH)D concentrations were detected to ensure that the vitamin D-deficient model was successfully established. Afterwards, all rats were mated with standard vitamin D diet-fed male SD rats (three rats and seven rats were matched successfully in the SC group and VDD group, respectively). During gestation, two groups of pregnant rats continued to be fed their previous diets, and serum 25(OH)D concentrations were detected on embryonic Day 18 (E18). During lactation, all of the dams in both groups were fed a standard vitamin D diet. At postnatal Day 21 (PND 21, weaning), 8 offspring rats were killed in both groups ($SC_{offspring}$ and $VDD_{offspring}$ groups) for blood sampling, after which we randomly selected 16 and 48 offspring rats from the $SC_{offspring}$ group and $VDD_{offspring}$ group, respectively, for subsequent experiments. Forty-eight offspring rats in the $VDD_{offspring}$ group were randomly assigned to the $VDD_{offspring}$, $VDD_{offspring}$-S_{3300} and $VDD_{offspring}$-S_{10000} groups according their body weights ($VDD_{offspring}$: 49.46 ± 1.00 g; $VDD_{offspring}$-S_{3300}: 49.76 ± 1.74 g and $VDD_{offspring}$-S_{10000}: 49.26 ± 1.35 g, $p > 0.05$), which were fed a diet containing 1,000 IU vitamin D_3/kg (standard dosage), 3,300 IU vitamin D_3/kg (medium dosage) and 10,000 IU vitamin D_3/kg (high dosage) (AIN-93G with 1,000/3,300/10,000 IU vitamin D_3/kg, Trophic Animal Feed High-tech

Co., Ltd., China) until the end of the experiment. To examine the biochemical index changes, 8 offspring rats were euthanized in four groups for blood sampling at week 8 (PND 56, near adulthood) and week 12 (PND 84, adulthood). All of the offspring rats were weighed on PND 1, PND 3, PND 7, PND 14, PND 21, PND 28, PND 42, PND 56, PND 70 and PND 84.

All experimental procedures followed the guidelines for the care and use of animals, which were established by Zhejiang Chinese Medical University and approved by the Animal Experimentation Ethics Committee of Zhejiang Chinese Medical University (Approval NO. IACUC-20200518-15).

Vitamin D measurements

Serum was collected after centrifugation of blood samples at 3,500 × g for 10 min and stored at −80°C until analysis, which was performed by using liquid chromatography–mass spectrometry (LC–MS/MS) as previously described (22).

Fasting variables

500 microliters of blood were collected from the lower mandibular vein after 12 h of fasting, and the serum was separated by centrifugation at 3000 rpm for 10 min. Then the concentrations of calcium, triacylglycerols (TGs), total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and fasting glucose (FG) were measured by using a fully automatic biochemical analyzer (Hitachi 3,100, Japan). The levels of fasting insulin (FIN) in the serum were determined via enzyme-linked immunosorbent assay kits (CUSABIO, Wuhan, China) according to the manufacturer's guidelines.

Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was performed in the 10th week after birth. Two-hour OGTT with gavage of 20% glucose (2.0 g/kg glucose solution) were given in groups, followed by collection of blood samples from tail vein at 0. 30, 60, and 120 min to measure plasma glucose levels by an automatic glucometer (Accu-Chek, Roche Diagnostics, Mannheim, Germany).

Statistical analysis

All of the statistical analyses were performed by using SPSS 23.0 software. Continuous variables were summarized as the mean with standard deviation (SD). Independent samples t tests were used to compare the differences in variables between two groups. One-Way ANOVA analysis and LSD test were applied to compare the differences among multiple groups (more than two groups). Pearson's correlation analysis was used to examine correlations between different variables (maternal vitamin D level on E18 and lipid metabolism). Moreover, a simple linear regression was used to predict the effects of maternal vitamin D level (E18) on glycolipid metabolism in the offspring. Two-tailed p values<0.05 were considered to be statistically significant.

Results

Maternal and offspring vitamin D status and calcium levels

Maternal 25(OH)D was measured on embryonic Day 0 (E0) and embryonic Day 18 (E18) to determine whether the prenatal vitamin D deficiency model was successfully established in rats. There were significant differences in the concentrations of maternal 25(OH)D between the SC group and VDD group on E0 (12.58 ± 1.18 μg/mL and 5.55 ± 0.75 μg/mL, respectively; $p < 0.001$) and on E18 (10.54 ± 0.77 μg/mL and 2.97 ± 0.59 μg/mL, respectively; $p < 0.001$), whereas the calcium levels were similar between the SC group and VDD group on E18 (2.55 ± 0.05 mmol/L and 2.47 ± 0.05 mmol/L, respectively; $p = 0.058$), which indicated the success of the model (Figure 1A).

For offspring, the serum 25(OH)D concentrations between the $SC_{offspring}$ group and the $VDD_{offspring}$ group were not significantly different ($p = 0.654$) on PND21, with the levels being 7.35 ± 0.85 μg/mL and 7.73 ± 2.19 μg/mL, respectively. After weaning with the intervention of different vitamin D dosages, rats in the $SC_{offspring}$ group and $VDD_{offspring}$ group maintained similar 25(OH)D levels, and the concentration of 25(OH)D in $VDD_{offspring}$-S_{3300} was significantly higher than that in the $SC_{offspring}$ group and $VDD_{offspring}$ group. Rats in the $VDD_{offspring}$-SC_{10000} group had the highest 25(OH)D concentrations on PND 56 and PND 84 (Figure 1B). Furthermore, the calcium levels among all groups remained similar at all of the time points ($p > 0.05$). Data were shown in Supplementary Table S1.

Body weight

As shown in Table 1; Figure 2, there were significant and different effects of maternal vitamin D deficiency during pregnancy on the body weights of their offspring at different life cycles. The birth weights and the weights on PND 4 and PND 7 in the $VDD_{offspring}$ group were significantly lower than those in the $SC_{offspring}$ group ($p < 0.05$); however, the weights of rats in the $VDD_{offspring}$ group seemed to increase at a faster rate. On PND 14, the weights in the two groups were similar; in addition, from PND 21 to PND 42, the body weights in the $VDD_{offspring}$ group were significantly higher than those in the $SC_{offspring}$ group. From the time of PND 56, there was no significant difference in body weights between the two groups; however, the weights in $VDD_{offspring}$ were still higher than those in the $SC_{offspring}$ group.

With medium and high dosages of vitamin D supplementation after weaning, the body weights in the $VDD_{offspring}$-S_{3300} and $VDD_{offspring}$-S_{10000} groups had no significant differences from those in the $VDD_{offspring}$ group at all of the time points ($p < 0.05$). Similarly, compared with $SC_{offspring}$, the weights in the $VDD_{offspring}$-S_{3300} and $VDD_{offspring}$-S_{10000} groups were still significantly higher from PND21 to PND42 (except for the weights in $VDD_{offspring}$-S_{10000} on PND42), after which it maintained a higher weight at later time points.

Lipid metabolism and related factors

The effect of maternal vitamin D deficiency during pregnancy on the lipid metabolism of the offspring rats was significant

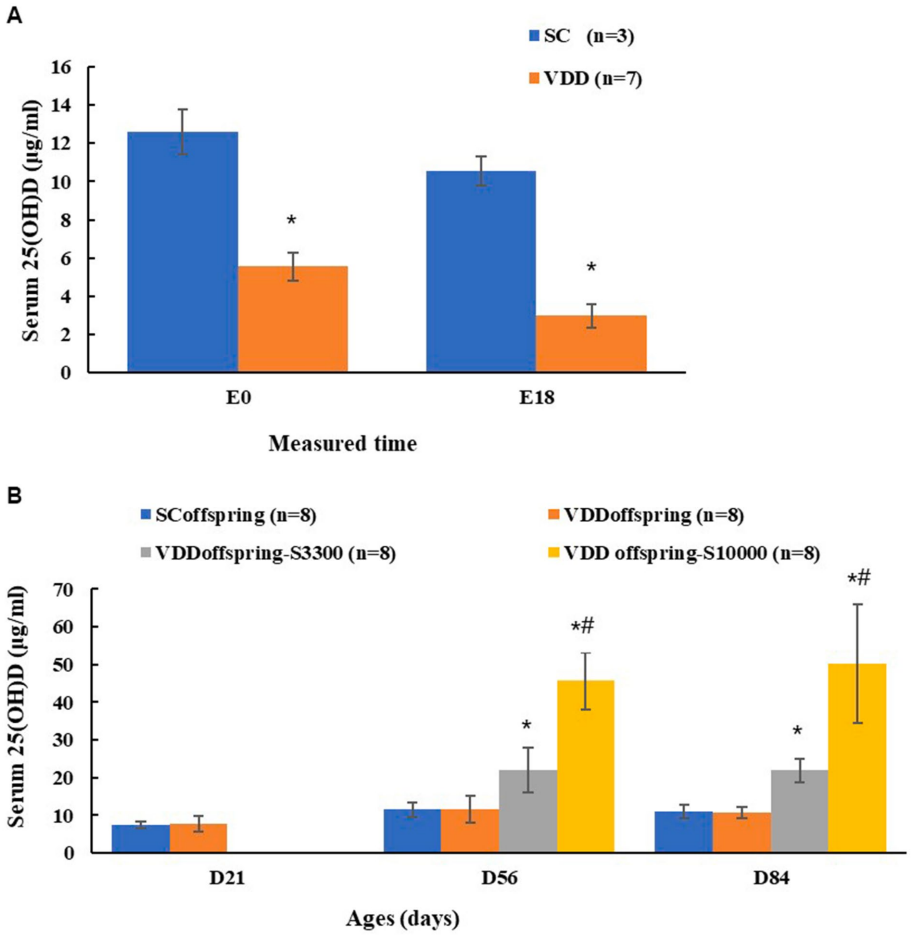

FIGURE 1
Maternal and their offspring vitamin D status at different time **(A)** Maternal 25(OH)D conentration of two groups on embryonic day 0 (E0) and embryonic day 18 (E18). *Compared to SC group, $p < 0.05$; **(B)** The 25 (OH) D conentration of offspring among four groups on PND21, PND56 and PND84. *Compared to $SC_{offspring}$ group, $p < 0.05$; #Compared to $VDD_{offspring\text{-}S3300}$ group, $p < 0.05$.

in early life. As shown in Table 1, the levels of TG, TC, LDL-C, TC/HDL and LDL/HDL were significantly increased in the $VDD_{offspring}$ group compared with the $SC_{offspring}$ group on PND 21 ($p < 0.05$), and these differences disappeared on PND56 and PND84 ($p > 0.05$).

The continuation of vitamin D supplementation at medium and high dosages after weaning did not cause any obvious change in lipid metabolism among the $VDD_{offspring}$, $VDD_{offspring}$-S_{3300} and $VDD_{offspring}$-S_{10000} groups ($p > 0.05$). We observed similar levels of TG, TC, HDL-C, LDL-C, TC/HDL and LDL/HDL among the three groups on PND56 and PND84.

Pearson correlation analysis showed that maternal vitamin D levels during pregnancy (E18) were negatively correlated with the levels of TC, TG, LDL-C, TC/HDL, and LDL/HDL in their offspring rats on PND21 (Table 2). Additionally, simple linear regression was calculated to determine the effects of maternal vitamin D levels during pregnancy (E18) on the lipid metabolism of offspring rats. We found that 81.4% of the TC/HDL cholesterol ratio and 66.8% of the LDL/HDL cholesterol ratio were determined by maternal vitamin D levels during pregnancy for offspring rats in early life (adjusted R^2 values were 0.814 and 0.668, respectively, $p < 0.05$). Data are shown in Supplementary Table S2.

Glucose metabolism and oral glucose tolerance test

Compared with the $SC_{offspring}$ group, no significant differences in glucose metabolism (fasting glucose, fasting insulin and HOMA-IR) were found in the $VDD_{offspring}$ group at all of the time points, although they were numerically higher (Table 1). However, OGTT showed that significantly higher blood glucose levels were detected in the $VDD_{offspring}$ group than in the $SC_{offspring}$ group at 30 min, 60 min, and 90 min, although blood glucose levels at 0 min and 120 min were similar between the two groups (Figure 2). Afterwards, simple linear regression was calculated to determine the effect of maternal vitamin D levels during pregnancy (E18) on the blood glucose levels of offspring rats, which indicated that 35.8, 50.8, and 20.4% of blood glucose levels at 30 min, 60 min, and 90 min were determined by maternal vitamin D levels during pregnancy, respectively (adjusted R^2 values were 0.358, 0.508, and 0.204, respectively, $p < 0.05$). Data are shown in Supplementary Table S2.

Unlike the change in lipid metabolism, vitamin D supplementation at different dosages after weaning had different effects on glucose metabolism and OGTT. Compared with the $SC_{offspring}$ group, the fasting blood glucose levels significantly increased in the

TABLE 1 Fasting variables among four groups at different time points.

Variable	PND21		PND56				PND84			
	SCoffspring (n = 8)	VDDoffspring (n = 8)	SCoffspring (n = 8)	VDDoffspring (n = 8)	VDDoffspring-S3300 (n = 8)	VDDoffspring-S10000 (n = 8)	SCoffspring (n = 8)	VDDoffspring (n = 8)	VDDoffspring-S3300 (n = 8)	VDDoffspring-S10000 (n = 8)
TG (mmol/L)	0.74 ± 0.17	0.92 ± 0.15*	0.74 ± 0.22	0.69 ± 0.47	0.81 ± 0.38	0.46 ± 0.14	0.73 ± 0.12	0.63 ± 0.29	0.59 ± 0.23	0.55 ± 0.13
TC (mmol/L)	2.14 ± 0.21	2.45 ± 0.20**	1.61 ± 0.18	1.76 ± 0.52	1.91 ± 0.34	1.68 ± 0.39	1.68 ± 0.15	1.65 ± 0.31	1.48 ± 0.20	1.52 ± 0.14
HDL-C (mmol/L)	0.62 ± 0.06	0.59 ± 0.04	0.52 ± 0.04	0.54 ± 0.10	0.58 ± 0.05	0.53 ± 0.07	0.58 ± 0.04	0.53 ± 0.04	0.50 ± 0.04	0.52 ± 0.06
LDL-C (mmol/L)	0.45 ± 0.04	0.55 ± 0.07**	0.24 ± 0.05	0.27 ± 0.09	0.29 ± 0.06	0.26 ± 0.09	0.26 ± 0.05	0.26 ± 0.07	0.22 ± 0.05	0.22 ± 0.03
TC/HDL	3.47 ± 0.19	4.15 ± 0.12**	3.08 ± 0.21	3.19 ± 0.34	3.26 ± 0.34	3.12 ± 0.37	2.91 ± 0.27	3.12 ± 0.37	2.95 ± 0.25	2.95 ± 0.22
LDL/HDL	0.72 ± 0.06	0.93 ± 0.09**	0.46 ± 0.07	0.49 ± 0.07	0.50 ± 0.07	0.48 ± 0.10	0.44 ± 0.09	0.48 ± 0.11	0.44 ± 0.07	0.43 ± 0.08
FG (mmol/L)	4.64 ± 0.95	5.11 ± 0.35	4.07 ± 0.38	4.55 ± 0.46	4.67 ± 0.68	4.09 ± 0.85	4.96 ± 0.23	5.29 ± 0.51	5.12 ± 0.91	5.68 ± 0.66*
FIN (ng/mL)	5.26 ± 2.22	6.17 ± 2.05	5.43 ± 0.95	4.27 ± 1.53	7.32 ± 2.25$^{\#\#}$	3.73 ± 1.50	3.82 ± 1.83	4.45 ± 1.97	3.37 ± 1.66	2.65 ± 1.39$^{\#}$
HOMA-IR	1.03 ± 0.33	1.38 ± 0.39	0.97 ± 0.41	0.88 ± 0.36	1.49 ± 0.42*$^{\#\#}$	0.69 ± 0.37	0.85 ± 0.42	1.06 ± 0.53	0.75 ± 0.33	0.65 ± 0.34

Data are presented as Mean ± SD; *Compared to $SC_{offspring}$ group, $p < 0.05$; **Compared to $SC_{offspring}$ group, $p < 0.01$. $^{\#}$Compared to $VDD_{offspring}$ group, $p < 0.05$; $^{\#\#}$Compared to $VDD_{offspring}$ group, $p < 0.01$.

TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC/HDL, the ratio of TC and HDL; LDL/HDL, the ratio of LDL and HDL; FG, Fasting glucose; FIN, fasting insulin; HOMA-IR, homeostatic model assessment of insulin resistance.

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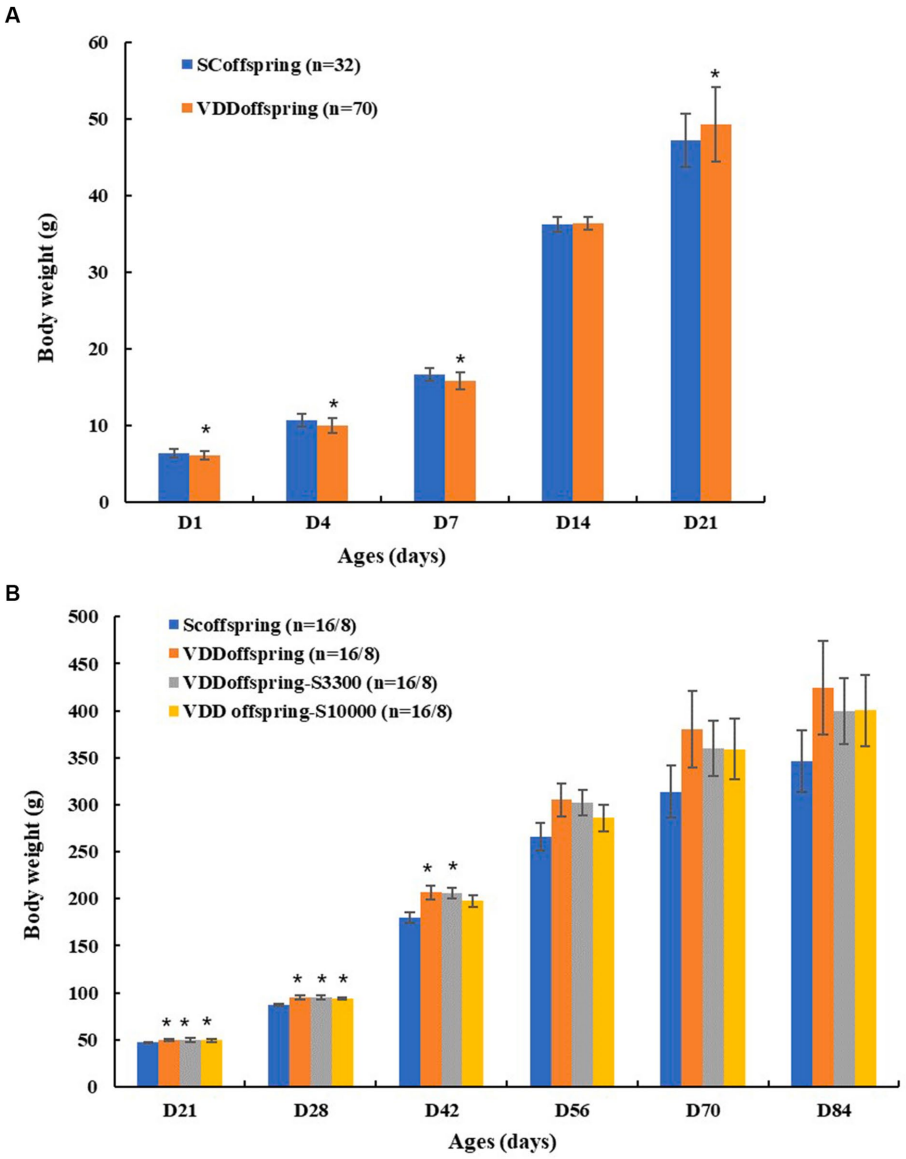

FIGURE 2
The weight of rats in different groups **(A)** The weight of rats in $SC_{offspring}$ and $VDD_{offspring}$ groups before weaning; **(B)** The weight of rats in $SC_{offspring}$, $VDD_{offspring}$, $VDD_{offspring}$-S_{3300} and $VDD_{offspring}$-S_{10000} groups after weaning (from PND21 to PND56, the number of rats in four groups was 16; after PND56, the number of rats in four groups was 8). *Compared to $SC_{offspring}$ group, $p < 0.05$.

TABLE 2 Pearson's correlation coefficients among related factors (maternal VD levels on E18 and VD of offspring on PND21) and lipid metabolism on PND21.

Related factors	TC	TG	HDL	LDL	TG/HDL	LDL/HDL
Maternal VD levels ($n = 5$)	**−0.586***	**−0.504***	0.325	**−0.658****	**−0.902****	**−0.817****
VD levels of offspring ($n = 16$)	0.425	0.355	0.318	0.346	0.154	0.133

Bold indicates the relationships are significant statistically. *$p < 0.05$; **$p < 0.01$.

$VDD_{offspring}$-S_{10000} group on PND70 (data from OGTT at 0 min) and PND84. Moreover, the levels of FIN and HOMA-IR were obviously increased in the $VDD_{offspring}$-S_{33000} group on PND56, whereas the fasting blood glucose levels in this group remained similar to those in the other groups. As shown in Figure 3, the OGTT showed that the glucose levels of offspring in the $VDD_{offspring}$-S_{3300} group were lowest at all five time points among the $VDD_{offspring}$, $VDD_{offspring}$-S_{3300} and $VDD_{offspring}$-S_{10000} groups, although the levels were still significantly higher than those in the $SC_{offspring}$ group (except at 120 min). In addition, we found that the highest blood glucose levels (fasting glucose level and postprandial blood glucose at 30 min, 60 min, and 90 min) among the four groups were in the $VDD_{offspring}$-S_{10000} group, which indicated that the highest dosages of vitamin D supplementation (10,000 IU) may negatively affect the blood glucose levels in offspring.

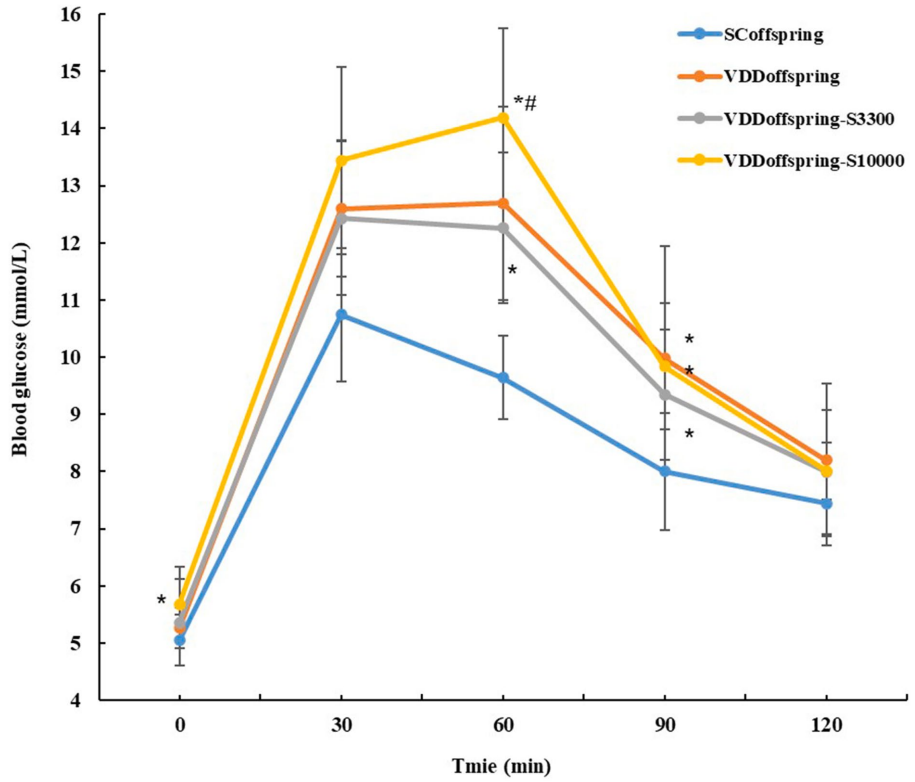

FIGURE 3
Oral glucose tolerance test (OGTT) in offspring rats aged 70 days (n = 8).

Discussion

Vitamin D deficiency during pregnancy is widespread worldwide, and as vitamin D plays an important role in maintaining normal glycolipid metabolism, the effect of maternal vitamin D deficiency during pregnancy on glycolipid metabolism in offspring has aroused researchers' attention. Emerging clinical evidence shows that maternal vitamin D deficiency during pregnancy may lead to abnormal growth (either low birth weight or largeness for gestational age) and lipid and glucose metabolism disorders in offspring (23). Some cohort studies showed that lower maternal vitamin D status was associated with higher triglyceride level in infant umbilical arterial blood (12), increased odds of overweight at the first year (24), greater fat mass at ages 4 and 6 years (13, 18). Up to now, however, relevant studies are few, and most of them focused on changes in early childhood, possibly due to follow-up time or confounding factors. Based on the Developmental Origins of Health and Disease (DOHaD) theory, the adverse health effects for their offspring may last longer than expected (23).Thus, taking advantage of animal experiments, we attempt to explore the long-term effects of maternal vitamin D deficiency during pregnancy on glycolipid metabolism of offspring rats.

In this study, we demonstrated an absolutely adverse effect of vitamin D deficiency during pregnancy on the lipid metabolism of their offspring rats in early life. At PND21, higher serum levels of TC, TG and LDL-C and higher ratios of TC/HDL and LDL/HDL were observed in the $VDD_{offspring}$ group than in the $SC_{offspring}$ group. The maternal vitamin D levels during pregnancy were negatively correlated with the levels of TC, TG and LDL-C, as well as with the TC/HDL cholesterol ratio and LDL/HDL cholesterol ratio, in offspring rats. These results were similar to a mother-infant cohort study (12). They determined that compared with the control group (25[OH]D ≥ 20 ng/mL), vitamin D-deficient pregnant women had higher concentrations of TC and lower concentrations of HDL-C in infant umbilical arterial blood, and there was a negative correlation between maternal serum 25(OH)D and the TC level in the umbilical artery. Moreover, linear regression models showed that 81.4% of the TC/HDL cholesterol ratio and 66.8% of the LDL/HDL cholesterol ratio were determined by maternal vitamin D levels during pregnancy for offspring rats at PDN 21. Recent studies have claimed that the TC/HDL and LDL/HDL cholesterol ratios, which can simultaneously evaluate TC, HDL-C and LDL-C levels, were deemed to be better indices for predicting the risk of metabolic-related diseases than a single lipoprotein (25, 26). This suggests that maternal vitamin D deficiency during pregnancy not only causes abnormal lipid metabolism in their offspring in early life but also may increase the risk of metabolic diseases in offspring, including obesity and diabetes. Indeed, in addition to lipid metabolism, the data from the rat weight monitoring and OGTT experiment mentioned below also seemed to support this speculation.

Data from population-based prospective cohort studies showed that maternal vitamin D deficiency significantly increased the risk of neonatal low birth weight and small-for-gestational-age (27–31). Consistent with these studies, we also found significantly lower birth weights in the $VDD_{offspring}$ group than in the $SC_{offspring}$ group. Low birth weight is considered a risk factor for metabolic syndrome in adulthood (32, 33). Interestingly, in addition, we observed that compared with vitamin D sufficiency during pregnancy, prenatal vitamin D deficiency likely accelerated the weight gain of offspring rats before near

adulthood and maintained their body weight at a relatively higher level after adulthood. We assumed that the observed accelerated growth at the infantile and juvenile stages may represent "catch-up growth" due to the removal of vitamin D deficiency in the uterus. Data have emerged to suggest that excessive catch-up growth may elicit deleterious effects over a long-term time period (32, 34). Thus, the low birth weight and accelerated preadult weight gain caused by maternal vitamin D deficiency during pregnancy, as well as the possible risk of metabolic disease, should be seriously considered.

Fortunately, rats in the $VDD_{offspring}$ group were given the same dosage of vitamin D (standard dosage: 1,000 IU vitamin D3/kg) as the $SC_{offspring}$ group after weaning, and all of the indices of lipid metabolism, including TC/HDL and LDL/HDL cholesterol ratios and the weight gain of rats, were effectively improved. From PND56, we no longer observed significant differences in body weight between these two groups. After near adulthood, all indices of lipid metabolism and body weight in the $VDD_{offspring}$ group were similar to those in the $SC_{offspring}$ group; however, it cannot be ignored that most of these altered indices were numerically still higher in the $VDD_{offspring}$ group. These results suggested that early supplementation of adequate vitamin D for offspring could ameliorate (rather than completely eliminate) the adverse effects of maternal vitamin D deficiency during pregnancy on lipid metabolism and weight gain.

Unlike lipid metabolism, we did not observe any significant differences in the indices of glucose metabolism (fasting glucose, fasting insulin and HOMA-IR) between the $VDD_{offspring}$ and $SC_{offspring}$ groups at any stage, which is in agreement with the results of other studies (20, 35–38). Some animal experiments to identify the impact of maternal vitamin D deficiency on glucose metabolism in normal and high-fat diets observed glucose homeostasis (fasting glucose, fasting insulin, HOMA-IR and insulin tolerance tests) of adult offspring mice with no modifications in the normal diet; however, the high-fat diet combined with maternal vitamin D deficiency could aggravate the disruption of glucose homeostasis in the male offspring (20, 35). These studies implied that maternal vitamin D deficiency during pregnancy likely had little effect on the baseline glucose metabolism (fasting glucose, fasting insulin and HOMA-IR) of their offspring when they were fed a normal diet; however, it may amplify the adverse effects of other adverse factors on glucose metabolism. In addition, Zhang et al. (37) reported that compared with the control group, vitamin D deficiency during pregnancy in rats led to a marked increase in fasting insulin and HOMA-IR levels at 16 weeks, but no significant differences were observed at 0, 3 and 8 weeks. In addition, glucose metabolism is age dependent. The adverse effects of maternal vitamin D deficiency on the baseline glucose metabolism of the offspring may well be observed after a certain period of accumulation. However, our study was only focused on PND84.

Notably, the OGTT demonstrated significantly higher blood glucose levels in the $VDD_{offspring}$ group at 30 min, 60 min, and 90 min, although blood glucose levels at 0 min and 120 min were similar between the $VDD_{offspring}$ and $SC_{offspring}$ groups. The linear regression model indicated that 35.8, 50.8 and 20.4% of blood glucose levels at 30 min, 60 min, and 90 min were determined by maternal vitamin D levels during pregnancy, respectively. The results indicated that maternal vitamin D deficiency during pregnancy was closely related to postprandial hyperglycemia of the offspring as adults, which was similar to the research of Pei Li et al. (35). In their study, they also found that the number of insulin-positive cells and amount of insulin secretion significantly decreased in the maternal VD-deficient group, which indicated the existence of impaired pancreatic β-cells in the maternal VD-deficient group. Regrettably, we did not measure insulin levels at the time corresponding to OGTT. However, we believe that maternal vitamin D levels during pregnancy likely affect postprandial blood glucose in offspring by facilitating the action and secretion of insulin (39, 40), which requires further studies to confirm.

In view of the important role of vitamin D in metabolic health, more and more vitamin D supplementation intervention studies are conducted. A follow-up study of 988 healthy adolescent girls in Iran showed that significant reductions in serum fasting blood glucose, total- and low-density lipoprotein-cholesterol were observed after high dosages of vitamin D (50,000 IU) supplementation for 9 weeks (41). A meta-analysis including 19 RCTs (1,550 participants) claimed vitamin D supplementation significantly reduced serum fasting plasma glucose, insulin concentration and HOMA-IR in women with GDM (42). Whether supplementation with higher dosages of vitamin D can effectively improve abnormal glycolipid metabolism caused by vitamin D deficiency during pregnancy? Unfortunately, little relevant researches have been done. To clarify this question, we continuously administered medium and high dosages of vitamin D to the offspring of vitamin D-deficient rats during pregnancy ($VDD_{offspring}$-S_{3300} and $VDD_{offspring}$-S_{10000} groups) after weaning.

However, no significant differences were observed in the indices of lipid metabolism and body weight among the $VDD_{offspring}$, $VDD_{offspring}$-$S_{3300,}$ and $VDD_{offspring}$-S_{10000} groups at all of the time points, which indicated that higher dosages of vitamin D supplementation did not further improve the effect of vitamin D deficiency during pregnancy on offspring lipid metabolism and body weight. This result was supported by a meta-analysis of randomized clinical trial. They claimed Vitamin D supplementation (the high dosages were greater than 4,000 IU) cannot effectively decrease the levels of LDL, TC, TG, and BMI (43). Interestingly, in addition, we found that supplementation with different dosages of vitamin D had different effects on improving postprandial hyperglycemia. According to the OGTT, we found that the glucose levels in the $VDD_{offspring}$-S_{3300} group were lowest at all five time points among the $VDD_{offspring}$, $VDD_{offspring}$-$S_{3300,}$ and $VDD_{offspring}$-S_{10000} groups, although they were still significantly higher than those in the $SC_{offspring}$ group. Unexpectedly, the highest dosages of vitamin D supplementation (10,000 IU) negatively affected blood glucose. Rats in the $VDD_{offspring}$-S_{10000} group exhibited the highest blood glucose levels (fasting glucose level and postprandial blood glucose) among the four groups. Although no studies have been conducted on the effects of such high-dosage vitamin D supplementation on glucose metabolism, some other studies have demonstrated a nonlinear association between the oral dosages of vitamin D and disease risk (44–47). Zittermann et al. demonstrated a U-shaped association between major cardiac and cerebrovascular events and a circulating 25(OH)D level of 20–120 nmol/L in cardiac surgical patients (48). A longitudinal analysis from the UK primary care database showed that the hazard ratios for cardiovascular disease and mortality were significantly increased in patients with 25(OH)D < 35 nmol/L or 25(OH)D ≥ 100 nmol/L (44). Therefore, we assumed that there was a nonlinear relationship between vitamin D levels and blood glucose levels on OGTT, and supplementation with 3,300 IU vitamin D may have been the most effective dosage in improving postprandial hyperglycemia in offspring caused by vitamin D deficiency during pregnancy; however, the concentration of vitamin D supplementation still did not reduce postprandial hyperglycemia to the levels in the control group.

According to the results of this study, it is not difficult to find that the adverse effects of vitamin D deficiency during pregnancy on metabolic

health of the offspring are long-term, including lipid metabolism abnormalities in early life, low birth weight, faster growth rate before adulthood, higher body weight in adulthood, and higher postprandial blood sugar levels in adulthood. What's more, giving adequate vitamin D supplementation to offspring after weaning can improve some adverse health outcomes, but it cannot completely eliminate them. This means timely and sufficient vitamin D intervention during pregnancy may be more effective than nutritional intervention in childhood. Thus, it is necessary for relevant departments to develop effective measures to increase the proportion of vitamin D sufficiency in women of childbearing age. In addition, there were some limitations in this study. First, the gender of the offspring was not classified, and the effects of maternal vitamin D deficiency during pregnancy may be different in male and female offspring. Second, considering that glucose metabolism is age-dependent, the effects of vitamin D deficiency during pregnancy on glucose metabolism in offspring may require longer observation times. We speculate that these factors should be considered in future studies.

Conclusion

In summary, hypovitaminosis D during pregnancy had different adverse effects on the body weight and glycolipid metabolism of the offspring at different stages compared to maternal vitamin D sufficiency. The adverse effects could indicate a higher risk of metabolic disease for offspring in adulthood, such as obesity and diabetes. Over a long period of time, adequate vitamin D supplementation after weaning is beneficial to glycolipid metabolism in the offspring of maternal vitamin D deficiency during pregnancy; however, the improvement is incomplete. In addition, higher dosages of vitamin D supplementation after weaning do little to improve these adverse effects (except for postprandial hyperglycemia). Therefore, maintaining optimal vitamin D status during pregnancy is especially important for the health of the offspring.

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

All experimental procedures followed the guidelines for the care and use of animals, which were established by Zhejiang Chinese Medical University and approved by the Animal Experimentation Ethics Committee of Zhejiang Chinese Medical University (Approval NO. IACUC-20200518-15).

Author contributions

ZC, TW, XQ, and YH were responsible for experimental operation. ZC was responsible for writing the first draft of the article and analyzed the data with YZ. WH revised and finalized 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1214040/full#supplementary-material

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Sucrose-induced hyperglycemia dysregulates intestinal zinc metabolism and integrity: risk factors for chronic diseases

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Objective: Zinc is an essential micronutrient that is critical for many physiological processes, including glucose metabolism, regulation of inflammation, and intestinal barrier function. Further, zinc dysregulation is associated with an increased risk of chronic inflammatory diseases such as type II diabetes, obesity, and inflammatory bowel disease. However, whether altered zinc status is a symptom or cause of disease onset remains unclear. Common symptoms of these three chronic diseases include the onset of increased intestinal permeability and zinc dyshomeostasis. The specific focus of this work is to investigate how dietary sources of intestinal permeability, such as high sucrose consumption, impact transporter-mediated zinc homeostasis and subsequent zinc-dependent physiology contributing to disease development.

