INSULIN RESISTANCE, METABOLIC SYNDROME, AND CARDIOVASCULAR DISEASE

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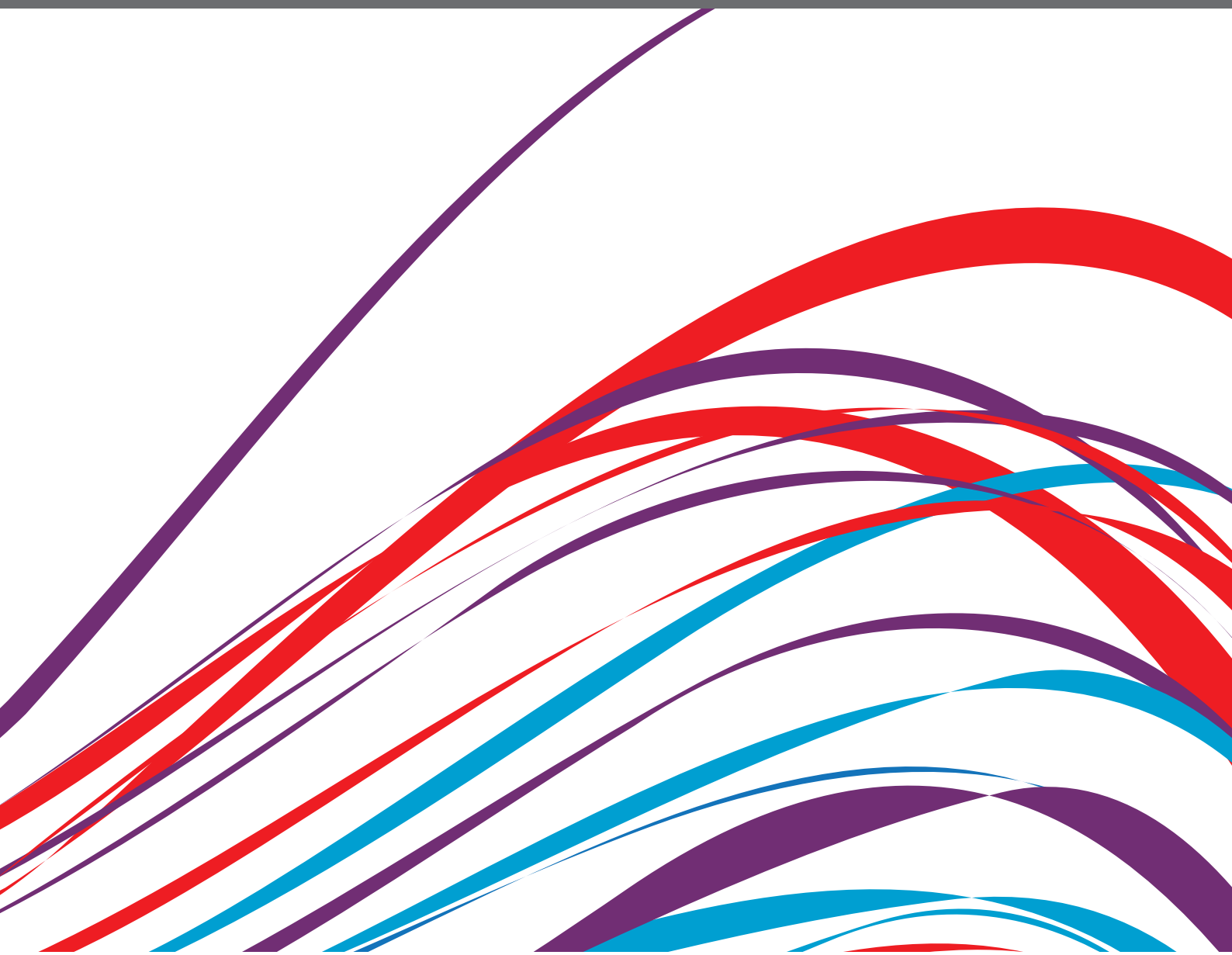

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INSULIN RESISTANCE, METABOLIC SYNDROME, AND CARDIOVASCULAR DISEASE

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EDITORIAL
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Editorial: Insulin Resistance, Metabolic Syndrome, and Cardiovascular Disease

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Editorial on the Research Topic

Insulin Resistance, Metabolic Syndrome, and Cardiovascular Disease

Two-hundred and eighty-six authors contributed 35 articles to the Research Topic, Insulin Resistance, Metabolic Syndrome, and Cardiovascular Disease, which received 70,189 views as of April 20 2022.

Heart Failure (HF) could be caused by multiple risk factors, and its treatments are still evolving. Six articles in this Research Topic studied the progression and outcomes of HF. Controversial evidence from prior observational studies indicated that the components of metabolic syndrome (MetS), including hypertension, obesity, and hypercholesterolemia, might have a protective effect on the prognosis in patients with established HF ("epidemiologic paradox"). Huang Z.-M. et al. found that MetS was not associated with all-cause or cardiovascular mortality in HF patients, but was with an increased risk of composite cardiovascular events. Findings from the meta-analysis therefore challenged the "epidemiologic paradox" in the relationship between HF and MetS. In the National Health and Nutrition Examination Survey (NHANES) 2007–2016, patients with chronic HF exhibited higher body mass index (BMI) with lower energy and macronutrient intake, lower physical activity level, and longer rest time and hemodilution (Xu et al.). In addition, the HF patients with BMI $\geq$ 30 kg/m^2 had higher water intake, sedentary time, and hematocrit, while intakes of energy and macronutrient, and physical levels were comparable with patients with BMI $<$30 kg/m^2. In a review article, Zeng Q. et al. assessed the mechanisms and perspectives of sodium-glucose cotransporter 2 (SGLT2) inhibitors, a novel class of oral antidiabetic medication, in patients with HF. Multiple clinical trials have shown a pronounced decrease in hospitalization due to HF shortly after starting use of SGLT2 inhibitors. SGLT2 inhibitors may exert their benefits in HF by targeting inflammation, oxidative stress, insulin resistance (IR), energy metabolism, advanced glycation end products, obesity, and hyperuricemia. In a retrospective case-control study of 642 patients with rheumatic heart disease (RHD) and HF, Liu C. et al. reported that continuous use of digoxin was significantly associated with increased all-cause mortality, cardiovascular disease (CVD), medium-/long-term HF re-hospitalization, and new-onset atrial fibrillation (AF) in RHD patients with HF, compared with the intermittent digoxin regimen. In a study among 277 patients with HF at various stages, Cao et al. showed that serum secreted frizzled-related protein 2, which may play an important role in the pathological mechanisms of MetS and CVD, was a risk factor for

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HF in patients with CVD, especially with type 2 diabetes. One novel article based on experiments using *Seipin* knockout mice reported that *Seipin* deficiency might promote the pathogenesis of cardiac hypertrophy and diastolic HF by inducing myocardial calcium handling impairment, endoplasmic stress, inflammation, and apoptosis (Wu X. et al.).

Acute myocardial infarction (AMI) and acute coronary syndrome (ACS) are major causes of death and disability and the primary cause of death in patients with type 2 diabetes. Five articles in the Research Topic addressed this condition. Cui et al. reviewed the latest developments in the research on type 2 diabetes and myocardial infarction (MI), including epidemiology, clinical features, and cardiorenal benefits of the newer class of hypoglycemic agents. Recent evidence of cardiovascular safety and benefits of hypoglycemic agents, including SGLT2 inhibitors, dipeptidyl peptidase-4 inhibitors, and glucagon-like peptide 1 agonists, was discussed in the review. In another review article, Li, Chen, et al. examined stress-induced hyperglycemia (SIH) in the setting of ACS. The article began with various methods used to define SIH in the context of ACS and their pros and cons in its definition, such as blood glucose on admission, glycemic GAP/SHR, and glucose variability. In addition, they summarized major clinical trials involving participants with both AMI and SIH that evaluated the effects of insulin-based therapy on outcomes of ACS, including the DIGAMI, HI-5 study, and BIOMArCS-2 trial. In terms of the mechanisms underlying the harmful consequences of SIH, oxidative stress could play an important role in myocardial reperfusion injury and remodeling. While diabetes is a risk factor for CVD, Zhang et al. reported that severe stress hyperglycemia ($\geq$200 mg/dL or 11.1 mmol/L) in patients without diabetes enrolled in a coronary care unit had an increased risk of the short-term death compared with patients with diabetes. Meanwhile, normal blood glucose and moderate stress hyperglycemia (140 mg/dL $\leq$ random blood glucose $\leq$ 200 mg/dL, or 7.8 mmol/L $\leq$ random blood glucose $<$ 11.1 mmol/L) had no adverse impacts on short-term outcomes in the patients. In a retrospective study (Li, Gao, et al.), new-onset AF occurred more frequently when complicated with elevated fasting glucose ($\geq$7 mmol/L) in patients with AMI, and every 1 mmol/L increase in fasting glucose was associated with a 5% higher rate of new-onset AF. Furthermore, patients with AMI complicated with elevated fasting hyperglycemia and AF had a worse prognosis in terms of in-hospital and long-term all-cause mortality. In addition to hyperglycemia, an article by Zheng S. et al. reported the impact of systolic blood pressure (SBP) on the prognosis of AMI. SBP showed a non-linear effect on AMI prognosis. Compared with normal SBP (90–140 mmHg), both low ($<$90 mmHg) and high ($>$140 mmHg) SBP had a worse prognosis in AMI patients, especially in those with a low SBP.

Three articles in the Research Topic were on percutaneous coronary intervention (PCI). Recent studies suggested that lipoprotein(a) and lipid parameters other than low-density lipoprotein cholesterol (LDL-C) could predict CVD, even in patients receiving statin therapy (1–3). However, in a retrospective study among 445 patients with a median follow-up of 36 months, Zeng, Ye, et al. found that baseline LDL-C showed a better predictive ability for major adverse cardiovascular events in statin-naïve patients after PCI than other non-LDL-C lipid parameters in Chinese. Hence, LDL-C remains a strong predictive factor for CVD events in CVD secondary prevention. Zhao et al. conducted a retrospective study to compare the differences in the long-term prognosis of diabetes and non-diabetes patients who underwent PCI for *de novo* lesions and late or very late stent thrombosis. Diabetes patients with *de novo* lesions showed a higher risk of composite clinical outcomes than their non-diabetes counterparts, and patients with late or very late stent thrombosis lesions had higher risks of long-term composite clinical outcome events compared with patients with *de novo* lesions. Finally, Li et al. introduced a study protocol for a retrospective, multicenter analysis to develop and validate the risk prediction model for new-onset diabetes after PCI.

A meta-analysis by Li X. et al. assessed the impact of MetS and its components on the prognosis in patients with CVD. Fifty-five studies with 162,450 patients from 25 countries and regions were included. MetS was associated with increased risks of all-cause mortality, CVD mortality, MI, stroke, target vessel revascularization, and HF in CVD patients. Regarding the impact of individual MetS components on CVD, higher BMI ($>$25 kg/m^2) was associated with lower all-cause mortality, which could be potentially caused by reserve causality, while dyslipidemia and abnormal glucose metabolism were the main risk factors for CVD outcomes.

Many metabolic conditions could worsen vascular lesions. Zheng H. et al. used clinical data of patients with acute thoracic aortic dissection (AD), an animal model of IR and AD, and an IR model of human aortic vascular smooth muscle cells (VSMCs) to explore the role of IR in the pathogenesis of AD. The authors reported that IR could induce the phenotypic switching of VSMCs from contractile to synthetic, which contributed to the occurrence of AD. It was well accepted that metabolic abnormalities could contribute to thoracic aorta calcification (TAC). Liang et al. reported that patients with metabolic abnormalities had higher TAC scores than those without metabolic abnormalities, and β-hydroxybutyric (BHB) was a protective factor for TAC after adjusting for potential metabolic parameters. Using calcification models with arterial rings and VSMCs, the study showed that BHB might serve as an effective intervention target for high phosphate-induced vascular calcification *via* regulating VSMC phenotypical transition. In addition, the enhancement of autophagy in VSMCs by BHB could be the mechanism contributing to the inhibition of arterial calcification.

Endothelial progenitor cells (EPCs) are involved in the regeneration and repairment of adult vessels, and inflammation could potentially impair the function of EPCs. In a clinical study of 34 patients with acute AD and 20 non-AD patients with essential hypertension, Huang Z. et al. reported that the EPC number, migration, and proliferation were significantly reduced in patients with acute AD, and inversely correlated with the AD detection risk score. In addition, the IL-6 and IL-17 levels were negatively correlated with circulating EPC number and migration or proliferation in acute AD, indicating a possible inflammatory mechanism

in circulating EPCs in the acute AD. In addition, Zeng, Zhang, et al. showed that the age-related decrement in the EPC function was related to the severity of non-ST elevation MI in men patients, which might also be due to systemic inflammation.

In a prospective population-based study of 7,104 Chinese individuals, Shi et al. reported that the level of proprotein convertase subtilisin/kexin type 9, a key LDL-C mediator, was positively associated with worse cardiometabolic risk profiles in women but not men, including high LDL-C, elevated triglycerides, hypertension, type 2 diabetes, and MetS.

Ectopic fat depots are associated with an increased cardiometabolic risk, and the accumulation of epicardial adipose tissue (EAT) underlies cardiac dysfunction in the population with obesity. Maimaituxun et al. studied the impact of epicardial adipose tissue volume (EATV) on the cardiac function. In this retrospective study among 180 patients with a mean BMI of 25 $\pm$ 5 kg/m^2, the authors found that EATV was independently associated with global longitudinal strain despite preserved left ventricular ejection fraction and a lack of obstructive coronary artery disease (CAD), which suggested that EAT might affect myocardial systolic function through impaired left ventricular longitudinal strain.

CVD and microvascular complications are leading causes of morbidity and mortality for individuals with type 2 diabetes, and four basic and one clinical studies addressed on diabetic complications. The hallmark of diabetic cardiomyopathy is calcium mishandling in cardiomyocytes. Deng et al. showed that calcium dobesilate (CaD), a drug commonly used to treat diabetic microvascular complications, could attenuate high glucose and high lipid-induced impairment in calcium signaling and reactive oxygen species (ROS) production in cardiomyocytes. Thus, CaD might offer a protective effect in diabetic cardiomyopathy. Yan et al. demonstrated that metformin activated the AMP-activated protein kinase (AMPK)—phosphorylating PDZ and LIM domain 5 (Pdlim5) pathway, which could interrupt the migratory machine of VSMCs and inhibit cell migration *in vitro* and *in vivo*. Metformin seemed to suppress diabetes-accelerated atherosclerosis by the maintenance of AMPK activity. In another study on diabetic cardiomyopathy, Liu M. et al. showed that Adropin, a regulatory polypeptide involved in energy hemostasis, lipid metabolism, and IR, could alleviate myocardial fibrosis and improve diastolic function in the diabetic cardiomyopathy rats. Due to the substantial consequences of CVD in patients with type 2 diabetes, early detection of subclinical myocardial changes has important clinical implications. Li W. et al. reported that the combination of real-time three-dimensional echocardiography and myocardial contrast echocardiography in the left ventricle could detect subclinical myocardial dysfunction and impaired myocardial microvascular perfusion. Another article on diabetic complications assessed the mechanism of diabetic retinopathy. Li H. et al. showed that CCN1, which is involved in angiogenesis and fibrosis of retina, activated the NOX4/ROS axis in microvascular endothelial cells and led to vascular leakage through inhibiting VE-cadherin expression. Hence, the up-regulation of CCN1 expression could contribute to the pathogenesis of diabetic retinopathy.

Adequate glycemic control is the cornerstone in the management of diabetes. Numerous clinical trials demonstrated that controlling the blood glucose within the target range would help prevent or attenuate diabetic complications. The most commonly used parameter for assessing glycemic control is the glycated hemoglobin A1c (HbA1c) measurement. Liu L. et al. reported a U-shaped relationship between HbA1c and long-term all-cause mortality among 37,596 patients with CAD during a median follow-up of 4 years. Both low ($<$ 5.7%) and high ($>$ 6.7%) HbA1c levels were associated with increased all-cause mortality compared with the reference group (5.7 $<$ HbA1c $\leq$ 6.1%). Besides HbA1c measurements alone, Huang D. et al. found that long-term visit-to-visit HbA1c variability was also associated with higher incidence of cardiovascular complications and all-cause mortality in type 2 diabetes in a *post-hoc* analysis of the ACCORD population. Over a median follow-up of 4.85 years, the hazard ratios for the primary outcome (the first occurrence of non-fatal MI, non-fatal stroke, or cardiovascular death) in the second, third, and the highest quartiles of the variability of HbA1c were 1.26 (95% CI 1.03–1.54), 1.24 (95% CI 1.01–1.52), 1.61 (95% CI 1.29–2.00). Although HbA1c is an index that reflects the mean blood glucose for the prior 2–3 months and the gold parameter for evaluating overall glucose control, mean blood glucose could only contribute to 60–80% of the variance in HbA1c. Hempe et al. (4) introduced the hemoglobin glycation index (HGI), a biomarker of population variation in HbA1c due to factors other than blood glucose concentration. Wu J.-d. et al. conducted a meta-analysis to examine the association between HGI and risks of CVD and all-cause mortality in 22,035 patients with type 2 diabetes during a median follow-up of 5.0 years. After adjusting for multiple traditional cardiovascular risk factors, the level of HGI was positively associated with risks of composite CVD and all-cause mortality, and the associations became non-significant when HbA1c was further adjusted for. Thus, HbA1c might drive the association between HGI and CVD in type 2 diabetes.

Five articles reported associations of triglyceride glucose (TyG) index, a novel surrogate marker of IR, with various cardiometabolic conditions. Chen et al. assessed the association between TyG and risk of new-onset diabetes among Chinese adults. Using data from the China Health and Retirement Longitudinal Study, the authors found that a higher TyG index was associated with an elevated risk of new-onset diabetes in middle-aged and older Chinese adults. The effect of the TyG index on diabetes was not modified by age, sex, BMI, glycemic status, fasting blood glucose, or triglyceride level. Liu X.-c. et al. using data from the NHANES 1999–2014 demonstrated non-linear relationships of the TyG index with all-cause mortality and mortality from CVD among the US general population. In an analysis of data from 409 patients who underwent septal myectomy due to hypertrophic obstructive cardiomyopathy, Wei et al. reported that postoperative atrial fibrillation occurred more frequently in the high TyG index group. In the receiver operating characteristic curve analysis,

the TyG index could provide a moderate predictive value for postoperative atrial fibrillation after septal myectomy. In a retrospective cohort study of 103 patients with HF who had received cardiovascular magnetic resonance (CMR) imaging examination, Yang et al. concluded that the TyG index was positively associated with myocardial fibrosis detected by CMR. A higher TyG index was also associated with poor prognosis in HF patients after adjusting for other risk factors, including diabetes. In a meta-analysis of 12 studies involving 28,795 patients with CAD, Luo et al. showed that the elevated TyG index was associated with an increased risk of major adverse cardiovascular events, MI, revascularization, and stroke in patients with CAD.

Li et al. a systematically reviewed the relationships of oral, tongue-coating microbiota with metabolic disorders. First, oral microbiota disorders have been reported to be related to diabetes, obesity, CVD, cancer, and other systemic diseases. Second, the mechanism by which the tongue coating microbiota affects metabolisms may be similar to intestinal microbiota, including but not limited to the biological barrier effect, involvement in the nitric oxide cycle, and taste production.

In conclusion, this Research Topic contains a wide range of articles that span from original research to reviews, from animal to human studies, from *in vitro* to *in vivo* experiments, and from small clinical studies to large population-based studies. Evidence in this collection makes a significant contribution to our understanding of the relations among IR, MetS, and CVD.

AUTHOR CONTRIBUTIONS

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

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Association Between Triglyceride Glucose Index and Risk of New-Onset Diabetes Among Chinese Adults: Findings From the China Health and Retirement Longitudinal Study

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The triglyceride glucose (TyG) index has been proposed to be a surrogate of insulin resistance. In the present study, we aimed to examine the relationship between TyG index and the risk of incident diabetes in middle-age and older adults in China using nationally representative data from the China Health and Retirement Longitudinal Study 2011–2015. Information on socio-demographics, medical background, anthropometric measurement, and laboratory information were collected. The association between TyG index and diabetes was examined by Cox proportional hazards models and restricted cubic spline regression, and the results were presented in hazard ratio (HR) with 95% confidence interval (CI). Subgroup analyses were also conducted to examine potential interactions between demographics and TyG index. Among 7,428 participants, 791 (10.6%) of them developed diabetes over 3.4 years of follow-up. The multivariate HR for every SD increment in TyG index was 1.22 (95% CI, 1.14–1.31). When comparing to the lowest quartile of TyG index, the multivariate HRs for new-onset diabetes were 1.22 (0.96–1.54) for Q2, 1.61 (1.28–2.01) for Q3, and 1.73 (1.38–2.16) for Q4 (*P* for trend <0.001). The restricted cubic spline regression also showed a linear association. No interaction was found between subgroup variables and the association between TyG index and the risk of diabetes. In conclusion, higher TyG index associated with the elevated risk of new-onset diabetes in middle-aged and older adults.

Keywords: diabetes mellitus, association, CHARLS, predict, triglyceride glucose index

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INTRODUCTION

Diabetes is associated with an increased risk of cardiovascular disease and all-cause mortality, imposing a huge burden to public health (1). In the past few decades, the number of adults with diabetes has considerably increased, especially in low and middle-income countries (2). According to the International Diabetes Federation, China has the largest number of diabetic patients, reaching 114.4 million in 2017 (3). Therefore, identifying individuals at high risk for developing diabetes is of major importance to reduce the incidence rate and related complications.

Insulin resistance (IR) is a major pathophysiological pathway of type 2 diabetes development and may appear about two decades prior to the formal diagnosis (4, 5). A recent study indicated that IR was more closely associated with the risk of incident diabetes among Chinese adults than β-cell dysfunction, which is another pivotal pathological feature of diabetes (6). Additionally, in the past four decades, China has experienced disruptive transitions of dietary patterns and has witnessed a massive rise in with the rate of obesity, which is major factor for the progression of IR (7, 8). Moreover, the assessment of IR status is essential to identify individuals with high risk of diabetes. The traditional approach to measure IR, such as the homeostasis model assessment of IR (HOMA-IR), is time-consuming and costly for daily practice and in large epidemiological studies.

Recently, the triglyceride glucose (TyG) index, the product of triglyceride (TG) and fasting blood glucose (FBG), has attracted increasing attention as a simple indicator of IR owing to its good correlation with HOMA-IR and better performance to examine insulin sensitivity (9). Several studies have examined the association of TyG index and diabetes in Asia and western populations (10–15). However, the results were inconclusive and limited due to either the cross-sectional design (14), had small sample size (12) or being performed among selected population (11, 15). Therefore, we designed a prospective cohort study using nationally representative data from the China Health and Retirement Longitudinal Study (CHARLS) to explore the relationship between TyG index and the risk of new-onset diabetes.

METHODS

Study Design and Participants

CHARLS is an ongoing longitudinal survey to examine the social, economic and health status of community residents aged 45 years or older in China. Details of the study design of CHARLS have been described elsewhere (16). Briefly, the CHARLS adopted a multistage probability sampling and investigated 17,708 individuals in 28 provinces through random selection of 10,257 households to cover the overall population in China in the first wave (W1, 2011–2012). The response rate by provinces was up to 81% in the baseline survey. Information on socio-demographics, physical and biological assessments, and health-related information of participants were collected via standardized interviews. To date, the follow-up surveys have been conducted twice, including the second wave (W2) in 2013 and the third wave (W3) in 2015. For the present study, 10,111 individuals have been enrolled with data in laboratory measurement at W1. Participants aged <45, with missing data on TG, FBG, and glycated hemoglobin were excluded. After further excluding 1,760 participants with diabetes at baseline we also excluded subjects who died ($n = 103$) or lost follow-up ($n = 372$) in the subsequent waves (W2 and W3) of the study. Finally, 7,428 participants were enrolled for the final analysis (**Figure 1**). The Ethics Review Committee of Peking University approved CHARLS (IRB00001052–11015) and all participants have provided informed consent before participation.

Data Collection and Definitions

Trained researchers interviewed participants in their homes using computer-assisted technology to collect sociodemographic information [including age, gender, education level (primary school or lower, secondary school, and higher), and marriage status (current married or not)], health behavior (including current habits of smoking and alcohol drinking), medical history [including self-reported hypertension, diabetes, and cardiovascular disease (CVD)] and medication usage (including antihypertensive drugs, antidiabetic drugs, and lipid-lowering drugs).

Anthropometric indicators included systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), and waist circumference (WC). Blood pressure were measured three times in a seated position by trained nurses using the HEM-7200 electronic monitor (Omron, Dalian, Japan). Hypertension was defined as SBP≥140 mmHg or DBP ≥90 mmHg or self-reported prior diagnosis of hypertension by a doctor or using antihypertensive drugs in the past 2 weeks (17). Height and WC measurement were accurate to 0.1 cm and 0.1 kg, respectively.

These samples were transported from all study sites to Beijing and were stored at −80°C at the Chinese Center for Disease Control and Prevention. The determination of FBG, Hemoglobin A1c, TG, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were conducted by trained research staff. TyG index was calculated as ln [TG (mg/dl) × FBG (mg/dl)/2] (18). At baseline and follow-up, diabetes was defined as FBG > 125 mg/dL or Hemoglobin A1c >6.5%, self-reported prior diagnosis of diabetes by a doctor or using antidiabetic medications. Participants whose FBG was at 100–125 mg/dL or Hemoglobin A1c was at 5.7–6.4% were classified as having prediabetes (19). Participants without diabetes or prediabetes were defined as normoglycemia.