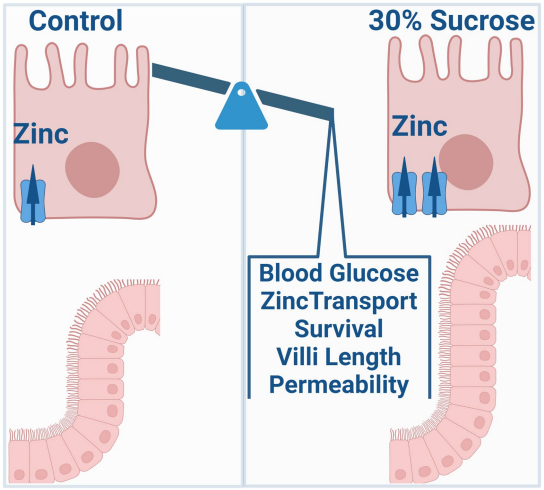
Method: We used *in vivo* subchronic sucrose treatment, *ex vivo* intestinal organoid culture, and *in vitro* cell systems. We analyze the alterations in zinc metabolism and intestinal permeability and metabolic outcomes.

Results: We found that subchronic sucrose treatment resulted in systemic changes in steady-state zinc distribution and increased ^{65}Zn transport (blood-to-intestine) along with greater ZIP14 expression at the basolateral membrane of the intestine. Further, sucrose treatment enhanced cell survival of intestinal epithelial cells, activation of the EGFR-AKT-STAT3 pathway, and intestinal permeability.

Conclusion: Our work suggests that subchronic high sucrose consumption alters systemic and intestinal zinc homeostasis linking diet-induced changes in zinc homeostasis to the intestinal permeability and onset of precursors for chronic disease.

KEYWORDS

permeability, barrier function, zinc transporter, ZIP14, Slc39a14, glucose, enteroid, organoid



GRAPHICAL ABSTRACT

1. Introduction

Zinc (Zn) is an essential micronutrient with crucial catalytic, structural, and regulatory functions (1). Zn is classified as a type II nutrient since all tissues need the micronutrient Zn for cellular division and growth (2). Despite its essentiality, there is no functional reserve or storage site for Zn in mammalian systems. Mainly, the two families of zinc transporters (SLC30/ZNT and SLC39/ZIP) maintain zinc homeostasis in the body, from intestinal absorption/excretion to the distribution of Zn to the target tissues, cells, or subcellular compartments. While the zinc status regulates some zinc transporters for maintaining homeostasis, others respond to cytokines, hormones, secondary messengers, and dietary components with consequent changes in zinc distribution (3–7). Therefore, zinc metabolism is very dynamic and requires careful assessment in normal- and pathophysiology.

Zinc is essential for intestinal health. Zinc deficiency increases the risk for gastrointestinal diseases, including inflammatory bowel diseases (IBDs). IBDs, including Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory disorders of the GI tract. According to a systematic analysis for the Global Burden of Disease Study, in 2017 there were 6.8 million cases of IBD globally and its prevalence is on the rise (8). The pathogenesis of IBD is not fully understood but involves a combination of genetic and environmental factors that disrupt the integrity of the intestinal epithelium (9, 10). This increases the permeability of the epithelial barrier, allowing both intestinal microbes and secreted endotoxins, such as lipopolysaccharides (LPS), to enter the circulation; a process known as metabolic endotoxemia (11, 12). Subsequent triggering of the immune response results in low-grade chronic inflammation, which can contribute to IBD development or worsen the current disease.

Furthermore, impaired intestinal barrier function and concurrent low-grade chronic inflammation are major factors contributing to obesity and diabetes, two of the most prevalent metabolic disorders whose incidence is increasing rapidly worldwide (13). Low-grade chronic inflammation activates immune reactions in metabolically important tissues such as adipose, muscle, and liver, causing insulin resistance and hyperglycemia (14, 15). A Western-type diet is characterized by a high content of refined sugar, fat, and animal proteins and has been linked to an increased incidence of chronic diseases, including type 2 diabetes, obesity, and inflammatory bowel disease (16). Clinical and experimental studies demonstrate that a high-fat diet can trigger these chronic disorders (17). Importantly, it has been shown in recent studies that dietary sugars may also contribute to preconditioning individuals for chronic disease (18, 19).

The major sugar used in processed food is sucrose, a disaccharide composed of the monosaccharides glucose and fructose. It has been shown that the consumption of sugar-sweetened beverages can alter serum levels of copper and zinc in healthy subjects (20). After consuming drinks that were sweetened by either glucose, fructose, high fructose corn syrup, or aspartate for 2 weeks, lower serum zinc levels were found in subjects who received glucose. Sucrose (30%) consumption in drinking water for 4 months lowered serum zinc concentrations in rats (21). Lumenal exposure to glucose has been known to alter intestinal permeability (22, 23), though more recent studies also have shown that hyperglycemia could be the driving factor for increased intestinal permeability (24). However, the impact of dietary sucrose on intestinal and systemic zinc homeostasis and subsequent zinc-dependent physiology remains unclear.

A western diet rich in fat and sugar is an environmental risk factor for IBD and metabolic disorders such as diabetes and obesity (17–19, 25, 26). Two common features of these chronic disorders are increased intestinal permeability and zinc dyshomeostasis. However, how high sugar consumption influences intestinal zinc metabolism and how altered zinc metabolism, in turn, affects intestinal permeability and the disease outcome has not been studied. Our results revealed that subchronic sucrose consumption altered intestinal and systemic zinc homeostasis. These alterations were concomitant with increased intestinal permeability.

2. Results

2.1. Sucrose consumption altered the metabolic phenotype in mice

High sugar treatment has been reported to cause alterations in systemic metabolism (27) and increase the risk of the development of

inflammatory bowel disease (28–30). To identify the metabolic phenotype following the high sucrose treatment, we first measured body weight every 2 weeks. At the beginning of the study, the average mouse weighed 22 grams. Both groups gained weight, but after 4 weeks, we observed significantly higher body weight in the sucrose-treated group (Figure 1A). Our body composition assessments by nuclear magnetic resonance (NMR) at 4 and 8 weeks revealed greater % body fat and lower % lean mass in the sucrose-treated group (Figure 1B). The blood glucose levels were higher in the fed state of sucrose treated animals at 8 weeks (Figure 1C). We observed significantly higher blood glucose levels in the sucrose-treated group in the glucose tolerance test via intraperitoneal injection (IPGTT) (Figure 1D). We also assessed changes in energy metabolism by using Comprehensive Laboratory Animal Monitoring System (CLAMs) (Figure 1E). Sucrose-treated mice had a higher respiratory exchange ratio and energy expenditure, indicating a shift towards carbohydrates as the preferred energy source and greater overall metabolic activity. Sucrose-treated mice consumed more water and less food compared to control mice. The related bar graphs were also provided in Supplementary Figure S1.

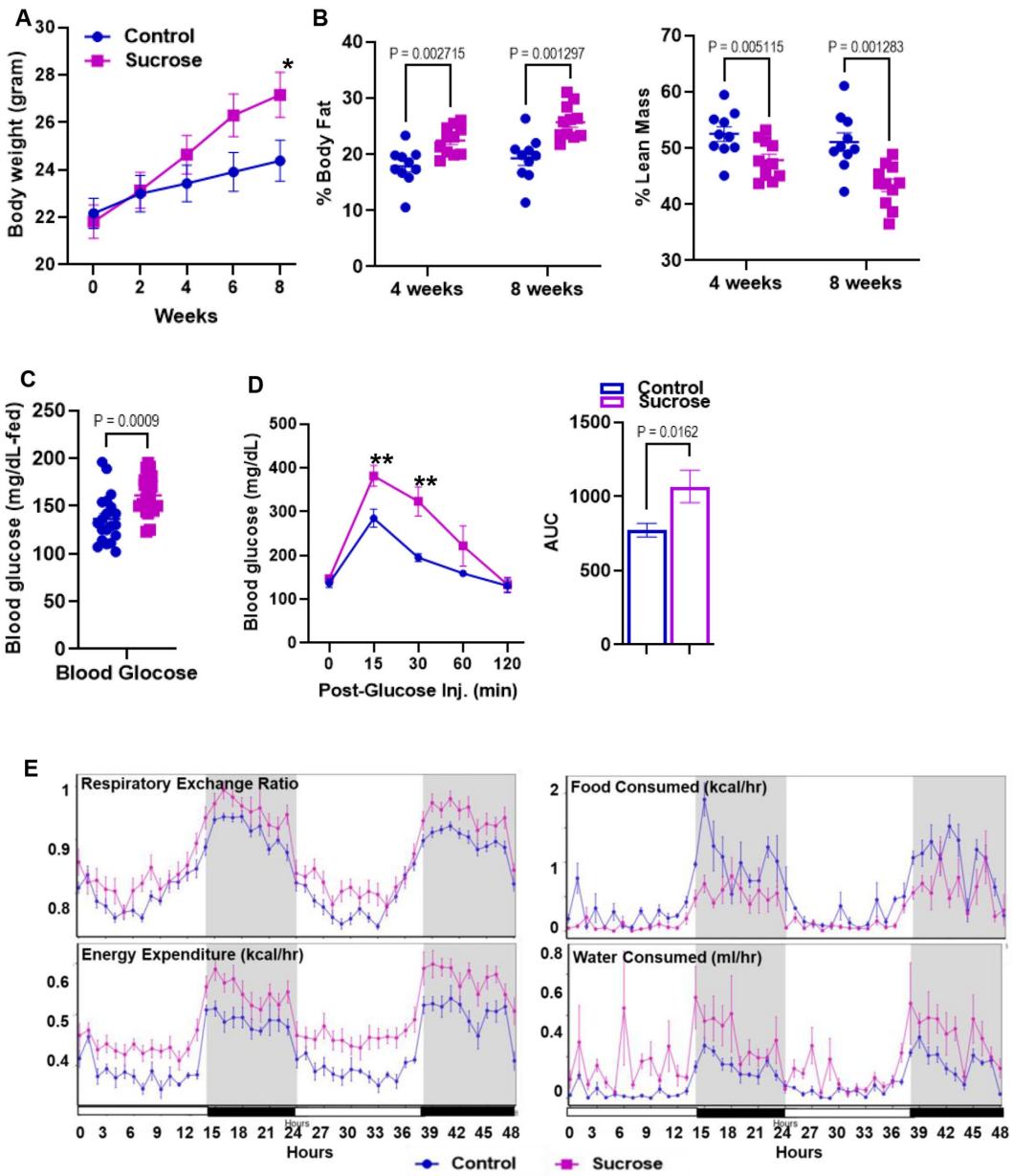


FIGURE 1
Subchronic liquid sucrose treatment induces metabolic dysregulation. Mice were given 30% sucrose in drinking water for 8 weeks. **(A)** Body weight was measured every 2 weeks up to 8 weeks. **(B)** Analysis of body composition using time-domain nuclear magnetic resonance using the Minispec LF65 Body Composition Mice Analyzer at 4 and 8 weeks of treatment. Blood glucose levels were measured by tail bleeding using the OneTouch® Ultra Blood glucose monitoring system at fed-state **(C)** and at 0, 15, 30, 60, and 120 min post glucose **(D)**. **(E)** Metabolic parameters of the mice were measured by the Comprehensive Laboratory Animal Monitoring System (CLAMS) by using Promethean (Sable Systems). Following 48 h of acclimation, data were collected for 48 h. The data were analyzed using Expedata software system (v1.9.27), Macro interpreter (v2.40), and then CalR software (v.1.2). Values are means ± SEM; $n = 4–8$ per group. *0.05, **0.01.

2.2. Sucrose consumption alters systemic zinc homeostasis

Disturbances in mineral homeostasis are associated with metabolic disorders. Having seen that sucrose treatment altered metabolism in mice, we measured the concentrations of Zn, Cu, and Fe in the plasma and liver. The plasma Zn concentrations were greater in the sucrose-treated mice compared to controls, while there was no change in plasma Cu and Fe levels (Figure 2A). Liver Zn and Cu concentrations were significantly lower in the liver of sucrose-treated mice, while there was no difference in the Fe levels (Figure 2B). Since the most consistent changes were observed in Zn, we concluded that sucrose treatment had the most significant impact on Zn metabolism during 8 weeks of sucrose treatment. Then we measured Zn levels in the peripheral tissues that are closely involved with both Zn and glucose metabolism. Zn concentrations were significantly lower in the pancreas and white adipose tissue of sucrose-treated mice, and there was no difference in the skeletal muscle (Figure 2C).

2.3. Sucrose consumption alters intestinal zinc transport and homeostasis

The changes in systemic Zn homeostasis led us to investigate further how subchronic sucrose consumption influences Zn metabolism in the intestine, the primary site for Zn regulation. We first measured Zn levels in isolated epithelial cells from the small intestine. We found significantly higher levels of Zn in the sucrose-treated mice (Figure 3A). To determine whether this accumulation of Zn in epithelial cells was due to increased dietary absorption or increased uptake of Zn from circulation, we administered ^{65}Zn either via subcutaneous injection or gavage. There was no difference in the level of ^{65}Zn in intestine tissue when administered via gavage (Figure 3C). However, ^{65}Zn levels were significantly higher in the small intestines of sucrose-treated mice when administered via subcutaneous injection (Figure 3B), suggesting that the increased Zn contents in small intestine epithelial cells are at least in part due to an increase in zinc transport in the serosal-to-mucosal direction. To identify Zn transport proteins at the basolateral localization that may be responsible for the changes in Zn transport, we measured the abundance of ZIP5 and ZIP14 in the proximal small intestine, where most Zn transport occurs. ZIP14 protein expression was upregulated, and ZIP5 was unchanged (Figure 3D). The increased abundance of ZIP14, along with the ^{65}Zn data, suggested that ZIP14 might facilitate the zinc transport to the intestinal epithelial cells (Figure 3E). Of note, there was no change in the Fe and Mn levels in the isolated enterocytes (Supplementary Figure S2).

2.4. Sucrose consumption alters intestinal homeostasis and increases intestinal permeability

Zn is essential for proper intestinal homeostasis. Therefore, having seen significant changes in intestinal Zn transport and homeostasis, we wanted to investigate how subchronic high sucrose consumption alters intestinal homeostasis further. Histological assessment revealed that sucrose treatment resulted in a significant increase in the length of the villi in both the proximal and distal small intestine (Figure 4A). The balance between the rates of proliferation and death of epithelial cells determines the intestinal villus length (31). Therefore, the increase in villi length led us to hypothesize that sucrose treatment either reduces cell death or increases cell proliferation. We found by TUNEL assay that sucrose treatment reduced apoptosis (Figure 4B). Furthermore, caspase-3 expression was lower in the intestine of sucrose-treated mice (Figure 4B). Epidermal growth factor receptor (EGFR) regulates cell proliferation and inhibition of apoptosis (32). Our results revealed an increased abundance of phosphorylated EGFR and downstream targets, AKT and STAT3 (Figure 4C), pathways associated with cell survival (33, 34).

High sugar consumption is associated with increased intestinal permeability (18, 19). Therefore, we tested whether 8 weeks of 30% sucrose treatment changes intestinal permeability. Mice were given FITC-Dextran following morning fasting, and the plasma concentration of FITC-Dextran was measured at 2 h post-FITC-Dextran gavage. We found a significantly higher amount of FITC-Dextran in the plasma of sucrose-fed mice (Figure 4D), indicating greater intestinal permeability. In the proximal intestine tissue, we found increased ZO-1 and decreased Claudin-1 (CLDN1) and Claudin-2 (CLDN2) protein expression (Figure 4E), suggesting dysregulation of tight junctions. Importantly, we did not find any significant changes in mRNA expression of *il-1β, il-6, Tnf-α, il-22, or il-23* in the intestine tissue (Figure 4F), consistent with that mice were fed a high-sugar diet (50% sucrose) (28) with no change in the cytokine expression and increased permeability. These data collectively suggested that subchronic sucrose treatment activated the EGFR-AKT-STAT3 pathway, and increased survival and permeability (Figure 4G).

2.5. Basolateral exposure to glucose drives dysregulation of the epithelial barrier in mouse intestine organoids

In order to more closely study the contributions of intestine epithelial cells to the alterations in permeability and zinc homeostasis following sucrose treatment, we treated organoids derived from mouse jejunum with both glucose and fructose for 24 h. Sucrose-treated mice were hyperglycemic (Figure 1D). Furthermore, it has been shown that hyperglycemia drives intestinal barrier dysfunction (17). Therefore, we administered treatment through the media to model the basolateral exposure to glucose experienced by intestinal epithelial cells (in the organoid/enteroid system) under hyperglycemic conditions. The composition of growth media for the enteroid culture contains the epidermal growth factor (EGF), therefore mimicking *in vivo* conditions where hyperglycemia and EGFR activation coexist. To assess the proliferation of the organoids following treatment, we measured the number of buds per organoid. Combined glucose and fructose treatment did not result in altered budding of organoids (Figures 5A,B). To determine whether glucose and fructose treatment resulted in increased permeability of the organoids, we administered FITC-Dextran following treatment with glucose and fructose (Figure 5C). In agreement with our *in vivo* data, treatment with glucose and fructose resulted in increased intensity of FITC fluorescence in the lumen of organoids, indicating greater permeability.

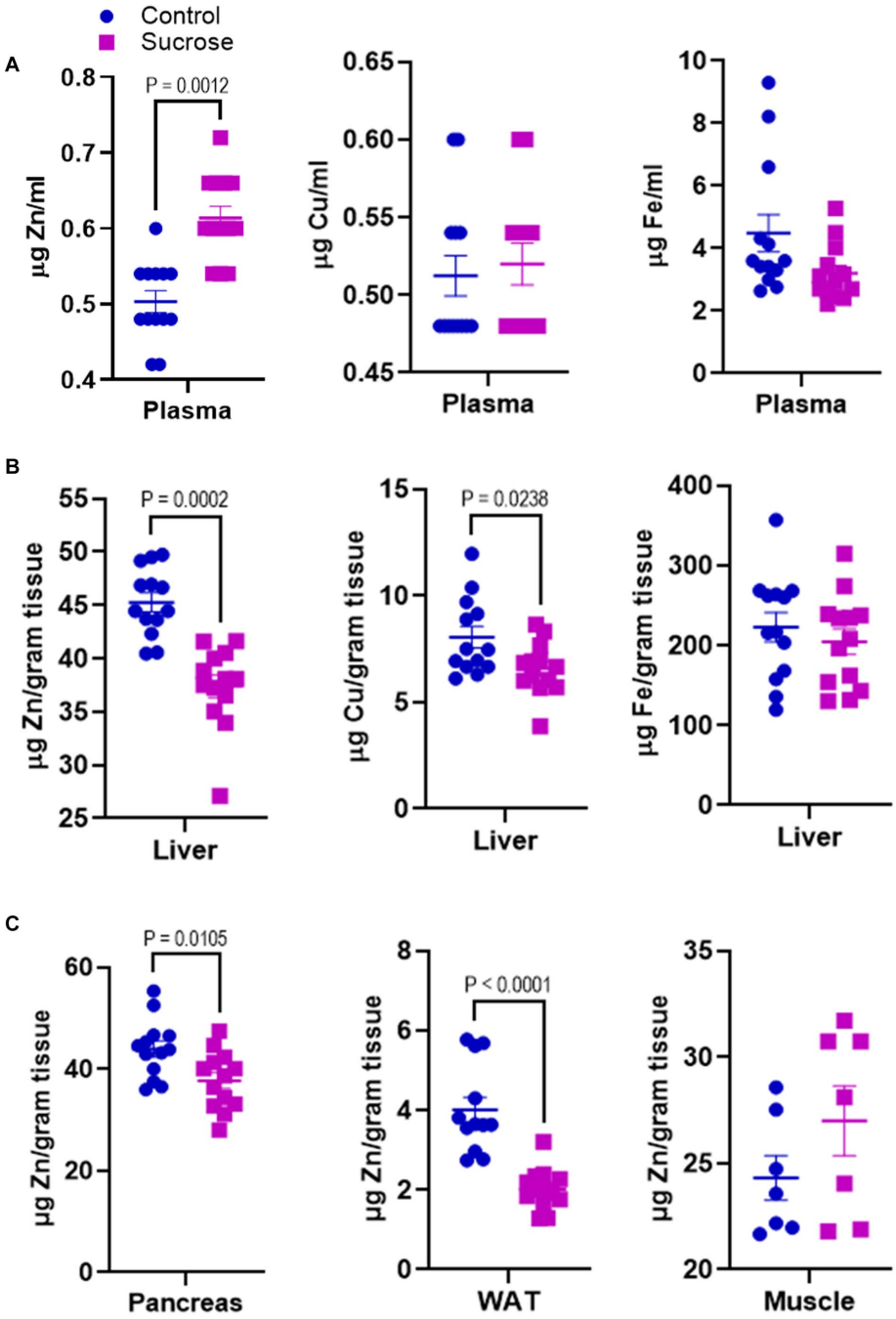


FIGURE 2
Subchronic liquid sucrose treatment alters systemic zinc homeostasis. Mice were given 30% sucrose in a drinking water for 8 weeks. **(A)** Plasma zinc, copper, and iron concentrations were measured using the microwave plasma-atomic emission spectrometer (MP-AES). Blood was collected in EDTA tubes and plasma was obtained by centrifugation. Plasma samples were diluted in deionized water (1/5) for MP-AES measurements. **(B)** Liver zinc, copper, and iron concentrations were measured using MP-AES. Liver tissues were digested in nitric acid and diluted in deionized water. Tissue weight was used as a normalizer. **(C)** Zinc concentrations in pancreas white adipose tissue (WAT) and muscle was measured using MP-AES. Tissues were digested in nitric acid and diluted in deionized water. Tissue weight was used as a normalizer. Values are means ± SEM; $n = 8–16$. Unpaired t-test between control and sucrose-treated groups.

To determine whether glucose or fructose alone is the driver of sucrose-induced alterations in epithelial barrier function, we treated organoids with either glucose, fructose, or both for 24, and 72 h. As an indicator of organoid growth and proliferation, we quantified the number of buds per organoid and found that different types of sugar treatment did not significantly alter the bud count (Supplementary Figure S3A). However, at both 24 and 72 h, glucose treatment resulted in significantly greater permeability than fructose treatment (Figure 6A; Supplementary Figure S3B), suggesting that basolateral exposure of small intestine epithelial cells may drive the increased intestinal permeability observed in mice following sucrose treatment. Glucose treatment resulted in increased ZIP14 as in *in vivo*

sucrose treatment (Figure 6B), while there was no change in the levels of ZIP4 and ZIP5. ZIP14 localization on the basolateral membrane was maintained in mouse organoids (Figure 6C). These data raised the question of whether glucose-induced increases in zinc concentrations were adaptive responses or dysregulation (Figure 6D). To test this, we treated organoids either with only glucose or glucose and supplemental zinc. Our result revealed reduced permeability with additional zinc when compared to glucose-only treated group, suggesting that the increased zinc concentration is an adaptive response (Figure 6E). In order to more closely study the impact of glucose exposure, Caco-2 cells were grown in a transwell system (35) until full confluency and were treated with high glucose (G) either basolaterally (G-BL), apically (G-Ap) or both (G-Both) for up to 48 h. Membrane resistance was measured, and changes in TEER values were plotted with time (Supplementary Figure S4A). We found the most significant reductions in TEER when high glucose was administered from the basolateral compartment. In agreement with our *in vivo* and *ex vivo* findings, *Zip14* was upregulated when glucose was administered from the basolateral side (Supplementary Figure S4B). Next, we treated Caco-2 cells with either high or low glucose media. Following treatment, high glucose-treated cells had greater total zinc content than cells grown in low glucose media (Supplementary Figure S4C). Notably, levels of Fe and Mn were unchanged (Supplementary Figure S4D).

2.6. Deletion of ZIP14 from intestinal epithelial cells results in greater intestinal permeability

Our *in vivo*, *ex vivo* and *in vitro* studies revealed sugar-induced upregulation of ZIP14. Furthermore, additional zinc to glucose treatment improved permeability in organoid culture. Therefore, we next tested intestinal permeability in the floxed control and intestine-specific *Zip14* KO (IKO) mice following 8 weeks of sucrose treatment. We found lower zinc concentrations in the IEC of IKO mice compared to floxed control (Figure 6F). Importantly, there was a significantly greater amount of FITC-Dextran in the plasma of sucrose-treated IKO mice (Figure 6G). We also found lower ZO-1 and Claudin1 protein abundance in the IEC from sucrose treated IKO compared to the sucrose-treated floxed mice, indicating greater permeability. We also tested metabolic parameters. There was no difference in body weight (Figure 6I) and blood glucose (in the fed state and in IPGTT) (Figure 6J), between sucrose-treated floxed and IKO mice. The only difference we observed was greater % body fat in sucrose-treated IKO mice when compared to sucrose-treated floxed mice (Figure 6I). Since there was no difference in blood glucose levels between sucrose-treated floxed and IKO mice, the result strongly suggests that the absence of ZIP14 from intestinal epithelial cells exacerbated intestinal permeability.

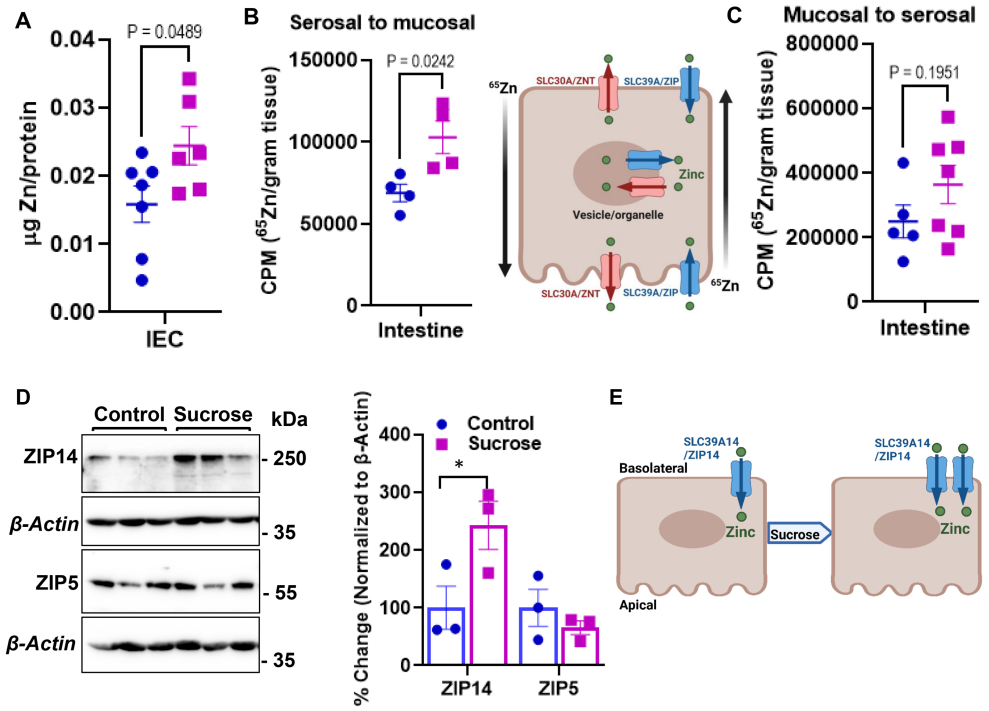


FIGURE 3
Subchronic liquid sucrose treatment alters intestinal zinc metabolism. **(A)** Zinc concentration in intestinal epithelial cells (IEC) using MP-AES. IECs were separated and digested in nitric acid. Total protein concentrations were used for normalization. **(B,C)** Following the morning fast, mice were administered ^{65}Zn via either gavage **(B)** or subcutaneous injection **(C)**. Three hours later, the amount of radioactivity in intestine tissue was measured. **(D)** Representative western analyses show intestinal ZIP5 and ZIP14 protein levels from control and sucrose-fed mice (n = 3 mice per group). **(E)** Depiction of proposed intestinal zinc transporter and zinc regulation based on the data in **A–D**. Values are means ± SEM; n = 4–7. Unpaired t-test between control and sucrose-treated groups.

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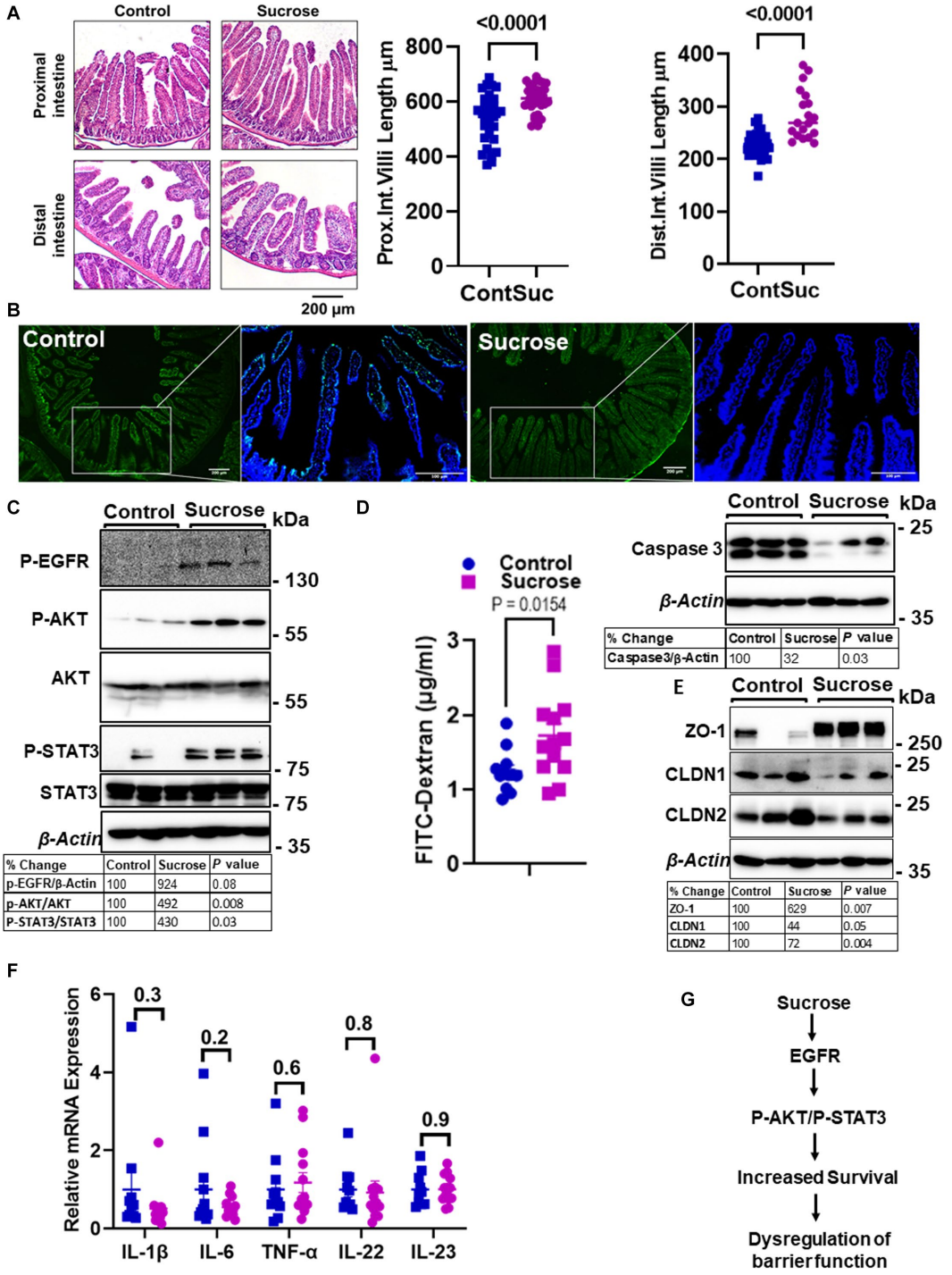

FIGURE 4
Subchronic liquid sucrose treatment dysregulates intestinal epithelial homeostasis and permeability. Mice were provided with either control or 30% sucrose-supplemented water for 8 weeks. **(A)** Representative H&E staining of the proximal and distal intestine from control and sucrose-fed mice. Villi length from both sections was quantified using ImageJ (further details are in the material methods section). **(B)** Apoptosis in the intestine tissues was assessed using the TUNEL assay (green). Representative western analyses showed intestinal caspase-3 protein levels (n = 3 mice per group). **(C)** P-EGFR, P-AKT/AKT, P-STAT3/STAT3, and β-actin protein levels from control and sucrose-fed mice (n = 3 mice per group). **(D)** Intestinal permeability was assessed by measuring plasma FITC-Dextran concentrations fluorometrically at 2 h post-gavage. **(E)** Representative western analyses showed intestinal ZO-1, CLDN1, CLDN 2, and β-Actin protein levels from control and sucrose-fed mice (n = 3 mice per group). **(F)** Intestine tissue cytokine expressions were measured using qPCR. **(G)** Depiction of the hypothesis. Values are means ± SEM; n = 3–12. The unpaired t-test between control and sucrose-treated groups.

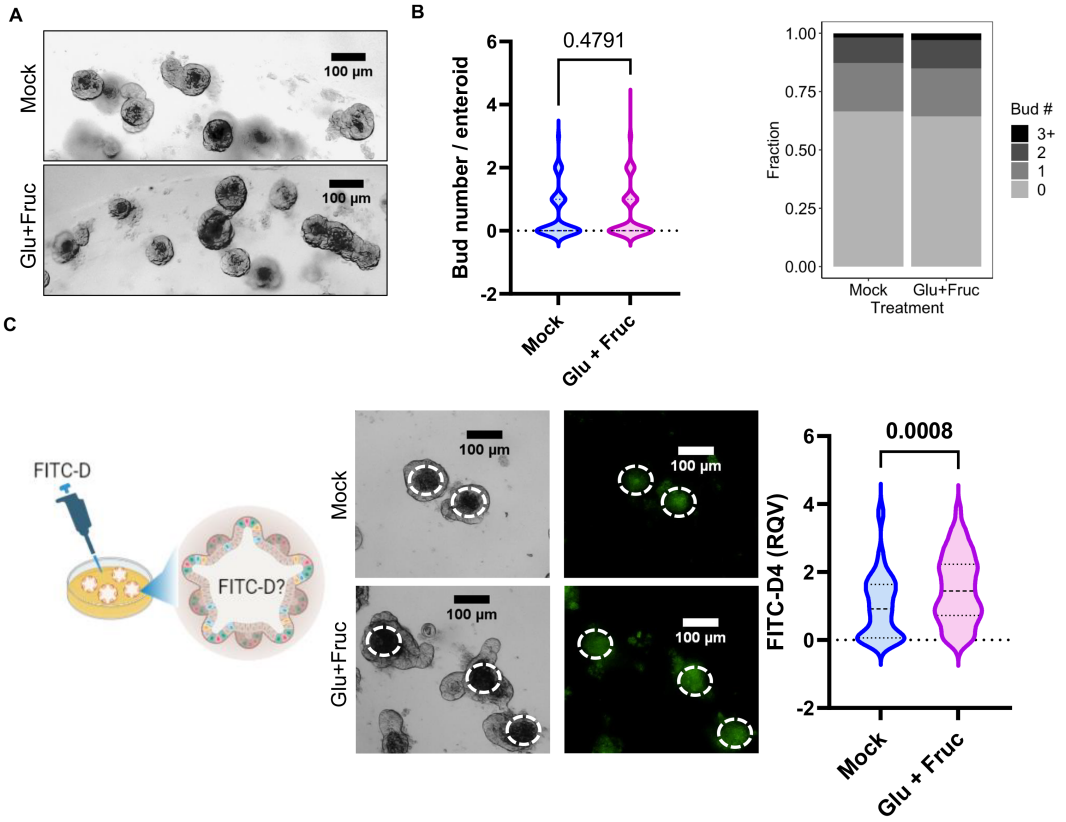

FIGURE 5
A combination of glucose and fructose treatment increases permeability in the *ex vivo* organoid system. **(A)** Representative bright field images of enteroid cultures at 24 h. post-glucose and fructose (GF) combination treatment. **(B)** Bud numbers of enteroids were quantified using the bright field images taken by OLYMPUS IX71 inverted microscope. **(C)** Representative FITC-Dextran (green fluorescence) images in enteroid cultures treated with either control or GF were acquired by OLYMPUS IX71 inverted microscope. Enteroid lumen was positioned by pairing bright field image and green fluorescence to quantify fluorescence intensity within an enteroid lumen by the software ImageJ. Values are means ± SEM; # of enteroid counted: mock, $n = 79$; GF, $n = 111$. Values are means ± SEM. The unpaired t-test between control and GF-treated groups.

2.7. Sucrose treatment preconditions mice for intestinal inflammation, which is partially rescued by zinc supplementation

The development of intestinal permeability and alterations in Zn homeostasis are both closely linked to inflammatory bowel disease. Having seen that sugar treatment increases intestinal permeability and alters Zn homeostasis in mice, mouse enteroids, and Caco-2 cells, we hypothesized that sucrose treatment in mice would precondition mice for the development of intestinal inflammation. To test this, we induced small intestinal inflammation using indomethacin. Following 8 weeks of sucrose only and sucrose combined with zinc supplementation in the last 4 weeks of sucrose treatment, we administered indomethacin (5 mg/kg/day) subcutaneously on two consecutive days (Figure 7A). Before indomethacin injection, metabolic assessments showed that mice receiving Zn supplementation following sucrose treatment had a significantly lower body fat % when compared mice without Zn supplementation (Figure 7B). No changes were found when comparing body weight, fed-blood glucose levels, and blood glucose levels following glucose injection (IPGTT) (Figures 7B,C). In control experiments, indomethacin resulted in the loss of BW, shortening of the small intestine, and increased permeability (Supplementary Figures S5A–C). Using the same conditions we administered indomethacin to both sucrose alone and sucrose and Zn-supplemented groups. As expected, Zn supplementation resulted in greater Zn accumulation in the small intestine (Figure 7D). Mice that received Zn supplementation along with sucrose treatment had greater small intestine lengths (Figure 7E) along with greater amount of phosphorylated EGFR, AKT and STAT3 in the sucrose and zinc combined group when compared to only sucrose group (Figure 7F). FITC-Dextran data revealed that Zn supplementation partially rescued sucrose-induced intestinal permeability but did not reach the statistical significance (Figure 7G). Importantly when we measured tight junction protein expression in intestinal epithelial cells, we found increased abundance of ZO-1 and CLDN1 and reduced abundance of CLDN2 in mice that received sucrose along with zinc (Figure 7F).

3. Discussion

In this study, we provide evidence linking sugar-induced hyperglycemia to intestinal permeability and zinc dyshomeostasis, which are risk factors for inflammatory chronic diseases.

Maintaining intestinal barrier function is essential for intestinal and systemic homeostasis and overall health. The impaired barrier

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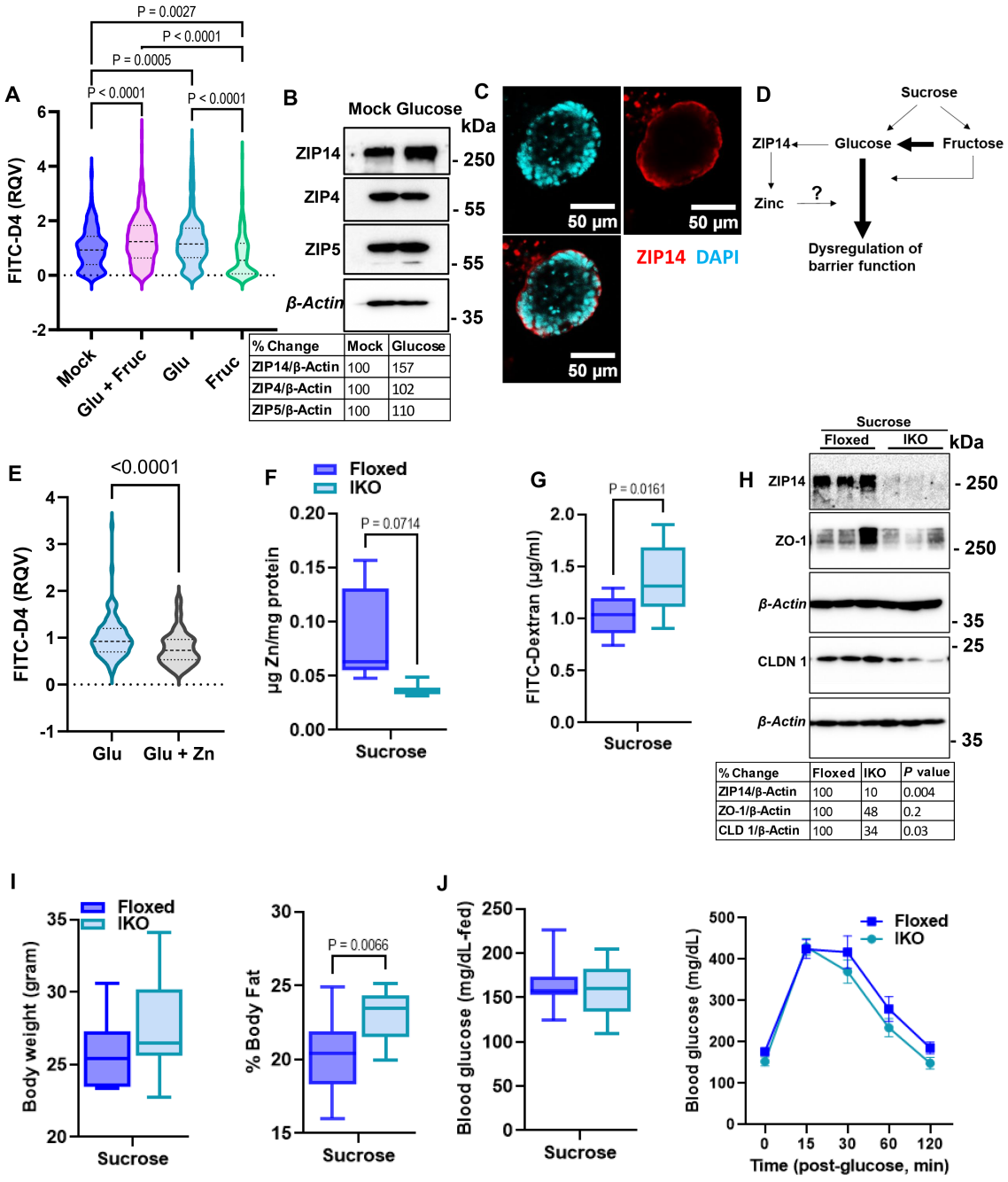

FIGURE 6
Glucose is the main driver of sucrose-induced dysregulation of intestinal epithelial barrier function. **(A)** Following 24 h of glucose (G), fructose (F), and GF treatments, FITC-Dextran (green) fluorescence intensity within the enteroid was quantified by software ImageJ. Values are means ± SEM; # of enteroid counted: mock, *n* = 322; GF, *n* = 293; G, *n* = 278; F, *n* = 253. One-way ANOVA. **(B)** Representative western analyses from two independent experiments showed ZIP14, ZIP4, and ZIP5 transporters. **(C)** Cellular location of ZIP14 at the basalateral side of the intestinal epithelium was shown in the mouse enteroid system. Images were obtained using Zeiss LSM880 confocal inverted microscope. Green: DAPI; red: ZIP14. **(D)** Depiction of the proposed mechanism of G or F-induced permeability. **(E)** Following 24 h of glucose (G) alone or combined with zinc treatments, FITC-Dextran (green) fluorescence intensity within the enteroid was quantified by software ImageJ. Values are means ± SEM; # of enteroid counted: (G), *n* = 102; G + zinc (Zn), *n* = 111. **(F)** Zinc levels in intestinal epithelial cells were measured by MP-AES. **(G,H)** Intestinal permeability was assessed by FITC-Dextran and western blot analysis for TJP. Following 8 weeks of sucrose treatment the body weight **(I)** % body fat **(J)** and blood glucose levels were measured from floxed control and intestine-specific *Zip14* KO (IKO-ZIP14 is deleted from villin-expressing cells) mice. Values are means ± SEM. *n* = 6–8 mice per group. The unpaired *t*-test between sucrose-treated floxed and IKO mice.

function has been linked to multiple gastrointestinal diseases, including inflammatory bowel disease. Similarly, zinc deficiency is reported in up to 50% of patients with inflammatory bowel diseases (26, 36, 37). However, it is unclear whether intestinal permeability or zinc deficiency are drivers or symptoms of the disease. The etiology of inflammatory bowel disease is not known, but diet is recognized as an

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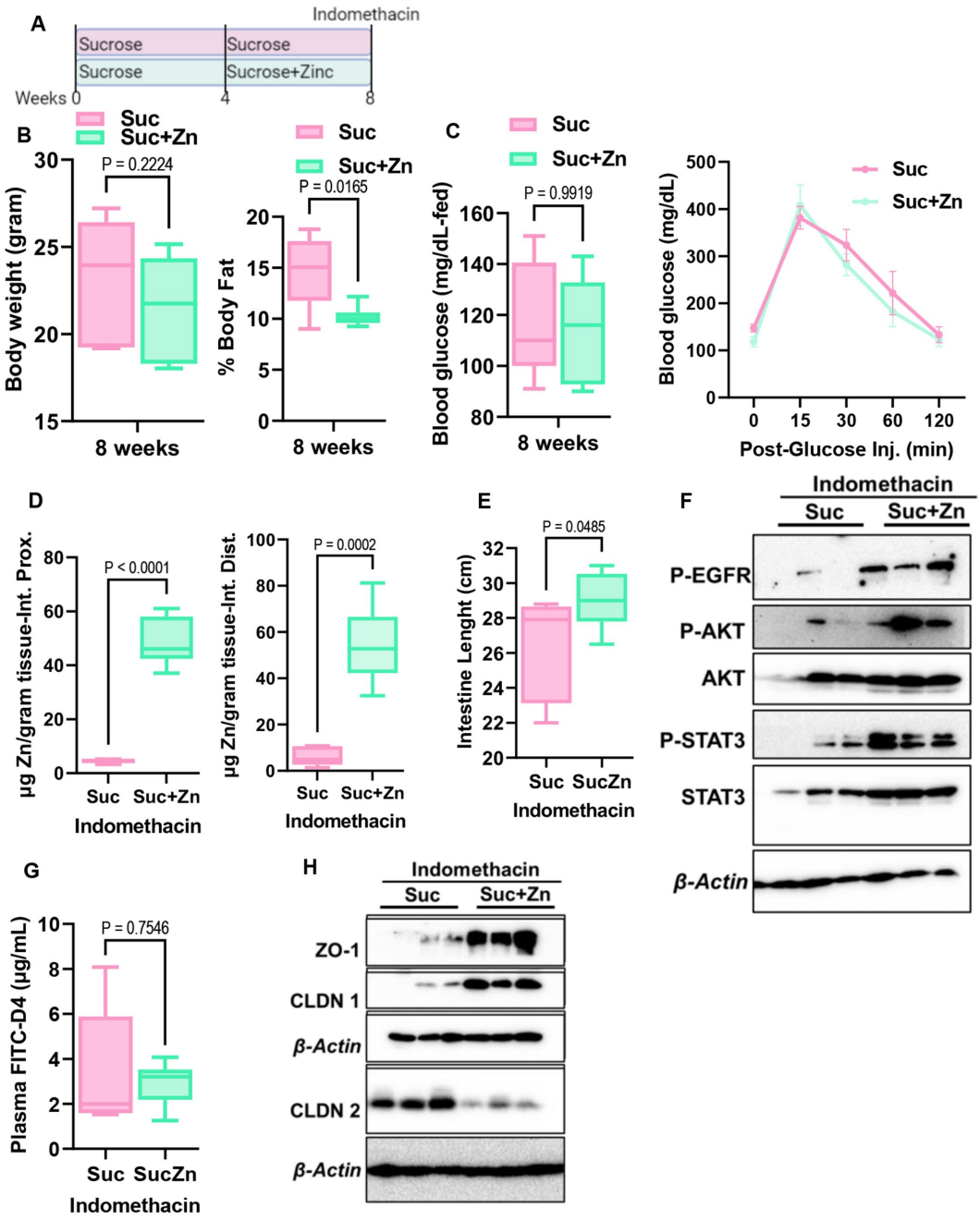

FIGURE 7
Effect of additional zinc in sucrose-fed and indomethacin-injected mice. **(A)** Mice were provided with sucrose only for 4 weeks and additional 4 weeks with both sucrose and zinc (Zn). At the end of 8 weeks, mice were injected with indomethacin for two consecutive days. **(B)** At the end of 8 weeks before indomethacin injection, body weight and percent body fat by NMR **(B)**, blood glucose levels at the fed state and following glucose injection (IPGTT) **(C)** were measured. **(D)** Following indomethacin injection, zinc concentrations in the proximal and distal intestines were measured by MP-AES. **(E)** Intestine lengths were measured at the time of tissue collection. **(F)** Western blot analysis was used to determine the protein abundance of indicated protein targets (n = 3 mice per group). **(G)** Intestinal permeability was measured by measuring plasma FITC-Dextran concentrations fluorometrically. **(H)** Western blot analysis was used to determine the protein abundance of indicated protein targets (n = 3 mice per group). Values are means ± SEM; n = 6–8 mice per group. The unpaired t-test between groups.

environmental risk factor. The association between IBD development and high sugar consumption has been shown in humans (29)—furthermore, high sugar consumption preconditions mice for colitis (28, 38). Dietary sugars alter zinc homeostasis, which is essential for intestinal health. However, how high sugar consumption influences intestinal zinc metabolism and how altered zinc metabolism, in turn, affects intestinal physiology to increase the risk of developing IBD has not been studied. Our *in vivo* studies revealed that subchronic liquid sucrose consumption caused hyperglycemia, intestinal permeability, altered intestinal zinc transport, and systemic zinc homeostasis. Using *ex vivo* enteroid culture and *in vitro* Caco-2 cells, we further showed intestinal permeability and altered zinc metabolism were in part

driven by basolateral glucose exposure. Mechanistically, high sugar consumption activated the EGFR-AKT-STAT3 pathway in the intestine, preventing cell death and consequent dysregulation of tight junction proteins. The sugar-induced increase in zinc transport to the intestine enhances survival.

A western diet rich in fat and sugar is an environmental risk factor for IBD (22) and metabolic disorders such as diabetes and obesity (25). It has been shown in rodent models that the mode of delivery of the sugar, rather than the amount, is the main factor in metabolic dyshomeostasis, suggesting that sugar-sweetened beverages are a potential driver of metabolic disorders. It has been shown that liquid sucrose consumption negatively affects metabolic health (7, 27). In agreement with these findings, our data revealed that consumption of liquid sucrose resulted in greater weight gain and fat/body ratio (Figures 1A,B). Furthermore, the liquid sucrose-treated group developed hyperglycemia and intestinal permeability (Figures 1C,D, 4D). Intestinal permeability shown to be driven by hyperglycemia (24), suggesting that in response to the sucrose, there were sequences of events starting with hyperglycemia leading to the intestinal permeability. Our data where enteroid cultures were treated with sugar basolaterally revealed increased permeability (Figures 5, 6), supporting that hyperglycemia, in part, could be driving impaired barrier function.

Altered systemic zinc homeostasis in response to sugar consumption was shown previously (20, 21). Here, we assessed systemic and intestinal zinc metabolism alterations related to metabolic dysregulation and intestinal permeability in response to sugar treatment. At the end of the 8 weeks of liquid sucrose treatment, we found increased ^{65}Zn transport in the serosal-to-mucosal direction resulting in higher zinc levels in the intestinal epithelial cells (Figure 3). The increase in the protein abundance of basolaterally localized metal transporter ZIP14 further supported the zinc transport in serosal-to-mucosal direction. We have previously shown glucose-induced upregulation of hepatic ZIP14 (39), suggesting zinc transport in intestinal epithelial cells could be mediated by ZIP14. These *in vivo* findings were confirmed in the Caco-2 cells, where high glucose treatment increased the expression of ZIP14 and intracellular zinc concentrations (Supplementary Figure S4). These endpoint measurements raised the important question of whether alterations in intestinal zinc metabolism were a dysregulation or adaptive tissue response to high sugar consumption. Our zinc supplementation studies both *in vivo* and *ex vivo*, supported the hypothesis that zinc was needed for normal physiology.

Gut permeability has been shown to be a preclinical biomarker of Crohn's disease onset (40). Our studies revealed that subchronic sucrose consumption caused intestinal permeability and altered intestinal zinc metabolism (Figures 3, 4). We have not found any changes in the intestinal cytokine expressions in response to sucrose consumption (Figure 4). These results are consistent with that mice were fed a high sugar diet (50% sucrose) (28) with no change in the cytokine expression and increased permeability. Similarly, mice that received 10% glucose or fructose in drinking water did not change their colon cytokine levels (38). Four months of 30% sucrose consumption in drinking water, however, increased inflammation, suggesting intestinal permeability and alterations in zinc metabolism occur before intestinal inflammation. These agree with the recent finding that intestinal permeability is associated with the later development of CD (40). Collectively, our interpretation is that high sugar consumption increases the risk of developing IBD due to increased intestinal permeability and subsequent systemic and intestinal chronic inflammation. First, high sugar consumption increases intestinal permeability due to disbalance between survival and cell death. Then, systemic and intestinal chronic inflammation is induced by the increased blood endotoxin levels resulting from intestinal leakage activating the immune system. Zinc is needed to maintain intestinal integrity and the proper function of immune cells; however, circulating zinc is depleted during inflammation. Our data with zinc supplementation suggest pretreatment may partially rescue the sugar-induced intestinal permeability. Further studies are warranted investigating the effect of zinc supplementation as a pretreatment or after disease development to test its therapeutic potential.

Chronic sucrose consumption for 16 weeks reduced serum zinc levels and increased inflammation in rats (21). We found greater serum zinc levels in the mice that received %30 sucrose for 8 weeks (Figure 2). One possible reason for the discrepancy between these studies and ours is the length of the sucrose treatment. In parallel to hyperglycemia and intestinal permeability, there could be sequential changes in zinc metabolism during subchronic sugar consumption. This possibility fits in with the fact that serum zinc levels were high along with hyperglycemia and intestinal permeability, but there was no sign of inflammation in our 8 weeks regimen. When the treatment was extended to 16 weeks, inflammation was present with lower serum concentrations (21). Similarly, low serum zinc levels and inflammation were found in IBD onset. These collectively support time-dependent alteration in zinc metabolism. This may provide important implications for the stage-dependent assessment of zinc status along with other indicators (e.g., hyperglycemia, permeability) in the development of chronic inflammatory diseases to establish better use of zinc in prevention/treatment strategies.

Zinc dyshomeostasis is closely associated with metabolic disorders such as type-2 diabetes and obesity. Hyperglycemia, inflammation, and zinc deficiency usually coexist in these chronic disorders. We previously showed inflammation-induced tissue redistribution of zinc (3, 41, 42); however, hyperglycemia-induced zinc redistribution has not been studied. The 8 weeks subchronic sucrose treatment allowed us to determine hyperglycemia-induced redistribution of zinc in the absence of inflammation. Our results revealed increased serum zinc levels and reduced liver and adipose tissue zinc concentrations. The liver and adipose tissue are metabolic organs that regulate glucose metabolism. Zinc is shown to be involved in the regulation of glucose uptake, gluconeogenesis, glycolysis, and glycogen synthesis and breakdown in the liver (7, 43). We have previously demonstrated that zinc inhibits glycogen synthesis and enhances gluconeogenesis (7). Reducing hepatic zinc might be an adaptive adjustment to limit the new glucose production and improve glycogen synthesis in subchronic high sucrose feeding without inflammation. In contrast, in high-fat diet feeding where hyperglycemia and inflammation were present hepatic zinc was increased (7). In adipose tissue, zinc is shown to be needed for the insulin response and management of the fat stores in both high-fat diet- and LPS-induced inflammation in mice (44–46). Our earlier studies found increased zinc levels in the adipose tissue in the inflammatory state. That increased zinc was needed for controlling the cytokine production in adipose tissue. Similar to the liver tissue, we found a reduction in adipose zinc levels in sucrose-treated mice without inflammation. These liver and adipose tissue findings

indicated that zinc regulation differs for hyperglycemic and inflammatory states. Further studies are needed to investigate the mechanistic link between sugar consumption, zinc, and metabolic alterations in liver and adipose tissues.

Sucrose is a disaccharide composed of monosaccharides, glucose and fructose. Emerging findings by isotope tracers and metabolomics studies have shown that the small intestine metabolizes most of the dietary fructose (47, 48). Furthermore, the major portion of the tracer-labeled fructose carbons was detected as glucose in circulation, indicating that dietary fructose was converted into glucose. The spill of fructose is found in circulation when high doses overwhelm the intestinal capacity. While our *in vivo* studies used liquid sucrose treatment to model sugar-sweetened drinks, we also investigated the individual effect of glucose and fructose on permeability using the intestinal organoid system. Treatment with glucose or fructose alone induced permeability at a greater extend with the former (Figure 6; Supplementary Figure S3). Above findings on fructose metabolism may explain the difference between glucose versus fructose-induced permeability and supports the attribution that the main driver of the permeability is high glucose.

Our results revealed elongated villus length both in the proximal and distal intestine in sucrose-fed mice (Figure 4). The balance between the rates of proliferation and death of epithelial cells determines the intestinal villus length (31). Our studies revealed that sucrose treatment prevented cell death. These findings agree with the elongated villi length due to reduced cell death in the fructose-treated mice (49). Epidermal growth factor receptor regulates cell proliferation and inhibition of apoptosis (32). In the intestine, in the sucrose-fed group, we found greater activation of EGFR. Furthermore, we found higher levels of STAT3 and AKT phosphorylation in the intestine of the sucrose-treated mice. Activation of the AKT-STAT3 pathway as the downstream target of EGFR was shown (50). The intestine epithelial barrier functions as a gatekeeper at the interface between what is outside the human body and the inside. Tight junction proteins (TJPs) were dysregulated in response to sucrose treatment. Downregulation of TJPs, Claudin1, and ZO-1, was shown in multiple disease models with the signature of intestinal permeability. Consistent with these studies, we found significant downregulation of intestinal Claudin1 in response to sucrose treatment. In contrast to these studies, we found upregulation of ZO-1 despite increased permeability. To our knowledge, there is no earlier report that ZO-1 protein expression was tested in sucrose treatment. However, it was shown in Caco-2 cells that zinc treatment alone upregulated ZO-1 protein and enhanced barrier function via an AKT-dependent pathway (51). Our findings, greater zinc levels in intestinal epithelial cells, activation of intestinal AKT, and upregulation of ZO-1 from sucrose-treated mice are consistent with these earlier studies except for intestinal permeability.

The one limitation of our studies is that our data from *in vivo* studies are endpoint measurements of 8 weeks of dietary intervention. Given that 16 weeks of interventions were shown to amplify the phenotype (21), conducting 8 and 16 weeks of sugar consumption in the same study would provide a more precise comparison. Further studies are warranted to expand the time frame between sugar-induced pre-symptoms and disease onset. The second limitation of our studies is related to gut microbial changes in response to sucrose treatment. Sucrose treatments have been shown to alter the gut microbial composition and metabolome, contributing to intestinal permeability phenotype (28, 38). Our studies focused on sucrose-induced hyperglycemia altering intestinal integrity and zinc metabolism. Following *in vivo* dietary interventions, we investigated microbiome-independent glucose-regulated paths using organoid culture and *in vitro* cell lines. Further studies are needed to explore the potential contribution of the gut microbiome to hyperglycemia, intestinal permeability, and zinc dyshomeostasis in subchronic and chronic sucrose consumption.

In conclusion, our data revealed that sucrose-induced high blood glucose impairs intestinal barrier function by activating the EGFR-AKT-STAT3 pathway preventing cell death and consequent dysregulation of tight junction proteins. A sugar-induced increase in intestinal zinc functions to enhance EGFR-AKT-STAT3 signaling.

4. Materials and methods

4.1. Animals and treatments

Floxed *Zip14* (*Zip14*$^{fl/fl}$) mice on the C57BL/6 background were generated via the targeting of introns 4 and 8 by Transposagen. The resulting *Zip14*$^{fl/fl}$ animals were bred with B6.Cg-Tg(Vil1-cre)997Gum/J (004586, Jackson Laboratory) to create villin-expressing cell-specific KO [intestine-specific KO (IKO)] animals (47). Animal colonies were maintained using standard rodent husbandry and received a commercial, irradiated chow diet (Harlan Teklad 7912, ENVIGO, Indianapolis, IN, with 60 mg Zn/kg as ZnO) and autoclaved tap water. Age-matched mice from both sexes were used as young adults (8–16 weeks of age) for all dietary interventions. Euthanasia was through exsanguination by cardiac puncture under isoflurane anesthesia. Injections and gavage were conducted on anesthetized mice. Protocols were approved by the Cornell University Institutional Animal Care and Use Committees.

Mice were provided 30% (weight/volume) sucrose in drinking water for 8 weeks. Sucrose water was changed twice a week. In some experiments, mice received either 30% (weight/volume) sucrose alone for 8 weeks or combined with zinc treatment (48) for the last 4 weeks of the 8 weeks intervention. To induce intestinal inflammation, we injected mice with indomethacin (5 mg/kg body weight, Sigma, 17378-10G).

4.1.1. Body composition

Measurements were conducted in awake animals by time-domain nuclear magnetic resonance using the Minispec LF65 Body Composition Mice Analyzer (Bruker, Germany).

4.1.2. Metabolic phenotyping

Metabolic parameters of the mice were measured by the Comprehensive Laboratory Animal Monitoring System (CLAMS) by using Promethion (Sable Systems, United States). Mice were transferred to the CLAMS metabolic cages and allowed to acclimate for 2–3 days with free access to food and water. Following the acclimation, measurements of VO_2 and VCO_2 gas exchanges and food and water consumption were collected for 2 days.

4.1.3. Fasting blood glucose

Following overnight fasting, blood glucose levels were measured by tail bleeding using a glucometer (OneTouch® Ultra, LifeScan Inc.).

4.1.4. Intraperitoneal glucose tolerance test (IPGTT)

Following morning fasting (4 h), blood glucose levels were measured by tail bleeding using a glucometer (OneTouch® Ultra, LifeScan Inc.) immediately prior and after administration of 2 mg/kg glucose by intraperitoneal injection at 0, 15, 30, 60, and 120 min post-treatment.

4.2. Isolation of intestinal epithelial cells

Lumenal contents of the proximal intestine were flushed by PBS prior to everting them using bamboo sticks with pointed ends (49). Everted intestines were incubated in PBS with 1.5 mM EDTA solution for 20 min and released epithelial cells were collected by centrifugation at 4°C at 500 × g for 5 min. Following two washes, the pellets were resuspended in lysis buffer (Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA) with protease and phosphatase inhibitors (Thermo Scientific, 1860932 and 78428, respectively) added along with the PMSF (Sigma-Aldrich, BP-481).

4.3. Metal assays

Following intestinal epithelial cell isolations, cell pellets were digested at 80°C overnight in HNO_3 to measure metal concentrations using microwave plasma-atomic emission spectrometry (MP-AES) (Agilent, Santa Clara, CA). Normalization was to total protein concentrations. Tissue metal concentrations were measured by MP-AES following overnight digestion at 80°C and appropriate dilutions for each tissue with deionized water. Normalization was to wet tissue weight. Blood was collected by cardiac puncture into EDTA tubes, and plasma was obtained by centrifugation at 3000 × g for 15 min. Following dilutions (1/5) in deionized water, plasma metal concentrations were measured by MP-AES.

4.4. ^{65}Zn transport

Following overnight fasting, mice were administered ^{65}Zn (Eckert & Ziegler, Valencia, CA) either by oral gavage (5 μCi/mouse/100 μL) or subcutaneous injection (2 μCi/mouse/100 μL). The tissue collection was at 3 h post-^{65}Zn administration. Intestine tissue was perfused with a metal chelating buffer (10 mM EDTA, 10 mM HEPES, and 0.9% $NaCl_2$). The amount of radioactivity in whole tissues was measured by the gamma counter and normalized by the wet tissue weight.

4.5. RNA isolation and qPCR

The total RNA of intestine tissues was isolated using TRI Reagent (Molecular Research Center Inc. # TR118). cDNA synthesis was performed using M-MLV reverse transcriptase (Invitrogen, #28025-013). Gene expression was measured using PowerTrack SYBR Green Master Mix (Invitrogen, #A46012). The quantitative PCR (qPCR) reactions were run in the Roche Lightcycler 480-II RT-PCR system. Primers used in this study were purchased from Integrated DNA Technologies, Coralville, IA. Primer sequences used in this study include: *il-1β* forward 5′-CAACCAACAAGTGATATTCTCCATG-3′, reverse 5′-GATCCACACTCTCCAGCTGCA-3′, *il-6* forward 5′-TAGTCCTTCCTACCCCAATTTCC-3′, reverse 5′-TTGGTCCT TAGCCACTCCTTC-3′, *Tnf-α* forward 5′-CAAAATTCGAGT GACAAGCCTG-3′, reverse 5′-GAGATCCATGCCGTTGGC-3′, *Il-22* forward 5′-ATGAGTTTTTCCCTTATGGGGAC-3′ reverse 5′-GCTGGAAGTTGGACACCTCAA-3′, *il-23* forward 5′-ATG CTGGATTGCAGAGCAGTA-3′ reverse 5′-ACGGGGCACATTAT TTTTAGTCT-3′. Data were normalized with Rplp0 measurements for relative quantitation. Rplp0 forward 5′-AGATTCGGGATA TGCTGTTGG-3′ reverse 5′-TCGGGTCCTAGACCACTGTTC-3′.

4.6. Histology

Animals were transcardially perfused with ice-cold 1x PBS. The intestines and colons were removed and fixed in formalin overnight at 4°C. Intestines were cut in half, proximal and distal. Both intestines and colon were cut into 1 cm pieces and collected into gut bundles (50). They were then placed in sucrose gradients (10, 20, 30%) until tissues sunk. Gut bundles were frozen in OCT (4583, Sakura) and cryosectioned at 10 μm thickness. The sections were stained with H&E (Abcam). All sections were longitudinal.

4.7. Histological analysis

Images of tissue sections were taken using the OLYMPUS IX71 microscope. Approximately 10 villi and 10 crypts from each animal were measured using ImageJ to determine villi length and crypt depth (44). Villi length was measured from base of villi to villi tip. Crypt depth was measured from base of crypt to mouth of crypt. Complete villi and crypts were chosen for this purpose. The measurements were pooled for each group ($n = 3$) and t-tests were performed (51). Crypt density was determined by calculating the number of crypts per mm-1 along submucosal circumference (51).

To conduct TUNEL staining, sections were stained with TACS 2 TdT-Flour *In Situ* Apoptosis Detection Kit (R&D Systems, 4812-30-K) according to manufacture's protocol. To visualize TUNEL-positive cells, 4× and 10× images of tissue sections were taken using the OLYMPUS IX71 microscope (Melvile, NY).

4.8. Western blotting

All the buffers were supplemented with protease inhibitors (AGScientific) during enterocyte separation. Solubilized proteins were separated by 10% SDS-PAGE. Visualization was by chemiluminescence (SuperSignal, Thermo Fisher, 34580) and digital imaging (Protein Simple, San Jose,). Rabbit anti-mouse ZIP14 antibody was custom-made by Genscript (Piscataway, NJ) (47). B-Actin (Cell Signaling, 8457), Claudin 1 (ThermoFisher, 71-7800), Claudin 2 (Cell Signaling, 48120), ZO-1 (ThermoFisher, 61-7300), P-EGFR (Cell Signalling, 3777), P-AKT (Cell Signalling, 4060), AKT (Cell Signalling, 4691), P-STAT3 (Cell Signalling, 9145), STAT3 (Cell Signalling, 30835), Cleaved Caspase 3 (Cell Signalling, 9661), SLC39A4/ZIP4 (Proteintech, 20625-1), SLC39A5/ZIP5 (Novusbio, NBP3-04949). Band intensities were measured by the software ImageJ. Values for

protein of interests were normalized to their control (pan-proteins or β-actin). The percentage of changes over control groups was presented.

4.9. Isolation of mouse jejunal crypts

Jejunal crypts were isolated from female floxed mice (8–12 weeks old) as previously described (51). Small intestine was excised from mice and divided into three equal segments. The jejunal segment (the middle segment) was processed for crypt isolation. Subsequent to luminal flushing with ice-cold PBS, the tissue was longitudinally cut and subjected to incubation in 3 mM EDTA in ice-cold PBS with 1% (v/v) Primocin™ (InvivoGen, ant-pm-1) for 15 min at 4°C. The mucosa of the intestinal pieces was gently scrapped of mucus, shaken in ice-cold PBS with 1% (v/v) primocin for 1 min, and incubated in fresh 3 mM EDTA in ice-cold PBS with 1% (v/v) primocin for 35 min at 4°C. The intestinal pieces were then shaken in ice-cold PBS with 1% (v/v) primocin for 2 min and filtered with a 70 μm cell strainer. The resulting purified crypts were pelleted with centrifugation at 110 × g for 10 min at 4°C. The isolated crypts were used for enteroid culture.

4.10. Mouse enteroid culture

Mouse enteroid culture was performed as previously described (50). The isolated jejunal crypts (day 0) were placed in Matrigel (density 500–700 crypts per 5 μL Matrigel dome) and grown in reduced growth factor Matrigel (Corning, 356231) and advanced DMEM/F12 (Gibco, 12634-028) containing GlutaMAX (Gibco, 35050-061), Penicillin-Streptomycin (15140122, Gibco), HEPES (Gibco, 15630-080), N2 supplement (Gibco, 17502-048), 50 ng/μL EGF (R&D Systems, 2028-EG), 100 ng/μL Noggin (PeproTech, 250-38), 250 ng/μL murine R-spondin (R&D Systems, 3474-RS-050), and 10 μM Y27632 (Enzo Life Sciences, ALX270-333). Cell culture media was changed every 2 days. To assess the impact of dietary sugar on enteroid culture, dietary sugar supplementation was treated daily (since day 1) for the following experimental groups: (1) glucose (G-10 mM), (2) fructose (F-10 mM), and (3) glucose and fructose (GF-5 mM each). To examine the effects of zinc supplementation on enteroid culture, enteroids were treated daily (since day 1) with $ZnCl_2$ at a final concentration of 10 μM. The enteroids from 24 h, 48 h or 72 h post-treatment were fixed in 2% paraformaldehyde for imaging analyses or collected for RNA/protein analyses. To assess enteroid growth, bud numbers of enteroids were quantified using the bright field images taken by OLYMPUS IX71 inverted microscope (Melvile, NY).

4.11. Enteroid permeability assay

The permeability of enteroids was examined using FITC-Dextran (4 kDa). Briefly, enteroids were washed with PBS and treated with FITC-4 kDa (Sigma, FDH1G) at a final concentration of 1 mg/mL for 30 min at 37°C. The enteroids were then washed by PBS and fixed in 2% PFA. The fixed FITC-4 kDa treated enteroids were processed through immunostaining procedures (described in the below "whole-mount enteroids immunostaining and imaging") to remove extra FITC-4 kDa that is not present in the enteroid lumen. Paired bright field (for positioning enteroid lumen) and green fluorescence images (for visualizing FITC-4 kDa signal) were acquired by OLYMPUS IX71 inverted microscope (Melvile, NY). Fluorescence intensity within an enteroid lumen was measured by the software ImageJ. Only enteroids of which the luminal areas were well-focused in an image were used to measure FITC-4 kDa intensity.

4.12. Whole-mount enteroids immunostaining and imaging

The immunostaining of enteroids was performed as previously described (50). Briefly, the fixed enteroids were permeabilized with 0.5% (v/v) Triton X-100/PBS, washed by PBS containing 0.1% (w/v) BSA/0.02% (v/v) Triton-X/0.05% (v/v) Tween 20, and blocked with 10% (v/v) normal goat serum. Primary antibodies were used to stain ZIP14 (at a final concentration 8 μg/mL; GenScript; Piscataway, NJ). The staining was visualized by fluorescent-conjugated secondary antibody (Alexa Fluor 594, A11012; 1:1000; Thermo Fisher Scientific). Nuclei were stained with DAPI (1:10000 dilution from 5 mg/mL stock). The immunofluorescent images were acquired by OLYMPUS IX71 (Melvile, NY) inverted microscope or Zeiss LSM880 confocal inverted microscope (White Plains, NY). Images were analyzed by the software ImageJ.

4.13. Statistical analyses

Data are presented as means ± SEM. Significance was assessed by student's *t*-test and ANOVA for single and multiple comparisons, respectively. Statistical significance was set at $p < 0.05$. Analyses were performed using GraphPad Prism (version 9.4.1).

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 animal study was approved by Cornell University Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TA conceived and designed research, drafted the manuscript, and edited and revised manuscript. TA, SM, Y-HH, TT, JZ, and FB performed experiments. TA, Y-HH, SM, TT, and SG analyzed data. TA, Y-HH, SM, and SG interpreted results of experiments. TA, SM, Y-HH, TT, JZ, and SG prepared figures. TA, Y-HH, SM, TT, JZ, FB, and SG approved the final version 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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Supplementary material

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Elevated first-trimester hepcidin level is associated with reduced risk of iron deficiency anemia in late pregnancy: a prospective cohort study

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Background: Iron deficiency (ID) and iron deficiency anemia (IDA) during pregnancy are highly prevalent worldwide. Hepcidin is considered an important biomarker of iron status. Currently, few longitudinal cohort studies have assessed the potential causal relationship between hepcidin and ID/IDA. Therefore, we aimed to investigate the association of first-trimester maternal serum hepcidin with third-trimester ID/IDA risk in a prospective cohort.

Methods: Total of 353 non-ID/IDA pregnant women at 11–13 weeks' gestation were enrolled in Southern China and followed up to 38 weeks of gestation. Data on demography and anthropometry were obtained from a structured questionnaire at enrollment. Iron biomarkers including hepcidin were measured at enrollment and follow-up. Regression models were used to evaluate the association of first-trimester hepcidin with third-trimester ID/IDA risk.

Results: Serum hepcidin levels substantially decreased from 19.39 ng/mL in the first trimester to 1.32 ng/mL in the third trimester. Incidences of third-trimester ID and IDA were 46.2 and 11.4%, respectively. Moreover, moderate and high levels of first-trimester hepcidin were positively related to third-trimester hepcidin (log-transformed $\beta = 0.51$; 95% CI = 0.01, 1.00 and log-transformed $\beta = 0.66$; 95% CI = 0.15, 1.17). Importantly, elevated first-trimester hepcidin was significantly associated with reduced risk of third-trimester IDA (OR = 0.38; 95% CI = 0.15, 0.99), but not with ID after adjustment with potential confounders.

Conclusion: First-trimester hepcidin was negatively associated with IDA risk in late pregnancy, indicating higher first-trimester hepcidin level may predict reduced risk for developing IDA. Nonetheless, given the limited sample size, larger studies are still needed.

KEYWORDS

hepcidin, iron deficiency, anemia, pregnancy, hemoglobin

1. Introduction

Iron deficiency (ID), and specifically iron deficiency anemia (IDA), has been well-recognized as one of the most common nutritional deficiencies during pregnancy globally (1). The prevalence of gestational anemia was reported to be 26% in developed regions such as the United States and Europe and reached 46%–48% in Southeast Asia and Africa (2). According to the WHO, 50% of anemia is attributed to ID (3). In China, the prevalence of maternal ID and IDA was reported to be 42.6% and 19.1%, respectively (4). Numerous studies have shown that antenatal ID and IDA are associated with increased risk of maternal mortality, preterm birth, small for gestational age, low birth weight, and long-term influence on cognitive function in the offspring (3, 5–7). Thus, it is essential to identify the risks of ID and IDA early.