Statistical Analysis

Data was presented as mean and standard deviation (SD) for continuous variables, and percentage for categorical variables. All continuous variables have followed a Gaussian distribution according to Kolmogorov-Smirnov test ($P > 0.1$). Baseline characteristics and the rate of diabetes development were grouped by quartiles of TyG index (Q1, Q2, Q3, Q4) and compared using the One-Way ANOVA, Kruskal-Wallis H test or chi-square tests, as appropriate. We initially built Cox proportional hazards models to estimate HR with 95%

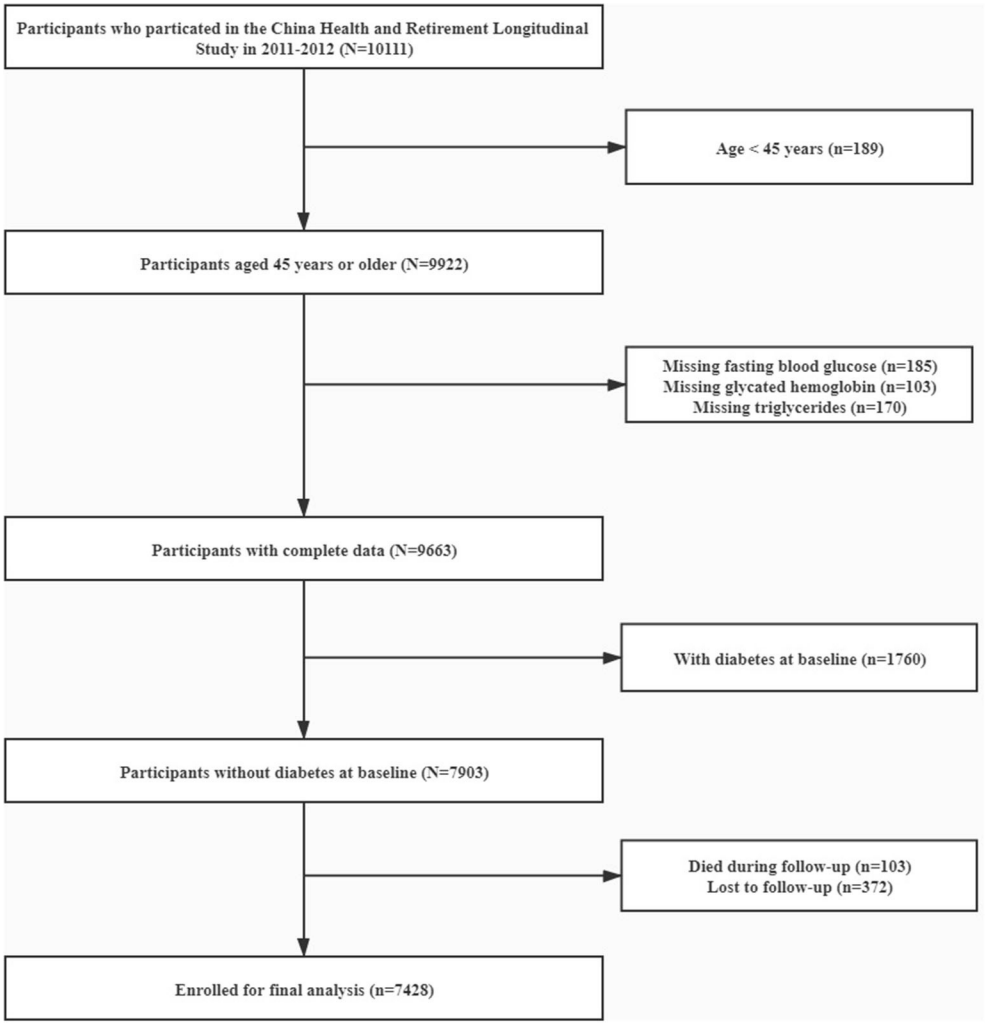

FIGURE 1 | Flow chart of study participants.

confidence interval (CI) of diabetes for TyG index as continuous (per SD increment) or categorical (quartiles) variables. Three Cox regression models were fitted. Model 1 only included TyG index. Model 2 was adjusted for age and gender. Fully adjusted model (Model 3) was adjusted for age, gender, education, marriage, smoking, alcohol drinking, BMI, WC, SBP, history of hypertension, history of CVD, and the usage of lipid-lowering drugs. Next, the shape of association between TyG index and incident diabetes was examined by multivariate restricted cubic spline model. We chose three knots at quartiles 25th, 50th, and 75th. Finally, we performed subgroup analyses of Cox proportional hazards models including age (<65 or ≥ 65 years), gender (male or female), BMI (<25 or ≥ 25 kg/m^2), glycemic status (normoglycemia or prediabetes at baseline), FBG (<100 or ≥ 100 mg/dL), and the level of TG (<100 or ≥ 100 mg/dL). $P < 0.05$ was considered statistically significant. R version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analyses.

RESULTS

Baseline Characteristics of Study Participants

The baseline characteristics of all participants according to quartiles of TyG index and the proportion of diabetes development were summarized in **Tables 1**, **2**, respectively. The present study included 7,428 participants (male: 46.5% and mean age: 59.3 years). The mean value TyG index was 8.56. Among quartiles of TyG index, we observed significant differences in all baseline covariates except for marriage status and education level (**Table 1**). When compared with participants without diabetes during follow-up, subjects who developed diabetes were older in age, having lower education level, less likely to be married, having higher levels of SBP, DBP, BMI, WC, TG, TC, LDL-C, HDL-C, FBG, Hemoglobin A1c, and TyG index, more likely to have hypertension and CVD, and more likely to use lipid-lowing drugs (**Table 2**).

TABLE 1 | Baseline characteristics of study participants according to quartiles of triglyceride glucose index.

	Q1	Q2	Q3	Q4	*P*-value
Number	1,857	1,857	1,857	1,857	
Age, years	59.56 ± 9.83	59.36 ± 9.36	59.60 ± 9.12	58.67 ± 9.11	0.008
Gender					<0.001
Male	1,007 (54.2)	921 (49.6)	782 (42.1)	747 (40.2)	
Female	850 (45.8)	936 (50.4)	1,075 (57.9)	1,110 (59.8)	
Education					0.338
Primary school or lower	1,325 (71.4)	1,307 (70.4)	1,332 (71.7)	1,281 (69.0)	
Secondary school	358 (19.3)	362 (19.5)	342 (18.4)	400 (21.5)	
Higher	173 (9.3)	187 (10.1)	183 (9.9)	176 (9.5)	
Current married	1,639 (88.3)	1,644 (88.5)	1,632 (87.9)	1,649 (88.8)	0.843
Current smoking	808 (43.5)	753 (40.5)	681 (36.7)	663 (35.7)	<0.001
Current drinking	548 (29.5)	498 (26.8)	403 (21.7)	441 (23.7)	<0.001
SBP, mmHg	126.70 ± 21.07	128.29 ± 20.75	131.30 ± 22.03	133.00 ± 21.63	<0.001
DBP, mmHg	73.69 ± 12.10	74.59 ± 11.96	76.34 ± 12.40	77.70 ± 12.52	<0.001
BMI, kg/m^2	22.01 ± 3.18	22.83 ± 3.73	23.61 ± 3.67	24.78 ± 3.67	<0.001
WC, cm	80.06 ± 10.74	82.55 ± 11.74	84.59 ± 12.75	87.71 ± 12.49	<0.001
TC, mg/dL	178.15 ± 33.52	188.61 ± 33.88	196.85 ± 36.74	205.84 ± 39.63	<0.001
TG, mg/dL	59.72 ± 13.51	86.81 ± 13.01	121.10 ± 18.03	217.93 ± 88.01	<0.001
LDL-C, mg/dL	107.93 ± 29.13	117.54 ± 30.86	124.06 ± 33.99	118.19 ± 39.17	<0.001
HDL-C, mg/dL	60.47 ± 14.97	55.65 ± 13.98	50.28 ± 12.89	42.27 ± 11.38	<0.001
FBG, mg/dL	93.57 ± 13.31	99.16 ± 10.40	101.03 ± 10.40	106.14 ± 9.98	<0.001
Hemoglobin A1c, %	5.05 ± 0.38	5.08 ± 0.39	5.11 ± 0.41	5.16 ± 0.40	<0.001
TyG index	7.90 ± 0.25	8.35 ± 0.10	8.70 ± 0.11	9.29 ± 0.33	<0.001
Hypertension	568 (30.8)	652 (35.5)	784 (42.7)	888 (48.2)	<0.001
Cardiovascular disease	197 (10.6)	233 (12.6)	249 (13.5)	265 (14.3)	0.006
Lipid-lowering drugs	34 (1.8)	52 (2.8)	76 (4.1)	107 (5.8)	<0.001
New-onset diabetes	123 (6.6)	166 (8.9)	232 (12.5)	270 (14.5)	<0.001

Data are presented as mean ± standard deviation or number (%).

Q, quartiles; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WC, waist circumference; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; TyG, triglyceride glucose.

Hazard Ratios for Incident Diabetes

Over a median of 3.4 years of follow-up, 791 (10.6%) participants have developed diabetes. After controlling for age, gender, education, marriage, smoking, drinking, BMI, WC, SBP, history of hypertension, history of CVD, and usage of lipid-lowering drugs (Model 3), every SD increase in TyG index was associated with 22% higher risk of developing diabetes (HR 1.22, 95% CI, 1.14–1.31). When comparing with the lowest quartile of TyG index, the multivariate HRs for new-onset diabetes were 1.22 (0.96–1.54) for Q2, 1.61 (1.28–2.01) for Q3, and 1.73 (1.38–2.16) for Q4 (*P* for trend <0.001) (**Table 3**). In the restricted cubic spline regression models, the relationship between TyG index and risk of incident diabetes was linear (**Figure 2**).

Subgroup Analyses

We performed subgroups analyses to stratify the association between TyG index and diabetes by age, gender, BMI, glycemic status, FBG and TG levels, as provided in **Table 4**. No interaction was found between subgroup variables and association of TyG index with the risk of diabetes (**Table 4**). The positive relationship remained consistent in both men and women, younger and elderly participants, lean and overweight/obese participants, normoglycemic and prediabetic participants, and participants with or without elevated FBG or TG levels (**Table 4**).

DISCUSSION

From the national data from the CHARLS, we have found a positive relationship between the simple surrogate of IR (TyG index) and the risk of new-onset diabetes in middle-aged and older Chinese adults. The effects of TyG index on diabetes did not interact with age, gender, BMI, glycemic status, FBG, or the level of TG.

Our results were consistent with previous studies that indicated a linear relationship between TyG index and the risk of diabetes. A recent study of 4,285 middle-aged and older Korean adults with BMI <25 kg/m^2 has found a positive association of TyG index and diabetes after 12 years of follow-up (15). Similarly, in lean Chinese individuals, Zhang et al. suggested that TyG index could predict the risk of incident diabetes (11). The authors claimed that lean people were more likely to suffer

TABLE 2 | Comparison of baseline characteristics of study participants who developed diabetes or not.

	Overall	Not developed DM	Developed DM	*P*-value
Number	7,428	6,637	791	
Age, years	59.30 ± 9.36	59.14 ± 9.37	60.68 ± 9.20	<0.001
Gender				0.210
Male	3,457 (46.5)	3,106 (46.8)	351 (44.4)	
Female	3,971 (53.5)	3,531 (53.2)	440 (55.6)	
Education				0.018
Primary school or lower	5,245 (70.6)	4,656 (70.2)	589 (74.6)	
Secondary school	1,462 (19.7)	1,319 (19.9)	143 (18.1)	
Higher	719 (9.7)	661 (10.0)	58 (7.3)	
Current married	6,564 (88.4)	5,888 (88.7)	676 (85.5)	0.008
Current smoking	2,905 (39.1)	2,595 (39.1)	310 (39.2)	0.993
Current drinking	1,890 (25.4)	1,709 (25.7)	181 (22.9)	0.088
SBP, mmHg	129.82 ± 21.51	129.29 ± 21.42	134.25 ± 21.78	<0.001
DBP, mmHg	75.58 ± 12.34	75.29 ± 12.24	77.98 ± 12.92	<0.001
BMI, kg/m^2	23.31 ± 3.71	23.16 ± 3.62	24.52 ± 4.23	<0.001
WC, cm	83.72 ± 12.28	83.31 ± 12.13	87.18 ± 12.94	<0.001
TC, mg/dL	192.36 ± 37.44	191.85 ± 37.38	196.62 ± 37.70	0.001
TG, mg/dL	121.39 ± 75.40	119.39 ± 73.93	138.21 ± 84.99	<0.001
LDL-C, mg/dL	116.93 ± 34.00	116.63 ± 33.98	119.46 ± 34.07	0.027
HDL-C, mg/dL	52.17 ± 14.98	52.46 ± 14.87	49.74 ± 15.68	<0.001
FBG, mg/dL	99.98 ± 11.98	99.54 ± 11.84	103.65 ± 12.47	<0.001
Hemoglobin A1c, %	5.10 ± 0.40	5.08 ± 0.39	5.25 ± 0.44	<0.001
TyG index	8.56 ± 0.56	8.54 ± 0.55	8.72 ± 0.56	<0.001
Hypertension	2,892 (39.3)	2,483 (37.7)	409 (52.1)	<0.001
Cardiovascular disease	944 (12.8)	807 (12.2)	137 (17.4)	<0.001
Lipid-lowering drugs	269 (3.6)	222 (3.3)	47 (5.9)	<0.001

Data are presented as mean ± SD or n (%).
Q, quartiles; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WC, waist circumference; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; FBG, fasting blood glucose; TyG, triglyceride glucose.

TABLE 3 | Cox-proportional hazard models for the association between triglyceride glucose index and incident diabetes.

	Case/total	Model 1	Model 2	Model 3
TyG index				
Per SD increase		1.34 (1.25, 1.43)	1.35 (1.26, 1.44)	1.22 (1.14, 1.31)
Quartiles				
Q1	123/1,857	Ref	ref	ref
Q2	166/1,857	1.37 (1.08, 1.73)	1.37 (1.08, 1.73)	1.22 (0.96, 1.54)
Q3	232/1,857	1.91 (1.54, 2.38)	1.90 (1.53, 2.37)	1.61 (1.28, 2.01)
Q4	270/1,857	2.27 (1.84, 2.81)	2.32 (1.87, 2.87)	1.73 (1.38, 2.16)
P for trend		<0.001	<0.001	<0.001

Data are presented as hazard ratio (95% confident interval). TyG, triglyceride glucose; SD, standard deviation; Q, quartiles.
Model 1 adjust for none.
Model 2 adjust for age and gender.
Model 3 adjust for age, gender, education, marriage, smoking, drinking, body mass index, waist circumference, systolic blood pressure, history of hypertension, history of cardiovascular disease, and usage of lipid-lowering drugs.

from hypertriglyceridemia because of the lack of subcutaneous fat, leading to subsequent IR and β-cell dysfunction (20). In a White European population, Navarro-Gonzalez et al. reported that the risk of developing diabetes was increased by 54% for per SD increase of TyG index, and the authors also suggested that TyG index was a better predictor of diabetes than TG or FBG separately (12).

In the subgroup analyses, we found that the positive relationship between TyG index and diabetes was consistent in all subgroup variables, and seemed to be more evident in

elderly, women, obese or prediabetic individuals. The reasons could be explained as follows. Visceral adiposity tissue increases with age and may lead to the higher incidence and risk of diabetes (21). In addition, the higher hepatocellular lipids in women may contribute to the increased risk of diabetes (22). Moreover, a recent study showed that TyG was an important mediator in the BMI-related diabetes development in both obese and non-obese individuals (23). Another retrospective study of 2,900 Korean adults reported that TyG index of 8.8 or higher significantly increased the risk of type 2 diabetes regardless of BMI range (13). Finally, prediabetes is more likely related to IR than normoglycemia, which explains the more pronounced risk of developing diabetes in this population (24–26).

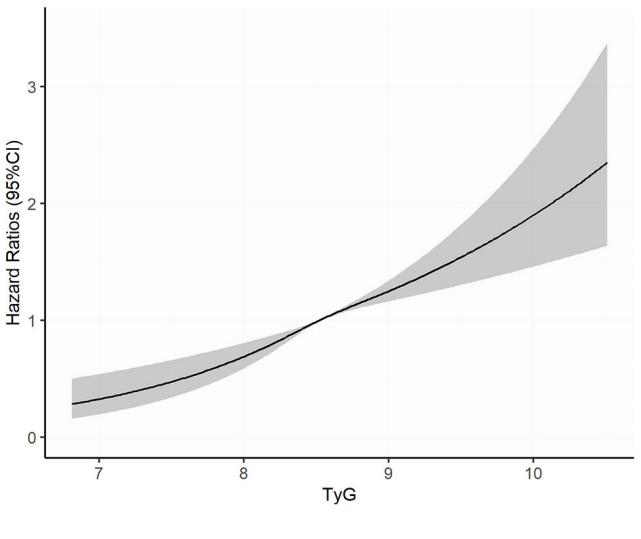

FIGURE 2 | Adjusted cubic spline model of the association between triglyceride glucose index and risk of new-onset diabetes. TyG, triglyceride glucose.

Several mechanisms have been reported to explain the relationship between TyG index and diabetes. On one hand, increased TG level in the blood contributes to the inhibited insulin activity, production of inflammatory cytokines, and muscle catabolism while overloaded TG in the pancreatic islet cells can disrupt the β-cell function (27). On the other hand, elevated glucose concentrations exerts toxic effects on β-cells by raising the level of reactive oxygen species (28). These mechanisms have been confirmed in an intervention study indicating that the capacity of insulin secretion and IR status were improved by the reduction in TG and FBG level (29). As a product of TG and FBG, high TyG index reveals both seriously decreased β-cells and the increased IR, which are considered to be the major stages of diabetes development (30). Despite the proposed pathways, more mechanistic researches are needed to reveal the role of TyG in the development of diabetes in different ethnicities.

Our findings have several clinical implications. First, TyG index was recently reported to be superior to traditional predictors of IR and diabetes such as TG/HDL-C and HMOA-IR (31). Second, several studies have shown that TyG index is a better predictor of diabetes compared with FBG or TG itself, as well as single lipid markers such as TC, LDL-C, and HDL-C

TABLE 4 | Multivariable-adjusted hazard ratios for the association between quartiles of triglyceride glucose index and incident diabetes by subgroups.

	Case/total	Q1	Q2	Q3	Q4	*P* for trend	*P* for interaction
Age, years							0.74
≥65	258/2,029	ref	1.22 (0.81, 1.83)	1.66 (1.13, 2.43)	1.82 (1.23, 2.69)	<0.001	
<65	533/5,399	ref	1.20 (0.89, 1.61)	1.55 (1.17, 2.05)	1.66 (1.26, 2.19)	<0.001	
Gender							0.14
Male	351/3,457	ref	1.15 (0.84, 1.58)	1.33 (0.97, 1.82)	1.56 (1.14, 2.14)	0.004	
Female	440/3,971	ref	1.34 (0.93, 1.94)	1.96 (1.40, 2.75)	1.99 (1.42, 2.79)	0.001	
BMI, kg/m^2							0.66
≥24	405/2,822	ref	1.53 (1.01, 2.33)	1.85 (1.25, 2.74)	2.03 (1.39, 2.97)	<0.001	
<24	377/4,493	ref	1.08 (0.80, 1.46)	1.55 (1.17, 2.06)	1.63 (1.20, 2.21)	<0.001	
Glycemic status							0.41
Normoglycemia	528/3,888	ref	1.16 (0.82, 1.63)	1.54 (1.12, 2.11)	1.38 (1.01, 1.89)	0.03	
Prediabetes	263/3,540	ref	1.17 (0.84, 1.65)	1.35 (0.95, 1.90)	1.80 (1.25, 2.60)	0.002	
FBG, mg/dL							0.602
≥100	508/3,762	ref	1.16 (0.88, 1.54)	1.26 (0.96, 1.66)	1.40 (1.07, 1.84)	0.013	
<100	283/3,666	ref	1.22 (0.84, 1.78)	1.29 (0.89, 1.87)	1.60 (1.12, 2.29)	0.001	
TG, mg/dL							0.089
≥200	123/825	ref	1.06 (0.61, 1.85)	1.23 (0.71, 2.12)	1.80 (1.09, 2.99)	0.016	
<200	668/6,603	ref	1.23 (0.96, 1.59)	1.44 (1.13, 1.84)	1.74 (1.37, 2.21)	<0.001	

Data are presented as hazard ratio (95% confident interval). Q, quartiles; BMI, body mass index; FBG, fasting blood glucose; TG, triglyceride.
Models are adjusted for age, gender, education, marriage, smoking, drinking, body mass index, waist circumference, systolic blood pressure, history of hypertension, history of cardiovascular disease, and usage of lipid-lowering drugs except the subgroup variable itself.

cross different ethnic groups (12, 32, 33). Third, TyG index is a simple, inexpensive and routine indicator for clinical practice. Finally, and most importantly, monitoring the TyG index can help to identify people at high risk of developing diabetes, even though their FBG or TG is not high or is at a borderline high level. For this group of people, timely lifestyle and diet adjustments are crucial (34).

Limitations

The strength of the current research was to include a nationally representative sample, using rigorous and standard protocol for data collection and follow-up. However, some limitations should be considered for cautious interpretation. First, residual confounding might exist such as physical activity and the family history of diabetes. Second, 2-h oral glucose tolerance test was not performed to detect cases of diabetes, which might underestimate the incidence. Third, we could not differentiate statins and fibrates from lipid-lowering drugs from the information in CHARLS. The impact of statins treatment on the association between TyG index and diabetes was not fully addressed, considering that statins treatment might increase the risk of developing diabetes (35). Fourth, the follow-up time was relatively short. Previous studies have suggested that prediabetes was associated with an increased risk of developing diabetes as well as CVD (36, 37), so studies with longer follow-up duration are needed to explore the association between TyG index and risk of CVD. Finally, all participants were Chinese people aged 45 years or older, caution should be made when interpreting our findings in younger individuals and in other ethnic populations.

In conclusion, TyG index was significantly associated with the risk of new-onset diabetes in middle-aged and older adults. TyG index might be a useful marker for predicting new-onset diabetes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: http://charls.pku.edu.cn/en.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Review Committee of Peking University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

C-lC, Y-qH, and Y-qF conceived and designed the study. C-lC, LL, Y-qH, and Y-qF analyzed and interpreted the data. C-lC and Y-qF wrote and/or 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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The Triglyceride-Glucose Index, an Insulin Resistance Marker, Was Non-linear Associated With All-Cause and Cardiovascular Mortality in the General Population

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Background: The triglyceride-glucose (TyG) index could serve as a convenient substitute of insulin resistance (IR), but epidemiological evidence on its relationship with the long-term risk of mortality is limited.

Methods: Participants from the National Health and Nutrition Examination Survey during 1999–2014 were grouped according to TyG index (<8, 8–9, 9–10, >10). Cox regression was conducted to compute the hazard ratios (HRs) and 95% confidence interval (CI). Restricted cubic spline and piecewise linear regression were performed to detect the shape of the relationship between TyG index and mortality.

Results: A total of 19,420 participants (48.9% men) were included. On average, participants were followed-up for 98.2 months, and 2,238 (11.5%) and 445 (2.3%) cases of mortality due to all-cause or cardiovascular disease were observed. After adjusting for confounders, TyG index was independently associated with an elevated risk of all-cause (HR, 1.10; 95% CI, 1.00–1.20) and cardiovascular death (HR, 1.29; 95% CI, 1.05–1.57). Spline analyses showed that the relationship of TyG index with mortality was non-linear (All non-linear $P < 0.001$), and the threshold value were 9.36 for all-cause and 9.52 for cardiovascular death, respectively. The HRs above the threshold point were 1.50 (95% CI, 1.29–1.75) and 2.35 (95% CI, 1.73–3.19) for all-cause and cardiovascular death. No significant difference was found below the threshold points (All $P > 0.05$).

Conclusion: Elevated TyG index reflected a more severe IR and was associated with mortality due to all-cause and cardiovascular disease in a non-linear manner.

Keywords: insulin resistance, all-cause mortality, cardiovascular mortality, NHANES, risk factors, triglyceride-glucose index

INTRODUCTION

Insulin resistance (IR) is an insensitivity state of the peripheral tissues and has been recognized as a major pathological feature of type 2 diabetes (1, 2). IR implies the defects in glucose uptake, reduction in glycogenesis synthesis, and decrement in suppressing lipid oxidation (3). In such a condition, to maintain glucose homeostasis, insulin secretion is increased and leads to chronic hyperinsulinemia, and therefore elevated oxidative stress and inflammatory responses (1, 4). IR may also impair lipid and serum urate metabolism, and therefore associated with metabolic syndrome (5, 6). Thus, quantifying IR quickly and accurately is important for clinical practice. The most established method to measure IR is euglycemic–hyperinsulinemic clamp. However, this method has limited applicability due to time and monetary cost (7). Another approach for measurement refers to Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), but is also expensive due to insulin measurement (8).

Previous studies have demonstrated that lipotoxicity and glucotoxicity are key factors in IR modulation (1, 3). The triglyceride-glucose (TyG) index is the logarithmized product of fasting triglycerides and fasting glucose and has been proposed as the alternative indicator of IR due to its relevance to lipotoxicity and glucotoxicity (9, 10). TyG index has demonstrated a close relationship with cardiometabolic outcomes, namely diabetes, arterial stiffness, hypertension, cardiovascular disease (CVD), stroke and obesity-related cancers in previous studies (11–18). However, epidemiological evidence on its relationship with all-cause mortality and CVD death is limited. Therefore, we will address the knowledge gap in the United States (US) general population.

MATERIALS AND METHODS

Study Design and Participants

We analyzed data from the National Health and Nutrition Examination Survey (NHANES) 1999–2014. The data cut-off period was 31 December 2015. NHANES is a nationwide study to assess the health status of US citizens. The surveys were approved by the National Center for Health Statistics Research. Details of study implementation are available for online access (19). After excluding participants <18 years and those without complete medical records, 19,420 were included in the final analyses (**Figure 1**).