Although there are some well-established methods to detect iron status, accurate identification of maternal ID and IDA with conventional markers still remains a challenge (8). Serum ferritin (SF) and hemoglobin (Hb) serve as common markers of ID and IDA. Hb is a sensitive indicator of ID, because IDA, as a type of Hb disorder, directly results from ID. However, Hb in the diagnosis of ID/IDA may be interfered with other Hb disorders such as thalassemia. Moreover, SF can be confounded by inflammation and infection (9). Therefore, a biomarker that enables accurate identification of the risk of iron status is needed.

Hepcidin, a cysteine-rich antimicrobial peptide hormone secreted by the liver, has been shown in previous studies that it exerts a crucial role in iron metabolism (10). Although both hepcidin and SF are correlated with inflammation, such as C-reactive protein (CRP), prior studies have shown that hepcidin is closely related to iron status during pregnancy (11–13). In detail, evidence from the studies have shown that the level of hepcidin in pregnant women with IDA was lower than that in controls (14, 15). Some cohort studies also revealed the significant association between hepcidin and iron status, but mostly within a single time period (16, 17). Furthermore, only one sub-analysis of a pooled dataset among pregnant women before 28 weeks' gestation found that baseline serum hepcidin >1.6 μg/L was associated with a reduced risk of IDA at delivery (11). Additionally, hepcidin is suppressed in the second and third trimester, especially the level of hepcidin was extremely low and hard to check out in the third trimester (18). Therefore, it is necessary to explore the hepcidin level at early pregnant stage with iron status in late pregnancy.

Our objectives were to investigate the associations of serum hepcidin levels in the first trimester with iron status and ID/IDA risk in the third trimester through a prospective cohort study, thus providing scientific evidence for identification of ID/IDA risk at an early stage.

2. Materials and methods

2.1. Study design and participants

This prospective cohort study was conducted among pregnant women at Shenzhen Nanshan Maternity and Child Healthcare Hospital in Shenzhen city, South China, from May 2019 to April 2020. Briefly, non-ID/IDA women with singleton pregnancy, aged 18 to 45 years, were recruited between 11 and 13 weeks of gestation (here non-ID/IDA was defined as the absence of SF < 20 ng/mL or Hb < 110 g/L). Any subjects with (1) a history of iron-supplementation intake during the past 3 months; (2) a history of hypertension and diabetes; or (3) unable to complete questionnaire or refused to sign the informed consent, were excluded. Written informed consents were obtained from all participants at the time of recruitment. The participants were invited to complete a structured questionnaire, undergo physical examinations, and provide blood samples after enrollment and were recalled for blood samples collection at 38 weeks of gestation. This study was approved by the Ethics Committee of School of Public Health Sun Yat-sen University, and conducted corresponding to the Declaration of Helsinki.

2.2. Laboratory tests

Each pregnant woman who met the eligible criteria had her venous blood drawn by a trained nurse in the morning after overnight fasting in the first trimester (12.14 ± 0.04 gestational weeks) and the third trimester (38.62 ± 0.13 gestational weeks), in accordance with the standard protocol. Serum iron biomarkers including serum hepcidin, SF, Hb and serum iron (SI), and inflammatory biomarkers like CRP were tested within 2 h by trained technicians in the hospital laboratory. Serum hepcidin levels were measured using a commercially available quantikine ELISA kit (DHP250; R&D system; USA) according to the manufacturer's instructions, which is a validated and highly sensitive enzyme immunoassay for quantitative *in vitro* diagnostic determination of hepcidin in human cell culture supernate, serum, plasma or urine. SF concentrations were determined by the enzyme immunoassay method, using commercial kits (FERRITIN ELISA; Diametra, Boldon, UK). Hb levels were quantified on an automated hematological analyser (TC Hemaxa 1,000; Teco Diagnostic, Anaheim, CA, USA), using a hemiglobin-cyanide method. SI levels were assessed by a commercial test, using a colorimetric method (Ferentest, bioMérieux® SA, France). Serum concentrations of CRP were determined using an immunoturbidimetric assay. For these parameters, the intra-assay and inter-assay CV (%) were below 5.7 and 6.7%, respectively. The remaining blood samples were stored at -80 C until assayed.

2.3. Exposure assessment

The exposure was serum hepcidin level in the first trimester, which was divided into tertiles based on its distribution among all participants. First-trimester serum hepcidin was classified into three categories: (1) low serum hepcidin, ≤11.85 ng/mL (reference); (2) moderate serum hepcidin, 11.86–27.43 ng/mL; and (3) high serum hepcidin, ≥27.44 ng/mL.

2.4. Assessment of outcomes

The primary outcomes were the incidences of ID and IDA in the third trimester. ID and IDA were classified according to SF and Hb. ID was defined as SF < 20 ng/mL and IDA was defined as ID plus low Hb (Hb < 110 g/L), in accordance with the definition proposed by Chinese Medical Association, 2014 (19). The secondary outcomes were iron status in the third trimester, including serum hepcidin, SF, Hb and SI.

2.5. Assessment of covariates

Data on demographic information and pregnancy history were collected from a structured questionnaire at enrollment, including maternal age (years), educational level, monthly income, participant source, gestational age, gravidity and parity. Anthropometric data at enrollment were obtained by experienced clinicians and nurses. Barefoot height was measured to the nearest 0.1 cm using a stadiometer (Yilian TZG, Jiangsu, PRC), and body weight was measured to the nearest 0.1 kg with a self-zeroing scale (Hengxing TGT-140, Jiangsu, PRC). Pre-pregnancy weight was measured and recorded in health booklets by professional staffs during the pre-pregnancy checkups. Pre-pregnancy BMI (pre-BMI) was calculated by dividing one's pre-pregnancy weight in kilograms by her height in meters and categorized as underweight (<18.5 kg/m2), normal (18.5–23.9 kg/m2), or overweight (≥24 kg/m2), according to Chinese criteria (20). Intake of iron supplementation during pregnancy was obtained from medical records.

2.6. Statistical analysis

Normality was assessed using the Shapiro–Wilk test and Q-Q plot. Data were presented as mean ± standard deviation or median (interquartile range) for continuous variables and number (percentage) for categorical variables. The difference among three groups were compared by One-way ANOVA (continuous variables with normal distribution), or Kruskal-Wallis H tests (continuous variables with skewed distribution), and Chi-square tests (categorical variables). We used linear regression model to evaluate the associations of first-trimester serum hepcidin with third-trimester iron biomarkers (serum hepcidin, SF, Hb and SI) and inflammatory biomarker (CRP). Right-skewed biomarkers including serum hepcidin, SF, SI and CRP in the third trimester, as dependent variables, were log(e)-transformed to normalize distributions prior to linear regression analysis. Resulting regression coefficients (β) expressed the change in log-transformed biomarker levels that are associated with moderate or high tertiles of first-trimester hepcidin, compared to low tertile of first-trimester hepcidin. In addition, logistic regression models were built to examine the associations between first-trimester serum hepcidin and third-trimester ID/IDA risk. Maternal age, pre-pregnancy BMI, parity, iron supplementation during pregnancy, and CRP or serum hepcidin in the third trimester were included as covariates in the regression models. All statistical analyses were performed using R 4.0; $p < 0.05$ was considered significant.

3. Results

3.1. Characteristics of the participants stratified by tertiles of first-trimester serum hepcidin

The flow chart of the study participants is shown in Supplementary Figure S1. Of the total 353 pregnant women recruited, 264 participants with full data were included in the final analysis. The characteristics of the participants stratified by tertiles of first-trimester serum hepcidin are presented in Table 1. The mean age of participants was 29.12 ± 4.45 years and the mean gestational age was 12.15 ± 0.64 weeks at enrollment. The median serum hepcidin concentration was 19.39 (9.69–33.59) ng/ml in the first trimester and 1.32 (0.52–6.67) ng/ml in the third trimester. In the first trimester, no significant differences were observed in demographic characteristics among pregnant women ($p > 0.05$), and significant differences were observed only in SF levels ($p < 0.05$). In the third trimester, significantly higher serum hepcidin, Hb and SI levels were observed among pregnant women with higher tertiles of first-trimester hepcidin compared to those with the lowest tertile ($p < 0.05$). In addition, 122 (46.2%) incident ID cases and 30 (11.4%) incident IDA cases were identified in the third trimester, respectively.

3.2. The relationships between tertiles of first-trimester serum hepcidin and third-trimester iron status

We evaluated the relationships between tertiles of first-trimester serum hepcidin and third-trimester iron status (Table 2). Significantly higher serum hepcidin levels in the third trimester were more likely to be observed in pregnant women with moderate first-trimester hepcidin (log-transformed $\beta = 0.51$; 95% confidence interval [CI] = 0.01, 1.00), as well as those with high first-trimester hepcidin (log-transformed $\beta = 0.66$; 95% CI = 0.15, 1.17), compared to those with low first-trimester hepcidin, after adjusting for maternal age, pre-pregnancy BMI, parity, iron supplementation during pregnancy and CRP. These β values, when calculated back to original scales of third-trimester serum hepcidin, mean that women with moderate and high first-trimester hepcidin, respectively, had 66.5% (i.e., $[e^{0.51}-1] \times 100\%$) and 93.5% (i.e., $[e^{0.66}-1] \times 100\%$) higher levels of serum hepcidin in the third trimester, compared to those with low first-trimester hepcidin. Moreover, in comparison to low first-trimester hepcidin, moderate first-trimester hepcidin was positively associated with SI (log-transformed $\beta = 0.20$; 95% CI = 0.06, 0.34) and Hb ($\beta = 5.43$; 95% CI = 1.17, 9.70) in the third trimester, after adjustment with the mentioned covariates. Similarly, these β values mean that women with moderate first-trimester hepcidin had 22.1% (i.e., $[e^{0.20}-1] \times 100\%$) and 5.43 ng/mL higher levels of SI and Hb in the third trimester than those with low first-trimester hepcidin, respectively. However, no significant associations were observed between first-trimester hepcidin and SF or CRP in the third trimester.

3.3. The relationships between tertiles of first-trimester serum hepcidin and ID/IDA risk in the third trimester

Table 3 shows the relationships between tertiles of first-trimester serum hepcidin and ID/IDA risk in the third trimester. In the unadjusted analyses, when compared to low first-trimester hepcidin, moderate and high first-trimester hepcidin was marginally associated with reduced risk of IDA in the third trimester (crude odds ratio [OR] = 0.40; 95% CI: 0.16, 1.03 and crude OR = 0.39; 95% CI: 0.15, 1.01, respectively). Furthermore, after additional adjustment with maternal age, pre-pregnancy BMI, parity, iron supplementation during pregnancy and CRP, first-trimester hepcidin was independently inversely associated with IDA risk in the third trimester (moderate vs.

TABLE 1 Characteristics of individuals stratified by tertiles of first-trimester serum hepcidin levels.

Characteristics	Total (N = 264)	Low hepcidin ≤ 11.85 (N = 89)	Moderate hepcidin 11.86–27.43 (N = 87)	High hepcidin ≥ 27.44 (N = 88)	P
Age, years	29.12 ± 4.45	29.02 ± 4.61	29.07 ± 4.35	29.27 ± 4.43	0.924
Educational level					
College or lower	74 (41.3)	23 (39.7)	26 (44.1)	25 (40.3)	0.871
University or higher	105 (58.7)	35 (60.3)	33 (55.9)	37 (59.7)	
Monthly income					
≤5,000 RMB	89 (50.0)	27 (46.6)	32 (55.2)	30 (48.4)	0.618
>5,000 RMB	89 (50.0)	31 (53.4)	26 (44.8)	32 (51.6)	
Participant source					
Rural	202 (76.5)	65 (73.0)	67 (77.0)	70 (79.5)	0.588
Urban	62 (23.5)	24 (27.0)	20 (23.0)	18 (20.5)	
Gestational age at enrolment, weeks	12.15 ± 0.64	12.14 ± 0.61	12.20 ± 0.66	12.11 ± 0.65	0.684
Gravidity					
1	86 (32.6)	23 (25.8)	26 (29.9)	37 (42.0)	0.057
≥2	178 (67.4)	66 (74.2)	61 (70.1)	51 (58.0)	
Parity					
Primiparous	112 (42.4)	33 (37.1)	33 (37.9)	46 (52.3)	0.072
Multiparous	152 (57.6)	56 (62.9)	54 (62.1)	42 (47.7)	
Height, cm	158.48 ± 5.18	158.84 ± 4.28	158.15 ± 6.48	158.42 ± 4.59	0.674
Weight at enrolment, kg	53.23 ± 8.23	53.62 ± 9.02	53.73 ± 7.34	52.48 ± 8.42	0.710
Pre-BMI, kg/m^2	21.31 ± 2.76	21.28 ± 2.81	21.47 ± 2.92	21.18 ± 2.54	0.781
Pre-BMI category					
Normal	189 (71.6)	63 (70.8)	63 (72.4)	63 (71.6)	0.870
Underweight	35 (13.3)	13 (14.6)	9 (10.3)	13 (14.8)	
Overweight	40 (15.2)	13 (14.6)	15 (17.2)	12 (13.6)	
Iron supplementation during pregnancy					
Received	59 (22.3)	25 (28.1)	19 (21.8)	15 (17.0)	0.209
Not received	205 (77.7)	64 (71.9)	68 (78.2)	73 (83.0)	
First trimester					
SH, ng/mL	19.39 (9.69–33.59)	8.03 (5.01–9.76)	19.43 (16.14–23.84)[a]	40.74 (33.76–58.53)[a]	<0.001*
SF, ng/mL	85.00 (54.85–124.00)	52.00 (41.00–80.75)	86.00 (61.50–116.00)[a]	123.00 (93.75–175.25)[a]	<0.001*
Hb, g/L	126.21 ± 8.08	125.31 ± 7.26	126.61 ± 8.31	126.72 ± 8.63	0.473
SI, umol/L	21.07 (17.32–25.06)	20.30 (16.76–24.09)	22.43 (17.88–25.73)	20.41 (17.28–25.55)	0.128
Third trimester					
SH, ng/mL	1.32 (0.52–6.67)	0.85 (0.37–3.25)	1.47 (0.67–7.94)	2.31 (0.76–7.48)[a]	0.007*
SF, ng/mL	20.00 (14.15–31.00)	20.00 (13.00–27.10)	20.00 (13.25–29.50)	20.85 (16.00–36.50)	0.201
Hb, g/L	118.17 ± 14.40	115.28 ± 14.44	120.79 ± 13.04[a]	118.50 ± 15.25	0.038*
SI, umol/L	12.90 (9.80–17.56)	12.73 (8.60–16.48)	13.82 (11.32–18.60)[a]	12.90 (9.38–16.97)	0.041*
CRP, mg/L	6.85 (3.77–15.47)	6.85 (3.67–11.83)	6.85 (3.50–14.30)	6.88 (4.73–26.91)	0.211
ID	122 (46.2)	43 (48.3)	41 (47.1)	38 (43.2)	0.774
IDA	30 (11.4)	16 (18.0)	7 (8.0)	7 (8.0)	0.054

RMB, Renminbi; pre-BMI: pre-pregnancy body mass index; SH, serum hepcidin; SF, serum ferritin; Hb, hemoglobin; SI, serum iron; CRP, C-reactive protein; ID, iron deficiency; IDA, iron deficiency anemia.

Data were presented as N (percentage) for categorical variables and mean ± standard deviation or median (inter-quartile range) for normal or skewed continuous variables.

*$p < 0.05$ among three groups, assessed by Chi-square test for categorical variables and one-way ANOVA and Kruskal-Wallis H test for continuous variables when appropriate. Post-hoc tests with Bonferroni.

[a]$p < 0.05$, compared to low hepcidin group.

TABLE 2 The associations between first-trimester serum hepcidin levels and third-trimester iron status.

Outcomes	Low hepcidin	Moderate hepcidin		High hepcidin	
	≤11.85 (*N* = 89)	11.86–27.43 (*N* = 87)	*P*	≥27.44 (*N* = 88)	*P*
SH[†]					
Crude	Reference	0.54 (0.03, 1.04)	0.038*	0.73 (0.22, 1.23)	0.005*
Adjusted[‡]	Reference	0.51 (0.01, 1.00)	0.046*	0.66 (0.15, 1.17)	0.011*
SF[†]					
Crude	Reference	0.02 (−0.23, 0.27)	0.857	0.19 (−0.06, 0.44)	0.145
Adjusted[‡]	Reference	0.01 (−0.24, 0.26)	0.958	0.17 (−0.08, 0.42)	0.183
Hb					
Crude	Reference	5.51 (1.27, 9.75)	0.011*	3.22 (−1.01, 7.45)	0.135
Adjusted[‡]	Reference	5.43 (1.17, 9.70)	0.013*	2.98 (−1.33, 7.30)	0.175
SI[†]					
Crude	Reference	0.20 (0.06, 0.34)	0.006*	0.04 (−0.10, 0.19)	0.532
Adjusted[‡]	Reference	0.20 (0.06, 0.34)	0.005*	0.03 (−0.11, 0.17)	0.637
CRP[†]					
Crude	Reference	0.15 (−0.28, 0.57)	0.489	0.34 (−0.08, 0.77)	0.110
Adjusted[§]	Reference	0.12 (−0.30, 0.54)	0.579	0.41 (−0.02, 0.84)	0.063

SH, serum hepcidin; SF, serum ferritin; Hb, hemoglobin; SI, serum iron; CRP, C-reactive protein. Data were presented as β (95% confidence interval).
*$p < 0.05$, estimated by liner regression model.
[†]The dependent varibles were log(e)-transformed prior to linear regression analysis. Thus, the β values expressed the change in log-transformed biomarker levels that are associated with moderate or high tertiles of first-trimester hepcidin, compared to low tertile of first-trimester hepcidin.
[‡]Adjusted for maternal age, pre-pregnancy BMI, parity, iron supplementation during pregnancy and CRP in the third trimester.
[§]Adjusted for maternal age, pre-pregnancy BMI, parity, iron supplementation during pregnancy and hepcidin in the third trimester.

TABLE 3 The associations between first-trimester serum hepcidin levels and third-trimester iron deficiency and iron deficiency anemia.

Outcomes	Low hepcidin	Moderate hepcidin		High hepcidin	
	≤11.85 (*N* = 89)	11.86–27.43 (*N* = 87)	*P*	≥27.44 (*N* = 88)	*P*
ID, *n* (%)	43 (48.3)	41 (47.1)		38 (43.2)	
Crude	Reference	0.95 (0.53, 1.72)	0.875	0.81 (0.45, 1.47)	0.493
Adjusted[†]	Reference	0.98 (0.54, 1.80)	0.950	0.82 (0.44, 1.51)	0.521
IDA, *n* (%)	16 (18.0)	7 (8.0)		7 (8.0)	
Crude	Reference	0.40 (0.16, 1.03)	0.056	0.39 (0.15, 1.01)	0.053
Adjusted[†]	Reference	0.38 (0.15, 0.99)	0.047*	0.38 (0.14, 0.99)	0.049*

ID, iron deficiency; IDA, iron deficiency anemia.
Data were presented as odds ratio (95% confidence interval).
*$P < 0.05$, estimated by logistic regression model.
[†]Adjusted for maternal age, pre-pregnancy BMI, parity, iron supplementation during pregnancy and CRP in the third trimester.

low hepcidin: adjusted OR = 0.38; 95% CI: 0.15, 0.99 and high vs. low hepcidin: adjusted OR = 0.38; 95% CI: 0.14, 0.99). However, when extreme tertiles were compared, first-trimester hepcidin was not substantially related to ID risk in the third trimester regardless of whether the selected confounders were controlled for.

4. Discussion

The prospective associations between maternal serum hepcidin in early pregnancy and subsequent risks of ID and IDA are still unknown. Our longitudinal cohort study showed that elevated first-trimester serum hepcidin was associated with increased serum hepcidin and higher iron status, as well as significantly lower risk of IDA in the third trimester, independent of potential confounders including CRP.

ID and IDA are prevalent nutritional deficiency disorders during pregnancy worldwide. Therefore, early identification of risk factors for ID and IDA is essential to prevent the occurrence of ID and IDA. It has been shown that hepcidin is a key regulator of iron metabolism. As known in previous studies, hepcidin level would continue to fall throughout pregnancy to allow more iron to be released into plasma to meet maternal iron requirements and the needs of fetal growth and

development (21). In the present study, we did observe a substantial decline of hepcidin from the first to the third trimester. Our finding is in accordance with previous findings that hepcidin undergoes apparent changes during pregnancy (18, 22). However, a prospective cohort study included 103 healthy Turkish women with normal pregnancies found no significant differences in hepcidin between early pregnancy and late pregnancy, probably because the effects of obstetrical complications such as anemia or ID on maternal hepcidin were not considered (23).

The association between hepcidin and iron status during pregnancy has been reported in previous researches (14, 17, 24). However, the relationships between hepcidin in early pregnancy and iron biomarkers in late pregnancy have rarely been analyzed. Of note, positive associations between first-trimester serum hepcidin and third-trimester serum hepcidin, Hb and SI were observed in our study. A plausible explanation is that although hepcidin decreases with the progress of pregnancy, hepcidin that is higher in the first trimester remains at a higher level in the third trimester, indicating a replete iron status, which partly counteracts suppression of hepcidin by pregnancy signals (9).

Numerous prior studies measured hepcidin during pregnancy when ID/IDA was already diagnosed (14, 15), whereas we investigated hepcidin of non-ID/IDA pregnant women in the first trimester and ID/IDA risk in the third trimester. We found that elevated first-trimester serum hepcidin was associated with diminished risk of third-trimester IDA, similar to some studies reporting a negative relationship between hepcidin and IDA (14, 15, 25). Previous studies have demonstrated that hepcidin inhibits iron efflux into plasma by degrading its only receptor, ferroportin (FPN) in hepatocytes, intestinal enterocytes, and macrophages, thereby leading to iron sequestration in cells (26). During iron replete pregnancy, maternal iron and hepcidin metabolism keep homeostatic, the mothers maintain constant SI levels and relatively high but not overexpressed serum hepcidin levels, despite increased iron utilization in advanced pregnancy. However, in iron-overloaded mothers, hepcidin production is overstimulated, resulting in hypoferremia and limiting the iron availability for both the mother and the fetus (9). SF is a stable and valid indicator reflecting iron stores, and it can be found in our study that first-trimester SF levels were higher among those women with higher first-trimester serum hepcidin levels but did not reach the threshold of iron overload. Therefore, a possible reason for our findings is that elevated hepcidin levels in early pregnancy implies replete iron stores, indicating that sufficient iron is available for Hb synthesize as pregnancy progresses, thereby decreasing IDA risks in the third trimester. Recently, Sangkhae et.al assessed maternal hepcidin suppression in different iron status using mouse models and found that compared with nonpregnant levels, hepcidin mRNA and protein levels in iron-replete pregnant mice were already almost suppressed at the earliest examined time point, whereas levels of liver iron were not yet largely decreased (9). The lowering of hepcidin is presumed to precede liver iron mobilization. This indicates that decline in hepcidin reflects a physiologic state of high iron requirement, although the onset of low iron stores may not yet occur at this time, when timely iron supplementation may effectively prevent the development of ID/IDA in late pregnancy (16). Except for iron stores, another important factor that affects hepcidin levels is anemia itself, through erythroferrone (ERFE). Studies have demonstrated that ERFE is a glycoprotein hormone secreted by erythroblasts in response to erythropoietin (EPO) stimuli such as hemorrhage, hypoxia, EPO therapy, β-thalassemia, and anemia of inflammation, and it suppresses the hepatic production of hepcidin, thereby mobilizing iron for erythropoiesis (27). Accordingly, it can be speculated in our study that lower levels of first-trimester serum hepcidin may imply a state of EPO-stimulated ERFE production, as well as a possible condition of high anemia risk, although all the participating women were below the diagnosis threshold of anemia at enrollment. Taken together, further studies is warranted to collect more information on iron storage indicators and erythroid regulatory factors, thereby elucidating the mechanisms underlying the association between higher levels of first-trimester serum hepcidin and lower risk of third-trimester IDA.

The relationship between hepcidin and ID was also investigated in the present study. No significant association was observed between first-trimester hepcidin and third-trimester ID risk, which is consistent with a Tanzanian study that found no relationship between baseline hepcidin (< 28 weeks) and ID at delivery (11). However, there are some studies with inconsistent findings (9, 11, 16). For example, an analysis among pregnant women based on an incorporative dataset of clinical trials and a prospective cohort found that hepcidin in individuals with ID was significantly lower than iron-replete individuals (11). Additionally, in another cohort study in Gambia, the prevalence of maternal ID increased, while hepcidin gradually decreased with progressive gestation (16). Furthermore, a follow-up study among iron-deficient Tanzania pregnant women taking iron supplements showed that the prevalence of ID dropped from 93 to 12%, while hepcidin increased form 1.0 μg/L at baseline to 12.3 μg/L at delivery (28). The discrepancy may be partially due to the fact that criteria to determine ID differ and that serum hepcidin levels are often undetectable or low in ID. Therefore, those inconsistent results between hepcidin and ID still call for further investigation.

The strengths in our study include a prospective cohort design and detailed information on potential confounders. We confirmed a negative association between first-trimester hepcidin and third-trimester IDA risk in Chinese population. Additionally, this is the first study to examine the associations between first-trimester hepcidin and third-trimester iron biomarkers within the Chinese setting. However, limitations should be acknowledged as followed. First, a major limitation in our study is the relatively small number of IDA women in each category of first-trimester serum hepcidin levels, and thus the EPV (events per variable) criterion was marginally met when performing logistic regression analysis. Given that pregnant women are usually advised a range of measures preventing ID/IDA (e.g., iron, folic acid and vitamin C supplementation) during routine pregnancy care, and that the study population was derived from a single hospital, it is not surprising that few women developed IDA in late pregnancy. Hence, although we revealed the potential negative association of first-trimester serum hepcidin with third-trimester IDA, our findings were mainly explorative and caution should be exercised when extrapolating to other groups. Second, the relationships between serum hepcidin levels in the second trimester and iron status in the third trimester were not evaluated in the present study. Finally, only CRP was collected in our study, yet hepcidin is affected by other inflammatory factors. Thus, further multi-center study with larger sample size is warranted to provide more evidence on the association between serum hepcidin and IDA

during consecutive trimesters of pregnancy while taking into account the effects of other inflammatory factors.

5. Conclusion

Our study demonstrated that elevated first-trimester serum hepcidin level was closely associated with decreased risk of IDA in the third trimester, which indicates that high hepcidin level in early pregnancy implies replete iron stores and therefore lower risk of IDA in late pregnancy. However, considering the relatively limited sample size, larger studies collecting more data on iron metabolism indicators and inflammatory factors during consecutive trimesters are still needed.

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 Ethics Committee of School of Public Health, Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YaZ and PS designed the study. PS, SX, YuZ, XW, XL, HL, ZL, LZ, and FH enrolled the participants. SX and YaZ analyzed the data. PS, SX, and YuZ wrote the article. SX, YuZ, and YaZ interpreted the results. PS, XL, LZ, ZL, and FH contributed intellectually to the manuscript. YaZ had primary responsibility for final content. All authors have 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1147114/full#supplementary-material

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Association of vitamin B1 with cardiovascular diseases, all-cause and cardiovascular mortality in US adults

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Background: The correlation between dietary vitamin B1 intake and cardiovascular diseases, as well as the all-cause and cardiovascular-associated mortality, is not well known. A large-scale data pool was used to examine the aforementioned correlations of Vitamin B1.

Methods: This paper analyzed the dietary data from the survey conducted by National Health and Nutrition Examination (NHANES; 1999–2018). The correlation of vitamin B1 intake in each quartile with cardiovascular diseases such as hypertension, coronary heart disease, myocardial infarction and heart failure was analyzed using multivariate logistic regression models. The hazard ratios for dietary vitamin B1 intake in each quartile, along with all-cause and cardiovascular-associated mortality, were performed using multivariate cox regression analysis, setting the lowest quartile (Q1) as a reference. The restricted cubic spline (RCS) method was used to study the nonlinear relationship. Subgroup stratification and sensitivity analyses were used to further investigate the association between them.

Results: The study enrolled 27,958 subjects (with a mean follow-up time of 9.11years). After multivariate adjustment, dietary vitamin B1 intake was significantly associated with hypertension, heart failure and cardiovascular mortality, with the most significant association in quartile 4 (Q4) of vitamin B1 intake. The results of the restricted cubic spline showed that vitamin B1 intake was nonlinearly associated with hypertension, whereas it was linearly associated with heart failure and cardiovascular mortality. Meanwhile, a dose–response correlation was observed, indicating that increased vitamin B1 intake leads to reduced risk of both cardiovascular prevalence and mortality. The stratified analysis showed that the correlation between age≥50years, overweight, smoking history, drinking history and dyslipidemia were more significant in male patients. The associations remained similar in the sensitivity analyses.

Conclusion: The large NHANES-based studies indicate a gradual trend toward decreasing the risk of hypertension and heart failure prevalence and cardiovascular mortality with increasing dietary vitamin B1 intake. This association is especially significant in elderly-aged men, overweight individuals, smokers, drinkers, and dyslipidemia patients.

KEYWORDS

dietary vitamin B1, cardiovascular diseases, all-cause mortality, cardiovascular mortality, NHANES

1. Introduction

Globally, cardiovascular diseases (CVD) are one of the foremost causes of death, with a mortality rate of 17.9 million deaths per year (32% of global deaths), and are recognized by the United Nations as a major global health burden (1, 2). Cardiovascular diseases include heart failure, hypertension, ischemic heart disease (stable angina and acute coronary syndrome), cerebrovascular diseases (stroke), valvular abnormalities (aortic stenosis), and arrhythmias (atrial fibrillation), and other various diseases (3–5). Recently, the American Society of Preventive Cardiology (ASPC) published *10 Things to Know About the 10 Major Risk Factors for cardiovascular diseases (2022)*. These 10 factors include unhealthy diet, lack of exercise, dyslipidemia, pre-diabetes/diabetes, hypertension, obesity, special population, thrombosis, renal insufficiency, family history/genetic factors/familial hypercholesterolemia (6). The American College of Cardiology (ACC) and the American Heart Association (AHA) formulated an extensive report on the prevention of cardiovascular diseases (7). The European Society of Cardiology (ESC) also issued strategies to prevent CVD incidence in clinical practice in 2016 (8). The aforementioned report mentioned that healthy nutrition is the cornerstone of cardiovascular disease prevention, and is often one of the most challenging factors in managing cardiovascular disease risk factors. Despite these challenges, nutritionists have determined that even small, but targeted health changes in dietary intake have the potential to improve cardiovascular health (9).

Vitamin B1, also known as thiamine, is an essential micronutrient (10). However, vitamin B1 itself is only stored in small amounts in the body and cannot be produced endogenously, and has a half-life of 1–3 weeks in the body (11). Its main sources are dietary (whole grains, legumes, nuts) or ingested from supplements. In recent years, with the improvement in living conditions, the consumption of white rice has increased, which has increased the incidence of vitamin B1 deficiency. In addition, vitamin B1 is an essential coenzyme required for glucose metabolism. The consumption of more high-calorie diets has increased nowadays which in turn increases the demand for vitamin B1 in the human body, resulting in vitamin B1 deficiency (12). In addition, Excessive alcohol consumption can affect the cellular transport of vitamin B1 and its intracellular phosphorylation (13). Chronic alcoholism has been found to be one of the main causes of vitamin B1 deficiency in clinical studies (14). Thiamine deficiency is also more prevalent in patients with advanced age, diuretic use and pregnancy (15). Although vitamin B1 deficiency is rare at present, it has attracted more and more attention as a risk factor for a variety of systemic diseases in recent years, as evidenced by the review of a large number of literature sources (16). Since the end of 2014, thiamine deficiency has re-emerged in the Pacific Islands, but the underlying cause of the re-emergence is still unknown, Therefore the study of vitamin B1 should not be overlooked (17). There is growing evidence that vitamin B1 supplementation can reverse CVD, diabetes, obesity, dyslipidemia, angina, myocardial infarction, and psychiatric disorders (18, 19). However, large population-based studies examining the association between vitamin B1 intake and cardiovascular disease and mortality are lacking. To address this gap, data was collected from the National Health and Nutrition Examination Survey (NHANES) from 1999 to 2018 and an analysis of large, nationally representative clinical studies was conducted to evaluate the association of dietary vitamin B1 intake with cardiovascular and all-cause mortality, and provide a theoretical basis for prevention.

2. Methods

2.1. Study population

The National Health and Nutrition Examination Survey (NHANES) is a large-scale, stratified, multi-stage sampling study conducted by the US Centers for Disease Control and Prevention (20). NHANES are widely used as large prospective cohorts with nationally representative samples through their association with follow-up mortality data (21). The NCHS Ethics Review Board approved all NHANES programs, and study members or their agents provided informed consent prior to participation (22). Detailed information about NHANES can be found at www.cdc.gov/nchs/nhanes/.

The data from 10 NHANES cycles from 1999 to 2018 was screened, involving 101,316 participants. Among these individuals, data with missing information on vitamin B1 intake ($n = 38,611$), as well as participants without documented cardiovascular diseases, all-cause death, and cardiovascular mortality ($n = 27,186$) were excluded. Confounding factors with missing values were also excluded ($n = 7,561$). Ultimately, a total of 27,958 participants were included in the analysis (Supplementary Figure S1).

2.2. Variables

Dietary data were obtained from the NHANES database, and all participants were interviewed about a dietary recall for two 24-h periods. The first recall interview was conducted at the NHANES Mobile Test Center, and the second was conducted by telephone 10 days later. Total daily amounts of all nutrients/food components were calculated, using the USDA Food and Nutrition Dietary Research Database and were input into the NHANES database (23). In the study, the mean intake of vitamin B1 was analyzed from two 24-h recalls. Cardiovascular diseases were identified depending on their answers to a questionnaire wherein the participants were asked if they had been diagnosed by a medical professional with coronary heart disease (24). Measurements for myocardial infarction and congestive heart failure were similar. Participants were considered to have high blood pressure if any of the following criteria were met: informed by a medical expert about their increased blood pressure, or were taking blood pressure medication and had a mean systolic blood pressure (SBP) of 140 mmHg or diastolic blood pressure (DBP) of 90 mmHg at the physical examination. Participants were considered to have diabetes if one of the following was true: an individual informed by a medical expert of their contracting of diabetes and was taking insulin, or taking diabetes medication to lower blood sugar (25).

The information regarding all-cause mortality and cardiovascular conditions was accessed in the 2019 NHANES Linked Mortality File (LMF), which is available for public use. This file contains data from previous surveys that include mortality factors and the status of the participants from 1999 to December 31st. 2019. The information from the death certificates was used to derive the Clinical Modification System codes I00-I78 to determine the mortality rate resulting from all-cause mortality and cardiovascular diseases by the International

Classification of Diseases, 10th Revision. Further details of the participants regarding the mortality factors can be accessed at https://www.cdc.gov/nchs/data-linkage/mortality-public.htm (26).

Based on existing information relevant covariates such as age, sex, level of education (less than 9th grade, grades 9–11, high school graduate/GED or equivalent record of formal schooling, partial college or AA degree or above, college graduate or above), BMI, smoking history (never smoked, smoking history), drinking history (less or more than 12 cups/year), aspirin use, family poverty income ratio (PIR:<1.0; 1.0–3.0; >3.0), physical activity, diabetes status, total energy intake, and laboratory measures of blood lipids were included. The energy intake of adults was calculated using the USDA Food and Nutrient Database, available at https://www.ars.usda.gov/nea/bhnrc/fsrg, which was designed to provide an effective and accurate method to collect intakes for large-scale national surveys (27). Detailed information on dietary vitamin B1 intake and other variables can be accessed at www.cdc.gov/nchs/nhanes/.

2.3. Statistical analysis

The information from NHANES (1999–2018) was merged and extracted on R Studio 4.1.3 (R Foundation for Statistical Computing, Vienna, Austria). The GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, United States), R (4.1.3) studio, and EmpowerStats[1] were used to analyze the graphic design and data. The Mann–Whitney *U* test, a nonparametric test, or the t-test was utilized to compare baseline characteristics for continuous variables, whereas categorical variables were compared with the chi-square or Fisher test. The Multivariate logistic regression models were utilized to estimate the correlation between the intake of dietary vitamin B1 and cardiovascular diseases (hypertension, coronary heart disease, myocardial infarction, heart failure) in each quartile. The hazard ratio of the mortality rate of all-cause and cardiovascular conditions to the intake of vitamin B1 in the diet was analyzed using multivariate Cox regression analysis. In each quartile, the hazard ratios (HRs) and 95% confidence intervals (CIs) were displayed using the lowest quartile (Q1) as a reference. Age, sex, body mass index (BMI), smoking history, drinking history and blood lipid were taken into consideration when performing stratified analysis. A 3-bar restricted cubic spline was constructed to visualize the dose–response relation of vitamin B1 intake and the risk of incidence of cardiovascular diseases along with all-cause, and cardiovascular mortality. To test the robustness of our findings, we conducted sensitivity analyses excluding the participants who died during the first year of follow-up (28). *p*-value <0.05 was regarded as statistically significant when all data were examined utilizing R studio v4.1.3.