Exposure

Blood specimen collection was conducted in the morning session after at least 9 h of overnight fasting. Hexokinase method was used to measure fasting blood glucose (20). The measurement of serum triglycerides (TG) and total cholesterol (TC) was performed with enzymatic assays. The level of high-density lipoprotein cholesterol (HDL-C) was

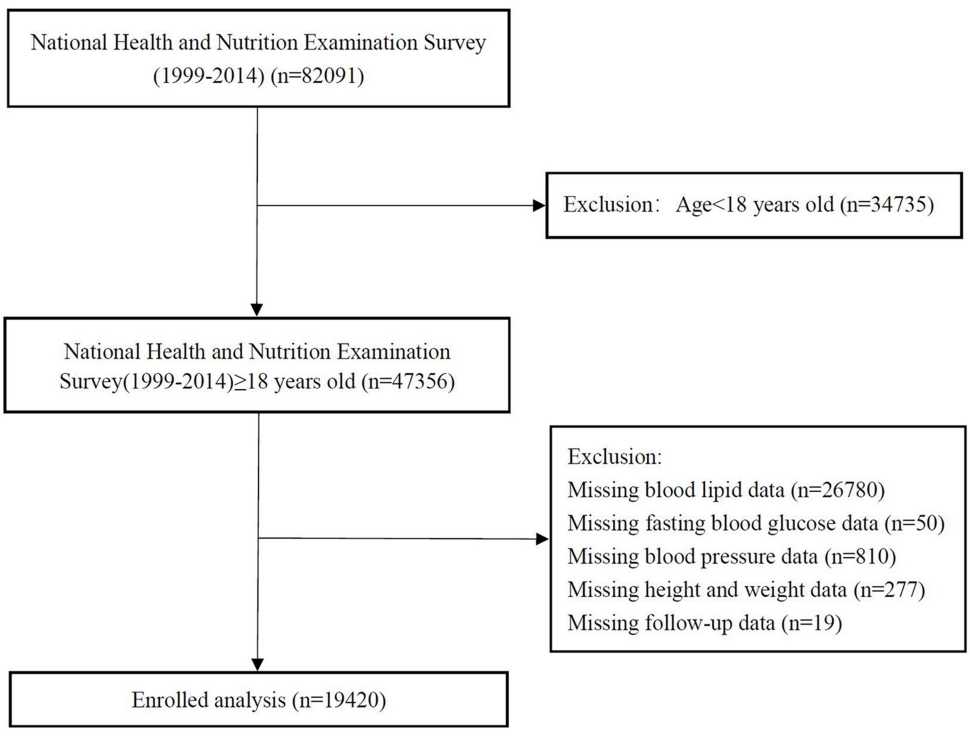

FIGURE 1 | The research flow chart.

determined by direct immunoassay or precipitation (21). If TG was ≤400 mg/dL, low-density lipoprotein cholesterol (LDL-C) was computed by the Friedewald formula (22). TyG index was defined as **TyG**=Ln[*fasting triglycerides* (*mg*/*dL*) × *fasting glucose* (*mg*/*dL*)/2] (9, 10).

Covariates

With the use of standardized questionnaires, participants provided information on age, sex, race (classify as white or other), smoking (classify as yes or no), self-reported medical history, and medication use. Body mass index (BMI) was measured by anthropometric measurement, Modification of Diet in Renal Disease formula was used to compute the estimated glomerular filtration rate (eGFR) (23). CVD history was self-reported by the onset of coronary artery disease, angina, heart attack, or stroke. Blood pressure was also measured to identify participants with hypertension. The definition of hypertension was having systolic blood pressures (SBP) ≥140 mmHg, diastolic blood pressure (DBP) ≥90 mmHg, reported the usage of hypotensive drugs or history of hypertension (24). Participants were classified as having diabetes if fasting blood glucose ≥126 mg/dl, hemoglobin A1c ≥6.5%, reported the usage of hypoglycemic drugs or history of diabetes (25). Full procedures on data collection are available on the official website (26).

Outcomes

In the present study, included outcomes were mortality cases due to all-cause and CVD. NHANES dataset was linked with the National Death Index (NDI) based on a probabilistic match to obtain mortality data. This match was performed by The National Center for Health Statistics with personal data, including birth date and social security number. Death certificate was used for confirmation whenever possible. These mortality files are available for online access (https://www.cdc.gov/nchs/data-linkage/mortality-public.htm). The definition of cardiovascular mortality in our study was adapted from the International Classification of Diseases, 10th Clinical Modification (ICD-10) System codes (I00–I09, I11, I13, I20–I51, I60–I69) (27).

Statistical Analysis

Baseline characteristics of participants were classified by TyG index (<8, 8–9, 9–10, >10). For continuous variables, data were presented as average value with standard deviations (SD). For categorical variables, data were presented as numbers with percentages. Depending on the nature of data, Chi-square, ANOVA, or Kruskal-Wallis *H*-test were performed to detect subgroup differences. To examine the association of TyG index with mortality, three sets of Cox regression models were constructed. Model 1 only included TyG index. Model 2 was adjusted for age, sex, and race. On top of the variables in Model 2, SBP, eGFR, TC, HDL-C, CVD, diabetes, hypertension, hypotensive drugs, hypoglycemic drugs, lipid-lowering medication, and antiplatelet drugs were additionally adjusted in Model 3. A test of the trend across TyG index groups was also performed. The differential onset of all-cause mortality and cardiovascular death according to TyG index was tested by Kaplan–Meier survival analyses. The statistical significance in subgroup differences was evaluated by the log-rank test. Restricted cubic spline models were built to detect any non-linear relationship between TyG index and mortality. If non-linear relationships were identified, we used two-piecewise linear regression models to elucidate how the associations differed by the threshold point. The threshold value was estimated by trying all possible value and choosing the threshold point with highest likelihood. Logarithmic likelihood ratio test was employed to compare the differences in associations when using one-line linear regression models vs. two-piecewise linear regression models. We also evaluated how the results from regression analysis differed by age (≥65 years or <65 years), sex (male or female), race (white or other), diabetes (yes or no), hypertension (yes or no), and BMI (≥25 or <25 kg/m^2). All analyses were performed with R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria), with statistical significance being identified at the level of $P < 0.05$.

RESULTS

Baseline Characteristics

Among 19,420 participants (48.9% men) in the present study, the average age was 47.1 (SD, 19.3) years. Across the average follow-up period of 98.2 (SD, 54.3) months, 2,238 (11.5%) death occurred and 445 (2.3%) died due to cardiovascular diseases. **Table 1** has presented the remaining details of the baseline characteristics. The subgroup differences were statistically significant for all variables (all $P < 0.001$).

The Relationship of TyG Index With All-Cause and Cardiovascular Mortality

Kaplan-Meier curves of the survival rate stratified by the TyG index were demonstrated in **Figure 2**. The cumulative incidence of death due to all-cause and CVD increased with TyG index (log-rank test, $P < 0.001$). **Table 2** presents the results from Cox regression analysis. When treating TyG index as a continuous variable, a positive association with all-cause (HR, 1.10; 95% CI, 1.00–1.20) and cardiovascular mortality (HR, 1.29; 95% CI, 1.05–1.57) was found in Model 3. When treating TyG index as a categorical variable, compared with the lowest category (TyG index ≤8), participants with the highest TyG index (>10) had increased risks of all-cause (HR, 4.92; 95% CI, 3.94–6.14) and cardiovascular death (HR, 6.11; 95% CI, 3.90–9.58) in univariate analysis (Model 1). The association with all-cause mortality persisted in Model 3 (HR, 1.51; 95% CI, 1.15–1.98), but the positive effect sizes becoming non-significant with cardiovascular death (HR, 1.37; 95% CI, 0.78–2.42).

The Analyses of Non-linear Relationship

As shown in **Figure 3**, restricted cubic spline models suggested that the relationship between TyG index and mortality was non-linear (all non-linear $P < 0.001$). According to two-piecewise linear regression models (**Table 3**), the threshold value was 9.36 and 9.52 for all-cause and cardiovascular mortality, respectively. Below the threshold point, TyG index did not associate with all-cause mortality nor cardiovascular mortality. However, above the threshold point, the elevation in TyG index was associated with an increased risk of all-cause (HR, 1.50; 95% CI, 1.29–1.75) and cardiovascular (HR, 2.35; 95% CI, 1.73–3.19) mortality.

TABLE 1 | Baseline characteristics according to triglyceride-glucose index.

	Total	Triglyceride-glucose index				*P*-value
		≤8	8–9	9–10	>10	
Number	19,420	3,056	10,995	4,686	683	
Age, years	47.1 ± 19.3	36.5 ± 17.1	47.6 ± 19.5	52.6 ± 17.9	53.5 ± 15.5	<0.001
Sex, *n* (%)						<0.001
Male	9,494 (48.9)	1,235 (40.4)	5,301 (48.2)	2,538 (54.2)	420 (61.5)	
Female	9,926 (51.1)	1,821 (59.6)	5,694 (51.8)	2,148 (45.8)	263 (38.5)	
Race, *n* (%)						<0.001
Other	10,465 (53.9)	1,927 (63.1)	5,831 (53.0)	2,329 (49.7)	378 (55.3)	
White	8,955 (46.1)	1,129 (36.9)	5,164 (47.0)	2,357 (50.3)	305 (44.7)	
Smoking, *n* (%)						<0.001
No	9,672 (53.5)	1,668 (64.5)	5,551 (54.2)	2,158 (47.3)	295 (44.0)	
Yes	8,392 (46.5)	918 (35.5)	4,694 (45.8)	2,404 (52.7)	376 (56.0)	
BMI, kg/m^2	28.4 ± 6.6	25.4 ± 5.8	28.1 ± 6.5	30.6 ± 6.2	31.2 ± 6.2	<0.001
SBP, mmHg	123.0 ± 19.1	115.9 ± 16.3	122.4 ± 18.9	127.8 ± 19.6	131.3 ± 20.5	<0.001
DBP, mmHg	68.8 ± 13.3	66.4 ± 11.8	68.6 ± 13.1	70.5 ± 13.9	71.1 ± 15.5	<0.001
eGFR, mg/min/1.73m^2	91.1 ± 29.6	99.3 ± 26.8	90.6 ± 29.0	87.3 ± 31.5	88.8 ± 30.9	<0.001
TC, mg/dL	194.9 ± 42.9	169.3 ± 33.7	193.0 ± 38.7	210.4 ± 44.1	232.5 ± 61.1	<0.001
HDL-C, mg/dL	53.4 ± 15.8	62.2 ± 16.6	55.0 ± 15.1	46.1 ± 12.7	38.6 ± 11.3	<0.001
LDL-C, mg/dL	114.9 ± 35.9	96.8 ± 28.0	117.0 ± 34.3	122.1 ± 39.8	114.3 ± 40.5	<0.001
TG, mg/dL	135.9 ± 118.7	51.8 ± 11.6	104.9 ± 29.5	212.8 ± 66.4	484.1 ± 383.1	<0.001
Fasting blood glucose, mg/dL	105.6 ± 34.6	90.7 ± 9.6	99.2 ± 15.4	116.9 ± 37.4	197.8 ± 96.1	<0.001
Triglyceride-glucose index	8.66 ± 0.68	7.73 ± 0.23	8.51 ± 0.28	9.35 ± 0.26	10.49 ± 0.50	<0.001
Comorbidities, *n* (%)						
Hypertension						<0.001
No	11,327 (58.4)	2,398 (78.6)	6,574 (59.9)	2,111 (45.1)	244 (35.8)	
Yes	8,073 (41.6)	655 (21.5)	4,411 (40.2)	2,570 (54.9)	437 (64.2)	
Diabetes						<0.001
No	16,302 (84.0)	2,954 (96.7)	9,877 (89.9)	3,269 (69.8)	202 (29.6)	
Yes	3,112 (16.0)	101 (3.3)	1,115 (10.1)	1,415 (30.2)	481 (70.4)	
Cardiovascular disease						<0.001
No	16,204 (90.3)	2,407 (95.5)	9,286 (91.2)	3,954 (86.8)	557 (83.0)	
Yes	1,733 (9.7)	114 (4.5)	902 (8.9)	603 (13.2)	114 (17.0)	
Treatment, *n* (%)						
Hypotensive drugs						<0.001
No	14,479 (74.6)	2,723 (89.1)	8,369 (76.1)	2,997 (64.0)	390 (57.1)	
Yes	4,941 (25.4)	333 (10.9)	2,626 (23.9)	1,689 (36.0)	293 (42.9)	
Hypoglycemic drugs						<0.001
No	17,801 (91.7)	3,004 (98.3)	10,443 (95.0)	3,946 (84.2)	408 (59.7)	
Yes	1,619 (8.3)	52 (1.7)	552 (5.0)	740 (15.8)	275 (40.3)	
Lipid-lowering medication						<0.001
No	16,888 (87.0)	2,910 (95.2)	9,676 (88.0)	3,790 (80.9)	512 (75.0)	
Yes	2,532 (13.0)	146 (4.8)	1,319 (12.0)	896 (19.1)	171 (25.0)	
Antiplatelet drugs						<0.001
No	19,060 (98.2)	3,033 (99.3)	10,817 (98.4)	4,549 (97.1)	661 (96.8)	
Yes	360 (1.9)	23 (0.8)	178 (1.6)	137 (2.9)	22 (3.2)	
Outcomes, *n* (%)						
Cardiovascular disease mortality						<0.001
No	18,975 (97.7)	3,023 (98.9)	10,777 (98.0)	4,537 (96.8)	638 (93.4)	
Yes	445 (2.3)	33 (1.1)	218 (2.0)	149 (3.2)	45 (6.6)	
All-cause mortality						<0.001
No	17,182 (88.5)	2,906 (95.1)	9,770 (88.9)	3,986 (85.1)	520 (76.1)	
Yes	2,238 (11.5)	150 (4.9)	1,225 (11.1)	700 (14.9)	163 (23.9)	

Values are presented as mean ± standardized differences or n (%).

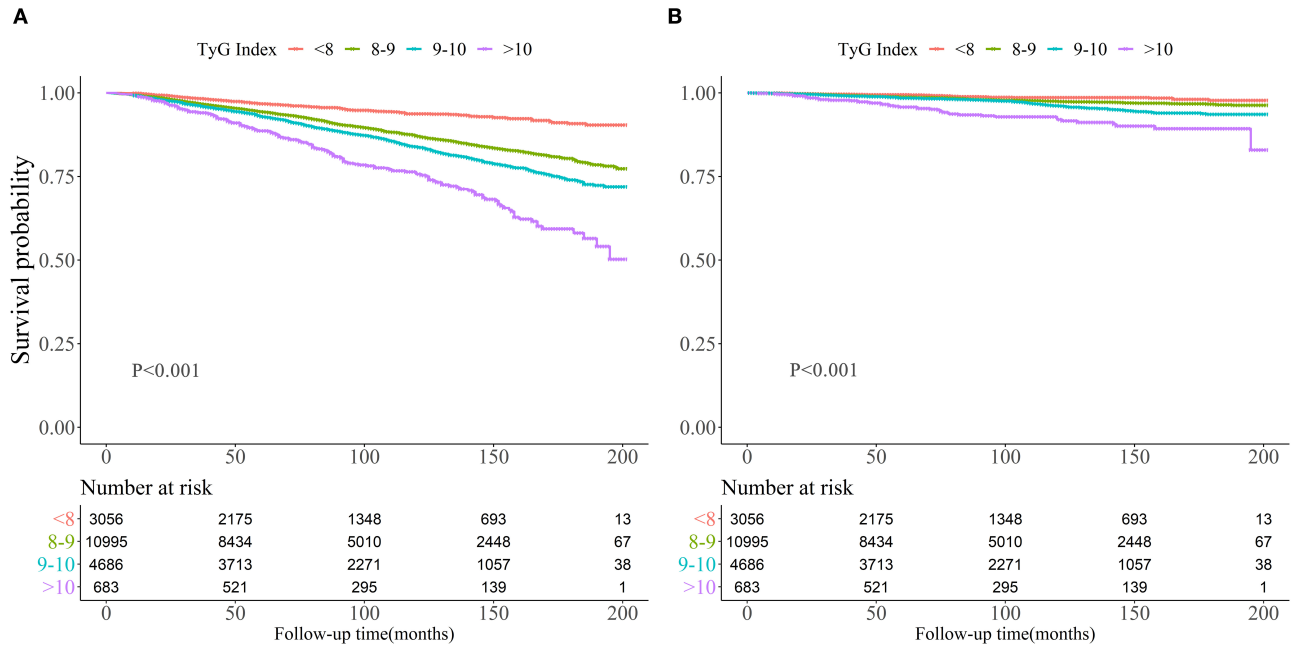

FIGURE 2 | Kaplan-Meier survival curve for all-cause mortality **(A)** and cardiovascular mortality **(B)** by Triglyceride-glucose index.

TABLE 2 | Cox regression analysis of Triglyceride-glucose index with cause-specific mortality.

	Number of events/ 1,000 person-years	Model 1	Model 2	Model 3
All-cause mortality				
Triglyceride-glucose index (per 1 unit increment)	14.09	1.56 (1.48, 1.65) <0.001	1.14 (1.07, 1.22) <0.001	1.10 (1.00, 1.20) 0.047
Triglyceride-glucose index group				
≤8	6.17	1.0	1.0	1.0
8–9	13.64	2.21 (1.87, 2.62) <0.001	0.91 (0.77, 1.08) 0.29	0.93 (0.77, 1.11) 0.41
9–10	17.78	2.87 (2.41, 3.43) <0.001	0.92 (0.77, 1.10) 0.39	0.88 (0.72, 1.09) 0.24
>10	30.15	4.92 (3.94, 6.14) <0.001	1.71 (1.37, 2.13) <0.001	1.51 (1.15, 1.98) 0.003
P for trend		<0.001	<0.001	0.12
Cardiovascular mortality				
Triglyceride-glucose index (per 1 unit increment)	2.80	1.82 (1.62, 2.04) <0.001	1.44 (1.25, 1.66) <0.001	1.29 (1.05, 1.57) 0.014
Triglyceride-glucose index group				
≤8	1.36	1.0	1.0	1.0
8–9	2.43	1.78 (1.24, 2.57) 0.002	0.66 (0.46, 0.96) 0.028	0.63 (0.43, 0.93) 0.021
9–10	3.79	2.77 (1.90, 4.04) <0.001	0.81 (0.56, 1.19) 0.28	0.64 (0.41, 1.00) 0.048
>10	8.32	6.11 (3.90, 9.58) <0.001	2.02 (1.29, 3.17) 0.002	1.37 (0.78, 2.42) 0.27
P for trend		<0.001	<0.001	0.24

Data are presented as hazard ratios, 95% confidence intervals, and P-value.
Model 1 adjusted for none.
Model 2 adjusted for age, sex, and race.
Model 3 adjusted for age, sex, race, smoking, BMI, SBP, eGFR, TC, HDL-C, comorbidities (cardiovascular disease, diabetes, and hypertension), and medicine use (hypotensive drugs, hypoglycemic drugs, lipid-lowering medication, and antiplatelet drugs).

Stratification Analysis

Table 4 showed the result of the stratification analysis. Above the threshold point, TyG index only associated with all-cause mortality among participants age ≥65 years (HR, 1.50; 95% CI, 1.29–1.75), those with hypertension (HR, 1.67; 95% CI, 1.41–1.99) or diabetes (HR, 1.55; 95% CI, 1.29–1.86). The association of TyG index with cardiovascular death was significant in male (HR, 2.59; 95% CI, 1.81–3.72), people suffering from

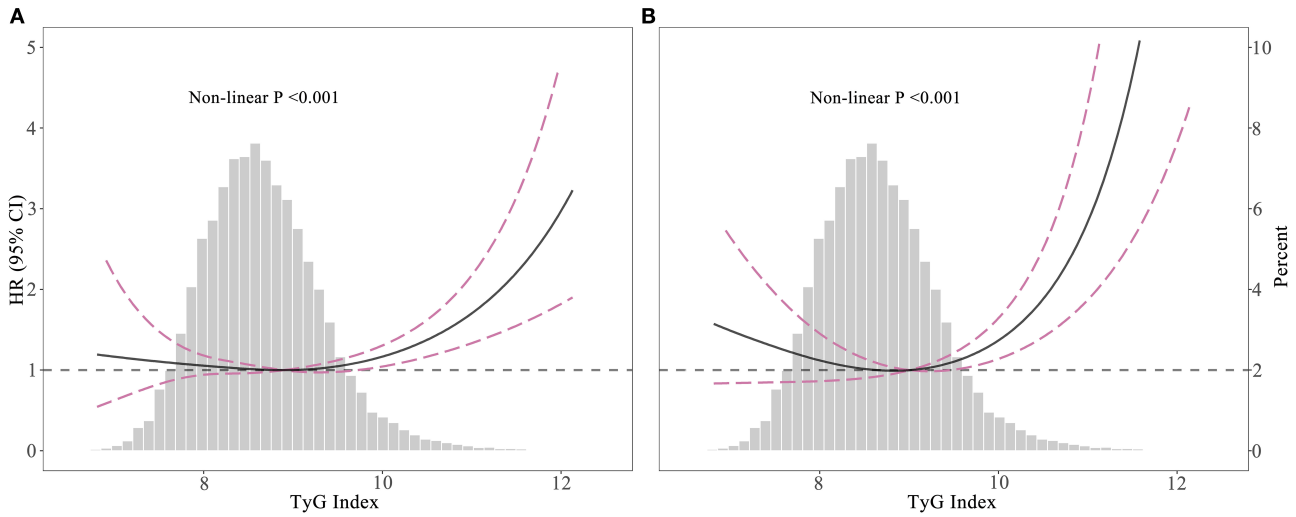

FIGURE 3 | Hazard ratios for all-cause mortality **(A)** and cardiovascular mortality **(B)** according to Triglyceride-glucose index and the histogram of probability distribution was presented in the background (hazard ratios were calculated by Cox models after adjusting for age, sex, race, smoking, BMI, SBP, eGFR, TC, HDL-C, CVD, diabetes, hypertension, hypotensive drugs, hypoglycemic drugs, lipid-lowering medication, and antiplatelet drugs).

TABLE 3 | The results of two-piecewise linear regression model between Triglyceride-glucose index and cause-specific mortality.

	All-cause mortality	Cardiovascular mortality
Threshold value	9.36	9.52
<threshold value	0.91 (0.81, 1.03) 0.14	0.90 (0.70, 1.16) 0.43
≥threshold value	1.50 (1.29, 1.75) <0.001	2.35 (1.73, 3.19) <0.001
P for log likelihood ratio test	<0.001	<0.001

Data are presented as hazard ratios, 95% confidence intervals, and P-value.
The two-piecewise linear regression models were adjusted for age, sex, race, smoking, BMI, SBP, eGFR, TC, HDL-C, comorbidities (cardiovascular disease, diabetes, and hypertension), and medicine use (hypotensive drugs, hypoglycemic drugs, lipid-lowering medication, and antiplatelet drugs).

hypertension (HR, 1.67; 95% CI, 1.41–1.99) or diabetes (HR, 1.55; 95% CI, 1.29–1.86).

DISCUSSION

Our analysis has demonstrated the non-linear relationship of TyG index with mortality due to all-cause and CVD among US population. We also identified the threshold points where the association of TyG index with mortality was further increased.

Patients with IR were often accompanied by lipid and glucose metabolism disorders (16). The phenomenon of lipid-induced insulin resistance can be explained by glucose-fatty acid cycle hypothesis (28). Therefore, TyG index can serve as a practical alternative of IR measurement (29). When compared to HOMA-IR, Lee et al. found that TyG index is a better indicator of arterial stiffness in Korean adults (18). Chiu et al. and Zhao et al. found that TyG index was associated with macrovascular and microvascular damage (30, 31). TyG index was also found to associate with CVD incidence (14, 32), hypertension, diabetes, as well as metabolic syndrome (11, 12, 33). Our analyses demonstrated the threshold values to detect mortality risk using TyG index. In a study of 2,531 subjects, Wang et al. found the threshold value of TyG index to predict adverse cardiovascular events was 9.32 among patients with diabetes and acute coronary syndrome (34), which was similar to the threshold values we have found. However, not all studies have found significant associations. Laura et al. failed to detect any significant associations between the TyG index and CVD for people with diabetes or hypertension (35). Cho et al. reported that TyG index had no significant relationship with coronary artery disease among diabetic participants after adjusting for covariates (36).

The detail in the mechanism accounting for the relationship between TyG index and death is still under investigation. Several speculations have been summarized as follows. First, TyG index had a close relationship with a broad range of adverse health conditions including obesity, diabetes, CVD, higher blood pressure, lower eGFR, and HDL-C levels (11, 12, 37). Elevated TyG index may reflect the adverse effects of impaired cardiometabolic health. In our stratified analysis, the positive association of the TyG index with mortality was more profound among older participants and people with pre-existing diseases. Second, endothelial dysfunction may have a role in the association of TyG index with mortality. Compared with other IR indicators, TyG index mainly quantifies

TABLE 4 | Stratification analysis of Triglyceride-glucose index with cause-specific mortality.