3. Results

3.1. Characteristics of participants

The mean age of the 27,958 participating individuals was 50.10 ± 17.61 years of which 49.50% were male ($n = 13,842$). The results showed that relative to quartile four (high intake) there was a higher likelihood of female participants being included in quartile one (low intake). Education level showed that 52.96% of participants had an educational background of university, AA education, college graduate or above. There were 3,887 deaths, including 974 deaths caused by cardiovascular diseases. All the baseline characteristics of the participating individuals were assessed (Table 1).

3.2. Prevalence of cardiovascular diseases and trends of all-cause and cardiovascular mortality

The NHANES database highlights a concerning trend in the prevalence of cardiovascular diseases in United States adults from 1999 to 2018, Hypertension saw a significant increase from 37.2% in 1999–2000 to 44.3% in 2017–2018. Additionally, within the aforementioned time range, coronary heart disease rose from 4.2 to 4.8% and myocardial infarction increased from 4.5 to 4.9%. The prevalence of heart failure did not increase significantly. However, the all-cause mortality rate decreased significantly from 25.6% in 1999–2000 to 2.4% in 2017–2018, and the cardiovascular mortality rate increased from 19.3 to 21.1%. Figure 1 shows the trends in the prevalence of various types of cardiovascular diseases and cardiovascular mortality as well as all-cause mortality in the United States adult population.

3.3. Effects of various factors on cardiovascular diseases, all-cause and cardiovascular mortality by univariate analysis

Most parameters were correlated with cardiovascular diseases, all-cause, and cardiovascular mortality. The risk of hypertension, coronary heart disease, myocardial infarction, heart failure, all- cause mortality, and cardiovascular mortality (95% confidence interval; *p*-value) among participants aged ≥50 years was 6.76 (6.41, 7.13; $p < 0.001$), 16.62 (13.08, 21.12; $p < 0.001$), 10.36 (8.50, 12.63; $p < 0.001$), 8.76 (7.06, 10.87; $p < 0.001$), 10.99 (9.91, 12.20; $p < 0.001$) and 13.92 (11.08, 17.48; $p < 0.001$), respectively compared with participants younger than 50 years. Except for hypertension, the odds ratios or hazard ratios (95% confidence interval; *p*-value) for this correlation were 0.44 (0.39, 0.50; $p < 0.001$), 0.47 (0.41, 0.53; $p < 0.001$), 0.72 (0.63, 0.82; $p < 0.001$), 0.74 (0.70, 0.79; $p < 0.001$) and 0.66 (0.58, 0.75; $p < 0.001$) for female relative to male, respectively. With the exception of 9-11th grade, all other parameters were associated with cardiovascular diseases, all-cause and cardiovascular mortality (Tables 2, 3).

3.4. Correlation analysis of vitamin B1 intake with cardiovascular diseases, all-cause, and cardiovascular mortality

Three multivariate logistic regression models were developed: Model 1, unadjusted; Model 2, adjusted for age, sex, and level of education; Model 3, adjusted for all covariates listed in Table 1. In unadjusted models, vitamin B1 intake was inversely correlated with all types of cardiovascular diseases, and the respective odds ratios for

1 http://www.empowerstats.com

TABLE 1 Baseline characteristics of total participants and stratification by the quartile of vitamin B1 intake.

Characteristic	Total subjects	Vitamin B1 Intake Quartile, mg/day				
		Q1 (Low) (0–1.04)	Q2 (1.04–1.43)	Q3 (1.43–1.94)	Q4 (High) (1.94–15.64)	*p*-value
Number	27,958	6,979	6,994	6,989	6,996	
Median intake	1.43	0.82	1.24	1.65	2.40	
Average (SD)	1.58 ± 0.81	0.78 ± 0.20	1.24 ± 0.11	1.66 ± 0.14	2.65 ± 0.81	
Demographics						
Age (years old)	50.10 ± 17.61	51.44 ± 17.74	51.50 ± 17.81	50.36 ± 17.53	47.10 ± 17.00	<0.001
<50	13,821 (49.43)	3,186 (45.64)	3,214 (45.95)	3,444 (49.28)	3,979 (56.88)	
≥50	14,137 (50.57)	3,794 (54.36)	3,781 (54.05)	3,545 (50.72)	3,017 (43.12)	
Sex, n (%)						<0.001
Male	13,840 (49.50)	2,254 (32.30)	2,902 (41.49)	3,622 (51.82)	5,062 (72.36)	
Female	14,118 (50.50)	4,725 (67.70)	4,092 (58.51)	3,367 (48.18)	1934 (27.64)	
Level of education, n (%)						<0.001
Less than 9th grade	2,758 (9.86)	934 (13.38)	760 (10.87)	577 (8.26)	487 (6.96)	
9-11th grade	3,935 (14.07)	1,177 (16.86)	979 (14.00)	872 (12.48)	907 (12.96)	
High school graduate/ GED or equivalent	6,457 (23.10)	1,642 (23.53)	1,638 (23.42)	1,621 (23.19)	1,556 (22.24)	
Some college or AA degree	8,220 (29.40)	2024 (29.00)	2068 (29.57)	2065 (29.55)	2063 (29.49)	
College graduate or above	6,588 (23.56)	1,202 (17.22)	1,549 (22.15)	1854 (26.53)	1983 (28.34)	
BMI (kg/m^2)						<0.001
<25	7,947 (28.42)	1862 (26.68)	1902 (27.19)	1999 (28.60)	2,184 (31.22)	
25–30	9,434 (33.74)	2,276 (32.61)	2,368 (33.86)	2,360 (33.77)	2,430 (34.73)	
≥30	10,577 (37.83)	2,841 (40.71)	2,724 (38.95)	2,630 (37.63)	2,382 (34.05)	
Smoking history, n (%)						<0.001
Never	15,037 (53.78)	3,683 (52.77)	3,817 (54.58)	3,875 (55.44)	3,662 (52.34)	
smoker	12,921 (46.22)	3,296 (47.23)	3,177 (45.42)	3,114 (44.56)	3,334 (47.66)	
Drinking history, n (%)						0.012
<12	8,599 (30.76)	2,574 (36.88)	2,259 (32.30)	2059 (29.46)	1707 (24.40)	
≥12	19,359 (69.24)	4,405 (63.12)	4,735 (67.70)	4,930 (70.54)	5,289 (75.60)	
Aspirin use, n (%)						<0.001
No	27,602 (98.73)	6,873 (98.48)	6,896 (98.60)	6,902 (98.76)	6,931 (99.07)	
Yes	356 (1.27)	106 (1.52)	98 (1.40)	87 (1.24)	65 (0.93)	
Poverty to income ratio, n (%)						<0.001
0.0–1.0	5,377 (19.23)	1,665 (23.86)	1,288 (18.42)	1,208 (17.29)	1,216 (17.38)	
1.01–3.0	11,781 (42.14)	3,087 (44.23)	3,060 (43.75)	2,882 (41.24)	2,752 (39.34)	
>3.0	10,799 (38.63)	2,227(31.91)	2,646 (37.83)	2,898 (41.47)	3,028 (43.28)	
Physical activity (MET-min/wk), n (%)						<0.001
<500	12,453 (44.54)	3,606 (51.66)	3,213 (45.94)	2,970 (42.50)	2,664 (38.08)	
500–999	3,140 (11.23)	766 (10.97)	798 (11.41)	843 (12.06)	733 (10.48)	
≥1,000	12,365 (44.23)	2,607 (37.36)	2,983 (42.65)	3,176 (45.44)	3,599 (51.44)	
Total energy intake (kcal/d), (SD)	2110.92 ± 992.86	1400.98 ± 588.07	1868.77 ± 649.40	2236.58 ± 760.23	2935.67 ± 1156.84	<0.001
Laboratory measurements						
TC (mmol/L)	5.05 ± 1.08	5.12 ± 1.09	5.08 ± 1.09	5.03 ± 1.08	4.97 ± 1.07	<0.001
TG (mmol/L)	1.73 ± 1.54	1.60 ± 1.16	1.73 ± 1.61	1.76 ± 1.71	1.81 ± 1.59	<0.001
HDL (mmol/L)	1.35 ± 0.41	1.39 ± 0.43	1.38 ± 0.41	1.35 ± 0.40	1.29 ± 0.38	<0.001
Diseases						

(Continued)

TABLE 1 (Continued)

Characteristic	Total subjects	Vitamin B1 Intake Quartile, mg/day				
		Q1 (Low) (0–1.04)	Q2 (1.04–1.43)	Q3 (1.43–1.94)	Q4 (High) (1.94–15.64)	*p*-value
HTN, n (%)						<0.001
No	15,915 (56.92)	3,703 (53.05)	3,931 (56.20)	4,044 (57.86)	4,392 (62.78)	
Yes	12,043 (43.08)	3,277 (46.95)	3,064 (43.80)	2,945 (42.14)	2,604 (37.22)	
DM, n (%)						<0.001
No	20,883 (74.69)	5,136 (73.58)	5,089 (72.75)	5,185 (74.19)	5,473 (78.23)	
Yes	4,960 (17.74)	1,348 (19.31)	1,377 (19.69)	1,230 (17.60)	1,006 (14.38)	
IFG + IGT	2,116 (7.57)	496 (7.11)	529 (7.56)	574 (8.21)	517 (7.39)	
CHD, n (%)						0.236
No	26,756 (95.69)	6,685 (95.77)	6,672 (95.38)	6,679 (95.56)	6,720 (96.05)	
Yes	1,204 (4.31)	295 (4.23)	323 (4.62)	310 (4.44)	276 (3.95)	
MI, n (%)						<0.001
No	26,763 (95.73)	6,637 (95.09)	6,679 (95.48)	6,701 (95.88)	6,748 (96.46)	
Yes	1,195 (4.27)	343 (4.91)	316 (4.52)	288 (4.12)	248 (3.54)	
HF, n (%)						<0.001
No	27,073 (96.83)	6,703 (96.03)	6,733 (96.25)	6,795 (97.22)	6,844 (97.83)	
Yes	885 (3.17)	277 (3.97)	262 (3.75)	194 (2.78)	152 (2.17)	
Mortality risk						
ACM, n(%)						<0.001
No	24,071 (86.10)	5,805 (83.18)	6,032 (86.25)	6,041 (86.44)	6,193 (88.52)	
Yes	3,887 (13.90)	1,174 (16.82)	962 (13.75)	948 (13.56)	803 (11.48)	
CVDM, n (%)						<0.001
No	26,984 (96.52)	6,677 (95.67)	6,741 (96.38)	6,747 (96.54)	6,819 (97.47)	
Yes	974 (3.48)	302 (4.33)	253 (3.62)	242 (3.46)	177 (2.53)	

BMI, Body Mass Index; TC, Total Cholesterol; TG, Triglyceride; HDL, High Density Lipoprotein; HTN, Hypertension; DM, Diabetes Mellitus; CHD, Coronary Heart Disease; MI, Myocardial Infarction; HF, Heart Failure; ACM, All-cause Mortality; CVDM, Cardiovascular Mortality.

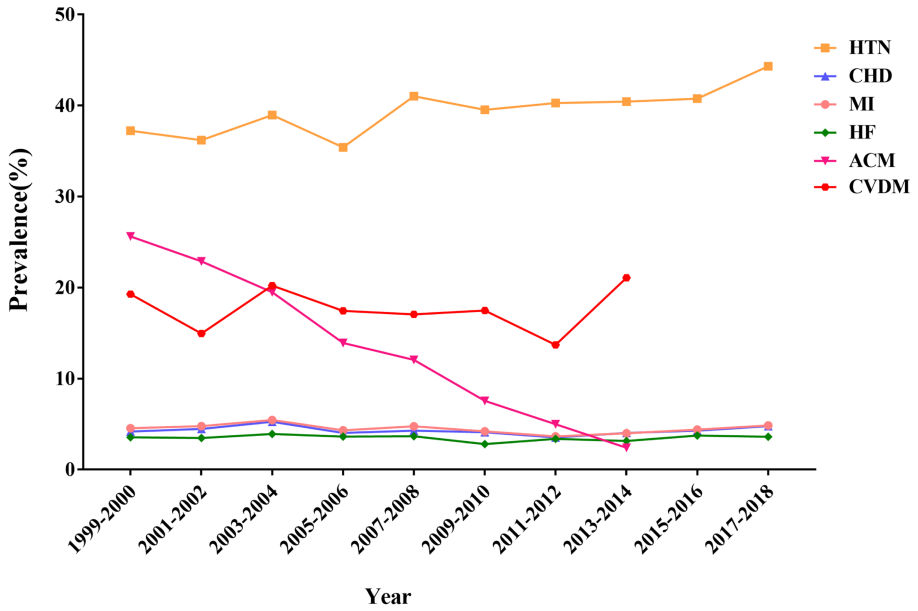

FIGURE 1
Trends in prevalence of cardiovascular diseases and all-cause and cardiovascular mortality among adults in the United States, 1999–2018.

TABLE 2 Effects of various factors on cardiovascular diseases by univariate analysis.

Variables	OR(95%CI) *p*-value			
	HTN	CHD	MI	HF
Age(years old)				
<50	1.0	1.0	1.0	1.0
≥50	6.76 (6.41, 7.13) <0.001	16.62 (13.08, 21.12) <0.001	10.36 (8.50, 12.63) <0.001	8.76 (7.06, 10.87) <0.001
Sex, n (%)				
Male	1.0	1.0	1.0	1.0
Female	1.00 (0.96, 1.05) 0.854	0.44 (0.39, 0.50) <0.001	0.47 (0.41, 0.53) <0.001	0.72 (0.63, 0.82) <0.001
Level of education, n (%)				
Less than 9th grade	1.0	1.0	1.0	1.0
9-11th grade	0.79 (0.71, 0.87) <0.001	0.79 (0.64, 0.98) 0.029	0.72 (0.58, 0.88) 0.001	0.84 (0.67, 1.05) 0.123
High school graduate/ GED or equivalent	0.77 (0.70, 0.84) <0.001	0.73 (0.60, 0.89) 0.002	0.66 (0.55, 0.79) <0.001	0.65 (0.52, 0.80) <0.001
Some college or AA degree	0.65 (0.61, 0.71) <0.001	0.59 (0.49, 0.72) <0.001	0.51 (0.43, 0.62) <0.001	0.54 (0.44, 0.67) <0.001
College graduate or above	0.50 (0.45, 0.54) <0.001	0.57 (0.46, 0.69) <0.001	0.39 (0.32, 0.48) <0.001	0.26 (0.20, 0.34) <0.001
BMI (kg/m^2)				
<25	1.0	1.0	1.0	1.0
25–30	1.86 (1.75, 1.99) <0.001	1.45 (1.24, 1.70) <0.001	1.32 (1.12, 1.56) <0.001	1.36 (1.12, 1.66) 0.002
≥30	3.05 (2.87, 3.25) <0.001	1.62 (1.39, 1.89) <0.001	1.73 (1.48, 2.01) <0.001	2.20 (1.84, 2.64) <0.001
Smoking history, n (%)				
No	1.0	1.0	1.0	1.0
Yes	1.33 (1.27, 1.40) <0.001	2.19 (1.94, 2.47) <0.001	2.40 (2.12, 2.71) <0.001	2.00 (1.74, 2.30) <0.001
Drinking history, n (%)				
No	1.0	1.0	1.0	1.0
Yes	0.70 (0.66, 0.73) <0.001	0.87 (0.77, 0.98) 0.023	0.95 (0.84, 1.07) 0.390	0.69 (0.60, 0.80) <0.001
Diabetes, n (%)				
No	1.0	1.0	1.0	1.0
Yes	4.85 (4.52, 5.18) <0.001	4.30 (3.80, 4.86) <0.001	3.95 (3.49, 4.46) <0.001	4.65 (4.04, 5.36) <0.001
IGT + IFG	2.18 (1.99, 2.38) <0.001	2.08 (1.69, 2.55) <0.001	1.71 (1.38, 2.13) <0.001	1.69 (1.30, 2.20) <0.001
Aspirin use, n (%)				
No	1.0	1.0	1.0	1.0
Yes	5.61 (4.31, 7.30) <0.001	5.50 (4.23, 7.27) <0.001	5.50 (4.20, 7.21) <0.001	4.49 (3.25, 6.20) <0.001
Poverty to income ratio, n (%)				
0.0–1.0	1.0	1.0	1.0	1.0
1.01–3.0	1.14 (1.07, 1.21) 0.001	1.21 (1.03, 1.43) 0.020	0.92 (0.79, 1.06) 0.258	0.97 (0.83, 1.15) 0.752
>3.0	0.92 (0.86, 0.99) 0.020	1.05 (0.89, 1.24) 0.587	0.59 (0.50, 0.70) <0.001	0.43 (0.35, 0.52) <0.001
Physical activity (MET-min/wk), n (%)				
<500	1.0	1.0	1.0	1.0
500–999	0.70 (0.65, 0.76) <0.001	0.88 (0.73, 1.06) 0.174	0.74 (0.61, 0.89) 0.002	0.50 (0.39, 0.64) <0.001
≥1,000	0.58 (0.55, 0.61) <0.001	0.59 (0.52, 0.67) <0.001	0.51 (0.45, 0.58) <0.001	0.37 (0.32, 0.43) <0.001
Total energy intake (kcal/d), (SD)	1.00 (1.00,1.00) <0.001	1.00 1.00,1.00) <0.001	1.00 (1.00,1.00) <0.001	1.00(1.00,1.00) <0.001
TC(mmol/L)	1.08 (1.05, 1.10) <0.001	0.60 (0.56, 0.63) <0.001	0.66 (0.63, 0.71) <0.001	0.68 (0.64, 0.73) <0.001
TG(mmol/L)	1.18 (1.15, 1.20) <0.001	1.06 (1.03, 1.08) <0.001	1.05 (1.02, 1.08) <0.001	1.06 (1.03, 1.09) <0.001
HDL(mmol/L)	0.92 (0.87, 0.97) 0.005	0.42 (0.36, 0.50) <0.001	0.50 (0.43, 0.59) <0.001	0.45 (0.38, 0.55) <0.001

hypertension, coronary heart disease, myocardial infarction and heart failure were 0.83 (0.80, 0.85; $p < 0.001$), 0.91 (0.85, 0.98; $p = 0.019$), 0.86 (0.79, 0.93; $p < 0.001$) and 0.69 (0.63, 0.77; $p < 0.001$) (Table 4; Figure 2). In the model adjusted for all covariates, vitamin B1 intake was significantly negatively associated with hypertension and heart failure, with odds ratios of 0.95 (0.90, 0.99; $p = 0.018$) and 0.83 (0.72, 0.95; $p = 0.006$), respectively, but not with coronary heart disease and myocardial infarction (Table 4; Figure 3). Restricted cubic spline analysis showed a nonlinear inverse association between vitamin B1 intake and hypertension and a linear association with heart failure (Figure 4).

Three multivariate cox regression models were developed: Model 1, unadjusted; Model 2, adjusted for age, sex, and level of education; Model 3, adjusted for all covariates listed in Table 1. In unadjusted models, vitamin B1 intake was inversely correlated with all-cause mortality and cardiovascular mortality, with hazard ratios of 0.83 (0.80, 0.87; $p < 0.001$)

TABLE 3 Effects of various factors on all cause mortality and cardiovascular mortality by univariate analysis.

Variables	HR(95%CI) *p*-value	
	ACM	CVD
Age(years old)		
<50	1.0	1.0
≥50	10.99 (9.91, 12.20) <0.001	13.92 (11.08, 17.48) <0.001
Sex, n (%)		
Male	1.0	1.0
Female	0.74 (0.70, 0.79) <0.001	0.66 (0.58, 0.75) <0.001
Level of education, n (%)		
Less than 9th grade	1.0	1.0
9-11th grade	0.77 (0.70, 0.86) <0.001	0.73 (0.60, 0.90) 0.003
High school graduate/ GED or equivalent	0.69 (0.63, 0.76) <0.001	0.68 (0.56, 0.82) <0.001
Some college or AA degree	0.49 (0.45, 0.55) <0.001	0.46 (0.37, 0.56) <0.001
College graduate or above	0.40 (0.35, 0.44) <0.001	0.39 (0.32, 0.49) <0.001
BMI (kg/m^2)		
<25	1.0	1.0
25–30	1.02 (0.94, 1.10) 0.694	1.32 (1.12, 1.55) 0.001
≥30	0.97 (0.90, 1.05) 0.433	1.24 (1.05, 1.45) 0.011
Smoking history, n (%)		
No	1.0	1.0
Yes	1.81 (1.69, 1.93) <0.001	1.64 (1.45, 1.87) <0.001
Drinking history, n (%)		
No	1.0	1.0
Yes	0.79 (0.74, 0.85) <0.001	0.73 (0.64, 0.83) <0.001
Diabetes, n (%)		
No	1.0	1.0
Yes	3.09 (2.88, 3.31) <0.001	3.70 (3.23, 4.24) <0.001
IGT + IFG	1.97 (1.76, 2.20) <0.001	2.05 (1.63, 2.58) <0.001
Aspirin use, n (%)		
No	1.0	1.0
Yes	2.88 (2.40, 3.45) <0.001	3.10 (2.18, 4.41) <0.001
Poverty to income ratio, n (%)		
0.0–1.0	1.0	1.0
1.01–3.0	1.18 (1.09, 1.28) <0.001	1.23 (1.04, 1.45) 0.015
>3.0	0.65 (0.59, 0.71) <0.001	0.65 (0.54, 0.78) <0.001
Physical activity (MET-min/wk), n (%)		
<500	1.0	1.0
500–999	0.62 (0.55, 0.68) <0.001	0.68 (0.56, 0.83) <0.001
≥1,000	0.53 (0.49, 0.57) <0.001	0.44 (0.38, 0.52) <0.001
Total energy intake (kcal/d), (SD)	1.00(1.00,1.00) <0.001	1.00(1.00,1.00) <0.001
TC(mmol/L)	0.98 (0.96, 1.01) 0.290	0.96 (0.90, 1.02) 0.187
TG(mmol/L)	1.03 (1.01, 1.04) <0.001	1.02 (0.98, 1.05) 0.303
HDL(mmol/L)	1.10 (1.02, 1.19) 0.012	0.88 (0.75, 1.04) 0.138

and 0.76 (0.70, 0.83; $p < 0.001$), respectively (Table 5; Figure 5). In the model adjusted for all covariates, vitamin B1 intake was significantly negatively associated with cardiovascular mortality, with a hazard ratio of 0.84 (0.74, 0.94; $p = 0.003$) (Table 5; Figure 6). Restricted cubic spline analysis showed a linear association with cardiovascular mortality and the dose–response correlation showed that the prevalence of cardiovascular diseases and mortality decreased with increasing vitamin B1 intake (Figure 4).

TABLE 4 Multivariable associations of vitamin B1 with cardiovascular diseases.

	Model 1	Model 2	Model 3
	OR(95% CI) *p* value	OR(95% CI) *p* value	OR(95% CI) *p* value
HTN			
Vitamin B1 (mg/d)	**0.83 (0.80, 0.85) < 0.001**	**0.94 (0.91, 0.98) 0.002**	**0.95 (0.90, 0.99) 0.018**
Vitamin B1 (mg/d) quartiles			
Q1	**1.0**	**1.0**	1.0
Q2	**0.88 (0.82, 0.94) 0.001**	**0.85 (0.79, 0.92) < 0.001**	**0.83 (0.76, 0.90) < 0.001**
Q3	**0.82 (0.77, 0.88) < 0.001**	**0.87 (0.80, 0.94) < 0.001**	**0.84 (0.77, 0.92) < 0.001**
Q4	**0.67 (0.63, 0.72) < 0.001**	**0.85 (0.78, 0.92) < 0.001**	**0.84 (0.76, 0.92) < 0.001**
CHD			
Vitamin B1 (mg/d)	**0.91 (0.85, 0.98) 0.019**	0.94 (0.86, 1.02) 0.134	1.02 (0.92, 1.14) 0.660
Vitamin B1 (mg/d) quartiles			
Q1	1.0	1.0	1.0
Q2	1.10 (0.93, 1.29) 0.261	1.01 (0.86, 1.20) 0.869	1.05 (0.88, 1.26) 0.564
Q3	1.05 (0.89, 1.24) 0.544	0.96 (0.80, 1.14) 0.604	1.06 (0.88, 1.28) 0.532
Q4	0.93 (0.79, 1.10) 0.401	0.94 (0.78, 1.12) 0.487	1.14 (0.91, 1.41) 0.252
MI			
Vitamin B1 (mg/d)	**0.86 (0.79, 0.93) < 0.001**	**0.88 (0.81, 0.96) 0.004**	1.00 (0.90, 1.11) 0.999
Vitamin B1 (mg/d) quartiles			
Q1	1.0	1.0	1.0
Q2	0.92 (0.78, 1.07) 0.269	0.85 (0.72, 1.00) 0.054	0.89 (0.75, 1.06) 0.201
Q3	**0.83 (0.71, 0.98) 0.024**	**0.76 (0.65, 0.91)0.002**	0.87 (0.72, 1.04) 0.126
Q4	**0.71 (0.60, 0.84) < 0.001**	**0.71 (0.59, 0.85) < 0.001**	0.88 (0.71, 1.09) 0.233
HF			
Vitamin B1 (mg/d)	**0.69 (0.63, 0.77) < 0.001**	**0.76 (0.68, 0.85) < 0.001**	**0.83 (0.72, 0.95) 0.006**
Vitamin B1 (mg/d) quartiles			
Q1	1.0	1.0	1.0
Q2	0.94 (0.79, 1.12) 0.494	0.93 (0.78, 1.11) 0.403	0.97 (0.81, 1.17) 0.771
Q3	**0.69 (0.57, 0.83) < 0.001**	**0.71 (0.58, 0.86) < 0.001**	**0.79 (0.64, 0.98) 0.031**
Q4	**0.54 (0.44, 0.66) < 0.001**	**0.64 (0.51, 0.79) < 0.001**	**0.77 (0.60, 1.00) 0.048**

Model 1: No adjustments made for confounding factors.
Model 2: Adjustments made for age, sex, level of education.
Model 3: Adjustments made for age, sex, level of education, BMI, smoking history, drinking history, aspirin use, diabetes mellitus, poverty to income ratio, physical activity, Total energy intake, TC, TG, HDL.
Bold values represent significant associations.

3.5. Subgroup and sensitivity analyses

To determine whether the association between vitamin B1 intake and cardiovascular disease, all-cause mortality, and cardiovascular mortality varied by age, sex, BMI, smoking history, drinking history and blood lipid profile, stratified analyses were performed. When stratified by age, vitamin B1 intake was more significantly associated with hypertension and heart failure in patients aged ≥50 years, with odds ratios of 0.94 (0.91, 0.98; $p = 0.002$) and 0.79 (0.69, 0.91; $p = 0.001$), respectively(Supplementary Table S1). Similarly, the subgroups including males (Supplementary Table S2), those with a $25 \leq \text{BMI} < 30$ (Supplementary Table S3), smokers (Supplementary Table S4), drinkers (Supplementary Table S5), and those with dyslipidemia (Supplementary Table S6) were more strongly associated with hypertension, heart failure and mortality. The correlations of vitamin B1 intake in Q4 were higher than those in Q1. In the sensitivity analysis, after further adjustment for energy intake at baseline, these associations remained similar and statistically significant (Supplementary Table S7).

4. Discussion

In a nationally representative sample of US adults, vitamin B1 intake were associated with risk for hypertension, heart failure and cardiovascular mortality, whereas were not associated with coronary heart disease, myocardial infarction and all-cause mortality. The study also provided trends in vitamin B1 intake, prevalence of cardiovascular-related disease, and cardiovascular and all-cause

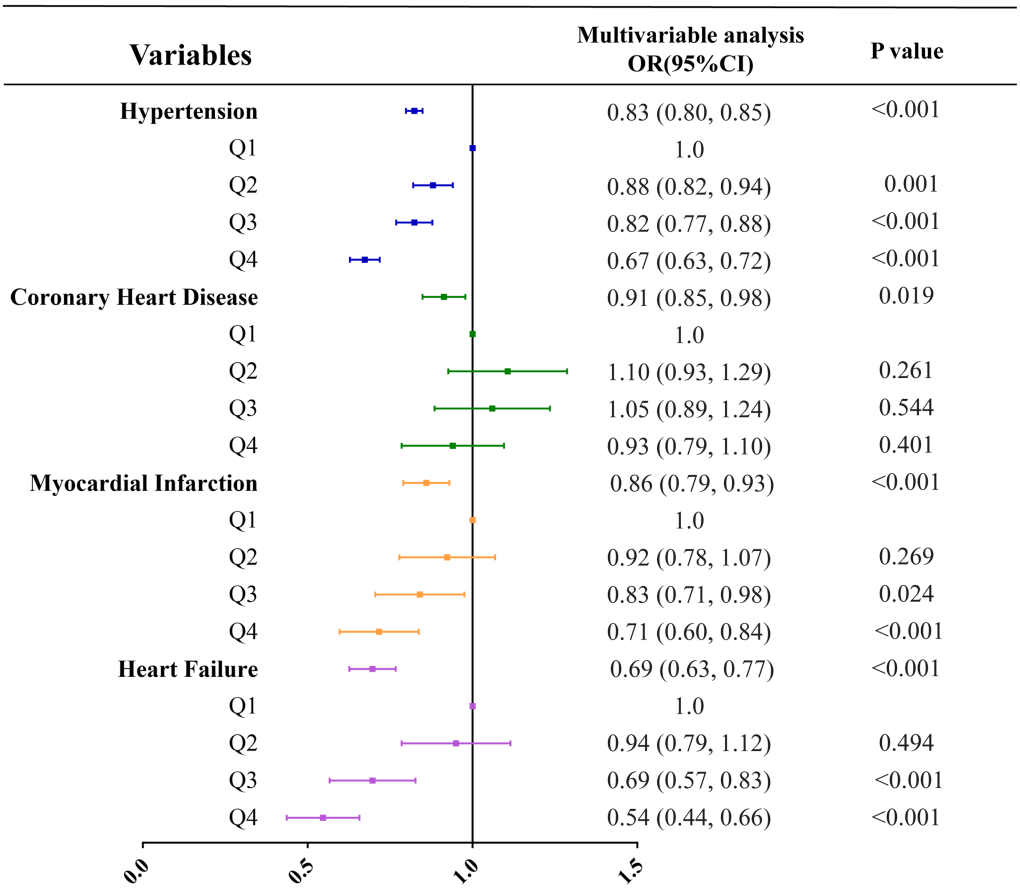

FIGURE 2
Correlation analysis between vitamin B1 and various types of cardiovascular diseases under unadjusted models.

mortality among U.S. adults from 1999 to 2018. From 1999 to 2018, the all-cause mortality of adults in the United States showed a significant downward trend, but the mortality and prevalence of cardiovascular disease showed an upward trend.

This study found a linear negative correlation between vitamin B1 intake and heart failure and cardiovascular mortality. Using restricted spline analysis, vitamin B1 intake was non-linearly associated with hypertension. In addition, the correlation was more significant in the high-intake group of vitamin B1 compared with the low-intake group. The findings of this research were consistent with those of a previous study that reported that daily intake of thiamine could reverse the risk of hypertension (OR 0.95; 95% CI 0.90, 0.99), myocardial infarction or angina pectoris (OR 0.84; 95% CI 0.74, 0.95), type 2 diabetes (OR 0.86; 95% CI 0.81, 0.93), depression (OR 0.90; 95% CI 0.83, 0.97) and dyslipidemia (OR 0.90; 95% CI 0.86, 0.95) (18). Vitamin B1 could be a potential low-cost and effective intervention, which could improve cardiac function (29, 30) and hemodynamic characteristics (31), as well as reduce systemic vascular resistance (32). In a previous study, thiamine, mediated by glucose and insulin, had a protective effect on the propagation of human smooth muscle cells of the inferior artery, which were reported to be crucial in the formation of atherosclerotic plaque (33). A trial conducted on 30 hyperglycemic participants (10 subjects each grouped into healthy, impaired glucose tolerance, and T2D) by Arora et al. examined the impact on endothelium-dependent vasodilation after using 100 mg of intravenous thiamine. All the groups depicted improvement. Arora et al. suggested that since the stability of endothelial function was closely correlated to the occurrence of coronary atherosclerotic heart disease, therefore, thiamine should be administered regularly to enhance endothelial function and slow the development of atherosclerosis (34). In patients with stable HF, several researchers had studied whether thiamine supplementation could improve their cardiac health. The randomized, double-blind controlled trial conducted by Schoenenberger et al. (35) demonstrated that the left ventricular ejection fraction (LVEF) was 3.30% (95% CI 0.63–5.97%) higher in patients taking thiamine than in those receiving a placebo. Furthermore, Shimon et al. (36) found in a double-blind randomized study that LVEF was increased by 2.20% in the group taking thiamine than in the placebo group, but their improvement was not statistically significant. Dinicolantonio et al. (15) performed mate analysis and the data indicated that compared with the placebo group, thiamine supplementation significantly improved LVEF (3.28, 95% CI 0.64–5.93%), which was important for the treatment of heart failure and prevention of recurrence. Moreover, it might be correlated to the improvement of energy metabolism in cardiomyocytes. Additionally, Jain et al. (37) performed a meta-analysis and depicted that deficiency of thiamine was more prevalent in patients with HF than in healthy individuals. Although thiamine supplementation is generally not recommended in patients with systolic heart failure, studies have shown that it can have a positive effect on LVEF in patients with systolic heart failure. Alattas et al. (38) found that supplementation of thiamine (100 mg/d) for 6 months could

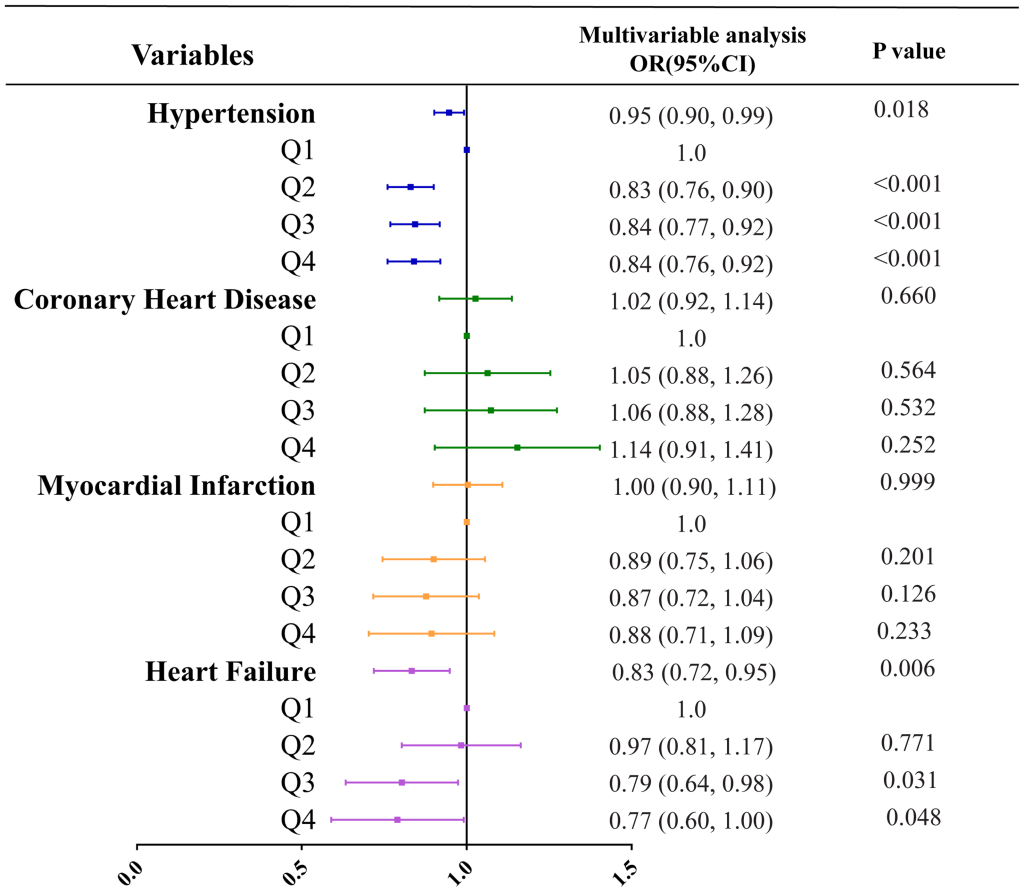

FIGURE 3
Correlation analysis between vitamin B1 and various types of cardiovascular diseases under all covariates adjusted models.

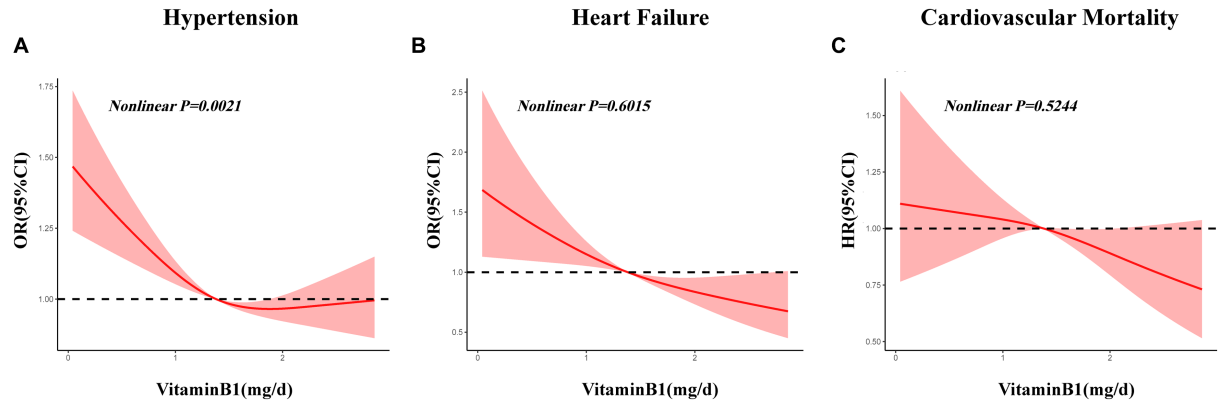

FIGURE 4
Restricted cubic spline analysis of associations between vitamin B1 intake and hypertension, heart failure and cardiovascular mortality. **(A)** Restricted cubic spline analysis of association between vitamin B1 intake and hypertension; **(B)** Restricted cubic spline analysis of association between vitamin B1 intake and heart failure; **(C)** Restricted cubic spline analysis of association between vitamin B1 intake and cardiovascular mortality.

significantly control and alleviate cardiovascular complications in diabetic patients. Common risk factors for thiamine deficiency in patients with heart failure include malnutrition, increased metabolic status, the severity of heart failure, and the use of diuretics (39). In a meta-analysis, LVEF was significantly improved by 3.28% (95% CI 0.64–5.93) in 34 patients treated with thiamine but the meta-analysis was limited by the quality of the original study and the small sample size (39). These findings suggested that thiamine had a protective effect against vascular diseases, of which heart failure might be more prominent, and are consistent with the results of the present study.