Threshold value, mmol/L	Number	All-cause mortality		*P* for log likelihood ratio test	Cardiovascular disease mortality		*P* for log likelihood ratio test
		<9.36	≥9.36		<9.52	≥9.52	
Age							
≥65	4,337	0.81 (0.70, 0.94) 0.005	1.47 (1.18, 1.82) <0.001	<0.001	0.82 (0.61, 1.10) 0.18	2.15 (1.34, 3.44) 0.001	0.003
<65	13,509	1.24 (1.01, 1.52) 0.037	1.15 (0.92, 1.45) 0.22	0.66	1.18 (0.72, 1.92) 0.51	1.76 (1.12, 2.79) 0.015	0.27
Sex							
Male	8,674	0.91 (0.78, 1.06) 0.23	1.52 (1.25, 1.86) <0.001	<0.001	0.99 (0.72, 1.36) 0.95	2.59 (1.81, 3.72) <0.001	<0.001
Female	9,172	0.92 (0.76, 1.11) 0.39	1.48 (1.16, 1.89) 0.002	0.006	0.82 (0.54, 1.24) 0.35	1.73 (0.94, 3.21) 0.080	0.080
Race							
Other	9,325	0.94 (0.78, 1.12) 0.48	1.37 (1.09, 1.71) 0.006	0.018	0.86 (0.59, 1.27) 0.46	2.91 (1.94, 4.36) <0.001	<0.001
White	8,521	0.91 (0.77, 1.07) 0.24	1.59 (1.28, 1.97) <0.001	<0.001	0.93 (0.66, 1.31) 0.68	1.77 (1.07, 2.93) 0.027	0.059
Hypertension							
No	9,908	1.05 (0.83, 1.35) 0.67	1.08 (0.76, 1.54) 0.68	0.93	1.54 (0.78, 3.05) 0.22	0.92 (0.34, 2.48) 0.88	0.40
Yes	7,938	0.85 (0.74, 0.98) 0.021	1.67 (1.41, 1.99) <0.001	<0.001	0.82 (0.63, 1.08) 0.16	2.79 (2.00, 3.88) <0.001	<0.001
Diabetes							
No	14,783	0.95 (0.82, 1.10) 0.49	1.37 (0.91, 2.08) 0.13	0.14	0.96 (0.69, 1.33) 0.80	0.39 (0.05, 2.85) 0.35	0.36
Yes	3,063	0.88 (0.70, 1.10) 0.25	1.55 (1.29, 1.86) <0.001	<0.001	1.04 (0.67, 1.61) 0.86	2.22 (1.55, 3.18) <0.001	0.018
BMI, kg/m^2							
<25	5,516	0.88 (0.71, 1.08) 0.22	2.67 (1.87, 3.82) <0.001	<0.001	0.79 (0.50, 1.26) 0.32	5.30 (2.82, 9.96) <0.001	<0.001
≥25	12,330	0.93 (0.80, 1.08) 0.33	1.38 (1.16, 1.65) <0.001	0.002	0.93 (0.69, 1.27) 0.66	2.07 (1.43, 2.99) <0.001	0.003

Data are presented as hazard ratios, 95% confidence intervals, and P-value.
When analyzing a stratification variable, age, sex, race, smoking, BMI, SBP, eGFR, TC, HDL-C, comorbidities (cardiovascular disease, diabetes, and hypertension), and medicine use (hypotensive drugs, hypoglycemic drugs, lipid-lowering medication, and antiplatelet drugs) were all adjusted except the variable itself.

IR in muscle and is a better indicator for peripheral IR. Therefore, TyG index probably relates to the level of endothelial dysfunction, oxidative stress and inflammatory response (1, 3, 38). Positive associations of the TyG index with white blood cells count and high-sensitivity C-reactive protein levels were also revealed in previous studies (16, 17). Inflammation may impair vascular endothelium. That can lead to the leakage of blood contents into the perivascular spaces and further cause vascular damage (39). Third, insulin may cause lipohyalinosis through the elevation of sympathetic activity, which may lead to diffuse hypoperfusion or blocks small perforating arterioles (37, 39). IR can also promote atherogenesis and advanced plaque progression (37). Fourth, TyG index predicts the incidence of prediabetes (40), while meta-analyses have confirmed that prediabetes was positively associated with all-cause and CVD death in general population (41, 42). Nevertheless, the precise role of the TyG index in mortality remains to be investigated.

Despite the important findings being mentioned, some limitations of this study need to be recognized. First, insulin clamp was not available in this study, and thus we could not use the most accurate methods to evaluate IR. Second, the present study does not imply causality due to the cross-sectional nature. Lastly, the finding was mainly applicable in the United States and may not be directly extrapolated to other regions and ethnic groups.

CONCLUSIONS

There is a non-linear relationship of TyG index with mortality due to all-cause and CVD among US population. Whether the

improvement of TyG index may reduce mortality risk in long run need to be further evaluated by intervention studies.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found at: https://www.cdc.gov/nchs/nhanes/index.htm.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Centers for Disease Control and Prevention (Protocol 98–12, 2005–06, and 2011–17). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-qH, G-dH, KL, Y-qF, and X-cL: conceptualization and methodology. Y-qH and X-cL: formal analysis. Y-qH and Y-qF: supervision and validation. X-cL and KL: writing and revision. 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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Deleterious Effects of Epicardial Adipose Tissue Volume on Global Longitudinal Strain in Patients With Preserved Left Ventricular Ejection Fraction

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Background: It is known that epicardial adipose tissue (EAT) volume is linked to cardiac dysfunction. However, it is unclear whether EAT volume (EATV) is closely linked to abnormal LV strain. We examined the relationship between EATV and global longitudinal strain (GLS), global circumferential strain (GCS), and global radial strain (GRS) in patients with preserved LV function.

Methods: Notably, 180 consecutive subjects (68 ± 12 years; 53% men) underwent 320-slice multi-detector computed tomography coronary angiography and were segregated into coronary artery disease (CAD) (≥1 coronary artery branch stenosis ≥50%) and non-CAD groups. GLS, GCS, and GRS were evaluated by 2-dimensional speckle tracking in patients with preserved left ventricular (LV) ejection fraction (LVEF) ≥50%.

Results: First, GLS, but not GRS and GCS, was lower in the high EATV group though the LVEF was comparable to the low EATV group. Frequency of GLS ≤18 was higher in the high EATV group. Second, multiple regression model showed that EATV, age, male sex, and CAD, were determinants of GLS. Third, the cutoff points of EATV were comparable (∼116–117 mL) in both groups. The cutoff of EATV ≥116 showed a significant correlation with GLS ≤18 in overall subjects.

Conclusions: Increasing EATV was independently associated with global longitudinal strain despite the preserved LVEF and lacking obstructive CAD. Our findings suggest an additional role of EAT on myocardial systolic function by impaired LV longitudinal strain.

Keywords: epicardial fat, global longitudinal strain (GLS), lipotoxicity, echocardiography, HFpEF (heart failure with preserved ejection fraction)

INTRODUCTION

The volume-based measurement of the left ventricular (LV) ejection fraction (LVEF) is a simple measure of the global systolic function that encompasses risk evaluation and the management of various cardiovascular diseases. However, this parameter is limited by pathophysiological entities where the ratio of the stroke volume to LV cavity size is preserved (1). This notion is well-applicable in the setting that patients with preserved EF (HFpEF) have a similar mortality rate to patients with reduced EF (HFrEF) (2, 3). A number of observational and interventional studies have validated the global strain of the LV myocardium measured by speckle tracking to be superior to both LVEF and LV filling parameters for predicting the outcome in HFpEF (4–6). Thus, adding global longitudinal strain (GLS), the most robust deformation marker, to LVEF increases the accuracy of predicting cardiovascular events in patients with ischemic or non-ischemic heart failure (4–6). GLS is often disturbed in preserved EF patients with hypertension (7), diabetes mellitus (8), LV hypertrophy (9), and cancer therapy-related cardiac dysfunction (CTRCD) (10, 11).

Poor GLS could be a marker of early cardiac dysfunction in obese individuals. There were reports that body mass index (BMI) (12–14) or visceral fat area (VFA) (15) was negatively associated with GLS. It has been suggested that the accumulation of epicardial adipose tissue (EAT) underlies cardiac dysfunction in obesity (16, 17). It is possible to hypothesize that the EAT volume (EATV) is more closely linked to an abnormal LV strain than other adiposity indices. However, studies evaluating this link are limited. Ng et al. showed that EATV is a determinant of LV strain independent of BMI and waist/hip ratio in patients without coronary artery disease (CAD) (18). LV strains can be affected by ischemic burdens in patients with preserved LVEF (1); therefore, the effects of CAD on LV strains are also to be elucidated.

In clinical imaging modalities, cardiac strain is represented by three principal directions (longitudinal, circumferential, circumferential, and radial) (19). LV myocardial fibers adjacent to the endocardium are longitudinally oriented and yield a longitudinal shortening; LV myocardial fibers in the middle layer are oriented circularly around the short axis and yield a radial shortening; and LV myocardial fibers adjacent to the epicardium are oriented obliquely and result in circumference shorting (19). Therefore, EATV might be linked differently to the strains of three layers. In this study, we examined the relationship between EATV and GLS, global circumferential strain (GCS), and the global radial strain (GRS) in CAD or non-CAD patients.

METHODS

Study Population

We retrospectively analyzed 482 consecutive Japanese patients who had undergone cardiac computed tomography (CT) for the purpose of suspected CAD between 2012 and 2015 at Tokushima University Hospital (**Supplement 1**, Participant recruitment flow chart). Subjects were divided into the coronary artery disease (CAD, if ≥1 coronary artery branch stenosis of ≥50%) and non-CAD groups. The major exclusion criteria include serum creatinine levels >1.5 mg/dL; class III or IV heart failure; iodine-based allergy; acute coronary events, stroke, or coronary revascularization within the preceding 3 months; overt liver disease; hypothyroidism; and severe valvular disease. We had excluded acute coronary events because LV strain may be largely variable during course of acute coronary events. To exclude acute coronary events, we had evaluated medical records before and after cardiac CT and selected only chronic and stable CAD. Since the data for the validity of systolic function indices during atrial fibrillation (AF) are limited, we excluded AF patients. Additionally, we excluded patients either with LVEF <50% or regional LV wall motion abnormality to detect early systolic abnormalities of LV strains. Altogether, 180 patients were included in the full analysis set.

Measurements

Trained staff measured the height, body weight, and blood pressure of the participants. Questionnaires were administered to record data on smoking history, use of anti-hypertensive drugs, anti-hyperglycemic drugs, and lipid-lowering drugs. A participant was recognized as having diabetes mellitus, when the fasting plasma glucose level was ≥126 mg/dL, or the HbA1c level was ≥6.5% (48 mmol/mol), or the participant was taking a regular medication of anti-hyperglycemic drugs. A participant was recognized as hypertensive, if systolic blood pressure was ≥140 mmHg, diastolic blood pressure was ≥90 mmHg, or if she/he was regularly taking antihypertensive drugs. A participant was recognized as having dyslipidemia, if the high-density lipoprotein (HDL)-cholesterol levels were <40 mg/dL (1.0 mmol/L), if low-density lipoprotein (LDL)-cholesterol levels were ≥140 mg/dL (3.6 mmol/L), or if triglyceride levels were ≥150 mg/dL (1.7 mmol/L), or if they were regularly taking lipid-lowering drugs.

Quantification of Epicardial Fat Volume

Cardiac CT was performed using a 320-slice CT scanner (Aquilion One; Toshiba Medical Systems, Tokyo, Japan) having 0.275-ms rotation and 0.5/320/0.25 collimation (20). CT images were acquired using a retrospective, non-helical electrocardiogram-triggered acquisition mode protocol (tube voltage, 120 kV; tube current, 450 mA × 5 ms) with a thickness of 5-mm slices. All reconstructed CT image data were transferred to an offline workstation (Synapse Vincent, ver. 4.4, Fuji Film, Tokyo, Japan). EATV and local EAT thickness were measured as previously reported (17, 21, 22).

Standard Echocardiographic Measurements

Echocardiography was performed using commercially available ultrasound diagnostic instruments in accordance with the guidelines issued by the American Society of Echocardiography (23). A complete 2D color, pulsed, and continuous-wave Doppler echocardiogram was performed. Imaging included apical two- and four-chamber views, from which LV and left atrial (LA) volumes were measured by the biplane method of disks using 2-dimensional images. The cavity dimension and wall thickness were measured in a parasternal long axis view. The left ventricular

mass was estimated using the formula recommended by the guidelines. The measurement of the left ventricular ejection fraction was performed in biplane apical (2- and 4-chamber) views using a modified Simpson's method.

2-Dimensional Strain Echocardiography

The peak systolic LV strains were analyzed offline using a computer software program by EchoInsight software as described (24, 25). Briefly, the endocardium was automatically tracked throughout the cardiac cycle in the apical 4-chamber, 2-chamber, and long-axis views, after the manual definition of the LV endocardial border. Horizontal long-axis cines were tracked to derive longitudinal displacement and strain, while short-axis cines were used to derive the circumferential and radial displacements and strain. The strain values for the 6 basal, 6 mid, and 6 apical segments of the LV were averaged for GLS, GCS, and GRS (19). Yang et al. adopted cutoff values of GLS in surveillance of cancer chemotherapeutic-related cardiac dysfunction: >18% normal, 16–18% borderline and <16% is abnormal (10). Since our participants included non-CAD in addition to CAD subjects were considered to be normal to mild in GLS dysfunction, the value of GLS ≤18 was set as abnormal GLS (10).

Statistical Analysis

The continuous and parametric values are expressed as mean ± standard deviation and categorical variables as percentage. The two-tailed unpaired student's *t*-test or chi-square test was used for group comparisons. Univariate or multivariate-adjusted regression analyses were performed to estimate the associations between potential determinants and longitudinal, radial, and circumference trains, the optimal cutoff values of EATV for predicting GLS ≤18 (10) were identified using receiver-operating characteristic (ROC) curves. Univariate or multivariate-adjusted odds ratios (OR) were also calculated to determine the clinical utility of the variables to estimate the GLS ≤18. Values of $P < 0.05$ were considered as statistically significant. Statistical analyses were conducted using the SPSS version 25 (SPSS, Inc., Chicago, Illinois, USA).

RESULT

General Characteristics

The general characteristics of patients with low EATV (<median EATV = 112 mL) and high EATV (≥median EATV = 112 mL) are shown in **Table 1**. EATV in the low and high EATV groups was 78 ± 25 and 162 ± 38 mL, respectively. In the high EATV group, the mean age and prevalence of male subjects were not statistically significant. There were no differences in the systolic and diastolic blood pressure and heart rate. The body weight and BMI were larger in the high EATV group. There was no difference between the total and LDL cholesterol; however, the HDL cholesterol was lower, and triglyceride was higher in the high EATV group. There was no difference between the fasting plasma glucose and HbA_{1C}. Although smoking history and obesity were higher in the high EATV group, there was no difference in the proportion of hypertension, diabetes mellitus, or CAD.

TABLE 1 | General characteristics of studied patients with low and high EATV.

Parameters	Overall	Low EATV (<112 mL)	High EATV (≥112 mL)	*P*-value
Numbers	180	90	90	
Age (years)	68 ± 12	66 ± 12	70 ± 11	0.051
Male gender, *n* (%)	96 (53%)	42 (47%)	54 (60%)	0.060
Systolic blood pressure(mmHg)	134 ± 21	134 ± 24	134 ± 17	0.956
Diastolic blood pressure(mmHg)	75 ± 13	74 ± 13	76 ± 13	0.417
Heart rate (beats/min)	70 ± 12	71 ± 13	70 ± 11	0.867
ANTHROPOMETRY				
Body weight (kg)	63 ± 15	59 ± 15	68 ± 14	<0.001
Body mass index (Kg/m^2)	25 ± 5	24 ± 5	26 ± 4	0.001
BLOOD MEASUREMENTS				
Total cholesterol (mg/dl)	196 ± 40	200 ± 40	191 ± 40	0.174
LDL cholesterol (mg/dl)	110 ± 32	111 ± 32	109 ± 32	0.788
HDL cholesterol (mg/dl)	61 ± 19	65 ± 20	58 ± 18	0.022
Triglycerides (mg/dl)	130 ± 66	116 ± 45	145 ± 82	0.008
Fasting plasma glucose (mg/dL)	120 ± 35	116 ± 35	125 ± 35	0.204
HbA1c (%)	6.1 ± 0.8	6.0 ± 0.9	6.1 ± 0.7	0.317
BNP (pmol/L)	49 ± 52	59.1 ± 61.5	38.4 ± 37.8	0.034
MEDICATIONS				
Antihypertensive medications, *n* (%)	82 (46%)	47 (52%)	35 (39%)	0.337
Lipid lowering medications, *n* (%)	45 (25%)	24 (27%)	21 (23%)	0.866
Antidiabetic medications, *n* (%)	35 (19%)	16 (18%)	19 (21%)	0.169
COMORBIDITIES				
Smoking history, *n* (%)	73 (41%)	27 (30%)	46 (51%)	0.003
Hyperlipidemia, *n* (%)	137 (76%)	67 (74%)	70 (78%)	0.507
Hypertension, *n* (%)	144 (80%)	71 (79%)	73 (81%)	0.597
Diabetes mellitus, *n* (%)	57 (32%)	25 (28%)	32 (36%)	0.262
Body mass index ≥25kg/m^2, *n* (%)	94 (52%)	34 (38%)	60 (67%)	<0.001
Coronary artery disease, *n* (%)	112 (62%)	50 (56%)	62 (69%)	0.065
EAT MEASUREMENTS				
EATV (mL)	120 ± 53	77.6 ± 24.9	161.6 ± 37.5	<0.001
LV MEASURES				
Interventricular septum wall thickness (mm)	9.0 ± 2.0	9.2 ± 2.6	8.7 ± 1.1	0.119
LV posterior wall thickness (mm)	8.6 ± 1.5	8.6 ± 1.7	8.6 ± 1.1	0.763
LVDd (mm)	46.0 ± 5.0	45.1 ± 5.7	47.0 ± 4.0	0.015
LVDs (mm)	28.6 ± 4.8	28.2 ± 5.2	29.1 ± 4.2	0.204
LVEDV (mL)	85.2 ± 23.2	81.6 ± 22.3	88.8 ± 23.7	0.040
LVESV (mL)	29.9 ± 10.3	28.7 ± 9.9	31.2 ± 10.6	0.109
LVEF (%)	65.2 ± 5.1	65.3 ± 4.9	65.1 ± 5.3	0.793
LV mass index (g/m^2)	83.9 ± 23.1	86.6 ± 25.7	81.3 ± 19.9	0.131
LV STRAIN				
GLS ≤18, GLS abnormality	53 (29%)	20 (22%)	33 (37%)	0.034

(Continued)

TABLE 1 | Continued

Parameters	Overall	Low EATV (<112 mL)	High EATV (≥112 mL)	*P*-value
Global longitudinal strain (%)	−19.1 ± 1.3	−19.4 ± 1.2	−18.8 ± 1.4	0.003
Global circumferential strain (%)	−30.1 ± 6.2	−30.6 ± 6.4	−29.6 ± 6.0	0.322
Global radial strain (%)	27.2 ± 8.8	27.0 ± 8.3	27.4 ± 9.4	0.794

Values are presented as the mean ± SD or n (%). Statistical significance were tested by independent t-test or chi-square test. n indicates of numbers of patients; EATV, epicardial adipose tissue volume; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF: left ventricular ejection fraction; GLS, global longitudinal strain.

Among the echocardiographic parameters, LVDd and LVEDV were significantly greater in the high EATV group than in the lower EATV group and other measurements including LVEF and LV mass index were comparable between two groups. For LV strain, the global longitudinal strain (GLS) was lower in the high EATV group; however, the global radial strain (GRS) and global circumference strain (GCS) were comparable. The frequency of GLS ≤18 was higher in the high EATV group (37 vs. 22%, $P = 0.034$). Representative measurements of the global longitudinal strain (GLS) in patients with lower and higher EATV are shown in **Figure 1**.

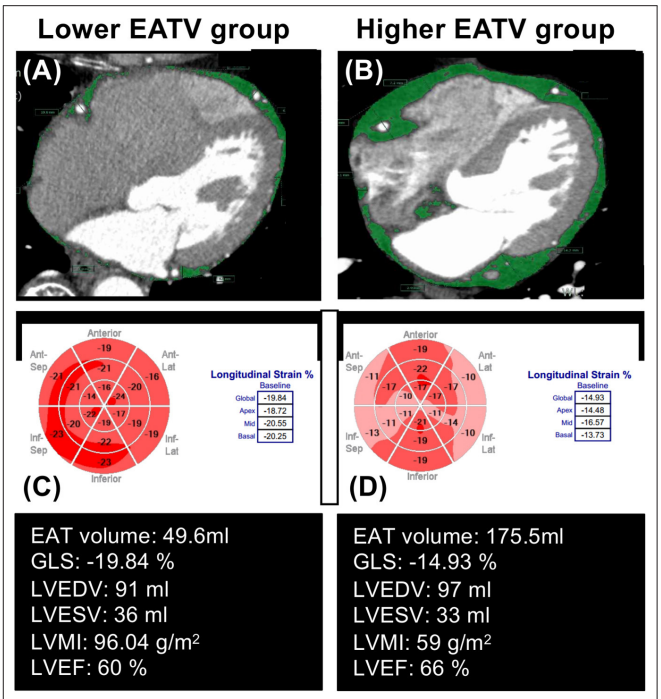

FIGURE 1 | Representative measurements of global longitudinal strain (GLS), in patients with lower and higher epicardial adipose tissue volume (EATV). EAT measurements was manually placed **(A,B)** along the visceral pericardium; **(C,D)** the EAT area was automatically acquired as a density range between −190 and −30 HU in cardiac computed tomography. Compared with the lower EATV group **(A)**, with the group with higher EATV **(B)** had an impaired GLS (−19.84 vs. −14.93%), despite other clinical parameters being similar.

EATV and LV Strain

The univariate regression analysis for the global longitudinal, radial, and circumferential strain in the overall group, non-CAD, and CAD patients is displayed in **Supplement 2** and **Figure 2**. In the overall patient group: GLS was observed to decrease with age, male sex, the presence of CAD, and EATV, but increased with LVEF; GRS was observed to decrease with IVS, LVPW, and LVMI; GCS was observed to decrease with BMI, but increased with IVS and LVEF. In the non-CAD subgroup, GLS was observed to decrease with EATV, but increased with LVEF; GRS was observed to increase with LVPW; GCS was observed to increase with smoking history and LVEF. In the CAD subgroup, GLS was observed to decrease with the male sex and EATV, but increased with the LVEF; GRS was observed to increase with IVS; GCS was observed to increase with LVEF.

The relationship between the LVEF and LV global strains and age, BMI, LVMI, and EATV was plotted in **Figure 2**. LVEF and GCS did not correlate with age, BMI, LVMI, or EATV. GLS was observed to decrease with EATV in both the non-CAD and CAD groups. GRS decreased with LVMI only in the CAD group. In **Supplement 3**, the explanatory factors of global strain by multivariate analysis were examined. GLS decline was significantly associated with the male sex, CAD, and EATV (**Model 4**). GRS correlated only with hypertension (Model 4) and GCS correlated with BMI. Since EATV was associated only with GLS, we focused the subsequent analysis on the association between EATV and GLS.

EATV Cutoff and OR for GLS Abnormality (GLS ≤18)

The general characteristics of patients with GLS > 18 and GLS ≤18 are shown in **Table 2**. The GLS ≤18 group was older and had higher prevalence of CAD. The body weight, BMI and blood pressure were comparable between two groups, while EATV was higher in the GLS ≤18 group. There was no difference in the fasting plasma glucose and HbA_{1C}. Among the echocardiographic LV measures and LV strain, only GLS and GRS were significantly different between two groups.

The cutoff points of EATV for predicting GLS ≤18% in the ROC curve analysis are shown in **Figure 3**. The cutoff points were comparable among the overall (116 mL), non-CAD (117 mL), and CAD (116 mL) groups, respectively, and showed a significant power, in the overall and non-CAD groups, but not in the CAD group (**Figure 3**, upper panel). EATV was significantly higher in the GLS <18% group than in the patients with GLS >18% only in the overall study group and non-CAD groups (**Figure 3**, lower panel).

Finally, we calculated the ORs of the EATV cutoff for GLS ≤18%. As shown in **Table 3**, in the overall study group, EATV ≥116 was correlated with GLS ≤18 (crude OR 2.54 [95% CI 1.31–4.92], $P = 0.006$, and multivariate-adjusted OR 2.22 [1.03–4.79], $P = 0.042$) as well as diabetes mellitus and CAD. In the non-CAD and the CAD groups, EATV ≥116 mL did not reach

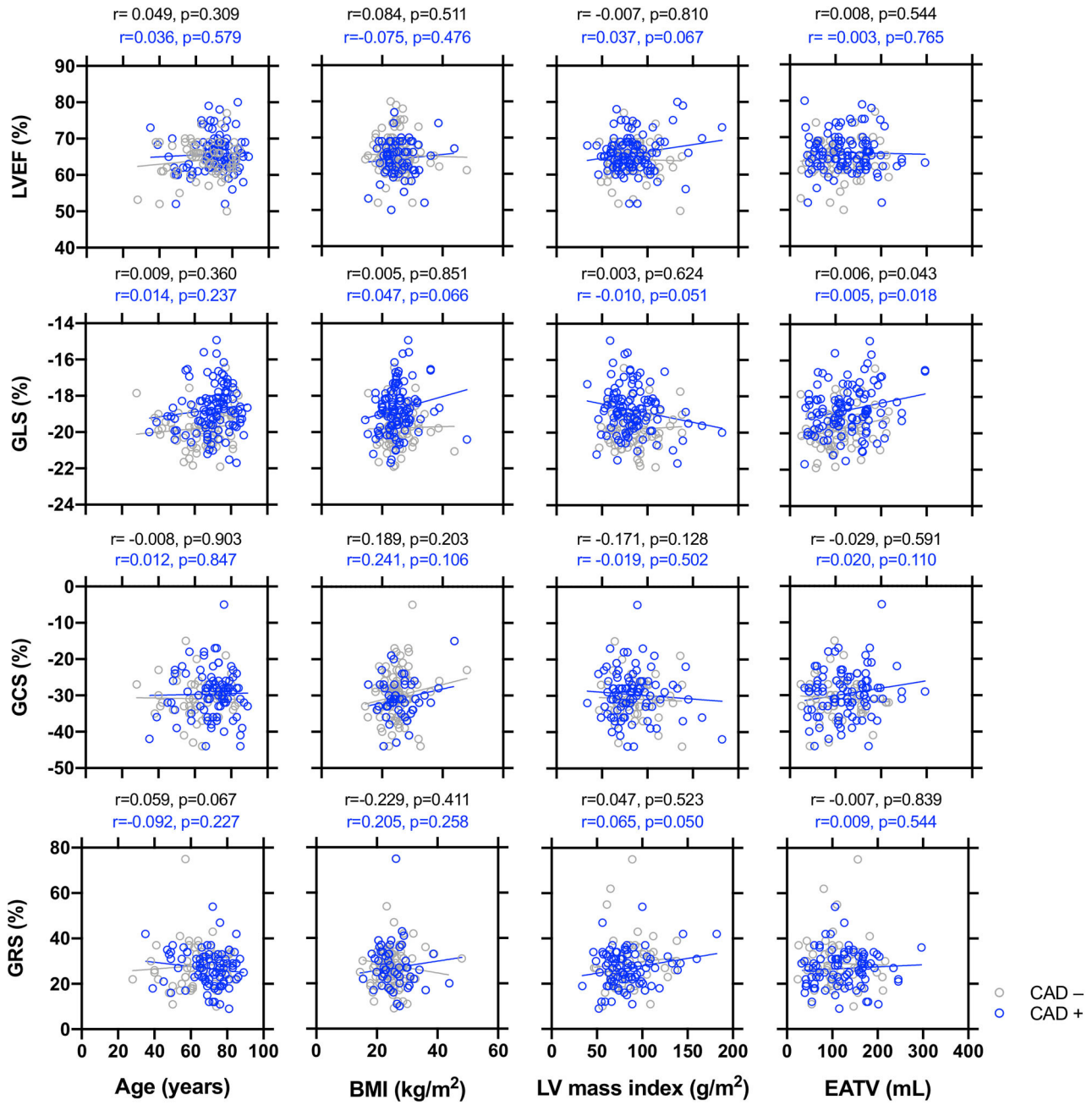

FIGURE 2 | Simple correlations between the echocardiographic parameters of end systolic global longitudinal strain (GLS), global circumferential strain (GCS), and global radial strain (GRS), respectively with age, body mass index (BMI), left ventricular mass index (LVMI), and epicardial adipose tissue volume (EATV) in patients with or without coronary artery disease (CAD). A simple regression analysis was made separately in non-CAD (black circles and lines) and CAD patients (blue circles and lines). The *R* and *P*-values are shown.

to a significant correlation with GLS ≤18. In the CAD group, only diabetes mellitus was correlated with GLS ≤18 (crude OR 2.54 [95% CI 1.05–6.12], $P = 0.038$, and multivariate-adjusted OR 2.22 [1.04–6.94], $P = 0.041$).

DISCUSSION

In this study, we examined the relationship between the EAT and LV strains in patients with preserved LVEF. We obtained three major findings. First, the GLS, but not the GRS and GCS, was lower in the high EATV group though LVEF values were comparable to the low EATV group. The frequency of GLS ≤18 was higher in the high EATV group. Second, the multiple regression model showed that EATV as well as age, male sex, CAD, were determinants of GLS (**Supplement 3**, Model 4). Third, the cutoff points of EATV were comparable (∼116–117 mL) in the overall, non-CAD, and the CAD groups. The cutoff of EATV ≥116 showed a significant correlation with

TABLE 2 | General characteristics of studied patients with GLS >18 and GLS ≤18.

Parameters	GLS >18	GLS ≤18	*P*-value
Numbers	127	53	
Age (years)	66 ± 12	72 ± 10	0.008
Male gender, *n* (%)	64 (50%)	32 (60%)	0.175
Systolic blood pressure(mmHg)	135 ± 21	132 ± 20	0.390
Diastolic blood pressure(mmHg)	75 ± 14	74 ± 12	0.759
Heart rate (beats/min)	69 ± 12	72 ± 12	0.314
ANTHROPOMETRY			
Body weight (kg)	63.4 ±15.2	63.2 ±14.0	0.937
Body mass index (kg/m^2)	25 ± 5	25 ± 4	0.906
BLOOD MEASUREMENTS			
Total cholesterol (mg/dl)	196 ± 38	195 ± 45	0.912
LDL cholesterol (mg/dl)	109 ± 30	111 ± 38	0.820
HDL cholesterol (mg/dl)	62 ± 19	61 ± 18	0.868
Triglycerides (mg/dl)	128 ± 66	134 ± 69	0.661
Fasting plasma glucose (mg/dL)	124 ± 38	110 ± 23	0.076
HbA1c (%)	6.1 ± 0.9	6.0 ± 0.8	0.774
BNP (pmol/L)	45 ± 51	60 ± 55	0.173
MEDICATIONS			
Antihypertensive medications, *n* (%)	64 (50%)	18 (34%)	0.352
Lipid lowering medications, *n* (%)	28 (22%)	17 (32%)	0.051
Antidiabetic medications, *n* (%)	27 (21%)	8 (15%)	0.846
COMORBIDITIES			
Smoking, *n* (%)	52 (41%)	21 (40%)	0.299
Hyperlipidemia, *n* (%)	94 (74%)	43 (81%)	0.139
Hypertension, *n* (%)	102 (80%)	42 (79%)	0.299
Diabetes mellitus, *n* (%)	46 (36%)	11 (21%)	0.042
Body mass index ≥25kg/m^2, *n* (%)	64 (50%)	30 (57%)	0.375
Coronary artery disease, *n* (%)	67 (53%)	45 (85%)	<0.001
EAT MEASUREMENTS			
EATV (mL)	114 ± 49	133 ± 60	0.029
EATV median ≥112 mL yes or no	59 (47%)	33(67%)	0.053
EATV cutoff ≥116 mL yes or no	50 (39%)	33(62%)	0.005
LV MEASURES			
Interventricular septum wall thickness (mm)	9.1 ± 2.3	8.7 ± 0.9	0.244
LV posterior wall thickness (mm)	8.6 ± 1.6	8.5 ± 0.9	0.647
LVDd (mm)	46.2 ± 5.2	45.5 ± 4.4	0.373
LVDs (mm)	28.9 ± 4.7	28.0 ± 4.8	0.287
LVEDV (mL)	85.3 ± 23.2	84.9 ± 23.4	0.931
LVESV (mL)	29.6 ± 9.9	30.6 ± 11.3	0.570
LVEF (%)	65.5 ± 4.8	64.4 ± 5.6	0.199
LV mass index (g/m^2)	85.7 ± 24.2	79.5 ± 19.6	0.111
LV STRAIN			
Global longitudinal strain (%)	−19.8 ± 0.8	−17.5 ± 0.8	<0.001
Global circumferential strain (%)	−31.1 ± 6.0	−27.9 ± 6.1	0.004
Global radial strain (%)	27.8 ± 8.8	25.8 ± 8.9	0.201

Values are presented as the mean ± SD or n (%). Statistical significance were tested by independent t-test or chi-square test. n indicates of numbers of patients; EATV, epicardial adipose tissue volume; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF: left ventricular ejection fraction; GLS, global longitudinal strain.

GLS ≤18 in overall, but did not in the non-CAD and CAD groups. Taken together, our results demonstrated that EATV was a determinant of GLS abnormality in overall patients with preserved LVEF.

EATV and General Characteristics

Although the LVEF values were comparable, the GLS, but not GRS and GCS, was altered in the high EATV group. Accordingly, the frequency of GLS ≤18 was higher in the high EATV group (37 vs. 22%, $P = 0.034$). Previous studies reported that patients with high EATV showed structural and functional alterations in the heart. Thus, high accumulations in EATV were reported to be correlated with a severity of CAD (21, 22, 26), progression of coronary high risk plaques (27), LV mass (16), and LV diastolic function (17). In contrast, the LVEF was not correlated with EATV (28) and patients with HFrEF would rather have a reduction in the EATV compared with normal controls (29). Collectively, the impact of EATV accumulation on cardiac indices can be various in patients' conditions and may be pronounced in preserved LVEF.

EATV and LV Strain

The relationship between EATV and LV strains remains to be elucidated. Previous studies indicated that an increase in the BMI (12–14) was correlated with reduced GLS. Further, the current study clarified that EATV was a determinant of LV GLS independent of BMI in patients with preserved LVEF. This finding was consistent with the results of the report by Ng et al. (18). It has been shown that EATV was correlated with markers of LV mass (16) and LV diastolic function (E/e) (17), independent of BMI and VFA. Taken together, with respect to our results and Ng. et al. (18), the accumulation of EATV can be related to reduced GLS more strongly than whole body adiposity (12–14).

EATV Cutoff and OR for GLS Abnormality (GLS ≤18)

For the first time, we evaluated the cutoff value of EATV for detecting global strain abnormalities and its diagnostic utility. The cutoff of EATV ≥116 mL showed a significant adjusted OR 2.22 [1.03–4.79] in overall subjects (**Table 3**). Reportedly, the cutoffs of EATV were 92 mL (CAD) (30), 100 mL (ACS) (31), 126 mL (cardiovascular events) (32), and 107 mL (high-risk

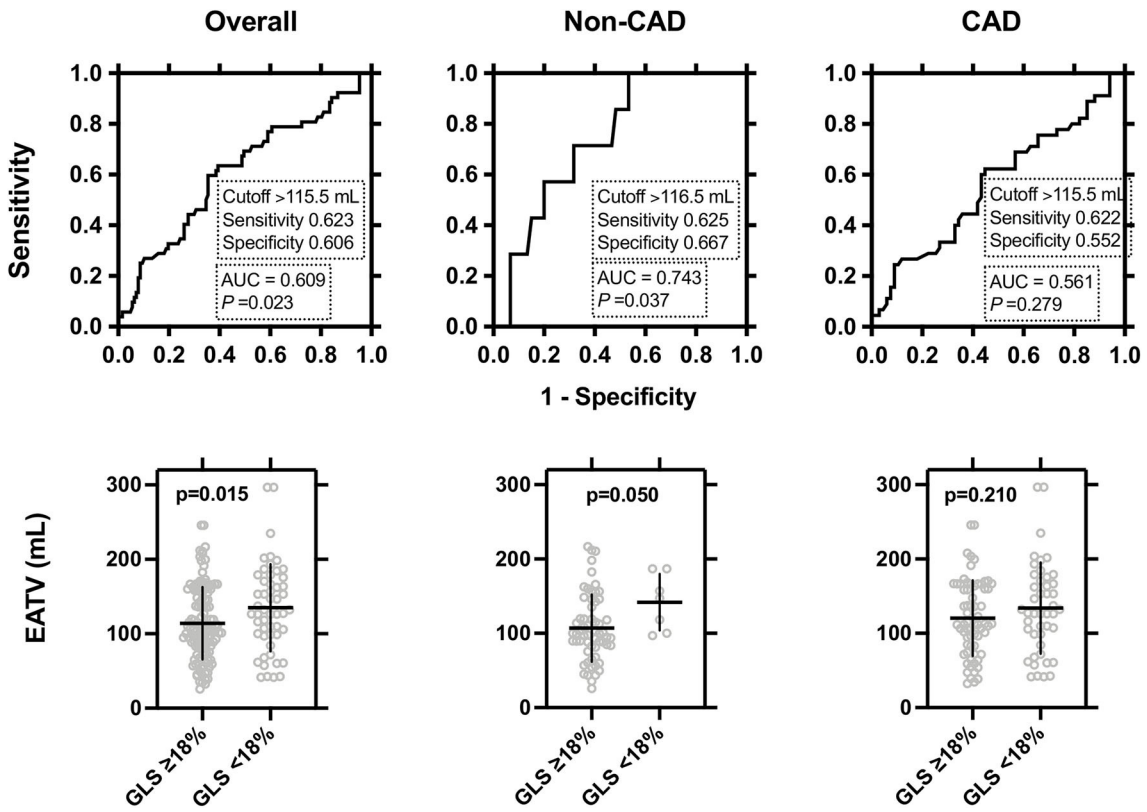

FIGURE 3 | Receiver-operating-characteristic (ROC) curve analysis evaluating the predictive accuracy of the left ventricular (LV) global longitudinal strain in the overall, non-CAD, and CAD patients. Upper panel: The cut-off point of epicardial adipose tissue volume (EATV) for predicting GLS ≤18 and its sensitivity and specificity are shown. Lower panel: Comparisons of EATV between GLS ≤18 vs. GLS >18 were made by two-tailed unpaired t-tests and the statistical significance was set at $P < 0.05$.

plaque) (27), respectively. Our cutoff value of 116 mL is close to these values; therefore, it may share hidden cardiovascular risks. In the overall subjects, CAD and EATV ≥116 mL were significant determinants and diabetes mellitus was the sole determinant in the CAD group for GLS ≤18. EATV might affect GLS as well as the presence of CAD and diabetes mellitus.

Potential Mechanisms

The mechanisms underlying the correlation between the accumulation of EATV and reduced GLS in patients with preserved LVEF remain to be elucidated. Three potential mechanisms were discussed below (33–35).

First, the accumulation of EATV may represent obesity-related systemic inflammatory disorders, which may promote cardiac dysfunction including GLS abnormality (33–35). Impaired LV global strain and/or heart failure (HFpEF) in obese individuals may be linked to systemic hemodynamic and hormonal abnormalities. It is noted that accumulated EATV closely related to visceral fat obesity (VFO) or central obesity, which is the potential risk of HFpEF (36, 37) through the development of diabetes mellitus, dyslipidemia, hypertension, and CAD (38). High EATV may be linked to reduced LV strains independent of the presence of diabetes, dyslipidemia, and hypertension as in VFO (33) via the activation of sympathetic nerve systems, production of reactive oxygen species (ROS) (39), chronic kidney disease (CKD), and proinflammatory immunometabolism (34). Moreover, our notion may be supported by the fact that increased EAT volume and insulin resistance were independently associated with increased myocardial fat accumulation and interstitial myocardial fibrosis (40).

Second, EAT may have local direct effects on the myocardium (33–35), which can modulate the LV strain. Hence, the accumulation of EATV may directly affect GLS via the paracrine action of EAT-derived cytokines. Notably, there are four components of lipids deposition in the heart: (1) circulatory and locally recruited fat, (2) intra- and extra-myocellular fat, (3) perivascular fat, and (4) pericardial fat, all of which are considered to modulate the LV strain via cellular cross-talk between the EAT and myocardium (lipotoxicity) (33, 41). Correlation of myocardial fat accumulation with GLS (40) supports this idea. Kramer et al. found that the subepicardial LV strain, as compared to the subendocardial strain, was largely impaired in the high-fat diet-induced obese model (42). A link between EAT accumulation and subepicardial strains might be suggested.

Third, EATV was associated with reduced GLS, but not with GRS and GCS, in the CAD and non-CAD groups. Haggerty et al. (43) demonstrated that EATV was negatively associated not only with GLS, but also with GCS and GRS. The reasons for this discrepancy could not be identified. Instead, our results agreed with Haggerty et al. (43) showing that the GRS was

TABLE 3 | Logistic regression analysis to predict GLS abnormality (GLS ≤18).

	Overall			non-CAD			CAD		
Variables	**Crude OR (95%CI)**	***P***	**Adjusted OR (95%CI)***	***P***	**Crude OR (95%CI)**	***P***	**Adjusted OR (95%CI)***	***P***	**Crude OR (95%CI)**	***P***	**Adjusted OR (95%CI)***	***P***
Age (per year)	1.04 (1.01–1.08)	0.010	1.03 (1.00–1.07)	0.094	1.05 (0.98–1.13)	0.172	1.02 (0.95–1.11)	0.555	1.03 (0.99–1.07)	0.200	1.04 (0.99–1.08)	0.106
Male sex (yes or no)	0.64 (0.33–1.23)	0.176	0.53 (0.22–1.29)	0.534	0.82 (0.19–3.58)	0.790	0.32 (0.05–2.17)	0.241	0.71 (0.32–1.54)	0.380	0.58 (0.20–1.62)	0.295
Body mass index (kg/m^2)	1.00 (0.94–1.07)	0.906	1.03 (0.94–1.12)	0.544	0.93 (0.79–1.11)	0.440	0.86 (0.66–1.11)	0.243	1.03 (0.96–1.12)	0.407	1.06 (0.96–1.17)	0.241
Smoking history (yes or no)	1.02 (0.53–1.98)	0.945	1.82 (0.75–4.42)	0.186	3.77 (0.43–32.7)	0.229	6.44 (0.58–71.6)	0.130	1.03 (0.48–2.22)	0.933	1.35 (0.48–3.76)	0.567
Dyslipidemia (yes or no)	0.60 (0.26–1.36)	0.217	0.60 (0.22–1.60)	0.306	1.30 (0.28–5.99)	0.741	0.82 (0.14–4.81)	0.825	0.60 (0.21–1.70)	0.334	0.44 (0.13–1.53)	0.198
Hypertension (yes or no)	0.97 (0.43–2.20)	0.945	1.22 (0.47–3.16)	0.689	1.21 (0.22–6.69)	0.831	0.88 (0.11–6.84)	0.906	0.67 (0.27–1.63)	0.374	1.27 (0.42–3.85)	0.676
Type 2 diabetes mellitus (yes or no)	2.17 (1.02–4.62)	0.045	2.33 (1.01–5.38)	0.047	1.50 (0.28–8.11)	0.638	2.29 (0.28–18.9)	0.442	2.54 (1.05–6.12)	0.038	2.69 (1.04–6.94)	0.041
Coronary artery disease (yes or no)	5.04 (2.20–11.5)	<0.001	3.99 (1.65–9.79)	0.002	–	–	–	–	–	–	–	-
EATV ≥116 ml (yes or no)	2.54 (1.31–4.92)	0.006	2.22 (1.03–4.79)	0.042	3.33 (0.72–15.4)	0.123	6.29 (0.89–44.7)	0.066	2.03 (0.94–4.39)	0.072	1.61 (0.67–3.87)	0.285

*OR, odds ratio. *OR adjusted for age, male sex, body mass index, smoking history, dyslipidemia, Hypertension, type 2 diabetes mellitus, coronary artery disease and EATV ≥116 ml. CAD, coronary artery disease; EATV, epicardial adipose tissue volume.*

positively associated with the LV mass index (**Figure 2**). Previous studies showed that GRS was higher and GLS was lower in hypertensive patients with LV hypertrophy (44). It may be suggested that LV functional and structural remodeling, which can be affected independently by hypertension and EATV, may affect the GLS and GRS with different time courses. The current study showed that the GCS was negatively associated with BMI, but not with EATV (**Supplement 2**). This result agrees with a previous report (45). Theoretically, myocardial contraction can be classified according to the involved myocardial layer into (1) contraction of the subendocardial fibers contributing to longitudinal shortening, (2) contraction of the subepicardial fibers contributing to circumferential shortening, and (3) transmural fibers contributing to radial thickening (46). Studies in obese individuals reported that the GLS was commonly impaired; however, the changes in GRS and GCS were inconsistent between 2D and 3D (47–49), suggesting that this layer-specific strain measurements are being useful but still under clinical validation.

Study Limitations

This study has potential limitations. First, the study design was cross-sectional, and it was conducted at a single center with a relatively small number of patients. Second, the patients consisted entirely of Japanese patients; therefore, the relevance of this study to other ethnic populations requires further research. Third, we did not consider the impact of patient medications or lifestyles on LV global strain. Fourth, we defined the impaired GLS as ≤18 based on the modification of Yang et al. (10), thereby limiting our results to moderate to severe GLS impairment. Fifth, previous studies show that accumulation of EATV is frequently linked to inflammatory status (26), suggesting that enhancement of chronic inflammation may underlie the link between EATV and GLS. However, because of the retrospective study design, we could not study the link in this study.

CONCLUSION

This study found that increasing EATV is independently associated with the global longitudinal strain despite the preserved LVEF and lack of obstructive CAD. Our finding suggests the additional role of EAT on the myocardial systolic function by impaired LV longitudinal strain. The finding may help further our understanding of the link between obesity and heart failure with preserved LVEF.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Fukushima Medical University and Tokushima

University ethics committees. Written informed consent was not provided because the study was done in a retrospective design.

AUTHOR CONTRIBUTIONS

MSh designed the research. GM collected data with the assistance of YT and NY. MSh and GM analyzed and interpreted data and wrote the manuscript with inputs from all other authors. KK and YT supervised the echocardiographic analysis. DF, SY, TS, HM, and MSa advised and discussed the study. MSh was the guarantor of this work, and as such, had full access to all the data in the study, takes responsibility for the integrity of the data, and the accuracy of the data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2020.607825/full#supplementary-material

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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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Calcium Dobesilate (CaD) Attenuates High Glucose and High Lipid-Induced Impairment of Sarcoplasmic Reticulum Calcium Handling in Cardiomyocytes

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Calcium dobesilate (CaD) is used effectively in patients with diabetic microvascular disorder, retinopathy, and nephropathy. Here we sought to determine whether it has an effect on cardiomyocytes calcium mishandling that is characteristic of diabetic cardiomyopathy. Cardiomyocytes were sterile isolated and cultured from 1 to 3 days neonatal rats and treated with vehicle (Control), 25 mM glucose+300 μM Palmitic acid (HG+PA), 100 μM CaD (CaD), or HG+PA+CaD to test the effects on calcium signaling (Ca^{2+} sparks, transients, and SR loads) and reactive oxygen species (ROS) production by confocal imaging. Compared to Control, HG+PA treatment significantly reduced field stimulation-induced calcium transient amplitudes (2.22 ± 0.19 vs. 3.56 ± 0.21, $p < 0.01$) and the levels of caffeine-induced calcium transients (3.19 ± 0.14 vs. 3.72 ± 0.15, $p < 0.01$), however significantly increased spontaneous Ca^{2+} sparks firing levels in single cardiomyocytes (spontaneous frequency 2.65 ± 0.23 vs. 1.72 ± 0.12, $p < 0.01$) and ROS production (67.12 ± 4.4 vs. 47.65 ± 2.12, $p < 0.05$), which suggest that HG+PA treatment increases the Spontaneity Ca^{2+} spark frequency, and then induced partial reduction of SR Ca^{2+} content and subsequently weaken systolic Ca^{2+} transient in cardiomyocyte. Remarkably, these impairments in calcium signaling and ROS production were largely prevented by pre-treatment of the cells with CaD. Therefore, CaD may contribute to a good protective effect on patients with calcium mishandling and contractile dysfunction in cardiomyocytes associated with diabetic cardiomyopathy.

Keywords: calcium dobesilate, cardiomyocytes, calcium sparks, hyperglycemia, laser scanning confocal microscope

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INTRODUCTION

The worldwide prevalence of diabetes mellitus increases markedly to cause various cardiovascular complications, which are the major cause of morbidity and mortality in this population (1, 2). Among patients with the same degree of cardiovascular diseases, the mortality rate from cardiovascular diseases in diabetic patients is 2–4 times higher than that of non-diabetic patients. In addition, diabetic cardiomyopathy can occur without any vascular pathogenesis (3–5); The disease progress from initial asymptomatic left ventricular (LV) diastolic dysfunction to impaired LV systolic function, and eventually advances to heart failure (6). Therefore, a better understanding of mechanisms of diabetes-associated cardiomyocytes impairments will aid the development of novel strategies for protecting heart function in these patients (7–9).

As we know, Ca^{2+} plays an essential role in cardiac EC-coupling. Ca^{2+} major stores in sarcoplasmic reticulum (SR) in the mammalian heart. At systole, instantaneous high concentration of Ca^{2+} is released from SR trigged by Ca^{2+} influx trough L-type Ca^{2+} channel, consequently Ca^{2+} transient that triggers myofilaments contraction in heart (10). Ryanodine receptors play essential role in Ca^{2+} release from SR *via* CICR mechanism. Spontaneous Ca^{2+} sparks, the elementary intracellular Ca^{2+} release from 4 to 6 neighboring RyRs, is sporadic occurs in the cadiomyocytes under physiological condition. The frequency of Ca^{2+} sparks is closely related to the activity of RyR at diastole, and control SR Ca^{2+} content. High Spontaneous Ca^{2+} sparks frequency, often happened in kind of diseases, have been reported to reduce SR Ca^{2+} content (11). RyR dysfunction has been shown to cause various of cardiac dysfunction, such as heart failure, hypertrophy (12, 13) and ischemia-reperfusion (14). Abnormal RyR function has also been shown in type 1 diabetes (15), and STZ-induced type-2 diabetes rat model (2), despite the pathological significance is not well-characterized.

Calcium dobesilate (CaD) is a vascular protective agent, highly effective in treating patients with various diabetes-associated microvascular disorders, retinopathy, nephropathy, and cataract (16–18). CaD has been shown to decrease intracellular ROS production, improve the biosynthesis of collagen in the basement membrane, and inhibit the high permeability caused by vasoactive substances. However, whether CaD has an effect on diabetes-associated or high glucose-induced cardiomyocytes dysfunction is currently unknown. In this study, we sought to examine the potential effects of CaD on calcium regulation of primary ventricular cardiomyocytes. We found that CaD efficiently protects high glucose-induced SR calcium leaks and depletion. Thus, CaD may be used to protect impairment of cardiomyocytes calcium signaling and contractile function associated with diabetic cardiomyopathy.

METHODS

Isolation, Culture, and Treatments of Neonatal Rat Ventricular Myocytes (NRVMs)

Sprague–Dawley rats of 1–3-days-old were purchased from the Animal center of Guangdong Experimental Animal Center and handled according to a protocol approved by the Institutional Care and Use Committee of Shenzhen University, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). NRVMs were isolated and cultured as previously described (19). After cultured for 24 h, the cells were stimulated with 25 mM glucose+300 μM PA (sigma, USA) for 24 h. In the case of CaD (Merck Serono Co., Ltd) treatment, NRVMs were pretreated with 100 μM CaD for 24 h.

Ca^{2+} Spark Recording

Briefly, NRVMs loaded with Fluo-4 AM (ThermoFisher Scientific, F14201, dissolved in DMSO contains 20% pluronic F-127) for 10 min at 37°C and then perfused with Tyrode's solution (137 mM NaCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 20 mM HEPES, 5.4 mM KCl, 1.8 mM $CaCl_2$, 10 mM glucose, pH 7.4) in a recording chamber. Confocal line-scan imaging was carried out in resting cells at 488 nm excitation and 505 nm collection with a Zeiss 780 inverted confocal microscope (Carl Zeiss) with a 40x oil immersion lens for detecting Ca^{2+} sparks as described previously (20). Line-scan images were acquired at a sampling rate of 3.84 ms per line.

Measurement of Action Potential (AP)-Elicited Ca^{2+} Transient

After cells loaded with Fluo-4 AM, Cells were superfused with a normal external solution containing (same as calcium spark recording) remove the dye and complete de-esterification. After the cardiomyocytes reached a steady-state with field stimulation (1 Hz), line-scan imaging was acquired along the longitudinal axis of the cells.