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TABLE 5 Multivariable associations of vitamin B1 with all-cause mortality and cardiovascular mortality.

	Model 1 HR(95% CI) *p* value	Model 2 HR(95% CI) *p* value	Model 3 HR(95% CI) *p* value
ACM			
Vitamin B1 (mg/d)	**0.83 (0.80, 0.87) < 0.001**	**0.95 (0.91, 0.99) 0.019**	0.95 (0.90, 1.01) 0.075
Vitamin B1 (mg/d) quartiles			
Q1	**1.0**	1.0	1.0
Q2	**0.88 (0.80, 0.95) 0.002**	**0.88 (0.81, 0.96) 0.004**	**0.88 (0.80, 0.96) 0.003**
Q3	**0.87 (0.80, 0.95) 0.002**	**0.90 (0.82, 0.98) 0.019**	0.92 (0.83, 1.01) 0.069
Q4	**0.67 (0.62, 0.74) < 0.001**	**0.85 (0.78, 0.94) 0.001**	0.87 (0.78, 0.97) 0.015
CVDM			
Vitamin B1 (mg/d)	**0.76 (0.70, 0.83) < 0.001**	**0.85 (0.77, 0.93) < 0.001**	**0.84 (0.74, 0.94) 0.003**
Vitamin B1 (mg/d) quartiles			
Q1	1.0	1.0	1.0
Q2	0.90 (0.76, 1.06) 0.200	0.89 (0.75, 1.05) 0.167	0.88 (0.74, 1.05) 0.144
Q3	0.86 (0.73, 1.02) 0.092	0.86 (0.72, 1.02) 0.093	0.88 (0.72, 1.06) 0.171
Q4	**0.58 (0.48, 0.70) < 0.001**	**0.71 (0.58, 0.86) < 0.001**	**0.72 (0.57, 0.90) 0.005**

Model 1: No adjustments made for confounding factors.
Model 2: Adjustments made for age, sex, level of education.
Model 3: Adjustments made for age, sex, level of education, BMI, smoking history, drinking history, aspirin use, diabetes mellitus, poverty to income ratio, physical activity, Total energy intake, TC, TG, HDL.
Bold values represent significant associations.

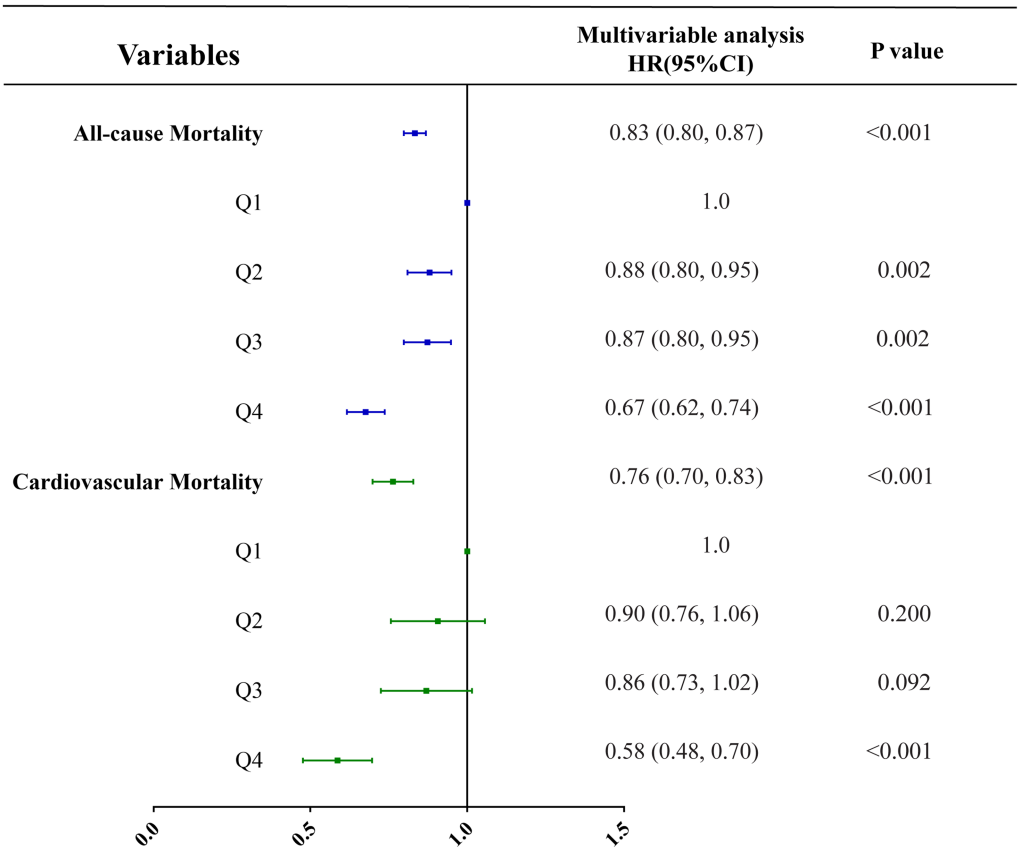

FIGURE 5
Correlation analysis between vitamin B1 and all-cause mortality and cardiovascular mortality under unadjusted models.

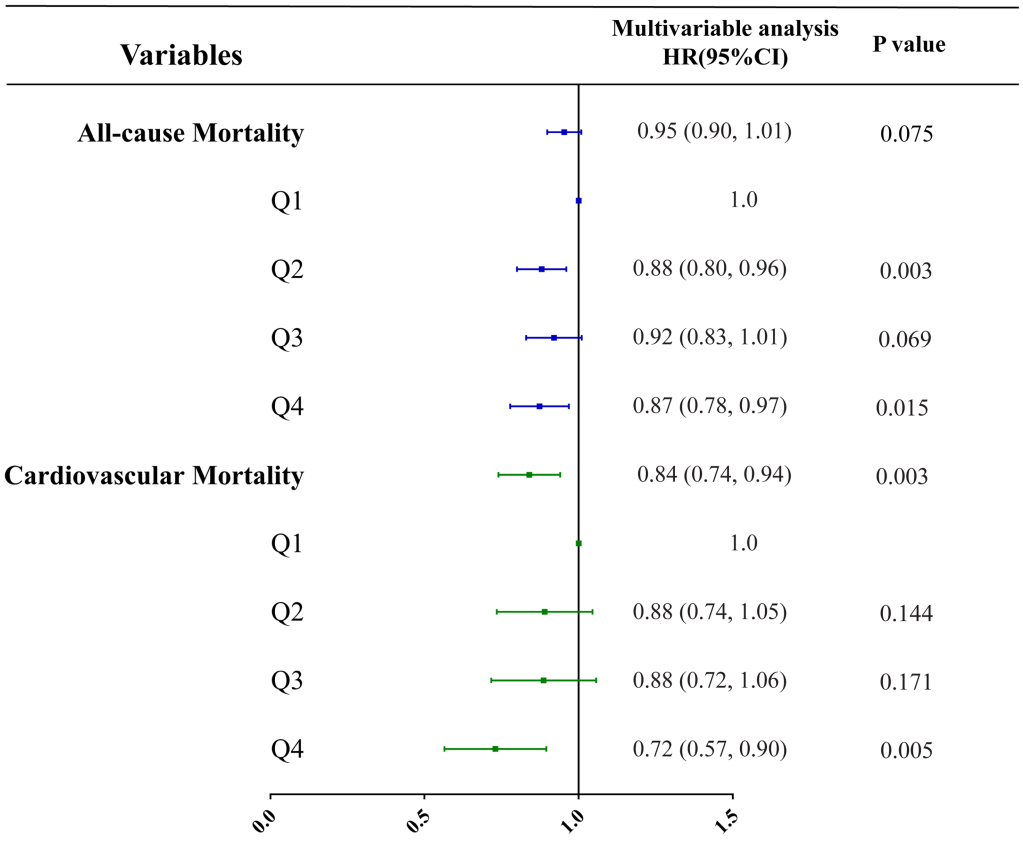

FIGURE 6
Correlation analysis between vitamin B1 and all-cause mortality and cardiovascular mortality under all covariates adjusted models.

It is worth noting that the associations of interest observed in the aforementioned studies were similar to those in the current analyses. However, the current study was based on a much larger sample size, providing greater statistical power for detecting modest associations. Furthermore, the current study conducted numerous subgroup analyses, exploring the impact of vitamin B1 intake on different populations. The findings indicated that vitamin B1 (thiamine) was more significantly correlated with heart failure in elderly people (whose age $\geq$ 50 years old). This could be due to the increased risk of micronutrient deficiency in the elderly caused by pathophysiological changes (40). The elderly are more susceptible to thiamine deficiency due to nutritional challenges and various potential comorbidities (41). Prior systematic reviews and meta-analyses of micronutrient intake in community-dwelling older adults showed that thiamine deficiency in food intake in older adults was mainly due to absorption and utilization factors (42). The subgroup analysis based on body mass index indicated that the effect of dietary vitamin B1 intake on heart failure was more significant in overweight people with a BMI of 25–30 kg/m^2. The possible explanation for this observation is that in individuals with high intake and obesity, glucose consumes most of the serum thiamine levels. Additionally, the surgical treatment of obesity may also impair thiamine absorption. Therefore, it appears that extra thiamine intake is needed for patients with any level of obesity (12). One study showed that smokers suffer from vitamin B1 deficiency more often than nonsmokers. Passive smoking can also affect their eating habits and nutritional status. Therefore, the poor nutritional status of smokers may lead to the appearance or deterioration of various conditions (cardiovascular disease, cancer, cataract, osteoporosis, etc.) related to smoking. Therefore, nutritional monitoring and correction of nutritional deficiencies may be beneficial to the health of smokers and passive smokers, which should be considered in the future (43). In addition, excessive alcohol consumption can affect the cellular transport of vitamin B1 and its intracellular phosphorylation, thereby inhibiting the absorption of vitamins and nutrients. This effect may be crucial for inducing specific nutritional deficiencies (such as thiamine) in alcoholics (13). The results of such prior experiments were consistent with those of our subgroup analysis study. The results of the current study indicated that vitamin B1 was more significantly associated with cardiovascular disease in dyslipidemia subjects. In a cross-sectional study involving diabetic patients, plasma thiamine levels were negatively correlated with TG and LDL levels and positively correlated with HDL levels in patients with DM (44). In healthy older adults, plasma thiamine concentrations were inversely related to total cholesterol concentrations (45).

This study was limited in several aspects. The studies on cardiovascular diseases were based on a cross-sectional design, which does not allow for causal inference. Hence, further prospective studies are needed to confirm these conclusions. Moreover, the number of participants with excessive dietary vitamin B1 intake was small, making it difficult to assess the correlation between excessive intake and cardiovascular diseases. Furthermore, the inclusion criteria for cardiovascular diseases relied on self-reported medical history, and its

impact on subtypes and stages of hypertension, coronary heart disease, myocardial infarction, and heart failure was unknown. Therefore, larger clinical studies on different stages and sub-types of cardiovascular diseases are required in the future. Finally, although this analytical model included many covariates, the potential impact of unknown confounders cannot be fully ruled out.

5. Conclusion

In this large cohort study, it was observed that higher vitamin B1 intake was associated with a trend toward lower risk of hypertension, heart failure and cardiovascular mortality. In addition, vitamin B1 intake was found to be more protective and pronounced in elderly-aged men, overweight individuals, smokers, drinkers and patients with dyslipidemia. Although the observed correlations require further validation in other populations and intervention studies targeting CVD risk factors, this study highlights the role of vitamin B1 in mitigating cardiovascular diseases. We aim to draw the attention of health policymakers and public health practitioners to include vitamin B1 in an overall healthy basal diet to promote the prevention of cardiovascular diseases.

Data availability statement

A full list of data sets supporting the results in this research article can be found at: https://wwwn.cdc.gov/nchs/nhanes/.

Ethics statement

The NCHS Ethics Review Board approved all NHANES programs, and study members or their agents provided informed consent prior to participation (22). Detailed information about NHANES can be found at www.cdc.gov/nchs/nhanes/.

Author contributions

HW proposed the strategy, implemented the extraction, collation of the data, and drafted the paper. XN and NS validated and analyzed the data. RZ and QW produced the figures. LM, YL, and WZ revised and finalized 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/fnut.2023.1175961/full#supplementary-material

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Association between iodine nutrition and cervical lymph node metastasis of papillary thyroid microcarcinoma

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We aimed to investigate the association between iodine intake and nodal metastasis stratified by central lymph node metastasis (CLNM) and lateral lymph node metastasis (LLNM) of papillary thyroid microcarcinoma (PTMC). Urinary iodine concentration (UIC) and clinicopathological characteristics were used to identify factors associated with CLNM and LLNM using logistic regression analysis. A sum of 3,858 PTMC patients were enrolled. The median UIC (MUI) of patients with CLNM or LLNM was not statistically different from those without nodal metastasis. Male patients had a higher MUI than females (183.4 μg/L vs. 173.6 μg/L). Female patients with extracapsular extension had a higher MUI than those without it (210.0 μg/L vs. 172.1 μg/L). Male patients with LLNM had a significantly lower MUI than those without LLNM (134.7 μg/L vs. 187.9 μg/L). Female patients with more than adequate iodine intake were more likely to present with CLNM and extrathyroidal extension than those with adequate iodine intake with an odds ratio (95% confidence interval) of 1.23 (1.01–1.51) and 1.59 (1.09–2.32) after adjustment. Iodine nutrition was not found to be associated with LLNM. In addition, patients with a younger age, larger tumors, extrathyroidal extension, and intrathyroidal spread were more likely to be CLNM, whereas nodular goiter presented with a protective factor; CLNM was the only factor associated with LLNM of PTMC in both genders. In conclusion, iodine nutrition has a much closer association with female than male patients, and high iodine intake may be associated with CLNM and extrathyroidal extension in female PTMC patients.

KEYWORDS

papillary thyroid microcarcinoma, iodine, iodine nutrition, urinary iodine concentration, lymph node metastasis

Introduction

The overall incidence of thyroid cancer increased by 3% annually in the United States from 1974 to 2013 (1). In China, the age-standardized incidence of thyroid cancer was $3.21/10^5$ in 2005 and $9.61/10^5$ in 2015 (2). The increasing incidence of thyroid cancer is due in large part to the epidemiology of surveillance and overdiagnosis but that there also appears to be a true increase in new cases of thyroid cancer (3). The increased incidence mainly attributes to papillary thyroid cancer (PTC), especially papillary thyroid microcarcinoma (PTMC) with its maximum tumor diameter ≤1 cm (4).

Iodine is an indispensable element for synthesis of thyroxine and is closely associated with thyroid diseases (5). High iodine intake was associated with the occurrence of Hashimoto thyroiditis, nodular goiter, and hyperthyroidism (6). However, the association between iodine intake and thyroid cancer remains controversial (7). Some studies reported that mild iodine deficiency may contribute to the exceptionally high incidence of thyroid cancer (8), whereas others reported that excessive iodine nutrition was associated with PTC and PTMC occurrence compared with adequate iodine intake (9, 10). We previously found that high iodine intake was not associated with PTC incidence in the general population and in patients with thyroid nodules (11). However, more than adequate iodine intake was independently associated with a larger tumor size of PTC patients (11). Thus, we expected that high iodine intake may be associated with the progression of PTC.

Central lymph node metastasis (CLNM) was not uncommon in PTMC patients with its incidence up to 51.7% (12). In addition, approximately 4.4% of patients with PTMC presented with lateral lymph node metastasis (LLNM) at presentation (13). Accumulating evidence suggested that cervical lymph node metastasis was a risk factor for PTC recurrence (14). Some scholars hold that low iodine intake was inversely associated with CLNM of PTC, particularly for PTMC (15). However, others reported that high iodine nutrition seemed to be associated with CLNM of PTC (16). We previously found that excessive iodine intake was marginally associated with CLNM in female PTC patients after defining CLNM as metastatic lymph nodes ≥2 (12). However, we failed to investigate the effect of iodine nutrition on PTMC progression.

Considering the limited and inconsistent results, we aimed to comprehensively investigate the association between iodine nutrition and clinicopathologic characteristics of PTMC. We divided patients into CLNM and LLNM subgroups and explored their associations with urinary iodine concentration (UIC). This study may provide new evidence of the effect of iodine nutrition on PTMC progression.

Patients and methods

Study population

Patients with PTMC were retrospectively enrolled from 2013 to 2018 in Union hospital, Wuhan, China. This study was approved by the Ethics Committee of Union hospital. The enrolled patients underwent thyroidectomy with central lymph node dissection (CLND), and lateral lymph nodes dissection (LLND) or not for the first time. CLND was routinely performed on PTMC patients. LLND was performed based on preoperative imaging suspicious for malignancy and/or fine-needle aspiration cytology preoperatively. Solitary focus means only one tumor in the thyroid, whereas multiple foci mean two or more foci limited to the thyroid. Bilaterality is defined as the presence of PTC foci in the left and right lobes of the thyroid. Capsular invasion means that one tumor invades the thyroid capsule but does not penetrate it, whereas one tumor penetrates the capsule into the strap muscle or perithyroidal fibrofatty tissues, which will be defined as extracapsular or extrathyroidal extension. Intrathyroidal spread means that the primary tumor spreads to the other parts of the thyroid through intrathyroidal lymph vessels. Intraoperative frozen section was routinely performed to determine thyroid malignancy. The final pathological types, tumor number, intrathyroidal spread, capsular invasion and extrathyroidal extension, central lymph node metastasis (CLNM), and lateral lymph node metastasis (LLNM) were determined by postoperative specimen. Hematoxylin and eosin staining with or without immunohistochemistry was performed to determine the pathology or nodal metastasis of PTMC. The pathological types included PTC, PTC and nodular goiter, PTC and Hashimoto's thyroiditis, and PTC and nodular goiter, and Hashimoto's thyroiditis. Patients with iodine pretreatment for hyperthyroidism, pathology other than PTC, and reoperation for PTC were excluded.

Iodine nutritional status

Every patient was asked to collect a fasting urine specimen (6). Urinary iodine concentration (UIC) was measured using Direction of Quantitative Test Kit type AR for urinary iodine (Wuhan, China) as previously described (11). Median UIC (MUI) was recommended to assess iodine nutritional status in population according to World Health Organization, and iodine nutritional status was divided into four categories: iodine deficiency (UIC <100 μg/L), adequate iodine intake (UIC: 100–199 μg/L), more than adequate iodine intake (UIC: 200–299 μg/L), and excessive iodine intake (UIC ≥300 μg/L).

Statistical analysis

Variables with skewed distribution were expressed as median (upper and lower quartile) and analyzed using the Mann–Whitney *U* test. Categorical data were analyzed with the chi-square test. Multivariate logistic regression analysis was performed to identify factors associated with nodal metastasis. All the analyses were performed using SPSS 22.0 software. A two-sided *P* value <0.05 was considered significant.

Results

The iodine nutrition of PTMC patients

Of the 3,858 patients enrolled, iodine deficiency was more common in female than male PTMC patients (20.0% vs. 13.4%).

Adequate iodine intake accounted for 39.7% of the total population. More than adequate and excessive iodine intake accounted for 41.4% (Figure 1).

The MUI was significantly higher in males than in females (MUI: 183.4 μg/L vs. 173.6 μg/L; P = 0.038). In addition, patients with extrathyroidal extension had a significantly higher MUI than those without it (MUI: 199.7 μg/L vs. 174.3 μg/L; P = 0.024). Patients with a younger age (<55 years) and intrathyroidal spread had a marginally higher MUI than the corresponding counterparts. The MUI in the CLNM and non-CLNM groups was 177.9 μg/L and 173.9 μg/L, respectively (P = 0.554). The MUI in the LLNM and non-LLNM groups of PTMC patients was 157.9 μg/L and 176.5 μg/L, respectively (P = 0.132) (Table 1).

We then evaluated the iodine nutrition in male and female PTMC patients, separately. We found that male patients with LLNM had a significantly lower MUI than those without LLNM (MUI: 134.7 μg/L vs. 187.9 μg/L; P = 0.002). In addition, female patients with extrathyroidal extension had a significantly higher MUI than those without it (MUI: 210.0 μg/L vs. 172.1 μg/L; P = 0.010) (Table 2).

We divided UIC into four categories: iodine deficiency, adequate iodine intake, more than adequate iodine intake, and excessive iodine intake. Similarly, we found that iodine nutrition status was not significantly different between patients with CLNM or LLNM and those without nodal metastasis (Supplementary Table 1). In addition, males were more likely to be adequate iodine intake and more than adequate iodine intake compared with females (70.1% vs. 63.0%). Additionally, patients with extrathyroidal extension were more likely to be more than adequate iodine intake and excessive iodine intake than those without it (50% vs. 40.9%) (Supplementary Table 1).

Association between iodine intake and CLNM in male PTMC

In the univariate analysis, iodine deficiency and more than adequate and excessive iodine intake were not associated with CLNM compared with adequate iodine intake. After adjustment, iodine nutrition status was not found to be associated with CLNM in male PTMC patients. In the multivariate model, a younger age, larger tumor size, extrathyroidal extension, intrathyroidal spread, and LLNM were associated with CLNM. Of note, PTMC patients accompanied by nodular goiter were inversely associated with CLNM compared with PTMC alone (Table 3).

Association between iodine intake and CLNM in female PTMC

In the univariate analysis, more than adequate iodine intake was associated with CLNM compared with adequate iodine intake with OR (95% CI) of 1.25 (1.04–1.50) in female PTMC patients. After adjustment, patients with more than adequate iodine intake were associated with CLNM than those with adequate iodine intake with OR (95% CI) of 1.23 (1.01–1.51). In addition, a younger age, lager tumor size, bilaterality, capsular invasion, intrathyroidal spread, and LLNM were positively associated with CLNM in female PTMC after adjustment. Of note, PTMC patients accompanied by nodular goiter, or nodular goiter and Hashimoto's thyroiditis were inversely associated with CLNM compared with PTMC alone (Table 4).

Association between iodine intake and LLNM in male PTMC

In the univariate analysis, more than adequate iodine intake was inversely associated with LLNM compared with adequate iodine intake with OR (95% CI) of 0.45 (0.21–0.96) in male PTMC patients. After adjustment, iodine nutrition status was not found to be associated with LLNM in male PTMC patients. In the multivariate model, results showed that multifocality, CLNM, and PTMC and nodular goiter, and Hashimoto's thyroiditis were independently associated with LLNM in male PTMC patients (Table 5).

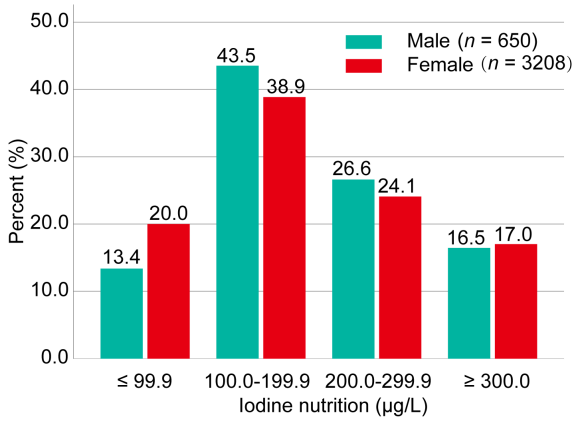

FIGURE 1
Iodine status of the study population. Iodine nutrition was classified into four categories: iodine deficiency (UIC <100.0 μg/L), adequate iodine intake (UIC: 100.0–199.9 μg/L), more than adequate iodine intake (UIC: 200.0–299.9), and excessive iodine intake (UIC ≥300.0 μg/L). Data were expressed as percentage (%). UIC, urinary iodine concentration.

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TABLE 1 Comparison of MUI in PTMC patients (*n* = 3,858).

Variables	*n*	MUI (μg/L)	*P*
Sex			
Male	650	183.4 (127.1–253.8)	0.038
Female	3,208	173.6 (111.4–262.9)	
Age (year)			
<55	3,175	177.2 (117.1–263.4)	0.058
≥55	683	165.9 (108.7–250.3)	
Tumor size (cm)			
D ≤ 0.5	1,996	173.1 (114.6–260.8)	0.387
0.5 < D ≤ 1.0	1,862	177.5 (115.8–261.9)	
Tumor number			
Solitary	2,208	178.2 (117.4–257.9)	0.818
Multiple	1,650	171.5 (112.7–265.5)	
Bilaterality			
No	2,711	178.2 (117.8–260.6)	0.475
Yes	1,147	167.4 (110.2–262.1)	
Capsular invasion			
No	1,878	172.5 (111.3–262.8)	0.366
Yes	1,980	178.2 (117.8–259.3)	
Extracapsular extension			
No	3,620	174.3 (114.5–260.8)	0.024
Yes	238	199.7 (130.0–269.8)	
Intrathyroidal spread			
No	3,744	174.7 (114.5–260.8)	0.076
Yes	114	203.7 (132.9–277.2)	
CLNM			
No	2,341	173.9 (114.5–263.1)	0.544
Yes	1,517	177.9 (116.0–257.2)	
LLNM			
No	3,623	176.5 (115.2–262.1)	0.132
Yes	235	157.9 (108.5–236.8)	
Pathology			
PTMC	1,122	179.8 (124.7–257.5)	0.105
PTMC&HT&NG	520	166.1 (105.6–257.1)	
PTMC&HT	1,446	173.1 (107.3–269.6)	
PTMC&NG	770	176.5 (118.9–256.2)	

MUI, median urinary iodine; PTMC, papillary thyroid microcarcinoma; D, diameter; CLNM, central lymph node metastasis; LLNM, lateral lymph node metastasis; HT, Hashimoto's thyroiditis; NG, nodular goiter.

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TABLE 2 Comparison of MUI in PTMC patients stratified by gender (n = 3,858).

Variables	Male			Female		
	n	MUI (μg/L)	*P*	*n*	MUI (μg/L)	*P*
Age (year)						
<55	550	184.2 (132.0–255.2)	0.109	2625	175.3 (111.7–265.5)	0.163
≥55	100	176.6 (107.1–251.7)		583	164.5 (110.0–248.3)	
Tumor size (cm)						
D ≤ 0.5	301	180.9 (123.8–252.8)	0.846	1695	171.3 (111.7–263.4)	0.694
0.5 < D ≤ 1.0	349	184.6 (127.8–255.6)		1513	176.0 (111.1–262.9)	
Tumor number						
Solitary	391	184.5 (127.7–251.0)	0.994	1817	177.2 (112.0–261.0)	0.810
Multiple	259	181.8 (122.5–260.8)		1391	169.0 (110.0–265.5)	
Bilaterality						
No	476	183.7 (125.9–254.9)	0.706	2235	177.2 (115.1–262.4)	0.312
Yes	174	181.9 (129.9–253.0)		973	165.9 (106.6–265.5)	
Capsular invasion						
No	306	187.5 (121.1–255.2)	0.787	1572	168.9 (108.5–265.2)	0.314
Yes	344	180.3 (131.4–252.1)		1636	177.8 (114.4–261.9)	
Extracapsular extension						
No	597	184.0 (127.8–255.0)	0.745	3023	172.1 (110.8–262.4)	0.010
Yes	53	173.0 (120.8–245.5)		185	210.0 (131.8–282.1)	
Intrathyroidal spread						
No	624	184.2 (127.3–253.3)	0.967	3120	172.7 (111.1–262.9)	0.068
Yes	26	175.4 (126.3–294.6)		88	211.3 (137.9–275.5)	
CLNM						
No	320	192.7 (131.4–259.3)	0.099	2021	171.4 (111.3–263.6)	0.271
Yes	330	177.4 (122.1–247.3)		1187	178.2 (112.5–261.6)	
LLNM						
No	591	187.9 (132.2–255.2)	0.002	3032	173.8 (111.3–263.9)	0.789
Yes	59	134.7 (107.1–207.2)		176	166.1 (120.0–240.7)	
Pathology						
PTMC	336	193.1 (136.3–261.0)	0.066	786	174.7 (119.7–251.3)	0.399
PTMC&NG, HT	22	146.3 (115.5–233.9)		498	166.2 (104.9–259.6)	
PTMC&HT	148	167.4 (118.0–250.7)		1298	174.6 (106.5–272.0)	
PTMC&NG	144	178.7 (119.8–251.9)		626	175.6 (118.6–257.3)	

MUI, median urinary iodine; PTMC, papillary thyroid microcarcinoma; D, diameter; CLNM, central lymph node metastasis; LLNM, lateral lymph node metastasis; HT, Hashimoto's thyroiditis; NG, nodular goiter.

Association between iodine intake and LLNM in female PTMC

Iodine nutrition status was not found to be associated with LLNM in female PTMC patients in the univariate and multivariate logistic regression analyses. After adjustment, larger tumor size, extrathyroidal extension, intrathyroidal spread, CLNM, and PTMC and Hashimoto's thyroiditis were independently associated with LLNM in female PTMC patients (Table 6).

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TABLE 3 Association between iodine intake and CLNM in male PTMC patients (n = 650).

Variables	Univariate		Multivariate	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (year)				
<55	Ref		Ref	
≥55	0.40 (0.25–0.63)	<0.001	0.45 (0.27–0.77)	0.003
Tumor size (cm)				
D ≤ 0.5	Ref		Ref	
0.5 < D ≤ 1.0	1.91 (1.39–2.60)	<0.001	1.55 (1.09–2.21)	0.016
Tumor number				
Solitary	Ref		Ref	
Multiple	1.45 (1.06–1.99)	0.020	1.29 (0.79–2.11)	0.308
Bilaterality				
No	Ref		Ref	
Yes	1.45 (1.02–2.05)	0.039	1.15 (0.67–1.98)	0.609
Capsular invasion				
No	Ref		Ref	
Yes	1.62 (1.19–2.21)	0.002	1.07 (0.75–1.53)	0.697
Extracapsular extension				
No	Ref		Ref	
Yes	2.93 (1.56–5.51)	0.001	2.65 (1.30–5.38)	0.007
Intrathyroidal spread				
No	Ref		Ref	
Yes	5.64 (1.92–16.56)	0.002	4.83 (1.52–15.35)	0.008
LLNM				
No	Ref		Ref	
Yes	10.01 (4.24–23.65)	<0.001	10.22 (4.02–25.94)	< 0.001
Pathology				
PTMC	Ref		Ref	
PTMC&NG&HT	0.64 (0.27–1.52)	0.313	0.38 (0.12–1.15)	0.087
PTMC&HT	0.96 (0.65–1.41)	0.815	0.85 (0.56–1.29)	0.437
PTMC&NG	0.38 (0.26–0.58)	<0.001	0.47 (0.30–0.74)	0.001
UIC (μg/L)				
≤99.9	1.16 (0.72–1.88)	0.548	1.21 (0.70–2.07)	0.495
100.0–199.9	Ref		Ref	
200.0–299.9	0.81 (0.56–1.18)	0.277	0.86 (0.57–1.29)	0.460
≥300.0	0.79 (0.51–1.23)	0.297	0.72 (0.44–1.18)	0.195

CLNM, central lymph node metastasis; PTMC, papillary thyroid microcarcinoma; OR (95% CI), odds ratio (95% confidence interval); D, diameter; LLNM, lateral lymph node metastasis; HT, Hashimoto's thyroiditis; NG, nodular goiter; UIC, urinary iodine concentration.

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TABLE 4 Association between iodine intake and CLNM in female PTMC patients (n = 3208).

Variables	Univariate		Multivariate	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (year)				
<55	Ref		Ref	
≥55	0.47 (0.38–0.58)	<0.001	0.50 (0.41–0.63)	<0.001
Tumor size (cm)				
D ≤ 0.5	Ref		Ref	
0.5 <D ≤ 1.0	2.58 (2.23–2.99)	<0.001	1.91 (1.62–2.24)	<0.001
Tumor number				
Solitary	Ref		Ref	
Multiple	1.74 (1.51–2.02)	<0.001	1.15 (0.91–1.45)	0.255
Bilaterality				
No	Ref		Ref	
Yes	1.97 (1.69–2.29)	<0.001	1.48 (1.15–1.90)	0.002
Capsular invasion				
No	Ref		Ref	
Yes	2.02 (1.75–2.34)	<0.001	1.41 (1.20–1.67)	<0.001
Extracapsular extension				
No	Ref		Ref	
Yes	1.91 (1.42–2.57)	<0.001	1.12 (0.80–1.57)	0.506
Intrathyroidal spread				
No	Ref		Ref	
Yes	5.03 (3.11–8.14)	<0.001	3.04 (1.80–5.14)	<0.001
LLNM				
No	Ref		Ref	
Yes	12.85 (8.24–20.05)	<0.001	8.56 (5.41–13.56)	<0.001
Pathology				
PTMC	Ref		Ref	
PTMC&NG&HT	0.49 (0.38–0.62)	<0.001	0.61 (0.47–0.79)	<0.001
PTMC&HT	0.98 (0.82–1.17)	0.819	0.97 (0.80–1.17)	0.735
PTMC&NG	0.58 (0.47–0.73)	<0.001	0.71 (0.56–0.90)	0.005
UIC (μg/L)				
≤99.9	1.02 (0.84–1.25)	0.816	1.03 (0.83–1.28)	0.775
100.0–199.9	Ref		Ref	
200.0–299.9	1.25 (1.04–1.50)	0.019	1.23 (1.01–1.51)	0.042
≥300.0	1.09 (0.89–1.35)	0.399	1.02 (0.81–1.28)	0.871

CLNM, central lymph node metastasis; PTMC, papillary thyroid microcarcinoma; OR (95% CI), odds ratio (95% confidence interval); D, diameter; LLNM, lateral lymph node metastasis; HT, Hashimoto's thyroiditis; NG, nodular goiter; UIC, urinary iodine concentration.

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TABLE 5 Association between iodine intake and LLNM in male PTMC patients (n = 650).

Variables	Univariate		Multivariate	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (year)				
<55	Ref		Ref	
≥55	0.99 (0.47–2.08)	0.977	1.14 (0.44–2.97)	0.786
Tumor size (cm)				
D ≤ 0.5	Ref		Ref	
0.5 <D ≤ 1.0	1.63 (0.93–2.84)	0.086	1.73 (0.88–3.40)	0.111
Tumor number				
Solitary	Ref		Ref	
Multiple	2.58 (1.49–4.47)	0.001	2.48 (1.12–5.47)	0.025
Bilaterality				
No	Ref		Ref	
Yes	1.86 (1.07–3.24)	0.028	0.77 (0.35–1.73)	0.530
Capsular invasion				
No	Ref		Ref	
Yes	1.56 (0.90–2.70)	0.116	1.08 (0.56–2.05)	0.827
Extracapsular extension				
No	Ref		Ref	
Yes	2.60 (1.23–5.49)	0.012	1.37 (0.57–3.30)	0.480
Intrathyroidal spread				
No	Ref		Ref	
Yes	3.23 (1.24–8.40)	0.016	2.18 (0.75–6.34)	0.153
CLNM				
No	Ref		Ref	
Yes	10.01 (4.24–23.65)	<0.001	9.23 (3.68–23.16)	<0.001
Pathology				
PTMC	Ref		Ref	
PTMC&NG&HT	7.11 (2.72–18.56)	<0.001	12.64 (3.48–45.90)	<0.001
PTMC&HT	1.51 (0.78–2.92)	0.222	1.44 (0.69–3.00)	0.328
PTMC&NG	0.93 (0.43–1.99)	0.848	1.30 (0.54–3.12)	0.564
UIC (μg/L)				
≤99.9	1.30 (0.64–2.66)	0.471	1.27 (0.57–2.82)	0.560
100.0–199.9	Ref		Ref	
200.0–299.9	0.45 (0.21–0.96)	0.039	0.57 (0.25–1.29)	0.177
≥300.0	0.57 (0.24–1.33)	0.195	0.57 (0.22–1.46)	0.237

LLNM, lateral lymph node metastasis; PTMC, papillary thyroid microcarcinoma; OR (95% CI), odds ratio (95% confidence interval); D, diameter; CLNM, central lymph node metastasis; HT, Hashimoto's thyroiditis; NG, nodular goiter; UIC, urinary iodine concentration.

Association between iodine nutrition and extrathyroidal extension in PTMC patients

In the univariate analysis, more than adequate iodine intake was associated with extrathyroidal extension with OR (95% CI) of 1.70 (1.18–2.44) in female PTMC patients. After adjustment, more than adequate iodine intake predicted a higher possibility for extrathyroidal extension with OR (95% CI) of 1.59 (1.09–2.32) compared with adequate iodine intake (Supplementary Table 2).