Measurement of SR Ca^{2+} Content

Short puffs of caffeine (20 mM) were applied to completely empty the SR, after a train of 1-Hz field stimulation to achieve steady-state SR Ca^{2+} loading. SR Ca^{2+} content was assessed by detecting the amplitude of the Ca^{2+} transient. Cells were superfused with normal external solution.

Measurement of Reactive Oxygen Species (ROS)

Cells were loaded with the probe 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, 10 μM, Molecular Probes) for 10 min at 37°C before the experiment. The 2′,7′-dichlorodihydrofluorescein (DCF) fluorescent

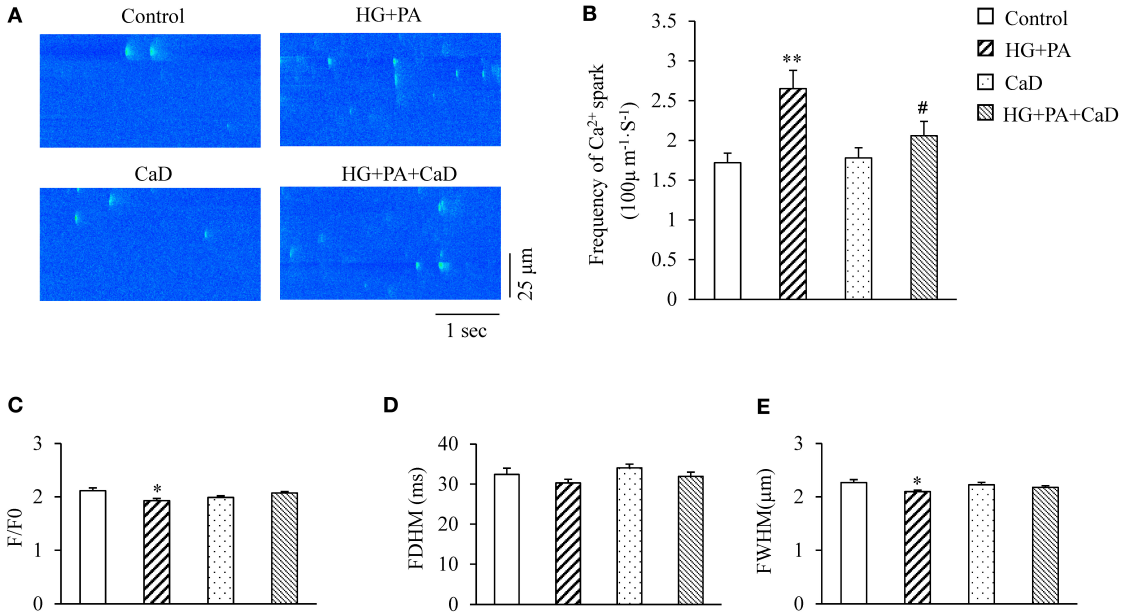

FIGURE 1 | HG+PA increases Ca^{2+} sparks frequency in cardiomyocytes. **(A)** Representative Ca^{2+} spark images. **(B–E)** Statistic analyses of the frequency **(B)**, amplitude (F/F0, **C**), full duration of half maximum (FDHM, **D**) and full width of half maximum (FWHM, **E**) of Ca^{2+} sparks in Control, HG+PA, CaD, and HG+PA+CaD treatments. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. HG+PA. $n = 103–258$.

intensity was measured by using LSM 780 (Zeiss) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (21).

Statistical Analysis

All values are given as mean ± SE. Unpaired Student's *t*-test between 2 groups was performed, one-way analysis of variance (ANOVA) followed by the Dunnett's *post-hoc* test was used for comparisons among four groups. In all tests, values of $p < 0.05$ were considered be statistically significant.

RESULTS

CaD Attenuates HG+PA–Induced Increase in Ca^{2+} Spark Frequency

We initiated our study by analyzing effects of HG+PA and CaD on the occurrence and properties of Ca^{2+} sparks in NRVMs. HG+PA increased the frequency of Ca^{2+} sparks by 1.54-fold (2.65 ± 0.23 vs. 1.72 ± 0.12 sparks/100 μm·s in Control, $p < 0.01$. **Figures 1A,B**) and to a lesser extent, decreased the amplitude(F/F0) of Ca^{2+} sparks (1.93 ± 0.04 vs. 2.12 ± 0.05 in Control, $p < 0.05$) (**Figure 1C**). In addition, HG+PA decreased full width of half maximum (FWHM) of Ca^{2+} sparks but did not alter the full duration of half maximum (FDHM) (**Figures 1D,E**). Remarkably, pre-treatment of cells with CaD restored HG+PA–induced alterations in Ca^{2+} spark frequency, amplitudes, and FWHM (**Figures 1A–E**). These results suggest that CaD normalizes HG+PA induced RyR dysfunction in the cardiomyocytes at baseline.

CaD Attenuates HG+PA–Induced Reduction in SR Ca^{2+} Content

SR Ca^{2+} content affects the frequency and amplitude of Ca^{2+} sparks in cardiomyocytes; the higher of SR Ca^{2+} content results in the higher frequency of spontaneous Ca^{2+}sparks (22). To determine whether HG+PA–increased increase in the spark frequency is due to an increased SR load, we analyzed the amplitude of caffeine-elicited Ca^{2+} transient (ΔF/F0) indicating SR Ca^{2+} content. High concentration of Caffeine (20 mM) was used to stimulate the change of calcium storage capacity in each group. We found that HG+PA treatment significantly decreased SR Ca^{2+} content (ΔF/F0, **Figure 2A**). In contrast, pre-treatment of cells with CaD completely prevented the HG+PA–induction of SR Ca^{2+} depletion (**Figure 2B**).

CaD Ameliorates HG+PA–Induced Reduction in Systolic Ca^{2+} Transient

Previous reports suggest that the reduction of systolic calcium transients in cardiomyocytes is the main cause of impaired contractility in diabetic cardiomyopathy (15). We elicited systolic Ca^{2+} transient by 1 Hz-field stimulation in the treatment groups. HG+PA treatment caused a significantly reduced amplitude (ΔF/F0, 2.22 ± 0.19 vs. 3.56 ± 0.21 in Control, $p < 0.01$, **Figures 3A,B**) and a significantly prolonged rise time (**Figure 3C**) of Ca^{2+} transient, suggesting an impairment of the synchrony of SR Ca^{2+} release. Treatment with CaD did not affect the calcium transient amplitude (3.68 ± 0.3 vs. 3.56 ± 0.21 in Control, $p > 0.05$), however, ameliorated HG+PA–induced impairment of SR Ca^{2+} release (ΔF/F0, 3.28 ± 0.25 vs. 2.22 ± 0.19 in HG+PA, $p < 0.05$; Rise time, 89.68 ± 2.81 vs. 78.59 ±

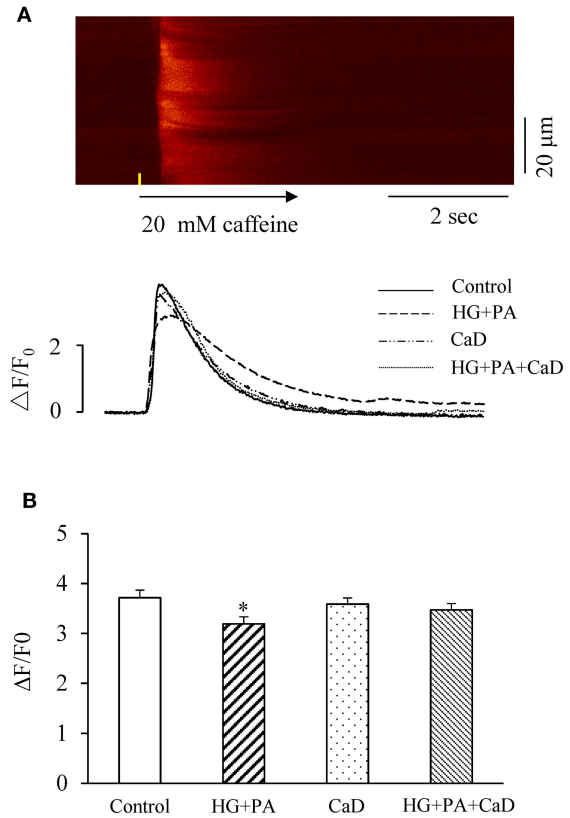

FIGURE 2 | HG+PA reduces SR Ca^{2+} content in cardiomyocytes. **(A)** Representative confocal image in control and representative time-courses of caffeine-elicited Ca^{2+} transient traces in Control, HG+PA, CaD, and HG+PA+CaD. **(B)** Average SR Ca^{2+} contents indicated by the amplitude of caffeine-elicited Ca^{2+} transient (ΔF/F0) in Control, HG+PA, CaD, and HG+PA+CaD. *$p < 0.05$ vs. control. $n = 10$–20.

2.12 in HG+PA, $p < 0.05$. **Figures 3A–C**). We did not observe a statistical significance in T50 between the four treatment groups (**Figure 3D**).

CaD Suppress HG+PA–Induced Oxidative Stress

Because oxidation and nitrosylation of cysteine residues on RyR are key post-translational modifications that make the channel open to Ca^{2+} leaks, whereas reversal of these modifications closes the channel (23, 24), we examined whether the HG+PA induced hyperactive Ca^{2+} sparks is accounted by the increased ROS level in cardiomyocytes. We found that HG+PA treatment dramatically increased superoxide concentration in cardiomyocytes, and this change was markedly attenuated by treatment with CaD (**Figure 4**). Thus, CaD suppresses HG+PA–induced ROS production.

DISCUSSION

In this study, we found that exposure of neonatal rat ventricular cardiomyocytes to high glucose and high lipid (HG+PA) results in a marked increase in the intracellular ROS and Ca^{2+} sparks the frequency, a partial depletion of the SR Ca^{2+} content, and contractile dysfunction. Treatment of these cells with CaD attenuates the ROS production, spontaneous Ca^{2+} sparks, and SR Ca^{2+} depletion, restoring cardiomyocyte function. Although the beneficial effects of CaD in diabetic microangiopathy (e.g., diabetic retinopathy, nephropathy, and neuropathy) have been well-known, we study for first-time documents that CaD protects cardiomyocytes from HG+PA induced injury and Ca^{2+} mishandling.

Our findings have a high translational value for the treatment of diabetic cardiomyopathy. Since the 1970's, CaD has been front-line medication for the prevention and treatment of diabetic retinopathy with an excellent safety profile. Our data suggest that it may have a direct protective effect on cardiomyocytes in diabetes and that its use may be extended to diabetic heart disease. This is particularly important because currently there is no drug to specifically target high-glucose and high-fat induced cardiomyocyte injury. Notably, our results indicate that CaD is effective after HG+PA treatment and does not affect cardiomyocyte function at baseline. Therefore, it is probably safe and consistent with its safety profile in the clinic.

Diabetes mellitus is a metabolic disorder characterized by abnormal glucose and lipid metabolism. Hyperglycemia and high fat can cause cardiac damage, leading to diabetic cardiomyopathy (3, 4, 25). Mechanistically, long-time high blood glucose leads to excessive protein glycosylation and accumulation of advanced glycation end products (AGEs), which in sync with an elevation of the receptor for AGEs (RAGE) in the HG milieu, induces the production of cytokines and free radicals, resulting in cardiomyocyte injury. Importantly, we have previously shown that superfluous AGEs can influence Ca^{2+} spark firing frequency in cardiomyocytes, and that inhibition of RAGE significantly reduces the calcium spark frequency and protects heart function (26). Additionally, high blood glucose may induce apoptosis of cardiomyocytes through the deposition of oxidative stress, and now ample evidence suggests an increase of RyR in diabetes that is associated with oxidative stress. In addition to hyperglycemia, high fat is also found to induce ROS production. For example, free fatty acid (FFA) can induce cardiomyocyte apoptosis by increasing the concentrations of ceramide and ROS. Because overproduction of ROS contributes to the pathogenesis of diabetic cardiomyopathy, treatment with antioxidants can reverse the cardiac dysfunction (27, 28).

Recently, a series of studies found that SR calcium leakage increases in the cardiomyocytes of heart failure patients and contributes to the pathogenesis of heart failure (29, 30). Notably, ROS has been shown directly associated with Ca^{2+} sparks in the cardiomyocytes (31, 32). Enhanced diastolic SR Ca^{2+} leak has been suggested to be a potential cause of reduction of SR Ca^{2+} content (12), and the systolic intracellular Ca^{2+} concentration determines cardiac contractility. In rodent heart, Ca^{2+} influx through LCC accounts for no more than 30% while SR Ca^{2+} release contributes to over 70% of Ca^{2+} transient in systolic (33). Our data suggested that HG+PA significantly decreased SR Ca^{2+} content, which is consistent with the alteration of the magnitude of the systolic Ca^{2+} transient. Our finding

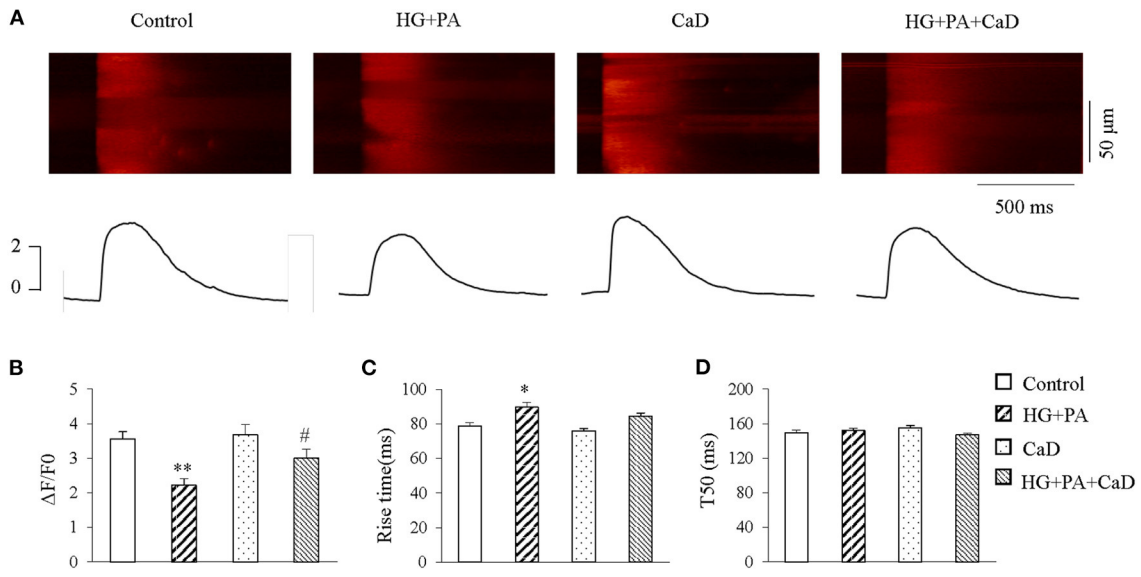

FIGURE 3 | HG+PA reduces systolic Ca^{2+} transient in cardiomyocytes. **(A)** Representative confocal line-scan images of Ca^{2+} transients (*upper panels*) along with the spatial average (*lower panels*) in Control, HG+PA, CaD, and HG+PA+CaD treatments. **(B–D)** Average amplitudes (ΔF/F0, **B**), Rise time **(C)** and half time of decay (T_{50}, **D**) of Ca^{2+} transient in Control, HG+PA, CaD, and HG+PA+CaD treatments. $^{**}p < 0.01$, $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. HG+PA. $n = 25–45$.

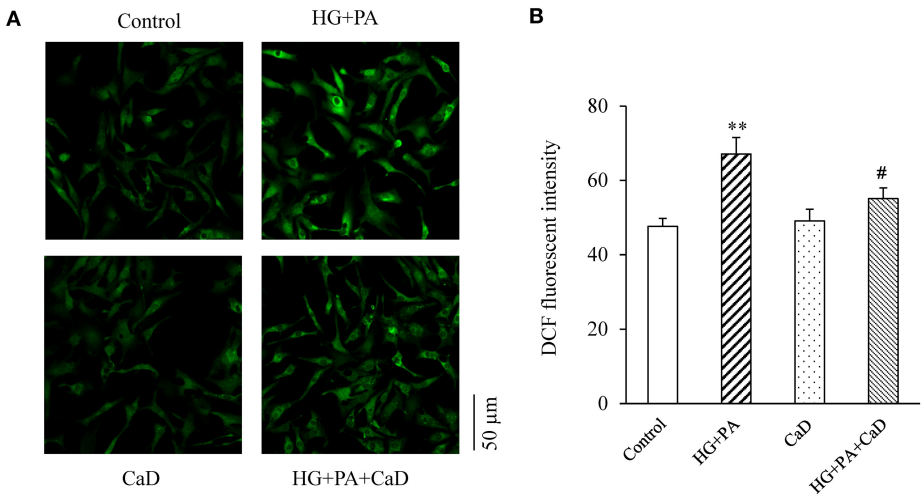

FIGURE 4 | HG+PA increases the level of ROS in neonatal cardiomyocytes. Representative fluorescence images **(A)** and quantification **(B)** of ROS production. $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. HG+PA.

indicates that HG+PA decreases cardiac contractile function, which may be an important mechanism for cardiac dysfunction in diabetic cardiomyopathy.

CaD, as an oxygen free radical scavenger, have been shown inhibits free radical production both *in vitro* and *in vivo* (34–37). In this study, we found that HG+PA significantly increases the oxidative stress level in cardiomyocytes, and this effect was effectively attenuated by CaD treatment. Thus, CaD reduces oxidative stress levels in the cells, thereby reducing the frequency of spontaneous calcium sparks, restoring the sarcoplasmic reticulum calcium pool, and stabilizing calcium transients in cardiomyocytes.

One limitation of this study is that our experimental system was primarily based on the cultured neonatal rat cardiomyocytes, which may have electrophysiological features different from the adult cells or the *in vivo* diabetic cardiomyopathy models. Thus, cautions should be taken when translating these findings into potential clinic use of the drug. In addition, the molecular targets of CaD that mediates its effects on ROS reduction and consequent Ca^{2+} signaling in cardiomyocytes are yet to be identified, which remains a subject of our future studies.

In conclusion, CaD protects cardiomyocytes from HG and PA-induced elevation in oxidative stress and spontaneous spark frequency and restores sarcoplasmic reticulum calcium levels. Thus, it may be utilized for the treatment of cardiac contractile dysfunction in diabetic cardiomyopathy.

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 reviewed and approved by Laboratory Animal Science Research Committee of Shenzhen University Health Science Center.

AUTHOR CONTRIBUTIONS

JD and DY conceptualized the study and contributed to resources. XC, ZC, MH, YL, HF, HC, HL, and JL collected data. JD and GQ wrote 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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REVIEW
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Mechanisms and Perspectives of Sodium-Glucose Co-transporter 2 Inhibitors in Heart Failure

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Heart failure (HF) is a common complication or late-stage manifestation of various heart diseases. Numerous risk factors and underlying causes may contribute to the occurrence and progression of HF. The pathophysiological mechanisms of HF are very complicated. Despite accumulating advances in treatment for HF during recent decades, it remains an intractable clinical syndrome with poor outcomes, significantly reducing the quality of life and expectancy of patients, and imposing a heavy economic burden on society and families. Although initially classified as antidiabetic agents, sodium-glucose co-transporter 2 (SGLT2) inhibitors have demonstrated reduced the prevalence of hospitalization for HF, cardiovascular death, and all-cause death in several large-scale randomized controlled clinical trials. These beneficial effects of SGLT-2 inhibitors can be attributed to multiple hemodynamic, inflammatory and metabolic mechanisms, not only reducing the serum glucose level. SGLT2 inhibitors have been used increasingly in treatment for patients with HF with reduced ejection fraction due to their surprising performance in improving the prognosis. In addition, their roles and mechanisms in patients with HF with preserved ejection fraction or acute HF have also attracted attention. In this review article, we discuss the possible mechanisms and applications of SGLT2 inhibitors in HF.

Keywords: sodium-glucose co-transporter 2 inhibitors, diabetes mellitus, heart failure, heart failure with preserved ejection fraction, insulin resistance

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INTRODUCTION

Heart failure (HF) has become an increasingly serious public-health problem worldwide (1). HF plagues not only high-income countries such as the USA and European countries, but also low- and middle-income countries (2). HF seriously affects the quality of life (QoL) and leads to significantly shortened life expectancy. Also, the HF-related economic burden is significant and will increase as populations age (1). Despite considerable progress in drug treatment and device therapies as well as widespread implementation of preventive measures, the prevalence of rehospitalization and mortality of HF patients remains unacceptably high (3).

Type 2 diabetes mellitus (T2DM) is also prevalent worldwide. Nearly 10% of the adult population worldwide (∼382 million) suffers from T2DM. More importantly, this number is increasing and has been projected to reach 592 million by 2035 (4).

There is a close relationship between T2DM and HF. Cardiovascular (CV) diseases remain the leading cause of disability and death among patients with T2DM. About two-thirds of people with T2DM eventually die from CV diseases, including atherosclerosis, myocardial infarction (MI), HF, or stroke (5). Conversely, T2DM is an important predictor of new-onset and recurrent HF (6, 7). More than 40% of hospitalized patients with HF also have T2DM. Furthermore, T2DM leads to significant increases in the risk of hospitalization, readmission to hospital, and death among patients with HF (8). A recent meta-analysis of 129 studies involving >10 million individuals demonstrated that prediabetes is also associated significantly with a higher risk of CV diseases and all-cause death in a healthy population and in people with established atherosclerotic CV disease (9). In a recent large-scale cohort study, patients with T2DM still had a higher risk of hospitalization for HF than the control group even if the five major risk factors (hypertension, smoking, albuminuria, elevated low-density lipoprotein cholesterol, elevated glycated hemoglobin) were under control (10). Several studies have shown that patients with both T2DM and HF have a worse outcome than those with one of these diseases only (11).

Traditional anti-HF pharmacological therapies are neuroendocrine inhibitors, which modulate the renin–angiotensin–aldosterone system (RAAS) or sympathetic nervous system (SNS). Such therapies can improve QoL significantly and reduce mortality in patients with heart failure with reduced ejection fraction (HFrEF) (12). However, these drugs do not show the same significant effects in patients with heart failure with preserved ejection fraction (HFpEF) (13–15). More frustratingly, the angiotensin receptor-neprilysin inhibitor sacubitril-valsartan also did not significantly reduce the risk of hospitalization and all-cause death among patients with HFpEF (16). So far, an "ideal" drug demonstrating a convincing prognosis-improving effect in patients with HFpEF is lacking (12–16).

Trials focusing on CV outcomes have demonstrated the significant benefits of sodium-glucose co-transporter 2 (SGLT2) inhibitors in improving clinical outcomes (17–22). Based on such overwhelming evidence, SGLT2 inhibitors are being recommended increasingly to treat patients with HFrEF or T2DM at high risk of CV diseases (23–26). Moreover, some studies have suggested that they may also be beneficial for patients with HFpEF or acute HF, which merits evaluation in large-scale studies.

SODIUM-GLUCOSE CO-TRANSPORTERS (SGLTS)

Under physiological conditions, glucose is filtered from glomeruli and reabsorbed in the S1 segment of the proximal convoluted tubule. Upon exceeding the absorptive capacity, redundant glucose is excreted through urine (27). Renal glucose reabsorption is mediated mainly by SGLTs. The latter are a family of membrane proteins found chiefly in the mucosa of the small intestine and in the proximal convoluted tubule of the kidney (28). Among the 12 members of the SGLT family, SGLT1 and SGLT2 are the two most important isoforms involved in renal glucose reabsorption. Characterized as a high-capacity and low-affinity transporter, SGLT2 is distributed mainly in segments S1/S2 of renal proximal convoluted tubule and mediates about 80–90% of filtered glucose reabsorption. In contrast, SGLT1 is a low-capacity and high-affinity transporter. SGLT1 is found mainly in the brush border of the small intestine and in the S3 segment of the proximal convoluted tubule. SGLT1 is responsible for the residual 10–20% of glucose reabsorption (29).

SGLT2 INHIBITORS

As a novel class of oral antidiabetic agents, SGLT2 inhibitors are used initially to reduce the serum level of glycated hemoglobin and improve glycemic control. Surprisingly, several large-scale randomized clinical trials have demonstrated that SGLT2 inhibitors reduce CV mortality dramatically, improve CV outcomes, and reduce HF hospitalization in T2DM patients whether they have pre-existing CV disease or not (17–22). Recently, investigators from Harvard Medical School (Boston, MA, USA) have suggested that SGLT2 inhibitors should be considered as one of the basic medications in patients with HFrEF, in addition to standard therapies (23). With the advent of more clinical trials to further evaluate the benefits and safety of SGLT2 inhibitors in different subtypes of HF, they may become increasingly promising agents for patients with HF.

The benefits of SGLT2 inhibitors upon clinical outcomes are attributable to several complex mechanisms in addition to lowering the serum glucose level (though they were developed initially as antihyperglycemic agents).

Hemodynamic Mechanisms of SGLT2 Inhibitors

A series of clinical trials have shown an obvious decrease in hospitalization due to HF shortly after beginning use of SGLT2 inhibitors, suggesting that they may improve hemodynamic status.

SGLT2 inhibitors have been reported to lower blood pressure by ∼4.0/1.6 mmHg without increasing the heart rate, which suggests that the SNS is not activated (and may even be inhibited) (30). SNS activation is unfavorable in HF, and is often accompanied by a worse clinical outcome (31). Therefore, compared with agents which may lead to SNS activation, SGLT2 inhibitors may be more beneficial to patients with HF (31, 32). In addition, unlike the gradually decreasing glucose-lowering effect of SGLT2 inhibitors in patients with chronic kidney disease, the effect of these agents on lowering blood pressure is similar in patients with different renal conditions, even those with a lower estimated glomerular filtration rate (eGFR) (33). Early theories and evidences have suggested that their antihypertensive effect might derive from a volume-reduction mechanism due to diuresis and natriuresis. In addition, other effects, such as calorie loss, fat-mass decrease, and weight loss, resulting from increasing diuresis and glucosuria also contribute to lowering of blood

pressure (34). Furthermore, recent studies have suggested that the ketogenic properties of SGLT2 inhibitors may be a reasonable explanation for their antihypertensive effects independent of renal function (35). However, the blood-pressure reduction observed with SGLT2 inhibitors cannot explain satisfactorily the rapid and effective improvement of clinical outcomes given that even those antihypertensive drugs do not demonstrate such impressive effects.