Discussion

In the present study, we comprehensively investigated the association between iodine intake and nodal metastasis of PTMC patients for the first time. The present evidence suggested that more than adequate iodine intake was associated with CLNM and extrathyroidal extension compared with adequate iodine intake in female PTMC patients. Iodine nutrition was not found to be associated with CLNM in males and was not associated with LLNM in either gender.

The association between iodine intake and PTC has been controversial. Some hold that a higher exposure to iodine intake was associated with PTC incidence (9, 10, 17–21). However, some scholars hold that high iodine intake was not associated with PTC occurrence and high UIC was just a specific characteristic of the disease (22). Fan et al. reported that the areas with a low consumption rate of qualified iodized salt and areas with a high incidence of thyroid goiter had a relatively high incidence of thyroid cancer (8). We previously found that the MUI was not significantly different between patients with benign thyroid nodules and PTC, which was consistent with other reports (23). However, more than adequate iodine intake might be associated with the growth of PTC (≥1 cm vs. <1 cm) compared with adequate iodine intake (11). We thus expected that a high iodine intake might be associated with the progression of PTC.

Extrathyroidal extension and intrathyroidal spread were aggressive features of PTC. Huang et al. found that excessive iodine exposure was associated with capsular invasion and extrathyroidal metastases of PTC (19). Similarly, we previously reported that more than adequate and excessive iodine intake were marginally associated with capsular invasion of PTC (12). In this study, the MUI of patients with extrathyroidal extension and intrathyroidal spread was higher than that in the corresponding patients. In addition, more than adequate iodine intake was associated with extrathyroidal extension of PTMC. Accumulating evidence suggested that extrathyroidal extension and intrathyroidal spread were positively associated with CLNM of PTC (24, 25). We can expect that high iodine intake may induce CLNM *via* promoting the aggressiveness of PTMC. High iodine intake within a certain concentration may promote PTC cell proliferation *via* upregulating growth signaling pathways (26). However, the potential mechanisms remain explored *in vivo*.

Cervical nodal metastasis was common in PTC, as well as in PTMC (12). However, the available data failed to find a definitive association between iodine take and CLNM. Fan et al. reported that the proportions of UIC >300 μg/L and of serum iodine concentration >90 μg/L were higher in the CLNM (n = 167) than that in the non-CLNM (n = 235) PTC patients (16). However, we did not find a statistical difference in MUI between CLNM (n = 1517) and non-CLNM (n = 2341) patients with PTMC. Similarly, Huang et al. found that iodine nutrition was not associated with CLNM with a sum of 97 PTC patients *via* UIC determination (19). We found that more than adequate iodine intake was associated with CLNM, but not excessive iodine intake. In our opinion, it may be easier to be more than adequate iodine status than excessive iodine intake. The recommended intake of iodine for adults is 150 μg/day, and the maximum tolerable iodine intake is 1,000 μg/day. In addition, the high iodine intake status will take a long time to produce adverse effects. The occasionally excessive iodine intake may not cause adverse results. Therefore, studies on the relationship between excessive iodine intake like high iodine area and thyroid cancer should be investigated. We found that patients with LLNM (n = 235) had a lower MUI than those without LLNM for the first time, whereas iodine intake was not found to be associated with LLNM, which should be validated in the following studies.

The MUI of male patients was significantly higher than that in female patients. In general, women's demand for iodine is greater than men's. This will lead to a higher proportion of women with iodine deficiency than men (11), which was consistent with the present result. However, we found that high iodine intake was associated with extrathyroidal extension and CLNM in women, but not in men. This indicated that women were more vulnerable to iodine nutrition than men. A recent study revealed that more than adequate and excessive iodine intake had an inverse relationship with thyroid peroxidase antibody, and they were predictors for elevated thyrotropin (27). Furthermore, the higher thyrotropin level was associated with extrathyroidal extension and cervical lymph node metastasis of PTMC (28). Therefore, we speculated that high iodine intake might induce thyroid antibody production, leading to elevated thyrotropin levels and contributing to the progression of thyroid cancer. In addition, the average serum thyrotropin concentration and prevalence of antithyroid antibodies are greater in women relative to men in the general population (29). This suggested that thyroid disease should be considered during routine evaluation of this susceptible population and should be followed by appropriate detection and treatment.

In this study, PTMC patients accompanied by nodular goiter (NG) were inversely associated with CLNM compared with PTMC alone in both men and women. Similarly, some reported that PTC patients with NG had a lower incidence of CLNM compared with those of PTC alone (30). We previously found that patients with NG had an obviously higher MUI than healthy controls, and high iodine intake was associated with a larger tumor size of NG (11). We expected that PTMC patients with NG may be treated at an earlier stage compared with patients with PTMC alone. Thus, these patients were more likely to be negative for cervical lymph nodes.

The mutation rate of $BRAF^{V600E}$ in PTC was up to 86.7%, whereas it was not found to be associated with CLNM (31), and LLNM of PTMC (13). However, some reported that $BRAF^{V600E}$ mutation was independently associated with CLNM when PTMC ≤5 mm (32). It was reported that low iodine intake (UIC <300 μg/L)

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TABLE 6 Association between iodine intake and LLNM in female PTMC patients (n = 3208).

Variables	Univariate		Multivariate	
	OR (95% CI)	*P*	OR (95% CI)	*P*
Age (year)				
<55	Ref		Ref	
≥55	0.63 (0.40–0.99)	0.046	0.93 (0.56–1.53)	0.774
Tumor size (cm)				
D ≤ 0.5	Ref		Ref	
0.5 < D ≤ 1.0	5.06 (3.46–7.41)	<0.001	2.83 (1.89–4.24)	<0.001
Tumor number				
Solitary	Ref		Ref	
Multiple	2.11 (1.55–2.88)	<0.001	1.21 (0.73–2.02)	0.459
Bilaterality				
No	Ref		Ref	
Yes	2.20 (1.62–2.99)	<0.001	1.18 (0.71–1.95)	0.521
Capsular invasion				
No	Ref		Ref	
Yes	2.62 (1.87–3.67)	<0.001	1.14 (0.78–1.68)	0.490
Extracapsular extension				
No	Ref		Ref	
Yes	3.64 (2.37–5.59)	<0.001	2.47 (1.50–4.05)	<0.001
Intrathyroidal spread				
No	Ref		Ref	
Yes	6.42 (3.86–10.68)	<0.001	3.37 (1.90–5.96)	<0.001
CLNM				
No	Ref		Ref	
Yes	12.85 (8.24–20.05)	<0.001	8.47 (5.35–13.41)	<0.001
Pathology				
PTMC	Ref		Ref	
PTMC&NG&HT	0.28 (0.13–0.61)	0.001	0.51 (0.23–1.12)	0.093
PTMC&HT	1.41 (0.98–2.04)	0.068	1.64 (1.10–2.45)	0.015
PTMC&NG	0.78 (0.48–1.28)	0.321	1.21 (0.71–2.06)	0.486
UIC (μg/L)				
≤ 99.9	0.91 (0.60–1.39)	0.668	0.83 (0.53–1.31)	0.427
100.0–199.9	Ref		Ref	
200.0–299.9	0.99 (0.67–1.46)	0.962	0.76 (0.50–1.16)	0.202
≥300.0	0.98 (0.63–1.52)	0.920	0.78 (0.49–1.25)	0.295

LLNM, lateral lymph node metastasis; PTMC, papillary thyroid microcarcinoma; OR (95% CI), odds ratio (95% confidence interval); D, diameter; CLNM, central lymph node metastasis; HT, Hashimoto's thyroiditis; NG, nodular goiter; UIC, urinary iodine concentration.

and more than excessive iodine intake (UIC ≥500 μg/L) were associated with $BRAF^{V600E}$ mutation of PTC (33). However, some reported that there were no significant differences in the prevalence of $BRAF^{V600E}$ mutation between PTC patients in iodine-rich areas and those in iodine-deficient areas (34). In addition, some researchers found that the MUI was not significantly different in patients with $BRAF^{V600E}$ mutation or not (21). Therefore, the relationship between iodine nutrition and $BRAF^{V600E}$ should be investigated further.

CLNM was positively associated with LLNM in both male and female PTMC patients, which was consistent with previous reports (24, 25). As far as we know, this is the largest study focusing on the association between iodine nutrition and PTMC progression. However, some shortcomings should be acknowledged. First, the retrospective study nature cannot demonstrate the cause and effect between iodine nutrition and nodal metastasis of PTMC. Second, the iodine nutrition of patients may not be evaluated accurately by a single determination of UIC. Third, we failed to assess the genetic alterations of PTMC. Last but not least, we cannot explore the underlying mechanisms between iodine intake and PTC initiation and progression.

In conclusion, we investigated the association between iodine nutrition and cervical lymph node metastasis of PTMC patients. We found that more than adequate iodine intake was associated with CLNM and extrathyroidal extension compared with adequate iodine intake in female PTMC patients. Iodine nutrition was more closely associated with tumor progression in female patients. The following studies with a larger sample size should be done to further illuminate the association between iodine nutrition and PTC progression.

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 humans were approved by Ethics Committee of Union hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

HZ and JH contributed the design of the study and writing of the manuscript. HZ, JH and LC collected the data and performed the analyses. YG and TH supervised the work. All authors reviewed and approved the final version of 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1164069/full#supplementary-material

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Free and bioavailable 25-hydroxyvitamin D thresholds for bone metabolism and their associations with metabolic syndrome in Chinese women of childbearing age

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Objective: The free hormone hypothesis suggests that free and bioavailable 25-hydroxyvitamin D [25(OH)D] may better reflect vitamin D bioactivity. This study aimed to determine the free and bioavailable 25(OH)D characteristics, estimate their thresholds based on parathyroid hormone (PTH) and bone turnover markers (BTMs), assess their associations with the risk of metabolic syndrome (MetS), and evaluate their potential advantages.

Methods: A cross-sectional study was conducted using a nationally representative database ($n = 1{,}505$, female, 18–45 years). Serum total 25(OH)D, vitamin D-binding protein, albumin, PTH, and BTMs [osteocalcin, β-CrossLaps of type 1 collagen containing cross-linked C-telopeptide (β-CTX), and procollagen type 1 N-terminal propeptide (P1NP)] were measured. Free 25(OH)D and bioavailable 25(OH)D were calculated. The threshold associations of 25(OH)D with PTH and BTMs were analyzed. The relationship between 25(OH)D and MetS risk was examined. An intervention study was then performed in 39 women (18–47 years) to assess the associations of increasing 25(OH)D with PTH and BTMs after vitamin D supplementation.

Results: In the cross-sectional study, the three forms of 25(OH)D were found to have similar distribution characteristics. Free and bioavailable 25(OH)D correlated well with total 25(OH)D. Significant total 25(OH)D cutoffs were observed for PTH (14.19 ng/mL and 18.03 ng/mL), osteocalcin (15.14 ng/mL), β-CTX (14.79 ng/mL), and P1NP (15.08 ng/mL). Free and bioavailable 25(OH)D cutoffs were only found for P1NP (3.47 pg/mL and 1.66 ng/mL, respectively). A total 25(OH)D of <15.14 ng/mL was marginally associated with a higher risk of reduced high-density lipoprotein cholesterol (HDL-C) [odd ratios (OR) = 1.371 (0.991–1.899)]. The ORs of higher versus lower free and bioavailable 25(OH)D levels for reduced HDL-C were 0.770 (0.621–0.956) and 0.772 (0.622–0.958), respectively. The results of the intervention study indicated that PTH and BTMs responded more sensitively to total 25(OH)D than to free or bioavailable 25(OH)D.

Conclusion: Free and bioavailable 25(OH)D only had a threshold effect on P1NP. The active 25(OH)D thresholds could be used for risk assessment of reduced HDL-C. However, no superiority of free or bioavailable 25(OH)D was found based

on the response of PTH and BTMs to changes in 25(OH)D in Chinese women of childbearing age following vitamin D supplementation.

Clinical trial registration: http://www.chictr.org.cn, ChiCTR2200058290.

KEYWORDS

free 25-hydroxyvitamin D, bioavailable 25-hydroxyvitamin D, parathyroid hormone, bone turnover markers, threshold, metabolic syndrome

1. Introduction

Vitamin D plays a critical role in skeletal health (1) and contributes to extra-skeletal effects, including metabolic syndrome (MetS) (2). The serum total 25(OH)D [Total-25(OH)D, mainly bound to vitamin D-binding protein (VDBP)] level is the best index of nutritional vitamin D status (3). The relationship between Total-25(OH)D and MetS has been investigated in populations worldwide, but the results were inconsistent (4). The free hormone hypothesis (5) suggests that the free and bioavailable forms of 25(OH)D [Free-25(OH)D and Bio-25(OH)D, respectively], which are free or albumin-bound, may better reflect the bioactivity of vitamin D. Because only the free fraction can passively cross the cell membrane, become hydroxylated to the active metabolite [1,25(OH)2D], and exert a biological action (6). In addition, VDBP and Total-25(OH)D are influenced by factors such as liver function, kidney diseases, pregnancy, and genetic background (7, 8), but free vitamin D is independent of these factors and has a better correlation with pathological conditions, especially with liver, kidney, and allergic diseases, and tumor as well as pregnancy. Therefore, it is recommended to determine Free-25(OH)D and/or Bio-25(OH)D under these pathological conditions (9).

Numerous studies have been conducted to confirm the free hormone hypothesis, but confusion remains regarding the superiority of Free-25(OH)D and Bio-25(OH)D in terms of prediction of health outcomes. Some studies have found that Free-25(OH)D and Bio-25(OH)D appear to be superior to Total-25(OH)D as predictive indices of bone health (10–12) or in a similar fashion (13, 14), whereas other have shown that vitamin D status assessed by Total-25(OH)D may better reflect bone health (15, 16). We speculated that these inconsistent conclusions may reflect differences in the ethnicity, sex, and age of the study populations and the methods used to determine Free-25(OH)D and Bio-25(OH)D. Therefore, more observational studies and clinical trials in various physiological and pathological conditions to define optimal Free-25(OH)D and Bio-25(OH)D concentrations are needed.

According to the presently used criteria, vitamin D deficiency is common in Chinese women of childbearing age (17). Studies have identified that there is a threshold association between Total-25(OH)D and serum parathyroid hormone (PTH) (18) and that a lower serum 25(OH)D is associated with some components of MetS (19) in this population. However, there have been no reports on the levels of Free-25(OH)D or Bio-25(OH)D and whether Free-25(OH)D or Bio-25(OH)D can be used to assess vitamin D nutritional status or bioactivity and evaluate the effectiveness of intervention in this population. Therefore, the aim of this research was to investigate whether Free-25(OH)D or Bio-25(OH)D also has a threshold effect on bone metabolism and whether it is more strongly linked to bone metabolism and MetS than Total-25(OH)D in Chinese women of childbearing women.

Two studies were performed. The first was a cross-sectional study based on the 2015 Chinese Chronic Diseases and Nutrition Survey (CCDNS) that was performed to determine the characteristics of Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D in women of childbearing age and to compare the threshold associations of these three forms of 25(OH)D with PTH and bone turnover markers (BTMs), which are indicators of bone remodeling. If the Free-25(OH)D or Bio-25(OH)D threshold was determined, it would be further used to assess the relationship between vitamin D and MetS risk. The second was an intervention study that was performed to clarify whether the associations of Free-25(OH)D and Bio-25(OH)D with PTH and BTMs were stronger than Total-25(OH)D after vitamin D supplementation.

2. Materials and methods

2.1. Study design and participants

2.1.1. The cross-sectional study

Women of childbearing age (18–45 years), who were not pregnant or lactating, were recruited for participation from the 2015 CCDNS. The investigation enrolled 1,568 women randomly from 279 survey areas, as described in our previous research (18). Participants with missing anthropometric measurements or other pertinent covariates ($n = 61$) were excluded. A total of 1,505 participants were ultimately included in the cross-sectional study. The study was approved by the Ethical Review Committee of Chinese Center for Disease Control and Prevention (CDC) (No. 201519-B).

2.1.2. The intervention study

Participants were recruited from November to December 2021 in Beijing, China. Women who met the following criteria volunteered for participation: (1) premenopausal women aged 18–50 years (non-pregnant or non-lactating); (2) no medical history of acute or chronic diseases such as hypertension, diabetes, cardiovascular disease, dyslipidemia, kidney disease, cancer, gastrointestinal disorders, and endocrine disorders; and (3) no use of vitamin D, vitamin K, or Ca supplements. Next, serum 25(OH)D concentration was assessed to screen volunteers with inadequate vitamin D status (less than 20 ng/mL). Finally, a total of 40 individuals (18–47 years) were included in the vitamin D supplementation trial. This intervention study was conducted in the Chinese CDC between December 2021 and April 2022.

This study was a 16 weeks, single-group repeated measures design trial. The allocation ratio was 5:3 to high-dose vitamin D3

supplementation (800 IU/d) versus low-dose groups (400 IU/d). A total of 40 participants were randomly assigned to 400 IU/d group ($n = 15$) or 800 IU/d group ($n = 25$). Participants took a commercialized 400 IU or 800 IU vitamin D3 capsule per day (provided by Xiamen Xingsha Pharmaceutical Group Co., Ltd). And no women took birth control pills or other medications that might affect vitamin D absorption and serum VDBP during the intervention period. Finally, 39 participants (97.5%) were available for the 16 weeks follow-up as one person failed to complete the trial. The study has been registered at http://www.chictr.org.cn (ChiCTR2200058290) and was approved by the Ethical Review Committee of Chinese CDC (No. 2018-009).

All procedures performed in the above two studies involving human participants were in accordance with the ethical standards of the committee. Written informed consent was obtained from all participants before their inclusion in the study.

2.2. Laboratory assay

Fasting blood samples were obtained between 8:00 and 10:00 AM, and serum samples were stored at −80°C until analysis. The biomarkers were measured following both the manufacturer's protocol and specialized laboratory assay quality control procedures. Total-25(OH)D, containing 25(OH)D2 and 25(OH)D3, was measured with the use of liquid chromatography-tandem mass spectroscopy (AB Sciex Pte. Ltd., Framingham, MA, United States). The National Institute of Standards and Technology of America (National Institute of Standards and Technology, NIST) standard reference material, SRM 972a, was used to verify the calibration of the assay. The average bias was 2.64% for 25(OH)D2 and 3.13% for 25(OH)D3 compared with Nist SRM 972a. Values of 25(OH)D were expressed in ng/mL (2.5 ng/mL equal to 1 nmol/L). Serum VDBP levels were measured using a polyclonal antibody enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (GenWay Biotech, Inc., San Diego, CA, United States). Values of VDBP were expressed in μg/mL (250 μg/mL equal to 4 μmol/L). The PTH, OC, β-CTX, and P1NP were measured by electronic chemiluminescence immunoassay (Roche e601, F Hoffmann-La Roche Ltd., CH4002 Basel, Switzerland). The plasma calcium (Ca) was detected by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer, NexION 350, Waltham, MA, United States). Commercially available quality control samples (Clincheck Level-2, Munich, Germany; Seronorm, Level-2, Billingstad, Norway) were used every 10 samples. Serum albumin (Alb), phosphorus (P), creatinine (CRE), high sensitivity C-reactive protein (hsCRP), fasting blood glucose (FBG), high-density lipoprotein cholesterol (HDL-C), total cholesterol, and triglyceride were measured using an automatic biochemical analyzer (Hitachi 7,600, Tokyo, Japan). Alb levels were expressed in g/L (1 g/L equal to 15 μmol/L). All inter- and intra-assay coefficients of variation (CVs) were <4.78%.

Method for selecting and detecting vitamin D metabolism-related single nucleotide polymorphisms (SNPs), including cytochrome P450-2R1 (CYP2R1) rs12794714, VDBP (GC) rs2282679, and vitamin D receptor (VDR) rs2228570 was described in our previous study (19). Then a genetic risk score (GRS) for each subject was obtained by adding the number of A alleles of rs12794714, T alleles of rs2282679, and G alleles of rs2228570 (ranging from 0 to 6).

2.3. Data calculation and collection, and potential confounders

Serum Free-25(OH)D and Bio-25(OH)D were calculated using Total-25(OH)D, VDBP, Alb concentrations, and affinity constants for VDBP and Alb, as the previous study reported (10). The mathematical formulas were as follows:

$$\text{Free}-25(\text{OH})\text{D} = \text{Total}-25(\text{OH})\text{D} / \left(\begin{matrix}1 + K_{\text{Alb}} \times \text{Alb} + \\ K_{\text{VDBP}} \times \text{VDBP}\end{matrix}\right)$$

$$\text{Bio}-25(\text{OH})\text{D} = \left[\left(K_{\text{Alb}} \times \text{Alb}\right) + 1\right] \times \text{Free}-25(\text{OH})\text{D}.$$

K_{alb} is the affinity constant for 25(OH)D and Alb binding (6×10^5/M), and K_{VDBP} is the affinity constant for 25(OH)D and VDBP binding (7×10^8/M). Total-25(OH)D, Free-25(OH)D, Bio-25(OH)D, Alb concentration, and VDBP levels are expressed in mol/L.

Height, weight, waist circumferences, systolic blood pressure, and diastolic blood pressure were medically examined. Body mass index (BMI) was calculated as weight kg/height m^2. As vitamin D status is partly dependent on sunlight exposure, the timing of health examination was divided into three subgroups (no health examination was carried out in summer in this survey): (1) spring (March to May), (2) autumn (September to November), and (3) winter (December to February). Dietary Ca intake was recorded using a Food Frequency Questionnaire (FFQ). Serum Alb-corrected Ca was calculated by the formula [serum Ca – 0.02 × (Alb-40)]. Age, BMI, geographical location, season of blood drawn, lifestyle, dietary Ca intake, time spent outdoors, serum Ca, P, CRE, hsCRP, and GRS were considered as the potential confounders.

2.4. Definition of metabolic syndrome

According to the American Diabetes Association (ADA) "Standards of Medical Care in Diabetes-2022" (20) and a joint interim statement (21), MetS was diagnosed when three or more of the following conditions were present: waist circumference >85 cm (women); serum triglyceride concentration >1.7 mmol/L or treated with lipid abnormalities; HDL-C concentration <1.3 mmol/L or treated; blood pressure ≥130/85 mmHg and/or diagnosed and treated with hypertension; or serum FBG concentration ≥5.6 mmol/L or diagnosed and treated with diabetes mellitus.

2.5. Statistical analysis

Data analyses were performed using IBM SPSS Statistics 23 and R version 4.0.3 statistical software. Normal distribution was assessed by the Kolmogorov–Smirnov test. If variables were considered non-normally distributed, they were expressed as median and interquartile range (P_{25}–P_{75}). Non-parametric statistic methods or the Student's *t*-test were used for the comparison of biochemical parameters between groups. An analytical procedure to predict the Total-25(OH)D, Free-25(OH)D, or Bio-25(OH)D thresholds was performed according to the method reported by Wu et al. (22). First, locally weighted regression and smoothing scatter plots (LOESS) were

used to visualize the nonlinear relationship between Total-25(OH)D, Free-25(OH)D, or Bio-25(OH)D and PTH or BTMs, and to obtain potential cutoff values for the three forms of 25(OH)D. Then, nonlinear least squares estimation (NLS) was used to determine the exact value based on the cutoff predicted by LOESS. Finally, the relationship between 25(OH)D and the four endpoints before and after the cutoff value was determined by segmented regression (SR), i.e., slope (β coefficient) and 95% confidence interval (CI), to further confirm the exact cutoff value. Before these procedures, Total-25(OH)D, Free-25(OH)D, or Bio-25(OH)D was adjusted for region, regional type, season, latitude, age, BMI, CRE, hsCRP, Ca intake, time spent outdoors, and GRS by generalized additive model (GAM). Once the thresholds were determined, multivariate logistic analysis was applied to analyze the relationship between the three forms of 25(OH)D and the risk of MetS as well as its components. In the intervention study, scatter plot and linear relationship between change in PTH or BTMs and change in each form of 25(OH)D was analyzed by GraphPad Prism 9. $p < 0.05$ was considered statistically significant.

3. Results

3.1. General characteristics in the cross-sectional study

In the cross-sectional study, the median age was 30.0 (24.0–37.8) years old. The median BMI was 22.7 (20.3–25.1) kg/m^2. The median Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D was 16.6 (12.0–22.6) ng/mL, 3.5 (2.3–5.0) pg/mL and 1.7 (1.1–2.4) ng/mL, respectively. The median PTH, OC, β-CTX, and P1NP concentrations was 34.2 (26.0–44.4) pg/mL, 17.2 (13.8–21.4) ng/mL, 0.4 (0.3–0.5) ng/mL, and 52.8 (40.1–68.4) ng/mL, respectively. The median Alb and VDBP was 53.1 (50.7–55.1) g/L and 390.7 (295.7–450.5) ug/mL, respectively. The median serum corrected Ca was 2.3 (2.2–2.4) mmol/L. The concentration of serum P was 1.4 (1.2–1.5) mmol/L. The prevalence of MetS was 12.6%. The results of renal function indexes and inflammatory markers were shown in Table 1 as well.

3.2. Free-25(OH)D, Bio-25(OH)D, and VDBP concentrations in different subgroups in the cross-sectional study

As shown in Table 2, the distribution characteristics of Free-25(OH)D and Bio-25(OH)D were similar to those of Total-25(OH)D. Free-25(OH)D and Bio-25(OH)D showed regional, latitudinal, regional type, seasonal, and age differences, with relatively higher serum Free-25(OH)D and Bio-25(OH)D levels in eastern regions, low-latitude regions, rural areas, autumn, and higher age groups ($p < 0.05$). Women with outdoor time ≥120 min/d had a higher Bio-25(OH)D concentration ($p < 0.05$). Women in the center and urban area, as well as in the younger age group and Han nationality group had higher VDBP concentrations ($p < 0.05$). Women with reduced HDL-C had lower Total-25(OH)D, Free-25(OH)D, Bio-25(OH)D, and VDBP ($p < 0.05$). In contrast, the three forms of 25(OH)D were higher in women with elevated glucose ($p < 0.05$). No differences of Total-25(OH)D, Free-25(OH)D, Bio-25(OH)D, and VDBP were found between women with and without MetS ($p > 0.05$).

3.3. Correlation between Free-25(OH)D or Bio-25(OH)D and Total-25(OH)D in the cross-sectional study

In the cross-sectional study, Free-25(OH)D and Bio-25(OH)D were positively associated with Total-25(OH)D ($p < 0.001$), with an estimated R^2 value of 0.526 and 0.507, respectively (Figure 1).

3.4. Threshold-associations of Free-25(OH)D and Bio-25(OH)D with PTH and BTMs in the cross-sectional study

In the predicted models, Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D were adjusted for region, regional type, season, latitude, age, BMI, CRE, hsCRP, Ca intake, time spent outdoors, and GRS. After adjustment, the LOESS scatter plots showed the non-linear associations of Total-25(OH)D with PTH or BTMs and their potential cutoffs, but such association was not found between Free-25(OH)D or Bio-25(OH)D and PTH (Figure 2).

Table 3 shows the exact 25(OH)D thresholds predicted by NLS and SR. The first adjusted cutoff of Total-25(OH)D for PTH, OC, β-CTX, and P1NP was 14.19 (12.23, 16.14) ng/mL, 15.14 (13.56, 16.71) ng/mL, 14.79 (12.11, 17.47) ng/mL, and 15.08 (12.89, 17.27) ng/mL, respectively. Below these cutoffs, Total-25(OH)D was negatively associated with PTH, OC, β-CTX, and P1NP ($p < 0.05$). Above these cutoffs, OC, β-CTX, and P1NP began to level off, while PTH declined again after the second cutoff [18.03 (15.11, 20.96) ng/mL]. Free-25(OH)D and Bio-25(OH)D cutoffs with their 95% CIs [$\text{cutoff}_{\text{Free-25(OH)D-1}} = 3.47$ (2.87, 4.08) pg/mL; $\text{cutoff}_{\text{Bio-25(OH)D-1}} = 1.66$ (1.37, 1.96) ng/mL] estimated by the NLS and SR were only significant for P1NP. Below the first cutoff, P1NP was negatively associated with Free-25(OH)D and Bio-25(OH)D levels [$\beta_{\text{Free-25(OH)D-1}} = -6.14$ (−11.09, −1.20), $p < 0.05$; $\beta_{\text{Bio-25(OH)D-1}} = -12.97$ (−23.28, −2.65), $p < 0.05$], and then entered a plateaus. Similarly, PTH showed a decreasing trend after the second Free-25(OH)D and Bio-25(OH)D cutoff in the models.

3.5. Risk of MetS and its components in subgroups stratified according to the thresholds determined in the cross-sectional study

In the cross-sectional study, women were divided into 3 subgroups based on the two predicted Total-25(OH)D cutoffs (15.14 and 18.03 ng/mL). The women were also stratified into two subgroups according to the threshold of Free-25(OH)D (3.47 pg/mL) or Bio-25(OH)D (1.66 ng/mL). In the analysis of model 2, with adjustments for age, race, education, physical activity, smoking, drinking, and BMI, and other components of MetS, women with Total-25(OH)D < 15.14 ng/mL had marginal higher risk of reduced HDL-C than those with Total-25(OH)D between 15.14 ng/mL and 18.03 ng/mL (OR: 1.371, 95% CI: 0.991–1.899, $p = 0.056$). And higher Free- or Bio-25(OH)D had a lower risk of reduced HDL-C ($\text{OR}_{\text{Free-25(OH)D}}$: 0.770, 95% CI: 0.621–0.956; $\text{OR}_{\text{Bio-25(OH)D}}$: 0.772, 95% CI: 0.622–0.958) than the lower group. But Bio-25(OH)D, Free-25(OH)D, and Bio-25(OH)D levels were not found significantly associated with the prevalence of MetS, elevated triglycerides, elevated waist circumferences, or elevated blood pressure.

TABLE 1 Characteristics of Chinese women of childbearing age ($n = 1,505$, data from the cross-sectional study).

Parameters	Median (P_{25}–P_{75}) or *N*(%)	Parameters	Median (P_{25}–P_{75}) or *N*(%)
Region		Total-25(OH)D, ng/mL	16.6 (12.0–22.6)
Eastern	522 (34.7)	Free-25(OH)D, pg/mL	3.5 (2.3–5.0)
Center	478 (31.8)	Bio-25(OH)D, ng/mL	1.7 (1.1–2.4)
Western	505 (33.6)	Alb, g/L	53.1 (50.7–55.1)
Latitude, °N	32.26 (27.8–38.0)	VDBP, ug/mL	390.7 (295.7–450.5)
Urban	891 (59.2)	PTH, pg/mL	34.2 (26.0–44.4)
Season		OC, ng/mL	17.2 (13.8–21.4)
Spring	118 (7.9)	β-CTX, ng/mL	0.4 (0.3–0.5)
Autumn	774 (51.4)	P1NP, ng/mL	52.8 (40.1–68.4)
Winter	613 (40.7)	Corrected Ca, mmol/L	2.3 (2.2–2.4)
Nationality		P, mmol/L	1.4 (1.2–1.5)
Han	1,309 (87.0)	CRE, nmol/L	76.0 (72.0–81.0)
Ethnic minorities	196 (13.0)	hsCRP, mg/L	0.7 (0.2–1.4)
Education		BMI, kg/m^2	22.7 (20.3–25.1)
Primary	375 (24.9)	Systolic blood pressure, mmHg	115.0 (107.7–123.3)
Medium	876 (58.2)	Diastolic blood pressure, mmHg	71.3 (65.7–76.8)
Advanced	254 (16.9)	Fasting plasma glucose, mmol/L	4.9 (4.6–5.2)
Cigarette smoker	22 (1.5)	Triglyceride, mmol/L	0.9 (0.6–1.2)
Alcohol consumer	322 (21.4)	HDL-C, mmol/L	1.3 (1.1–1.5)
Moderate or high physical activity	228 (15.2)	Total cholesterol, mmol/L	4.2 (3.7–4.8)
Age, years	30.0 (24.0–37.8)	Waist circumference, cm	75.8 (70.0–82.5)
Ca intake, mg/d	286.1 (169.5–430.8)	Elevated triglycerides	186 (12.4)
Time spent outdoors		Reduced HDL-C	716 (47.6)
≤120 min/d	216 (14.4)	Elevated glucose	91 (6.0)
>120 min/d	286 (19.0)	Elevated waist circumferences	492 (32.7)
Unclear	1,003 (66.6)	Elevated blood pressure	102 (6.8)
GRS		MetS	189 (12.6)
0–1	333 (22.1)		
2–3	900 (59.8)		
4–6	272 (18.1)		

25(OH)D, 25-hydroxyvitamin D; Total-25(OH)D, total 25(OH)D; Free-25(OH)D, free 25(OH)D; Bio-25(OH)D, bioavailable 25(OH)D; Alb, albumin; VDBP, vitamin D-binding protein; PTH, parathyroid hormone; OC, osteocalcin; β-CTX, β-CrossLaps of type 1 collagen containing cross-linked C-telopeptide; P1NP, procollagen type 1 N-terminal propeptide; Ca, calcium; P, phosphorus; CRE, creatinine; hsCRP, high sensitivity C-reactive protein; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; GRS, genetic risk score.

Although an increase in serum Bio-25(OH)D, Free-25(OH)D, and Bio-25(OH)D was significantly associated with an increased risk of elevated glucose, no significant associations were found between elevated glucose and Total-25(OH)D, Free-25(OH)D, or Bio-25(OH)D levels after stratification (Table 4).

3.6. General characteristics in the intervention study

In the intervention study, the average age was 31.2 ± 7.5 years and the average BMI was 21.5 ± 2.5 kg/m^2. Age and BMI were not significantly different between 400 IU/d and 800 IU/d groups. Baseline Total-25(OH)D (9.9 ± 3.0 ng/mL vs. 11.7 ± 4.0 ng/mL; $p = 0.154$), Free-25(OH)D (2.2 ± 0.7 pg/mL vs. 2.4 ± 0.9 pg/mL, $p = 0.602$), and Bio-25(OH)D (1.0 ± 0.3 ng/mL vs. 1.1 ± 0.4 ng/mL, $p = 0.611$) showed no significant differences between the 400 IU/d and 800 IU/d group (data not shown).

3.7. Total-25(OH)D, Free-25(OH)D, or Bio–25(OH)D associations with PTH and BTMs after vitamin D supplementation

In the intervention study, after vitamin D supplementation, the three forms of 25(OH)D increased in both groups, with no group differences. As Free-25(OH)D turned to be more responsive to vitamin D supplementation than Total-25(OH)D in week 4

TABLE 2 Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D and VDBP concentrations of Chinese childbearing women aged 18–45 y (n = 1,505, data from the cross-sectional study).