Under physiological conditions in proximal convoluted tubule, glucose and sodium ions (Na^+) are reabsorbed together from the glomerular filtrate. By inhibiting Na^+ reabsorption in proximal tubule, SGLT2 inhibitors promote natriuresis and reduce extracellular fluid and plasma volume. After taking SGLT2 inhibitors, the early manifestation is an increased urine volume in the first few days, then the urine volume returns gradually to a baseline level in several weeks, and a reduction of plasma volume of ∼7.3% is observed after 12 weeks (36). Interestingly, compared with loop diuretics, SGLT2 inhibitors tend to remove more fluid from the interstitial space than from the circulation, resulting in more electrolyte-free water clearance (37). In consideration of an increased volume load in HF, with inadequate arterial perfusion due to impaired cardiac function, the reduction of interstitial volume may be more favorable to patients with HF. In this sense, natriuresis and reduction in volume load are likely to explain (at least in part) the protective effects of SGLT2 inhibitors in HF (22).

Arterial stiffness is recognized as an important predictive factor of the morbidity and mortality associated with HF (38) because it can lead to an increased cardiac load and further deterioration of heart function. Some cardioprotective agents, such as RAAS inhibitors, have been demonstrated to ameliorate arterial stiffness, reduce cardiac load, and improve CV outcomes. Endothelial function also plays an important part in maintaining myocardial function, hemodynamics, the systemic circulation and pulmonary circulation. Endothelial dysfunction can lead to disturbed production and utilization of nitric oxide (NO), which increases vascular resistance further (39). Pulse wave velocity (PWV) is the major parameter of arterial stiffness (40). In an observational study, dapagliflozin significantly reduced the aortic PWV (41). Similarly, in subsequent randomized controlled trials (RCTs), PWV was also decreased by empagliflozin or canagliflozin compared to placebo (42, 43). These results suggested that SGLT2 inhibitors can alleviate arterial stiffness. In addition, the improvement of endothelial function was also observed by using SGLT2 inhibitors in these studies. These beneficial effects may be mediated by increasing NO production, reducing oxidative stress, or activating voltage-gated potassium ion (K^+) channels and protein kinase G (41, 44, 45). However, unlike the three well-recognized SGLT2 inhibitors (empagliflozin, dapagliflozin, canagliflozin), luseogliflozin did not show a similar beneficial effect on arterial stiffness in LUSCAR study (46). This may indicate that the improvement of arterial stiffness with SGLT2 inhibitors is due to specific drug rather than class effect.

Sodium–hydrogen exchangers (NHEs) transfer Na^+ into the cell in exchange for proton export. NHEs are involved in maintenance of Na^+ homeostasis and physiological pH (47). NHE1 is expressed in cardiomyocytes and its activation may lead to increased intracellular Na^+ and calcium ions (Ca^{2+}) (48). This effect is involved in abnormal myocardial hypertrophy and ischemia–reperfusion injury (49). NHE3 is expressed in proximal tubule and mediates tubular reuptake of Na^+. Upregulated expression of NHE1 and NHE3 can be observed in a failing heart (50). Despite absent expression of SGLT2 in the heart, SGLT2 inhibitors can reduce intracellular concentrations of Na^+ and Ca^{2+} and protect the heart from intracellular Ca^{2+} overload by inhibiting activation of NHE1 receptors (51). Similarly, SGLT2 inhibitors can also block NHE3, which further enhance natriuresis, restores whole-body sodium homeostasis, and improves cardiac function (52). Therefore, inhibition of NHE1 and NHE3 contributes to the cardioprotective effects of SGLT2 inhibitors.

Studies have demonstrated a slight increase in the hematocrit upon initial treatment with SGLT2 inhibitors (36). Changes in the hematocrit and hemoglobin concentration have been thought to be associated with decreased plasma volume and increased erythropoietin production. However, in the RED-HF trial, an increase in the hematocrit by erythropoietin injection showed no association with prognostic improvement, indicating that this effect could be attributed to reduction in plasma volume rather than increasing the circulating concentration of erythropoietin (53).

Cardio–Renal Protection of SGLT2 Inhibitors

Cardio–renal interactions are critical for the occurrence and progression of HF. Impaired heart function leads to inadequate perfusion and subsequent decreased renal function. Renal dysfunction furthers exacerbate HF deterioration, and is often accompanied by a worse prognosis (54).

In T2DM patients, the initial use of SGLT2 inhibitors may induce an acute, mild, dose-dependent reduction of the eGFR over the 1st weeks. Then, the eGFR returns toward baseline gradually and remains stable over a long time (55, 56). This early decline in the eGFR may be associated with vasoconstriction of afferent arterioles in glomeruli resulting from increased tubule–glomeruli feedback (57). In hyperglycemia condition, due to upregulation of SGLTs in the kidney, renal Na^+ reabsorption increases dramatically. In addition, activated NHE3 causes increased SGLT2 expression and leads to oxidative stress, acidosis, RAAS activation, SNS activation, and high reabsorption of Na^+. This alteration causes a remarkable decrease in Na^+ delivery to the macula densa, which may be misperceived as a decrease in the circulating volume, thereby leading to inappropriate afferent arteriolar vasoconstriction and subsequent increase of intraglomerular pressure and hyperfiltration. Increased intraglomerular pressure and hyperfiltration are involved in nephropathy progression.

Therapy using SGLT2 inhibitors can hinder the reabsorption of glucose and Na^+ in proximal convoluted tubule, and may increase Na^+ secretion by the S3 segment of proximal tubule. As a result, the Na^+ concentration transferred to the distal macula densa increases. Thus, by regulating tubule–glomerular feedback, SGLT2 inhibitors improve glomerular

afferent arteriolar adaption, alleviate abnormal intraglomerular hypertension and hyperfiltration, and achieve protection of renal function (58). This renal-protective mechanism is different from that elicited by RAAS inhibitors, which reduce intraglomerular pressure and alleviate hyperfiltration by efferent arteriolar vasodilatation. Therefore, combined use of these two agents may be more beneficial for the protection of cardio–renal function. This effect has been demonstrated in clinical and experimental studies (59, 60).

In a meta-analysis involving 48 randomized controlled trials, compared with placebo or other antidiabetic drugs, SGLT2 inhibitors exerted renal protection by decreasing albuminuria, slowing the progression from microalbuminuria to macroalbuminuria, and reducing the risk of end-stage renal disease (61).

Because of the close interaction between HF and renal dysfunction, use of SGLT2 inhibitors (especially in combination with RAAS inhibitors) can break this "vicious cycle" and provide cardio–renal protection.

Inflammatory and Metabolic Mechanisms of SGLT2 Inhibitors

Inflammation

Chronic systemic inflammation plays an important part in the development of cancer, chronic kidney diseases, CV diseases, DM, and non-alcoholic fatty liver disease (62). In several clinical trials, an increased level of C reactive protein (CRP) has been observed in patients with HF (acute and chronic) (63, 64), which suggests that inflammation is prevalent in HF. In fact, systemic inflammation has become a crucial pathophysiological feature of HF, and it is thought to be closely related to the occurrence, progression, and severity of HF (65). Inflammation contributes to HF development and regulation of heart function (66). These effects are achieved through a series of signaling pathways, and numerous proinflammatory cytokines, immune-response mediators, and inflammasomes are involved (67, 68).

To be specific, endothelial inflammation can cause a decline in NO production and increased generation of reactive oxygen species (ROS) (69). Subsequently, impaired metabolism of NO can cause an obvious reduction in activity of protein kinase G. This action leads to inhibition of phosphorylation in cytoskeletal proteins, and eventually results in increased myocardial stiffness and abnormal myocardial hypertrophy (69). In addition, endothelial inflammation can upregulate expression of intercellular adhesion molecules, thereby leading to collagen deposition, microvascular dysfunction, and myocardial fibrosis (69). These adverse effects result in deterioration of heart function (especially myocardial diastolic function).

Activation of the immune system is also involved in the inflammatory process in HF. As a toll-like receptor (TLR) expressed predominantly in the heart, TLR4 is closely associated with myocardial inflammation. Upon binding with its specific ligands, TLR4 can trigger a series of signaling responses, including expression of related proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1, and activation of inflammasomes (70). Although possibly beneficial in the short term to patients with HF, activation of the immune system is very detrimental in the long term due to the subsequent pathological cardiac remodeling and progressive deterioration of heart function. In addition, inflammatory responses affect the functions of other tissues and organs (e.g., skeletal muscles, lungs, kidneys) and lead to hypoxia, increased pulmonary arterial pressure, as well as retention of water and Na^+, which have adverse effects on patients with HF. In view of the important role of inflammation in HF, anti-inflammatory therapy has become desirable against HF. However, in clinical trials, most of the direct anti-inflammatory or anti-cytokine therapies have not demonstrated significant benefits for improving outcomes in patients with HF (71, 72).

Several experimental studies have shown that SGLT2 inhibitors decreased the concentrations of inflammatory factors such as CRP, TNF-α, and IL-6 (73). Subsequent clinical trials confirmed the anti-inflammatory effects of SGLT2 inhibitors (74). Recently, a meta-analysis involving 23 heterogeneously designed clinical trials showed that therapy using SGLT2 inhibitors could reduce the level of CRP, IL-6, TNF-α significantly, and increase the adiponectin level significantly (75). Those results suggested that SGLT2 inhibitors can suppress inflammation, but the exact mechanisms remain unclear. Experimental studies have indicated that SGLT2 inhibitors may exert their anti-inflammatory effect through different signaling pathways, such as the nuclear factor-kappa B (NF-κB) signaling pathway (76). Other signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway and TLR4 pathway, may also be involved in the anti-inflammatory effect of SGLT2 inhibitors. These effects need to be determined in further studies.

Oxidative Stress

Oxidative stress is another common important mechanism involved in the pathophysiology of CV disease. Oxidative stress is characterized by excessive generation of ROS and insufficient endogenous antioxidants (77). In health, a small quantity of ROS is produced by mitochondria, including xanthine oxidase, nitric oxide synthase, and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which maintain a relative balance with endogenous antioxidants. However, under pathological conditions, ROS generation increases significantly due to mitochondrial dysfunction, enhanced activity of NADPH oxidase, xanthine oxidase, and nitric oxide synthase, while the endogenous antioxidants are in serious deficiency (78). This shift causes an obvious accumulation of ROS. Then, an overabundance of ROS leads to DNA damage, protein peroxidation, cellular-microenvironment disorders, subsequent cellular dysfunction, and even cell death. In the failing heart, excessive ROS leads to an increased intracellular Ca^{2+} concentration by affecting the function of Na^+/Ca^{2+} exchangers and L-type Ca^{2+} channels, resulting in further myocardial electrophysiological abnormalities. Increased ROS also leads to enhanced activity of ryanodine receptor-2 and inhibited activity of Ca^{2+}-adenosine triphosphatase-2 in sarcoplasmic reticuli, thereby resulting in Ca^{2+} overload and reduced sensitivity to Ca^{2+}. In addition, accumulated ROS cause mitochondrial dysfunction, inefficiency of energy metabolism, and increased myocardial

fibrosis. These seriously detrimental effects lead eventually to cardiac remodeling, contractile and diastolic dysfunction, and progressive HF (79). Some experimental studies have suggested that anti-oxidative stress therapy can improve heart function and reduce myocardial fibrosis. Unfortunately, in a series of clinical trials, therapy targeting oxidative stress did not yield encouraging results in patients with HF (80, 81).

Experimental studies have shown that SGLT2 inhibitors have the potential for antioxidative stress because they can lower free-radical production and increase expression of antioxidants such as manganese superoxide dismutase and catalase (82). In addition, because of the close relationship between hyperglycemia and oxidative stress, the antihyperglycemic effects of SGLT2 inhibitors are thought to be an important mechanism of antioxidative stress. Moreover, SGLT2 inhibitors may also exert antioxidative effects by ameliorating RAAS activity, reducing expression of proinflammatory cytokines, and improving mitochondrial function (83, 84). In recent clinical studies, SGLT2 inhibitors reduced levels of 8-iso-prostaglandin-F2α and 8-hydroxy-2′-deoxyguanosine, which are considered to be biomarkers of oxidative stress (75). That result provides evidence to the antioxidative-stress effect of SGLT2 inhibitors in patients with T2DM. Similarly, this effect may also contribute (at least in part) to the CV benefits of SGLT2 inhibitors.

Insulin Resistance

Insulin resistance is a prevalent pathological mechanism hidden under a series of metabolic diseases represented by T2DM. The close relationship between insulin resistance and HF has been recognized (85). In a considerable proportion of patients with HF, insulin resistance coexists and further exacerbates the deterioration of HF. In turn, HF or MI may also increase the risk of insulin resistance or T2DM significantly. Insulin exerts its biological effects through two signaling pathways: phosphatidylinositol-3 kinase/protein kinase B (PI3K/Akt) and MAPK (86).

In health, when bound to insulin, the insulin receptor auto-phosphorylates. Then, the activated insulin receptor phosphorylates insulin receptor substrate (IRS) proteins. Phosphorylated IRS binds to PI3K and activates it, which further promotes activation of its downstream signaling molecules and, ultimately, leads to Akt phosphorylation. As a result, glucose transporter (GLUT)-4 translocates to the cell membrane and mediates glucose uptake into cardiomyocytes and skeletal muscle cells. When phosphorylated IRS binds to growth factor receptor bound protein-2, the MAPK signaling pathway is activated, which is associated with myocardial hypertrophy, fibrosis, and endothelial dysfunction. In insulin resistance, oxidative stress often coexists and leads to impaired function of Na^+/Ca^{2+} exchangers and L-type Ca^{2+} channels, resulting in impaired uptake of Ca^{2+}. Then, a Ca^{2+}-regulation disorder impairs cardiac contractile and diastolic function. In addition, resistance to cardiac insulin reduces the activity of the PI3K/Akt signaling pathway, which also contributes to the abnormality in Ca^{2+} uptake. In endothelial cells, insulin resistance decreases the production of NO and increases release of endothelin-1, contributing to myocardial hypertrophy, fibrosis, and apoptosis. Insulin resistance and oxidative stress also cause dysfunction in mitochondria and endoplasmic reticuli, which leads to myocardial apoptosis and metabolic insufficiency (87). These detrimental effects of insulin resistance promote the progression and deterioration of HF together.

SGLT2 inhibitors have various beneficial effects upon insulin resistance. Their antihyperglycemic effects are caused by increased urinary glucose excretion and are independent of the insulin level, so SGLT2 inhibitors can reduce excessive insulin secretion due to renal glucose absorption. This process can decrease lipogenesis and lipolysis, which leads to a reduction of fat mass and bodyweight. It can also increase glucose uptake and ketone production, which improves metabolic efficiency. In addition, SGLT2 inhibitors have been reported to reduce hyperglycemic toxicity and protect pancreatic function (88, 89). Moreover, several studies have shown that SGLT2 inhibitors can prevent mitochondrial dysfunction and improve cell viability (90). These effects of SGLT2 inhibitors contribute to an increase in insulin sensitivity and reduction of insulin resistance.

Energy Metabolism

Under physiological conditions, ∼60–90% of cardiac energy is derived from oxidation of fatty acids. Carbohydrate is responsible for the residual 10–40% of adenosine triphosphate (ATP) production. However, in patients with HF, oxidation of fatty acids may be reduced due to insulin resistance and oxidative stress. As a result, glucose utilization increases. Due to insufficient oxygen supply, the route of glucose metabolism shifts to anaerobic glycolysis accompanied by a small amount of ATP production. As a result, in a failing heart, impaired oxidation of fatty acids and a shift in glucose-metabolism pathways lead to a significant reduction in cardiac metabolic efficiency with inadequate ATP production (91, 92).

SGLT2 inhibitors have several beneficial effects on energy metabolism in patients with HF. As mentioned above, SGLT2 inhibitors can ameliorate oxidative stress and insulin resistance, thereby reducing their detrimental effects on energy metabolism. In diet-induced obese mice, empagliflozin showed to promote fat utilization by upregulating the expression of genes related to fatty acid oxidation, and also attenuate inflammation and insulin resistance (93). In another high-fat diet mice model, canagliflozin suppressed lipid synthesis and reduced body weight. In addition, recent studies suggested that SGLT2 inhibitors can not only regulate glucose metabolism and enhance glucose tolerance by improving insulin resistance (94), but also improve the function of pancreatic beta cells (95). These effects are beneficial to regulate glucose oxidation and increase energy metabolic efficiency. Besides, as an alternative energy source, the utilization capacity of ketones depends mainly on the levels of succinyl-CoA:3-ketoacid CoA transferase (SCOT) in different tissues. In this process, β-hydroxybutyrate is converted to acetoacetate, which is oxidized further to acetoacetyl-CoA by SCOT. Then, in mitochondria, acetoacetyl-CoA is converted to acetyl-CoA, which subsequently enters the tricarboxylic-acid cycle to produce ATP. In health, ketone metabolism contributes a very small proportion of energy production. However, in a failing heart, ketones may become an important and preferred

energy source due to the significant increase of SCOT when treated with SGLT2 inhibitors. Moreover, ATP is not consumed in ketone metabolism, so this process may increase metabolic efficiency and ATP production. In addition, SGLT2 inhibitors can improve mitochondrial function. These mechanisms may explain the metabolic benefits of SGLT2 inhibitors in patients with HF (96, 97).

Advanced Glycation End Products

Advanced glycation end products (AGEs) have been shown to be involved in diabetic nephropathy and HF. AGEs can increase arterial stiffness by interacting directly with the extracellular matrix (98). In addition, by interacting with the receptor for advanced glycation end products (RAGEs), AGEs can induce the inflammatory response, immunoreaction, oxidative stress, and fibrosis (99, 100). In a unicentric observational clinical study, Paradela-Dobarro et al. suggested that activation of the AGE-RAGE signaling pathway may contribute to HF development, and that soluble RAGEs may be critical predictors of adverse long-term outcome in HF (101). Some experimental studies have indicated reduced production of AGEs and suppression of the AGE-RAGE signaling pathway in mice after treatment with SGLT2 inhibitors (83, 102, 103). However, whether there is a similar effect on the AGE-RAGE signaling pathway using SGLT2 inhibitors in patients with HF must be studied further.

Obesity

Obesity is defined as a body mass index (BMI) >30 kg/m^2. Obesity has become an increasingly prevalent social problem in developed and developing countries. It is widely recognized that obesity (especially morbid obesity) can result in an increased risk of CV complications such as hypertension, arterial atherosclerosis, ventricular hypertrophy, and HF (104, 105). However, in several clinical studies, researchers were surprised to observe that in patients with CV diseases, those who were obese tended to have better clinical outcomes and survival compared with those in a leaner group. This is known as the "obesity paradox" (106). Although the exact mechanisms of the obesity paradox are incompletely understood, most investigators have recognized that it may be inappropriate to use BMI exclusively as the evaluation factor for obesity. Subsequent studies have suggested that adjusted cardiorespiratory fitness may be a more favorable indicator to evaluate the CV risk in an obese population. In general, fat distribution may be more closely associated with HF, and abdominal adiposity has been thought to be an important predictive factor of HF (107).

Several mechanisms may be involved in the close relationship between obesity and HF. First, compared with a non-obese population, obese people have a higher tendency of ventricular hypertrophy, which results in impaired adaptive regulation of cardiac output (even if the ejection fraction appears to be in the normal range). In addition, accumulation of epicardial fat in obese patients may lead to a further decline in left-ventricular compliance and exacerbate diastolic dysfunction. Similarly, adaption of their respiratory function also decreases. Second, higher levels of proinflammatory factors, including CRP, IL-6 and TNF-α, have been observed in obese patients compared with their non-obese counterparts, suggesting that obesity tends to induce inflammation. Obesity-related inflammation can cause oxidative stress, microvascular injury, and myocardial fibrosis. In addition, obese patients have a greater plasma volume load, which may increase the excess burden to the heart and kidneys, especially if pre-existing cardio–renal decline is present. Third, the RAAS activation and inflammation induced by obesity may lead to reduced vascular compliance and volume redistribution, which may play an important part in CV congestion and cardiac dysfunction.

Several oral antidiabetics agents and insulin may lead to weight gain, which is unfavorable for patients with HF. In contrast, SGLT2 inhibitors show a weight-loss effect owing to the increased excretion of glucose (108). This effect is observed from the 1st weeks of therapy, and the bodyweight reduces further in subsequent months and is then maintained. The reduction of bodyweight results primarily from fat loss, with a significant decrease in visceral and subcutaneous adipose tissue (109). The leptin–aldosterone–neprilysin axis is thought to have a distinct role in HF patients suffering from obesity (110). SGLT2 inhibitors have been observed to alleviate the detrimental effects of leptin. As mentioned above, SGLT2 inhibitors suppress RASS activity, ameliorate systemic inflammation, and improve insulin sensitivity. In addition, these agents are also thought to diminish fat accumulation and adipokine generation. These mechanisms help to prevent the structure and function of the heart from the harmful effects induced by obesity.

Epicardial adipose tissue (EAT) is a subtype of white adipose tissue. EAT is distributed around the heart. During recent decades, the relationship between EAT and CV diseases has attracted considerable attention. Recently, EAT has been recognized as a special type of fat with peculiar anatomic, biomolecular, and genetic features. In health, EAT appears as an "organ" for storing free fatty acids. However, overabundant free fatty acids may lead to excessive production of ROS and alteration of the intracellular Ca^{2+} concentration, which are components of diastolic dysfunction. EAT can also induce insulin resistance and inflammatory reactions, which are closely associated with a series of CV diseases (especially HF). Moreover, EAT is a crucial secretory organ. It can release a range of adipokines (IL-6, IL-1β, TNF-α) that can cause myocardial lipo-toxicity and impair heart function further. EAT can also secrete adiponectin (a cardioprotective adipocytokine), which achieves beneficial effects by reducing the inflammatory response and apoptotic activity. In addition, EAT has been shown to secrete various microRNAs, which are necessary in the formation and development of the heart. Numerous microRNAs demonstrate different effects on regulating the metabolism of glucose and lipids, myocardial hypertrophy, and cardiac fibrosis. Due to its metabolic and secretory activity, EAT is thought to have a critical role in the pathophysiological process of several CV diseases, including coronary heart disease, cardiomyopathy, and HF (111).

Experimental studies have indicated that use of SGLT2 inhibitors is associated with a significant increase in the adiponectin concentration, inhibited activities of inflammatory cells, and suppressed expression of proinflammatory cytokines. In a small clinical study, treatment with SGLT2 inhibitors

reduced the EAT volume and decreased the TNF-α level. Several other studies have also demonstrated that SGLT2 inhibitors reduce the accumulation and alter the distribution of epicardial fat (112–114). Those data suggest that SGLT2 inhibitors exert their benefits (at least in part) in patients with HF by regulation and distribution of EAT.

Hyperuricemia

Hyperuricemia is prevalent in patients with HF or T2DM. The serum level of uric acid is an important predictor of a poor prognosis in HF. An increased level of uric acid is thought to be associated with RAAS activation, oxidative stress, systemic inflammation, and endothelial dysfunction. On the one hand, the products of purine degradation increase significantly due to increased glycolysis and insufficient production of ATP during hypoxemia. On the other hand, reduced cardiac output and inadequate renal perfusion cause RAAS activation, which leads to increased reabsorption and decreased excretion of uric acid. Conventional diuretic therapy often causes an increased level of uric acid, which is unfavorable for patients with HF (115, 116). Differently, SGLT2 inhibitors have been shown to reduce the serum level of uric acid and contribute to reduce the CV risk (117, 118). As mentioned above, SGLT2 inhibitors can improve energy metabolism and increase ATP generation, which are beneficial to reduce purine metabolites. Furthermore, because of increased excretion of glucose in urine, the functions of GLUT-9 and uric acid transporter-1 (expressed in renal tubular epithelial cells) are inhibited. Thus, suppressed reabsorption and enhanced excretion of uric acid finally result in the reduction of the serum level of uric acid.

In brief, SGLT2 inhibitors exert CV-protective effects through various complex mechanisms, which merit further study.

SGLT2 Inhibitors in HFpEF

As a special phenotype of HF, HFpEF has received increasing attention due to its rapidly rising incidence and numerous complications. Compared with HFrEF, HFpEF is a more complicated clinical syndrome with multiple etiologies and unclear pathogenesis. HFpEF accounts for more than half of hospitalized patients with HF. First, the major risk factors of HFpEF are more diverse and complicated. In addition to coronary heart disease, hypertension, T2DM, and obesity, other factors are involved in the pathological process of HFpEF: anemia, chronic obstructive pulmonary disease, inflammation, and being female. Second, in patients with HFrEF, the manifestation of cardiac remodeling is enlarged ventricular volume and reduced ejection fraction. In contrast, cardiac remodeling is usually characterized by myocardial hypertrophy, decreased cardiac compliance, and subsequent diastolic dysfunction. Third, in HFrEF patients, inflammation may be a consequence of HF only, whereas inflammation is thought to be a crucial basis of HFpEF and closely associated with myocardial stiffness, fibrosis, and impaired diastolic function mediated by multiple cellular signaling pathways (119). In addition, RAAS activation and EAT are also involved in the occurrence and development of HFpEF. Moreover, unlike the obvious advances in drug and device therapies in HFrEF, treatments for HFpEF are more difficult and challenging. Current treatments for HFpEF are limited to alleviating symptoms and controlling the primary disease. To date, no convincing treatment, not even RAAS or SNS inhibitors, has been shown to reduce morbidity or mortality significantly in patients with HFpEF (120).

In view of the broad biological effects, including lowering of serum levels of glucose and blood pressure, reducing plasma volume, suppressing inflammation and oxidative stress, improving insulin resistance and increasing energy metabolism, SGLT2 inhibitors can maintain cardiac hemodynamic and electrophysiological stability, alleviate abnormal hypertrophy and remodeling, and improve myocardial systolic and diastolic function which are, theoretically, beneficial to patients with HFpEF.