Characteristic	N (%)	Total-25(OH)D (ng/mL)	P_1	Free-25(OH)D (pg/mL)	P_2	Bio-25(OH)D (ng/mL)	P_3	VDBP(ug/mL)	P_4
Region			<0.001		0.002		0.007		<0.001
Eastern	522 (34.7)	18.07 (12.94–24.2)#		3.68 (2.47–5.35)a		1.74 (1.17–2.55)a		396.64 (304.24–477.08)	
Center	478 (31.8)	15.84 (11.58–21.75)		3.46 (2.32–4.92)		1.64 (1.12–2.35)		354.34 (291.17–413.81)#	
Western	505 (33.6)	15.86 (11.33–21.19)		3.21 (2.18–4.85)		1.53 (1.06–2.31)		381.2 (295.58–478.76)	
Latitude, °N			<0.001		<0.001		<0.001		0.144
<23.5	141 (9.4)	24.99 (20.96–29.29)#		5.37 (4.02–7.28)#		2.51 (1.82–3.42)#		371.05 (297.04–452.98)	
23.5 ~ 32	602 (40.0)	20.16 (15.57–24.62)#		4.14 (2.96–5.56)#		1.98 (1.41–2.63)#		386.3 (311.84–462.49)	
32 ~ 40.5	524 (34.8)	12.74 (10.11–16.70)		2.69 (1.88–3.87)		1.30 (0.92–1.88)		360.41 (279.16–462.14)	
≥40.5	238 (15.8)	13.20 (9.75–17.49)		2.63 (1.87–3.9)		1.23 (0.91–1.81)		379.67 (296.4–449.28)	
Regional type			<0.001		<0.001		<0.001		0.033
Urban	614 (40.8)	15.24 (11.40–20.88)		3.14 (2.19–4.45)		1.49 (1.05–2.14)		383.13 (322.54–448.63)	
Rural	891 (59.2)	17.61 (12.55–23.87)		3.72 (2.37–5.39)		1.76 (1.15–2.58)		363.66 (280.86–467.18)	
Season			<0.001		0.001		<0.001		0.130
Spring	118 (7.9)	16.16 (11.83–21.83)		3.18 (2.22–4.88)		1.53 (1.02–2.31)		379.42 (309.78–497.45)	
Autumn	774 (51.4)	17.6 (12.79–23.39)b		3.65 (2.51–5.17)b		1.72 (1.2–2.49)#		379.26 (301.98–462.49)	
Winter	613 (40.7)	15.42 (11.17–21.63)		3.28 (2.1–4.84)		1.53 (1.01–2.31)		372.38 (286.41–451.35)	
Age group, y			0.021		<0.001		<0.001		0.005
18 ~ 24	491 (32.6)	15.77 (11.64–20.88)d		3.08 (2.13–4.52)c,d		1.48 (1.02–2.17)c,d		389.58 (315.48–461.15)d	
25 ~ 29	257 (17.1)	16.91 (12.35–23.77)		3.47 (2.43–5.28)		1.69 (1.15–2.52)		364.27 (286.67–458.21)	
30 ~ 34	263 (17.5)	16.26 (11.48–22.00)		3.67 (2.24–4.99)		1.77 (1.11–2.33)		373.7 (294.78–458.48)	
35 ~ 39	213 (14.1)	16.94 (12.29–22.98)		3.47 (2.19–5.06)		1.66 (1.05–2.46)		377.76 (298.47–483.63)	
≥40	281 (18.7)	17.49 (12.45–23.95)		3.84 (2.61–5.69)		1.80 (1.23–2.67)		353.02 (267.85–441.63)	
BMI			0.794		0.996		0.942		0.422
Underweight	136 (9.0)	16.18 (11.94–22.09)		3.48 (2.34–5.07)		1.72 (1.16–2.33)		357.61 (295.19–450.03)	
Normal weight	839 (55.8)	16.75 (12.07–22.97)		3.46 (2.24–5.02)		1.66 (1.09–2.39)		377.8 (298.4–464.36)	
Overweight	377 (25.0)	16.26 (11.98–22.93)		3.39 (2.32–5.09)		1.61 (1.14–2.45)		374.94 (293.98–456.08)	

(Continued)

TABLE 2 (Continued)

Characteristic	*N* (%)	Total-25(OH)D (ng/mL)	P_1	Free-25(OH)D (pg/mL)	P_2	Bio-25(OH)D (ng/mL)	P_3	VDBP(ug/mL)	P_4
Obesity	153 (10.2)	16.81 (11.79–20.85)		3.48 (2.31–4.9)		1.67 (1.09–2.31)		379.56 (273.18–461.17)	
Nationality			0.542		0.971		0.871		0.043
Han	1,309 (87.0)	16.63 (12.25–22.29)		3.45 (2.32–4.97)		1.66 (1.12–2.36)		377.76 (299.96–461.19)	
Ethnic minorities	196 (13.0)	16.72 (10.60–24.03)		3.43 (2.16–5.37)		1.59 (1.04–2.53)		348.18 (270.45–456.32)	
Education			0.286		0.972		0.966		0.083
Primary	375 (24.9)	16.46 (11.68–22.27)		3.42 (2.24–5.03)		1.63 (1.06–2.39)		357.78 (282.36–452.55)	
Medium	876 (58.2)	16.88 (12.27–23.04)		3.45 (2.31–5.05)		1.66 (1.12–2.39)		381.37 (296.43–461.95)	
Advanced	254 (16.9)	16.39 (11.99–21.89)		3.48 (2.32–4.91)		1.66 (1.12–2.34)		380.26 (308.16–465.26)	
Time spent outdoors			0.143		0.052		0.046		0.104
≤120 min/d	216 (14.4)	16.96 (12.27–22.3)		3.37 (2.23–4.96)		1.64 (1.13–2.37)		395.49 (300.81–468.34)	
>120 min/d	286 (19.0)	17.89 (12.3–23.67)		3.79 (2.45–5.23)		1.80 (1.18–2.49)#		364.85 (281.46–453.59)	
Unclear	1,003 (66.6)	16.16 (11.86–22.27)		3.37 (2.25–5.00)		1.61 (1.08–2.36)		377.16 (295.22–460.28)	
GRS			<0.001		<0.001		<0.001		0.369
0~1	333 (22.1)	18.28 (12.62–25.08)#		3.83 (2.35–5.55)		1.82 (1.12–2.66)		384.94 (300.27–474.47)	
2~3	900 (59.8)	16.72 (12.02–22.3)#		3.48 (2.34–5.05)		1.67 (1.13–2.38)		373.35 (293.08–457.72)	
4~6	272 (18.1)	14.68 (10.97–20.2)#		3.08 (2.1–4.31)#		1.46 (1.02–2.03)#		374.4 (298.8–461.22)	
MetS			0.343		0.656		0.612		0.050
Yes	189 (12.6)	16.47 (11.80–21.97)		3.47 (2.29–5.18)		1.70 (1.13–2.49)		352.62 (270.70–453.28)	
No	1,316 (87.4)	16.69 (11.99–22.78)		3.45 (2.29–5.01)		1.65 (1.11–2.37)		377.78 (297.96–461.42)	
Elevated triglycerides			0.367		0.904		0.931		0.957
Yes	188 (12.5)	16.39 (11.21–22.99)		3.46 (2.27–4.98)		1.66 (1.12–2.35)		378.70 (287.67–460.76)	
No	1,317 (87.5)	16.65 (12.08–22.44)		3.45 (2.31–5.05)		1.65 (1.11–2.39)		375.94 (295.36–460.76)	
Reduced HDL-C			<0.001		0.013		0.013		0.043
Yes	736 (48.9)	15.81 (11.50–21.77)		3.25 (2.24–4.90)		1.58 (1.10–2.35)		372.78 (286.53–451.93)	
No	769 (51.1)	17.41 (12.55–23.77)		3.61 (2.33–5.16)		1.73 (1.12–2.42)		378.58 (301.16–470.53)	
Elevated glucose			0.014		0.016		0.014		0.265
Yes	91 (6.0)	17.40 (13.56–25.75)		3.90 (2.64–5.95)		1.92 (1.24–2.80)		356.64 (289.86–436.98)	

(Continued)

TABLE 2 (Continued)

Characteristic	*N* (%)	Total-25(OH)D (ng/mL)	P_1	Free-25(OH)D (pg/mL)	P_2	Bio-25(OH)D (ng/mL)	P_3	VDBP(ug/mL)	P_4
No	1,414 (94.0)	16.51 (11.88–22.35)		3.44 (2.26–4.98)		1.64 (1.10–2.36)		376.98 (295.43–461.30)	
Elevated waist circumferences			0.189		0.821		0.764		0.197
Yes	492 (32.7)	16.21 (11.86–21.90)		3.46 (2.30–5.00)		1.66 (1.11–2.38)		368.48 (288.21–454.12)	
No	1,013 (67.3)	16.75 (12.05–23.02)		3.45 (2.28–5.05)		1.64 (1.11–2.38)		377.76 (298.40–462.49)	
Elevated blood pressure			0.839		0.236		0.194		0.069
Yes	223 (14.8)	16.50 (12.19–22.34)		3.59 (2.43–5.26)		1.70 (1.17–2.51)		354.66 (280.20–448.03)	
No	1,282 (85.2)	16.69 (11.93–22.55)		3.44 (2.27–4.96)		1.64 (1.10–2.36)		377.84 (296.94–462.56)	
Total	1,505 (100.0)	16.63 (11.96–22.55)		3.45 (2.29–5.03)		1.65 (1.11–2.38)		376.36 (295.36–460.72)	

25(OH)D, 25-hydroxyvitamin D; Total-25(OH)D, total 25(OH)D; Free-25(OH)D, free 25(OH)D; Bio-25(OH)D, bioavailable 25(OH)D; VDBP, vitamin D-binding protein; BMI, body mass index; GRS, genetic risk score; MetS, metabolic syndrome. #, compared with other groups; a, Eastern vs. Western; b, Autumn vs. Winter; c, 18~24 y vs. 25~30 y; d, 18~24 y vs. ≥40 y; $p < 0.05$.

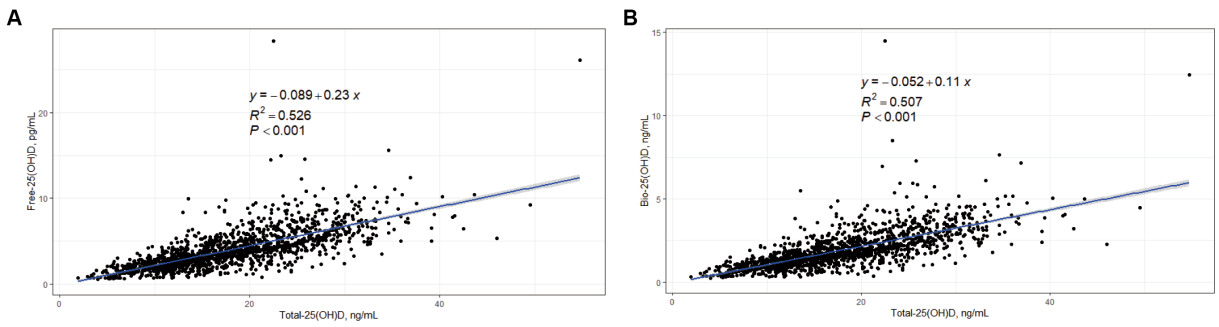

FIGURE 1
Correlations between Free-25(OH)D, Bio-25(OH)D, and Total-25(OH)D in women of childbearing age (data from the cross-sectional study). **(A)** Correlation between Free-25(OH)D and Total-25(OH)D; **(B)** Correlation between Bio-25(OH)D and Total-25(OH)D.

(Supplementary Figure S1), we further examined the associations between percent change in PTH (or BTMs) and percent change in Total-25(OH)D vs. Free-25(OH)D or Bio-25(OH)D for the 39 participants, after 4 weeks of supplementation. As shown in Figure 3, percent change in β-CTX and P1NP was significantly associated with percent change in Total-25(OH)D after adjustment for vitamin D supplementation regimen, baseline 25(OH)D, age, and BMI. Meanwhile, percent change in β-CTX was significantly correlated with percent change in Bio-25(OH)D. However, no association was found between percent change in any of these endpoints with percent change in Free-25(OH)D after adjustment for the same covariates.

4. Discussion

This study is the first to use a nationally representative database to determine the characteristics of serum Free-25(OH)D and Bio-25(OH)D and to analyze their threshold associations with PTH and BTMs in Chinese women of childbearing age. The thresholds of Free-25(OH)D (3.47 pg/mL) and Bio-25(OH)D (1.66 ng/mL) found in terms of P1NP were further used for assessment of the MetS risk in this population. Lower Free-25(OH)D and Bio-25(OH)D levels were associated with reduced HDL-C. However, as markers of vitamin D metabolism, Free-25(OH)D and Bio-25(OH)D did not show superiority over Total-25(OH)D in this study.

Several studies have evaluated the levels of Free-25(OH)D or Bio-25(OH)D in Chinese adults (23), postmenopausal women (24), and patients (25, 26). The median Total-25(OH)D (16.6 ng/mL) and median Bio-25(OH)D (1.65 ng/mL) levels in our cross-sectional study were slightly higher than the adults (aged 20–45 years) levels [mean Total-25(OH)D, 13.12 ng/mL; mean Bio-25(OH)D, 1.08 ng/mL] measured in winter in Shanghai, China, which is at a latitude between 30°40′ N and 31°53′ N (23). We suspect that this difference may be due to the latitude and season during which sampling was

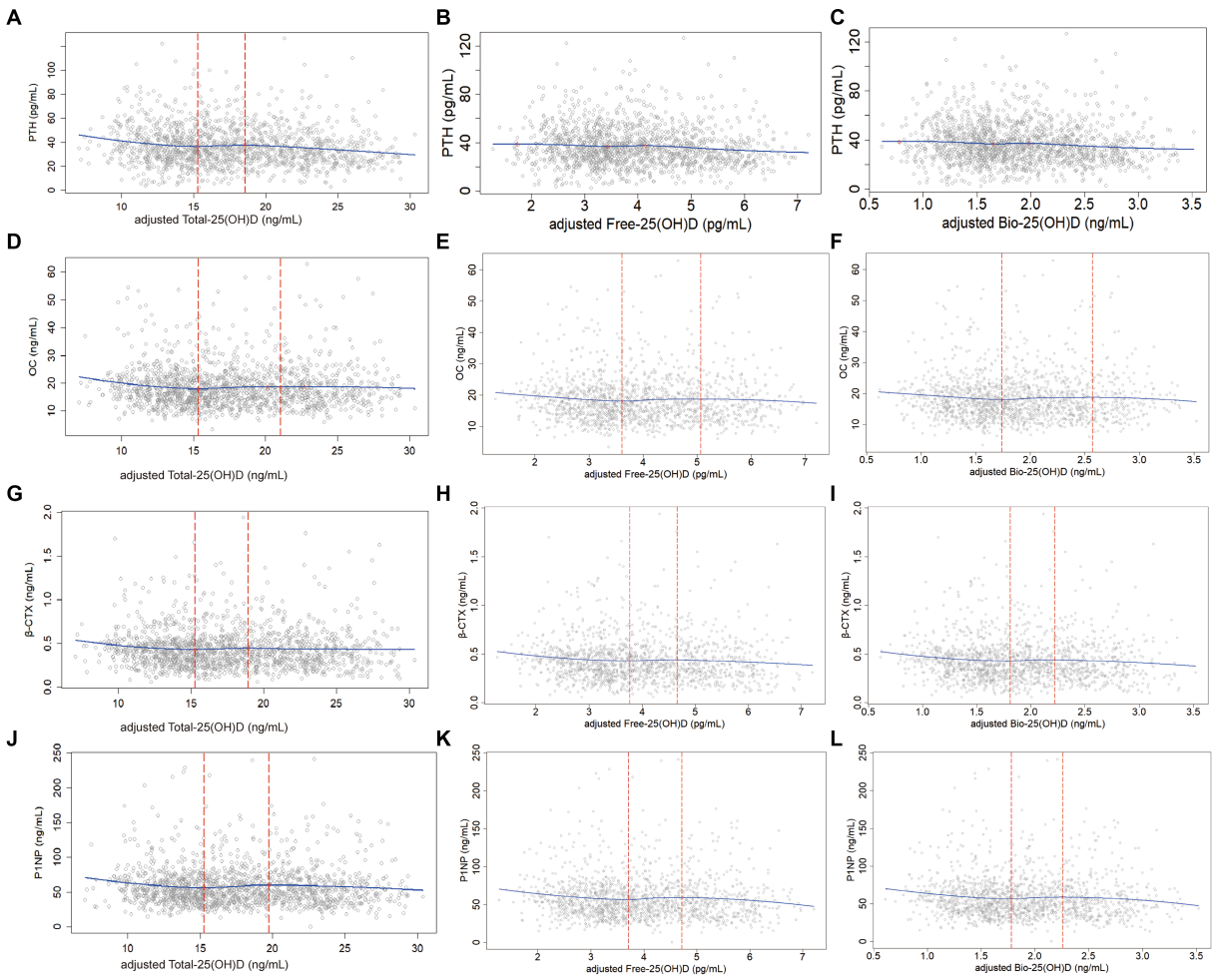

FIGURE 2
Non-linear associations (LOESS) between adjusted Total-25(OH)D, Free-25(OH)D or Bio-25(OH)D and PTH as well BTMs (data from the cross-sectional study): y-PTH, OC, β-CTX, or P1NP; x-adjusted Total-25(OH)D, Free-25(OH)D, or Bio-25(OH)D; **(A)**, **(D)**, **(G)**, and **(J)**: Non-linear associations of Total-25(OH)D with PTH as well as BTMs; **(B)**, **(E)**, **(H)**, and **(K)**: Non-linear associations of Free-25(OH)D with PTH as well as BTMs; **(C)**, **(F)**, **(I)**, and **(L)**: Non-linear associations of Bio-25(OH)D with PTH as well as BTMs. In the analysis for PTH, one sample was removed because the PTH concentration exceeded 150 pg/mL.

performed because a large proportion of our participants were from low-latitude regions or sampled in autumn, resulting in longer ultraviolet B exposure. As shown in our study, participants at lower latitudes or sampled in autumn had higher Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D concentrations. An epidemiological survey showed that the mean serum 25(OH)D level increased slightly with age (from 18 to 65 years) (27), which is consistent with our finding that younger women had lower Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D concentrations. Moreover, the Bio-25(OH)D level in this study was lower than that in postmenopausal women [2.91 (2.11–4.17) ng/mL], sampled in Shanghai (24). Furthermore, we found that Free-25(OH)D or Bio-25(OH)D showed characteristics similar to those of Total-25(OH)D in terms of longitudinal, urban–rural, and genetic profiles.

It has been confirmed that Free-25(OH)D is strongly correlated with Total-25(OH)D in most normal populations whether measured directly or indirectly (28). Similarly, in our study, Free-25(OH)D and Bio-25(OH)D were positively correlated with Total-25(OH)D. Previous studies have compared the associations of Free-/Bio-25(OH)D and Total-25(OH)D with various markers of vitamin D bioactivity, including serum Ca (29), PTH (30), Ca absorption, BTMs (31), bone mineral density (11, 15), and endogenous antimicrobial peptides (32). Some but not all of these comparisons suggest that Free-25(OH)D is a better biomarker of the effects of vitamin D. For example, a review from Bikle et al. concluded that measurement of the free level might provide a better index of vitamin D status than the total level in some clinical situations (6). However, Michaelsson et al. concluded that vitamin D status assessed by direct measurement of Free-25(OH)D was not a better indicator of bone mineral density than Total-25(OH)D (15). Therefore, the free hormone hypothesis regarding 25(OH)D remains unproven.

Based on the association of vitamin D with bone health, the optimal threshold of Total-25(OH)D is mainly determined by skeletal outcomes, such as bone mineral density (22, 33), PTH (34), and BTMs (35) because serum Total-25(OH)D has been shown to be positively/negatively associated these parameters at lower but not at higher Total-25(OH)D levels. Our previous study in Chinese women of childbearing age explored the threshold of vitamin D sufficiency (15.25–16.75 ng/

TABLE 3 Associations between serum Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D level and PTH or BTMs below and above the adjusted cutoffs of 25(OH)D, estimated by nonlinear least squares estimation and segmented regression (data from the cross-sectional study).

	Below cutoff$_1$ β (95%CI)	Cutoff$_1$	Above cutoff$_1$ β (95%CI)	Cutoff$_2$	Above cutoff$_2$ β (95%CI)
Total-25(OH)D					
PTH	−1.35 (−2.35,-0.34)	14.19 (12.23,16.14)	0.63 (−0.74,2.00)	18.03 (15.11,20.96)	−0.69 (−1.13,-0.25)
OC	−0.55 (−0.91,-0.19)	15.14 (13.56,16.71)	1.03 (−2.18,4.25)	16.39 (13.91,18.86)	−0.001 (−0.16,0.15)
β-CTX	−0.01 (−0.02,-0.00003)	14.79 (12.11,17.47)	0.01 (−0.03,0.05)	17.22 (11.83,22.62)	−0.002 (−0.007,0.004)
P1NP	−1.62 (−3.03,-0.21)	15.08 (12.89,17.27)	1.208 (−0.52,2.94)	20.08 (16.62,23.54)	−0.83 (−1.92,0.26)
Free-25(OH)D					
PTH	−2.29 (−4.87, 0.29)	3.61 (3.16, 4.05)	10.65 (−26.27, 47.57)	3.85 (3.42, 4.29)	−2.57 (−4.17, −0.98)
OC	−1.23 (−2.65, 0.19)	3.38 (2.46, 4.29)	0.21 (−0.33, 0.75)		
β-CTX	−0.04 (−0.08, 0.002)	3.42 (2.52,4.31)	0.01 (−0.04, 0.06)	4.74 (3.24,6.24)	−0.02 (−0.07, 0.02)
P1NP	−6.14 (−11.09, −1.20)	3.47 (2.87,4.08)	4.08 (−3.73, 11.89)	4.65 (3.75,5.55)	−4.00 (−8.82, 0.83)
Bio-25(OH)D					
PTH	15.70 (−18.90, 50.29)	1.09 (0.78, 1.40)	−7.19 (−22.13, 7.74)	1.51 (0.74, 2.29)	−2.38 (−4.53, −0.23)
OC	−2.68 (−5.72, 0.36)	1.60 (1.19, 2.01)	0.51 (−0.60, 1.61)		
β-CTX	−0.08 (−0.16, 0.001)	1.66 (1.26, 2.06)	0.04 (−0.09, 0.17)	2.21 (1.58, 2.83)	−0.05 (−0.13, 0.03)
P1NP	−12.97 (−23.28, −2.65)	1.66 (1.37, 1.96)	8.16 (−8.36, 24.67)	2.21 (1.74, 2.67)	−7.06 (−16.86, 2.73)

Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D were adjusted for region, regional type, season, latitude, age, BMI, CRE, hsCRP, Ca intake, time spent outdoors, and GRS. Total-25(OH)D, total 25(OH)D; Free-25(OH)D, free 25(OH)D; Bio-25(OH)D, bioavailable 25(OH)D; PTH, parathyroid hormone; OC, osteocalcin; β-CTX, β-CrossLaps of type 1 collagen containing cross-linked C-telopeptide; P1NP, procollagen type 1 N-terminal propeptide; GRS, genetic risk score. In the analysis of the 25(OH)D cutoff for PTH, one sample was removed because the PTH concentration exceeded 150 pg/mL.

mL) in terms of Total-25(OH)D, during which serum PTH plateaued (18). However, no consensus on the optimal serum Total-25(OH)D concentration for the PTH platform and/or maximal suppression has emerged from the various studies (34), with some studies finding no such threshold or plateau (36). Some studies have explored the Free-25(OH)D and Bio-25(OH)D threshold for vitamin D deficiency (23, 37). For example, Celik et al. estimated the cutoff values for all forms of vitamin D in terms of vitamin D deficiency based on PTH in obese and healthy adolescents using local polynomial regression models. The Free-25(OH)D and Bio-25(OH)D threshold for the entire study population were 13 pg/mL and 5.4 ng/mL, respectively (37). Yao et al. also reported a PTH-based Bio-25(OH)D threshold of 5.8 nmol/L (2.32 ng/mL) in Chinese adults in Shanghai using LOESS (23). However, no Free-25(OH)D or Bio-25(OH)D threshold for PTH was found in our study. Despite the limited research available, we have identified differences in the threshold association of Free-25(OH)D or Bio-25(OH)D with PTH in different populations. Therefore, more studies are needed to identify appropriate Free-25(OH)D and Bio-25(OH)D concentrations in specific populations.

Vitamin D has direct effects on bone cells, including simultaneous activation of both osteoblasts and osteoclasts, since the bone cells express VDR. But it is somewhat unclear whether the result is bone formation, bone resorption, or a neutral effect (38). PTH treatment also concomitantly stimulates new bone formation and bone resorption (39). It was reported that 25(OH)D levels in healthy premenopausal women were positively related with serum CTX and negatively with serum PTH and P1NP (40, 41). Cross-sectional studies also showed that the nonlinear relationship between vitamin D and PTH and BTMs was not entirely consistent (35, 42). We then analyzed the nonlinear relationships between the active forms of 25(OH)D and BTMs in this study population, and compared the results with those for Total-25(OH)D. The 25(OH)D level has been shown to be associated with seasonality, latitude (43), age, BMI (44), serum Ca, P, and CRE levels (45), inflammatory markers (46), genetic factors (47), and other factors. Thus, we further controlled the influence of time spent outdoors, serum P, CRE, and hsCRP levels, and gene polymorphisms based on the preliminary analysis (18). In the present study, the adjusted Total-25(OH)D thresholds were observed for each BTM by LOESS, NLS, and SR. The Total-25(OH)D thresholds obtained for BTMs were relatively concentrated, ranging from 14.79 ng/mL to 15.14 ng/mL. However, the Free-25(OH)D and Bio-25(OH)D thresholds were only obtained for P1NP. An alternative explanation for this finding is that the response of different BTMs to changes in active forms of vitamin D may be inconsistent among women of childbearing age with relatively healthy bones. NLS and SR suggested that the significant Free-25(OH)D and Bio-25(OH)D cutoff were 3.47 pg/mL and 1.66 ng/mL, respectively. The Bio-25(OH)D cutoff was lower than the value of 5.8 nmol/L (2.32 ng/mL) in another Chinese study performed in adults of both sexes, which showed that PTH reached a virtual plateau at this cutoff value (23). This finding might reflect differences in study populations and endpoints. Of course, these results suggest that Free-25(OH)D and Bio-25(OH)D might also be indicators that can be used to explore vitamin D deficiency in terms of bone remodeling in women of childbearing age. Nevertheless, more studies are warranted to verify these findings.

Many studies have reported that vitamin D levels are significantly associated with blood lipid levels. Liu et al. found that deficiency of vitamin D [serum 25(OH)D < 20 ng/mL] might be a risk factor for elevated TG and reduced HDL-C after adjustment for other components in elderly Chinese individuals (48). Similarly, our previous analysis also found that the serum 25(OH)D levels showed a significant association with elevated TG and reduced HDL-C (19).

TABLE 4 Multivariate logistic regression for the association of MetS and individual components of MetS with vitamin D status (data from the cross-sectional study).

	Total-25(OH)D[OR (95%CI)]				Free-25(OH)D[OR (95%CI)]			Bio-25(OH)D[OR (95%CI)]		
	Continuous	<15.14 ng/mL	15.14–18.03 ng/mL	≥18.03 ng/mL	Continuous	<3.47 pg/mL	≥3.47 pg/mL	Continuous	<1.66 ng/mL	≥1.66 ng/mL
MetS										
Model 1	0.986 (0.962–1.01)	1.031 (0.612–1.736)	reference	0.811 (0.479–1.372)	0.978 (0.903–1.058)	reference	0.857 (0.602–1.221)	0.963 (0.819–1.131)	reference	0.908 (0.638–1.292)
Elevated triglycerides										
Model 1	0.993 (0.972–1.015)	1.667 (0.987–2.814)	reference	1.416 (0.836–2.398)	0.969 (0.902–1.042)	reference	0.983 (0.718–1.347)	0.952 (0.823–1.102)	reference	0.976 (0.713–1.337)
Model 2	1.002 (0.98–1.025)	1.536 (0.892–2.645)	reference	1.531 (0.887–2.643)	0.979 (0.909–1.054)	reference	1.066 (0.768–1.479)	0.971 (0.836–1.128)	reference	1.051 (0.758–1.458)
Reduced HDL-C										
Model 1	0.969 (0.955–0.984)	1.444 (1.049–1.987)	reference	0.975 (0.709–1.342)	0.962 (0.918–1.008)	reference	0.78 (0.631–0.964)	0.924 (0.84–1.016)	reference	0.784 (0.634–0.968)
Model 2	0.969 (0.954–0.983)	1.371 (0.991–1.899)	reference	0.933 (0.675–1.291)	0.963 (0.919–1.01)	reference	0.77 (0.621–0.956)	0.925 (0.839–1.019)	reference	0.772 (0.622–0.958)
Elevated glucose										
Model 1	1.046 (1.018–1.075)	0.659 (0.347–1.251)	reference	0.951 (0.518–1.745)	1.122 (1.039–1.211)	reference	1.311 (0.845–2.033)	1.263 (1.085–1.47)	reference	1.45 (0.933–2.253)
Model 2	1.048 (1.019–1.077)	0.646 (0.339–1.231)	reference	0.954 (0.519–1.753)	1.123 (1.041–1.212)	reference	1.323 (0.852–2.055)	1.265 (1.087–1.473)	reference	1.463 (0.94–2.277)
Elevated waist circumferences										
Model 1	0.984 (0.964–1.005)	1.609 (0.988–2.622)	reference	1.212 (0.742–1.981)	0.972 (0.907–1.043)	reference	1.062 (0.776–1.452)	0.947 (0.819–1.095)	reference	1.13 (0.826–1.545)
Model 2	0.986 (0.966–1.007)	1.601 (0.97–2.642)	reference	1.245 (0.753–2.059)	0.978 (0.91–1.05)	reference	1.102 (0.801–1.517)	0.956 (0.824–1.109)	reference	1.173 (0.853–1.615)
Elevated blood pressure										
Model 1	1.006 (0.986–1.026)	0.86 (0.55–1.344)	reference	0.792 (0.507–1.237)	1.01 (0.946–1.079)	reference	1.026 (0.757–1.39)	1.033 (0.905–1.18)	reference	1.006 (0.743–1.363)
Model 2	1.006 (0.986–1.027)	0.794 (0.504–1.251)	reference	0.743 (0.473–1.168)	1.015 (0.949–1.086)	reference	1.008 (0.74–1.374)	1.042 (0.909–1.195)	reference	0.989 (0.726–1.348)

Model 1 was adjusted for age, race, education, physical activity, smoking, drinking, and BMI. Model 2 was adjusted for age, race, education, physical activity, smoking, drinking, BMI, plus additionally adjusted for other components of the MetS. Total-25(OH)D, Total 25(OH)D; Free-25(OH)D, free 25(OH)D; Bio-25(OH)D, bioavailable 25(OH)D; HDL-C, high-density lipoprotein cholesterol.

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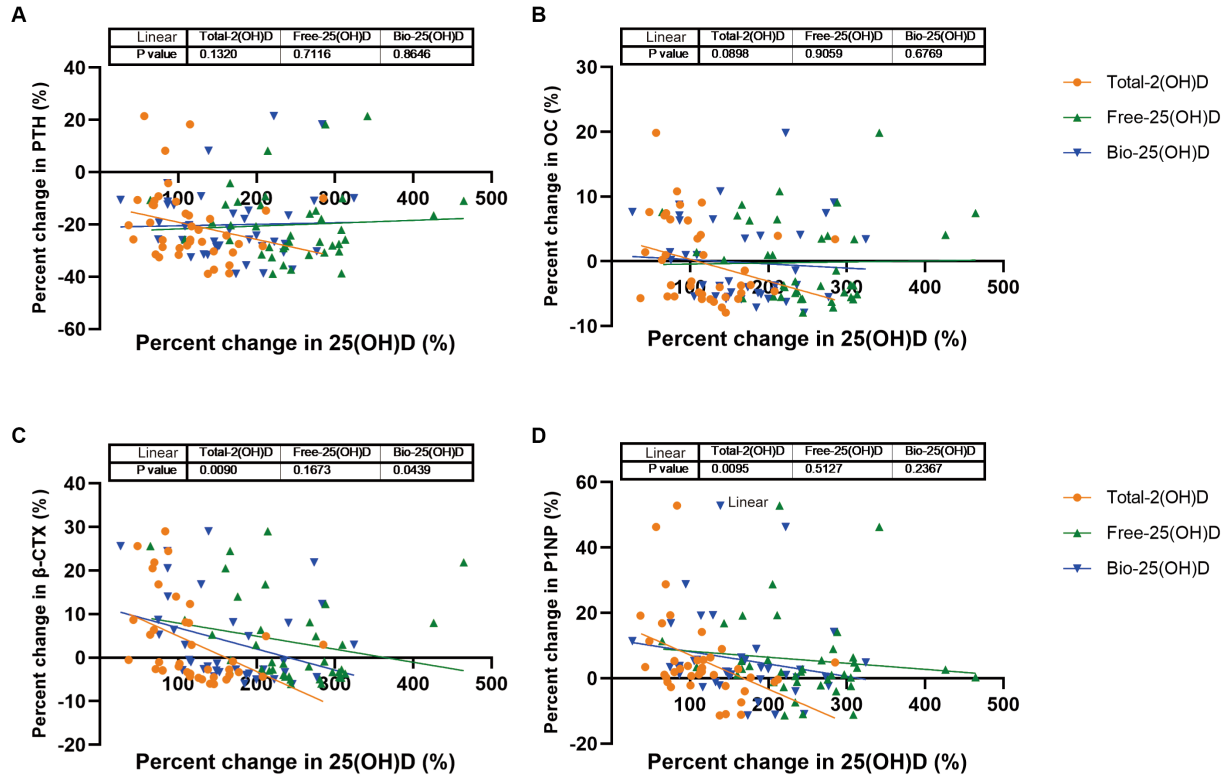

FIGURE 3
PTH and BTMs response to change in Total-25(OH)D vs. Free-25(OH)D or Bio-25(OH)D following vitamin D supplementation (data from the intervention study). **(A)** PTH response to change in Total-25(OH)D vs. Free-25(OH)D or Bio-25(OH)D; **(B)** OC response to change in Total-25(OH)D vs. Free-25(OH)D or Bio-25(OH)D; **(C)** β-CTX response to change in Total-25(OH)D vs. Free-25(OH)D or Bio-25(OH)D; **(D)** P1NP response to change in Total-25(OH)D vs. Free-25(OH)D or Bio-25(OH)D.

Possible mechanisms include the following. First, vitamin D and cholesterol share a common metabolic substrate, namely 7-dehydrocholesterol (49). Second, active 25(OH)D metabolites can regulate mitochondrial activity, lipid metabolism, and adipogenesis via VDR signaling (50). In this study, we also found that the three forms of 25(OH)D were lower in women with reduced HDL-C, while no differences were found between women with and without MetS. Lower Total-25(OH)D, Free-25(OH)D, and Bio-25(OH)D, which were stratified according to thresholds determined by NLS and SR, were associated with reduced HDL-C. The potential mechanism underlying the association between Free-25(OH)D and Bio-25(OH)D and HDL-C may be the same as Total-25(OH)D. Although an increase in serum Free-25(OH)D of 1 pg/mL and an increase in Total/Bio-25(OH)D of 1 ng/mL were significantly associated with an increased risk of elevated glucose, no associations were found after stratification. Free-25(OH)D, Bio-25(OH)D, and Total-25(OH)D thresholds act in the same way with regard to assessment of the risk of MetS. These findings also suggested that Free-/Bio-25(OH)D did not offer additional advantages over Total-25(OH)D regarding its association with metabolic traits, similar to a Mexican study (51).

Furthermore, we performed a 16 weeks intervention study to clarify whether Free-25(OH)D and Bio-25(OH)D would respond more sensitively to vitamin D supplementation in vitamin D-deficient women and whether the associations of Free-25(OH)D and Bio-25(OH)D with PTH and BTMs would become stronger in response to an increase in Free-25(OH)D and Bio-25(OH)D. The results within 4 weeks suggested that suppression of BTMs may be more strongly driven by Total-25(OH)D, which is consistent with the findings of another intervention study in participants aged >65 years, namely, that Total-25(OH)D but not Free-25(OH)D or Bio-25(OH)D (calculated) had a positive relationship with percent change in bone mineral density at the femoral neck after 12 months of vitamin D supplementation (52). However, the results of such studies have been inconsistent. A study by Smith et al. showed that there was a progressive decrease in serum PTH with increasing doses of vitamin D and that the percentage change in PTH was similar for Free-25(OH)D (directly measured) and Total-25(OH)D (53). Another study in adults aged ≥18 years with baseline 25(OH)D levels <20 ng/mL showed that an increase in Free-25(OH)D but not in Total-25(OH)D was significantly associated with a decrease in PTH early in the repletion course (baseline to 4 weeks) when 25(OH)D levels increase most rapidly (54). These inconsistencies may reflect differences in study populations and in the methodology used to determine Free-25(OH)D and Bio-25(OH)D levels. Therefore, further studies in specific populations using standardized and consistent measuring methods for Free-25(OH)D and Bio-25(OH)D are warranted.

This research has some limitations. First, the Free-25(OH)D and Bio-25(OH)D levels may have been overestimated because they were calculated based on the Total-25(OH)D, Alb, and VDBP levels in serum. Future comparisons should include direct measurement of Free-25(OH)D and Bio-25(OH)D. Second, although we considered the influence of season and time spent outdoors on the results, we lack detailed data on dietary habits, sunlight exposure, and use of sunscreen preparations.

5. Conclusion

In this research, which included a cross-sectional study and another with an interventional design, we investigated Free-25(OH)D and Bio-25(OH)D thresholds based on PTH and three BTMs with adjustment for environmental and genetic confounders, assessed the risk of MetS according to these thresholds, and compared the possible advantages of using Free-25(OH)D and Bio-25(OH)D over Total-25(OH)D. Unlike Total-25(OH)D, only the P1NP platform was found for both active forms of 25(OH)D. Although the thresholds determined for all three forms of 25(OH)D could also predict the risk of reduced HDL-C, no superiority of Free-25(OH)D or Bio-25(OH)D was found based on the PTH and BTMs response to changes in 25(OH)D in Chinese women of childbearing age during the intervention study. However, more cohort and intervention studies are needed to confirm our findings.

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 humans were approved by the Ethical Review Committee of Center for Disease Control and Prevention (CDC) (No. 2018-009 and No. 201519-B). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XS: data curation, formal analysis, methodology, software, visualization, writing – original draft, and writing – review and editing. YC, HZ, XZ, and SL: resources, methodology, and software. YH: investigation and conceptualization. LY: conceptualization, funding acquisition, project administration, 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1131140/full#supplementary-material

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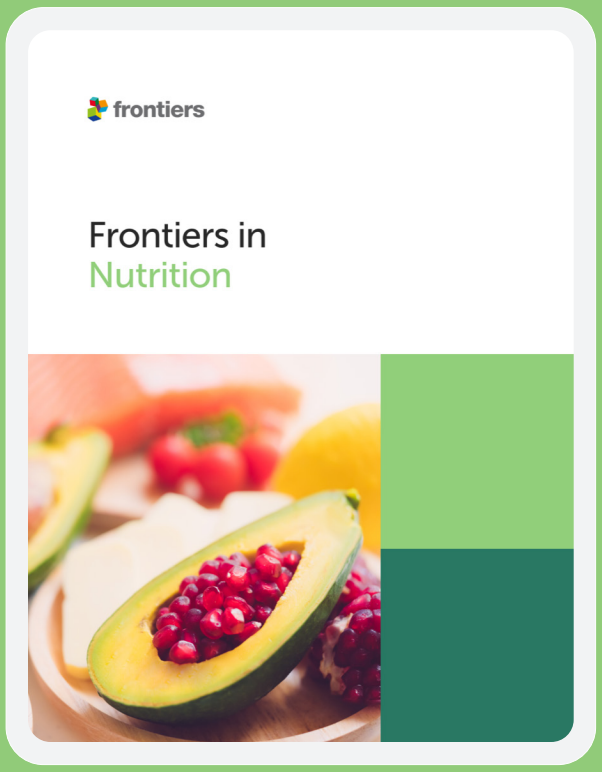

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