In a female diabetic rodent model, empagliflozin showed to improve diastolic function and reduce fibrosis (121). Similarly, in a non-diabetic rodent model of HFpEF, empagliflozin improved cardiac diastolic function and reduced wall stress. The potential pathophysiological mechanisms included reduced preload and altered hemodynamics (122). In a multi-hit mouse model of HFpEF, dapagliflozin also improved cardiac function and tissue fibrosis (123). Tofogliflozin, another SGLT2 inhibitor, ameliorated cardiac hypertrophy and fibrosis in dahl salt-sensitive and salt-resistant rats fed a high-fat diet (124). These experimental studies suggest that SGLT2 inhibitors may be beneficial for patients with HFpEF. HFpEF is a more prevalent phenotype of HF in T2DM patients. In large-scale clinical studies such as EMPA-REG OUTCOME, the consistent and significant benefits of different SGLT2 inhibitors for T2DM patients carrying a high CV risk suggest the potential therapeutic value of these agents for HFpEF. However, in a single-center, retrospective study, SGLT2 inhibitors did not appear to improve left ventricular reverse remodeling in patients with T2DM and HFpEF (125). But, considering the relatively short observation time, this study is not enough to suspect the effect of SGLT2 inhibitors for HFpEF. In the MUSCAT-HF study, although BNP concentration decreased after treatment of luseogliflozin or voglibose, no significant difference in the degree of reduction in BNP was observed between these two groups (126). But, in this study, the most of patients enrolled were at low risk, with a low degree of HF and a low baseline level of BNP. In addition, about 40% patients did not have pre-existing atherosclerotic CV disease. Since SGLT2 inhibitors may be more effective in patients at high CV risk, these biases may lead to an underestimated effect of SGLT2 inhibitors for HFpEF (126). Until now, whether patients with HFpEF will benefit from SGLT2 inhibitors remains controversial. The ongoing EMPEROR-Preserved trial will assess the effects of empagliflozin on morbidity and mortality in patients with HFpEF. In addition, other several studies such as DELIVER and PRESERVED-HF are also underway to evaluate the benefit of SGLT2 inhibitors in patients with HFpEF. If the expected result is achieved, SGLT2 inhibitors may prove to be efficacious treatment for patients with HFpEF (127).

SGLT2 Inhibitors in Acute HF

Acute HF is characterized by the sudden occurrence or rapidly worsening symptoms and/or signs of HF. As an

extremely dangerous and potentially life-threatening clinical condition, acute HF requires urgent diagnosis and treatment. Acute decompensated HF, which is frequently induced by acute myocardial ischemia, severe infection, severe arrhythmia, uncontrolled hypertension, or severe disorders in the internal environment, tends to be associated with high mortality and expensive healthcare costs during and after hospitalization (128). Diuretics and vasodilators can relieve the clinical symptoms of acute HF, but pharmacological agents known to improve the prognosis significantly in patients with acute HF are lacking.

Given that they increase natriuresis, reduce cardiac load, improve renal function, and have excellent performance in treatment of chronic HF, SGLT2 inhibitors are hypothesized to be beneficial in acute HF as well. A *post-hoc* analysis of the EMPA-REG OUTCOME trial showed that empagliflozin may reduce risk of the post-acute HF rehospitalization and mortality, which provide a cogent rationale for SGLT2 inhibitors in patients hospitalized with decompensated HF (129). In a single-center prospective study of acute HF, dapagliflozin was associated with decreased all-cause mortality and readmission despite the small sample size (130). In the EMPA-RESPNSE-AHF trial (the first study to evaluate the effects and safety of SGLT2 inhibitors in acute decompensated HF), despite the small sample size, these agents reduced the risk of rehospitalization for HF or death within 60 days, *vs.* placebo (131). In a recent retrospective analysis of a series of patients with acute decompensated HF, the researchers found that SGLT2 inhibitors improved weight loss, urine output, and diuretic efficiency without adverse change in renal function, blood pressure or electrolytes when used in combination with loop diuretics (132). This may partly explain the benefit of SGLT2 inhibitors in patients with acute HF, although the exact mechanism of action of SGLT2 inhibitors in acute HF is not known. However, due to the limitations of sample size or study method, it is not completely determined whether SGLT2 inhibitors can be safely and effectively applied in patients with acute HF. Large-scale randomized clinical trials are required to further evaluate the possible beneficial role of SGLT2 inhibitors in patients with acute HF (133).

Differences Between SGLT2 Inhibitors

Since phlorizin was first isolated from the root of an apple tree in 1,835, more than a dozen SGLT inhibitors have been developed (134). Due to the significant reduction of CV risk with empagliflozin, dapagliflozin, and canagliflozin in patients with T2DM, these benefits were once considered as class effects of SGLT2 inhibitors (135). However, recent studies showed inconsistent results, suggesting that SGLT2 inhibitors may have heterogeneity (136).

The differences in selectivity of SGLT2 inhibitors for SGLT2 vs. SGLT1 are summarized in **Table 1** (134, 137, 138), which may be the main factor leading to their different pharmacological profiles and clinical effects. For example, several SGLT2 inhibitors such as empagliflozin, dapagliflozin, canagliflozin, tofogliflozin showed to alleviate arterial stiffness (41–43, 139), while luseogliflozin and ipragliflozin did not demostrate the similar beneficial effect (46, 140). In previous studies, empagliflozin, dapagliflozin, canagliflozin, luseogliflozin, and ipragliflozin reduced the biomarkers of inflammation and oxidative stress (73–75, 112, 114), however, whether ertugliflozin and sotagliflozin had the similar effect has not been reported.

TABLE 1 | Differences between SGLT inhibitors in selectivity for SGLT2 vs. SGLT1.

SGLT inhibitor	IC_{50} for SGLT1 (nM)	IC_{50} for SGLT2 (nM)	Selectivity (SGLT2 vs. SGLT1)
Empagliflozin	8,300	3.1	∼2,680
Dapagliflozin	1,400	1.2	∼1,200
Canagliflozin	710	2.7	∼260
Ertugliflozin	1,960	0.87	∼2,250
Sotagliflozin	36	1.8	∼20
Tofogliflozin	12,000	6.4	∼1,875
Luseogliflozin	3,990	2.3	∼1,730
Ipragliflozin	3,000	5.3	∼560
Phlorizin	290	21	∼10

IC_{50} inhibitor concentration at half-maximal response.

TABLE 2 | Cardiorenal outcomes of SGLT2 inhibitors.

SGLT2 inhibitor (clinical trial)	MACE HR (95% CI)	CV death HR (95% CI)	HHF HR (95% CI)	Composite renal outcomes HR (95% CI)
Empagliflozin (EMPA-REG OUTCOME)	0.86** (0.74–0.99)	0.62** (0.49–0.77)	0.65** (0.50–0.85)	0.54** (0.40–0.75)
Dapagliflozin (DECLARE-TIMI 58)	0.93 (0.84–1.03)	0.98 (0.82–1.17)	0.73** (0.61–0.88)	0.53** (0.43–0.66)
Canagliflozin (CANNAS program)	0.86** (0.75–0.97)	0.87 (0.72–1.06)	0.67** (0.52–0.87)	0.60** (0.47–0.77)
Ertugliflozin (VERTIS CV)	0.97 (0.85–1.11)	0.92 (0.77–1.11)	0.70** (0.54–0.90)	0.81 (0.63–1.04)

*HR, hazard ratio; CI, confidence interval; ** statistical significance.*

In four large-scale clinical trials, although SGLT2 inhibitors showed significant benefits in HF hospitalization (HHF), other outcomes including MACE were inconsistent. The results are summarized in **Table 2** (17, 19, 21, 141). In newly published SCORED trial, sotagliflozin showed to reduce the risk of HHF and urgent visits for HF compared to placebo, however there was no significant difference in CV death between the two groups (142).

Differences in chemical structure and pharmacological characteristics may partly explain the inconsistent results of clinical trials. However, the detailed heterogeneity among SGLT2 inhibitors needs to be further studied.

CONCLUSIONS

Due to a series of favorable hemodynamic and metabolic effects, SGLT2 inhibitors have been transformed gradually into an important class of anti-HF drugs instead of just a novel group of antidiabetic agents. Increasingly, SGLT2 inhibitors are being recommended to improve clinical outcomes in patients with HFrEF or T2DM carrying a high CV risk. Researchers from Harvard Medical School have suggested that SGLT2 inhibitors should also be used as a new cornerstone drug for HFrEF, in addition to RAAS inhibitors, β-receptor blockers, and aldosterone antagonists. On this basis, in consideration of their multiple beneficial mechanisms and impressive performance in HFrEF, SGLT2 inhibitors have also attracted increasing attention in the field of HFpEF and acute HF. While some small-scale studies have shown encouraging results of SGLT2 inhibitors in patients with HFpEF or acute HF, others have shown inconsistent results. Although controversial, SGLT2 inhibitors are considered as a promising treatment for patients with HFpEF or acute HF. Ongoing or upcoming clinical studies will further evaluate their safety and efficacy in patients with HFpEF or acute HF, and the results will be awaited eagerly.

AUTHOR CONTRIBUTIONS

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Type 2 Diabetes and Myocardial Infarction: Recent Clinical Evidence and Perspective

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Type 2 diabetes mellitus (T2DM) and its complications are seriously affecting public health worldwide. Myocardial infarction (MI) is the primary cause of death in patients with T2DM. T2DM patients without a history of coronary artery disease (CAD) have the same risk of major coronary events as those with CAD; T2DM patients with a history of MI have >40% risk of recurrence of MI. Thus, CAD in patients with T2DM needs to be treated actively to reduce the risk of MI. The cardiology community focused on the role of T2DM in the development of CAD and on the related issues of T2DM and MI with respect to comorbidities, prognosis, drug therapy, and heredity. In this mini review, the latest progress of clinical evidence-based research between T2DM and MI in recent years was reviewed, and the possible research directions in this field were considered and prospected.

Keywords: type 2 diabetes, myocardial infarction, evidence-based medicine, drug, sodium-glucose cotransporter-2 inhibitor

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is one of the leading chronic non-communicable disease, and its prevalence has significantly increased globally. In 2017, the prevalence of adult T2DM accounted for 8.8% of the world population, and this proportion is expected to increase to 9.9% by 2045 (1, 2). With the increasing number of cases, T2DM and its complications are seriously affecting the quality of human life and have become a serious global public health problem. A pooled analysis of 22 prospective cohort studies encompasses more than one million subjects in Asia found that Asian patients with T2DM are at a higher risk of death than patients from the Western countries, with an 89% increase in mortality compared with that of those without T2DM (3). China has become a "hardest hit area" by T2DM; among Chinese adults, the estimated overall prevalence of T2DM is 10.9%, and the prevalence of pre-T2DM is 35.7% (4). Myocardial infarction (MI) is the primary cause of death in T2DM patients, and the risk of major coronary events in T2DM patients without a history of coronary artery disease (CAD) is equal to that in patients with CAD, with the >20% risk of a first MI within 10 years of developing T2DM, which is equal to the risk of a second MI within 10 years in non-T2DM patients with a history of MI, while the risk of recurrence of MI in the future in T2DM patients with MI history exceeds 40% (5). In a scientific statement published in *Circulation* on April 13, 2020 (6), the American Heart Association (AHA) noted that compared with CAD in patients without T2DM, CAD in patients with T2DM needs to be treated more aggressively to reduce the risk of MI. Although cardiologists have been treating patients with CAD and associated

T2DM for a long time, T2DM has traditionally been considered a comorbidity that only affects the development and progression of the CAD. In the past decade, many factors have changed, forcing the cardiology community to reconsider the important role of T2DM in the development and progression of CAD. In addition to being associated with increased cardiovascular (CV) risk, T2DM may influence the choice of multiple treatments for CAD. Thus, glycemic control is recommended as part of the comprehensive risk factor management for patients with CAD; there has been growing evidence that the mechanisms of glycemic control have a significant impact on CV outcomes (7).

With the publication of the results of several large clinical trials on oral hypoglycemic drugs with CV benefits in recent years, people are more concerned about the comorbidities, prognosis, drug treatment, genetics, and other issues related to T2DM and MI.

In this review article, by mainly retrieving PubMed, MEDLINE, EMBASE, and Web of Science, we identified and critically analyzed nearly 5 years (from January 1, 2016, until December 30, 2020) of published clinical studies [randomized controlled trials (RCTs) and cohort studies] focusing on T2DM and MI. The search terms were "diabetes, type 2 diabetes mellitus, myocardial infarction, MI, cardiovascular disease, cardiovascular safety, cardiovascular events, cardiovascular risk, cardiovascular outcomes." The retrieval formula was appropriately adapted to different databases.

In this paper, the latest progress in evidence-based clinical research on T2DM and MI in recent years has been reviewed, and the possible research directions in this field in the future have been considered and prospected.

COMORBID FEATURES OF TYPE 2 DIABETES MELLITUS AND MYOCARDIAL INFARCTION

In 2019, the *Guidelines on Diabetes, Pre-Diabetes and Cardiovascular Diseases* jointly issued by the European Society of Cardiology (ESC) and European Association for the Study of Diabetes (EASD) recommends that all patients with cardiovascular disease should be screened for T2DM and that patients with cardiovascular disease complicated by T2DM should undergo comprehensive risk factor management, including control of blood pressure, serum glucose, and lipid levels; management of antiplatelet therapy regimens; and lifestyle interventions (8). The trends of mortality and morbidity in MI patients after 1 year suggested that long-term trends in survival and CV outcomes have improved considerably in patients with MI; however, their risk of mortality and morbidity in MI remains higher than that of the general population, especially when additional risk factors such as T2DM, hypertension, or advanced age are present (9, 10).

The prevalence of unrecognized abnormal glucose tolerance (AGT) and the incidence of recurrent CV events in patients with MI have not been systematically assessed. A meta-analysis of the prevalence of AGT in MI patients without a history of DM as well as the risk of recurrent major adverse cardiac events (MACEs) and mortality in MI patients was conducted. In the 19 clinical studies included ($n = 541{,}509$ with a median follow-up of 3.1 years), the prevalence of newly discovered AGT in patients with MI was 48.4%. Patients with prediabetes had a higher risk of death and MACE than did patients with normal glucose tolerance (NGT). Newly diagnosed T2DM cases showed a higher risk of death and MACE occurrence than NGT cases (11). Clinical research on the prevalence and prognosis of MI in asymptomatic T2DM patients has also been conducted to determine the prevalence of unrecognized MI in asymptomatic T2DM patients using delayed-enhancement MRI (DE-MRI), and the results of up to 5 years of follow-up in 460 T2DM patients showed that the incidence of death or MI was significantly higher in unidentified T2DM patients and that unidentified MI was prevalent in asymptomatic T2DM patients without a history of heart disease (12).

Researchers are increasingly concerned about the relationship between prediabetes and the risk of CV disease (CVD) and mortality (13–15). A meta-analysis of the association between prediabetes and the risk of CVD and mortality including 129 studies with a total of 10,069,955 patients showed that prediabetes increased risk of all-cause mortality and CVD and that prevention of prediabetes was important for patients with CVD (16).

A systematic review and meta-analysis of the correlation between T2DM and long-term (≥1 year) post-MI mortality was conducted, including 10 RCTs and 56 cohort studies (714,780 patients), with a total of 202,411 deaths over a median follow-up time of 2.0 (range, 1–20) years; it was found that the high long-term mortality of patients with T2DM was significant over time, independent of the phenotype of MI and modern treatments, and the long-term mortality was approximately 50% higher in patients with T2DM than in those without T2DM (17). Patients with T2DM had worse short- and long-term prognoses than those without T2DM, and undiagnosed T2DM was significantly correlated with higher mortality, especially in patients still with undiagnosed T2DM at the time of hospital admission (5).

Intracoronary drug-eluting stent (DES) percutaneous coronary intervention (PCI) is currently one of the standard treatments for patients with acute coronary syndrome (ACS), including those with MI, and T2DM also has a negative impact on the treatment and outcome of patients after PCI. Early-stage arterial healing after DES-PCI makes short-term dual antiplatelet therapy (DAPT) possible. A study (18) used coronary angiography [coronary artery stenosis (CAS)] data to compare the intravascular status of T2DM patients ($n = 149$) and non-T2DM patients ($n = 188$) in the early post-DES-PCI period, and it found that 3–5 months after DES implantation, DM patients showed more uncovered stent wires than non-DM patients, suggesting that the recent ultrashort DAPT strategy may not be applicable to patients with concomitant T2DM. Meanwhile, new-onset T2DM [new onset of DM (NODM)] after DES-PCI is receiving increasing attention. A study (19) used a retrospective cohort design to report the incidence, predictors, and long-term clinical outcomes of NODM after DES-PCI in patients with MI. The study reviewed 6,048 patients after PCI, grouped according to the presence or absence of T2DM before PCI, and found

that 436 (11.8%) of 3,683 patients with ACS who did not have a diagnosis of T2DM before PCI had developed NODM over the 3.4 ± 1.9 years of follow-up, with independent predictors including high-dose statin therapy, high body mass index (BMI), and high fasting plasma glucose (FPG) and triglyceride levels. The cumulative MACE rate over 8 years of follow-up were significantly lower in the group with NODM after PCI (19.5%) than in the group with preoperatively diagnosed T2DM (25%, $P = 0.003$) and comparable with the group without T2DM (20.5%, $P = 0.467$). A retrospective cohort study conducted in Taiwan that included a larger number of patients (20) (30,665 patients diagnosed with ACS undergoing PCI) found a significant 27% increased risk of NODM in patients using statins than in those not using statins. The benefits of statins in preventing morbidity and mortality in patients with ACS have been validated in several clinical trials, and the clinical decision to recommend statin therapy for patients with pre-existing CVD should not be altered.

Knowledge about the mechanisms responsible for diabetes accelerating MI has increased enormously in recent years. The mechanisms by which hyperglycemia and insulin resistance increased mortality after MI were increasingly understood (21). Most diabetic patients are complicated with insulin resistance, hyperinsulinemia, and vascular calcification, which not only promote the occurrence of atherosclerosis but also accelerate the progression of stable plaques to unstable plaques or plaque rupture leading to thrombosis, thus leading to the occurrence of coronary adverse events (22). Contributing factors [including diabetes-induced overexpression of reactive oxygen species (ROS); secretion of inflammatory cytokines; increased aldose reductase (AKR1B1) substrate conversion; and activation of protein kinase C β, δ, and θ] accelerate the occurrence of MI (21).

Not only is T2DM strongly associated with MI, but its complications are also closely related with MI. A systematic review and meta-analysis of cohort studies on association between diabetic retinopathy (DR) and CVD included a total of 13 studies representing 17,611 patients, which suggested that DR is remarkably related with increased risk of CVD and CVD-associated mortality in diabetes (23). In addition, a hospital-based cross-sectional study in China included 949 patients (700 males and 249 females) with T2DM both non-proliferative DR (NPDR) and proliferative DR (PDR) independently associated with increased cardio-ankle vascular index (CAVI) (24). Other studies have also shown that central atherosclerosis is associated with the presence and severity of DR in patients with T2DM (25).

It is generally acknowledged that T2DM is an independent risk factor of acute kidney injury (AKI). Meanwhile, AKI predicts poor prognosis in patients with MI. The data from a multicenter factorial RCT included 10,251 participants who showed an incremental graded risk for CVD outcomes and all-cause mortality with the development of chronic kidney disease (CKD) and/or CVD in individuals with T2DM (26). Experimental evidence suggests that treatment with the sodium-glucose cotransporter-2 (SGLT2) inhibitor protects the diabetic kidney from MI-induced AKI (27).

SHIFT FROM "CARDIOVASCULAR SAFETY" TO "CARDIORENAL BENEFIT" OF ORAL HYPOGLYCEMIC AGENTS

The discovery and clinical application of insulin as well as the subsequent introduction of various oral hypoglycemic agents (OHAs) have greatly prolonged the survival time of T2DM patients and transformed T2DM into a major chronic non-communicable disease. The effect of CV-related complications on the prognosis of patients with T2DM has attracted more attention and finally became the primary problem to be solved in improving the clinical prognosis of patients with T2DM. Few large-scale CV outcome trials (CVOTs) have been conducted to verify the CV safety of traditional OHAs. Since 2007, when rosiglitazone was found to significantly increase the risk of MI in T2DM patients (28), the CV safety of OHAs has received more attention. In 2008, the US Food and Drug Administration (FDA) developed a guidance document (29) to clarify the CV safety of innovative OHAs for the treatment of T2DM, which requires clinical evaluation of CV safety for all agents used for the treatment of T2DM prior to marketing. Therefore, CVOTs have been conducted for all OHAs marketed after 2008 to evaluate their CV safety.

SGLT2 inhibitor is an innovative oral hypoglycemic drug marketed in recent years, and its main mechanism of action is to lower blood glucose by inhibiting the reabsorption of glucose by the renal proximal convoluted tubules and promoting urinary glucose excretion. In 2015, the results of the EMPA-REG OUTCOME trial (30, 31) were published, making empagliflozin the first oral hypoglycemic drug with definite CV benefits. In T2DM patients with comorbid CVDs, empagliflozin significantly reduced the risk of three-point MACE (3P-MACE) by 14%, and the risk of CV death and hospitalization for heart failure (HF) by 38 and 35%, respectively. Over the past 5 years, results from several large clinical trials on SGLT2 inhibitors have been published (30, 32–38) (see **Table 1**), and the expectation for OHAs has gradually changed from "cardiovascular safety" to "cardiovascular benefits." Among the renal outcome studies, EMPA-REG OUTCOME was the first to report a 39% reduction in the risk of developing a renal composite endpoint with empagliflozin, suggesting that empagliflozin may delay the progression of renal diseases (39). A clinical study evaluating the effect of canagliflozin on renal events of patients with DM complicated by renal diseases with renal outcomes as a primary endpoint also demonstrated that canagliflozin reduced the risk of composite endpoints by up to 30% (34). The therapeutic focus of T2DM has also changed, from an exclusive focus on glucose-lowering parameters, to comprehensive management, and then to the current therapeutic focus on cardiac benefits and renal outcomes (see **Figure 1**).

SGLT2 inhibitors exhibited superiority; thus, the cardiologist approve that SGLT2 inhibitors should be used in great property, at least in T2DM patients with high CV risk (40). What is more, major international guidelines all highly recommend the use (or combined use) of SGLT2 inhibitors in patients with T2DM with comorbid CVDs (or high risk

TABLE 1 | Characteristics of the RCT studies about SGLT2 inhibitors.

Clinical trials	EMPA-REG outcome (30)	CANVAS (32)	DECLARE–TIMI 58 (33)	Credence (34)	DAPA-HF (35)	Emperor-reduced (36)	Vertis (37)	DAPA-CKD (38)
ClinicalTrials.gov number	NCT01131676	NCT01032629/ NCT01989754	NCT01730534	NCT02065791	NCT03036124	NCT03057977	NCT01986881	NCT03036150
Year	2015	2017	2019	2019	2019	2020	2020	2020
Participants (*n*)	7,020	10,142	17,160	4,401	4,744	3,730	8,246	4,304
Age, years (mean)	63.1	63	63.9	63	66	67	64	62
Men (%)	71.2	64.9	63.1	66	77	76	70	67
SGLT2 inhibitor agent	Empagliflozin	Canagliflozin	Dapagliflozin	Canagliflozin	Dapagliflozin	Empagliflozin	Ertugliflozin	Dapagliflozin
Dose ($mg \cdot day^{-1}$)	10, 25	100, 300	10	100	10	10	5, 15	10
Eligibility criteria	≥18 years of age; clinical diagnosis of type 2 diabetes; established cardiovascular disease, glycated hemoglobin level of 7∼10%, eGFR ≥ 30 $ml \cdot min^{-1} \cdot (1.73\ m^2)^{-1}$	≥30 years of age with a history of symptomatic atherosclerotic cardiovascular disease or ≥50 years of age with two or more of the following risk factors for cardiovascular disease: clinical diagnosis of type 2 diabetes; glycated hemoglobin level 7.0∼10.5%, eGFR ≥ 30 $ml \cdot min^{-1} \cdot (1.73\ m^2)^{-1}$	≥40 years of age; clinical diagnosis of type 2 diabetes; multiple risk factors for atherosclerotic cardiovascular disease or had established atherosclerotic cardiovascular disease glycated hemoglobin level 6.5∼12%; creatinine clearance ≥ 60 $ml \cdot min^{-1}$	≥30 years of age; clinical diagnosis of type 2 diabetes; glycated hemoglobin level of 6.5–12.0%; eGFR 30∼90 $ml \cdot min^{-1} \cdot (1.73\ m^2)^{-1}$; urine albumin: creatinine ratio 300∼5,000 $mg \cdot g^{-1}$	≥18 years of age; New York Heart Association (NYHA) class II, III, or IV symptoms, ejection fraction ≤ 40%; NT-proBNP ≥ 600 $pg \cdot ml^{-1}$ (or ≥ 400 $pg \cdot ml^{-1}$ if they had been hospitalized for heart failure within the previous 12 months, or NT-proBNP ≥ 900 $pg \cdot ml^{-1}$ if they are with atrial fibrillation or atrial flutter on baseline electrocardiography regardless of their history of hospitalization for heart failure)	≥18 years of age; NYHA class II, III, or IV symptoms; ejection fraction ≤ 40%	≥40 years of age; clinical diagnosis of type 2 diabetes; established atherosclerotic cardiovascular disease involving the coronary, cerebrovascular, or peripheral arterial systems; glycated hemoglobin level of 7∼10.5%	≥18 years of age, eGFR 25∼75 $ml \cdot min^{-1} \cdot (1.73\ m^2)^{-1}$, urine albumin: creatinine ratio 200∼5,000 $mg \cdot g^{-1}$
Follow-up, years (median)	3.1	2.4	4.2	2.6	1.5	1.3	3	2.4
Percentage of patients with confirmed cardiovascular disease at baseline (%)	99.4	71.2	40.5	50.4	100	100	76.1	37.4
Percentage of patients with heart failure at baseline (%)	9.9	14	9.9	15	100	100	24	11
Proportion of patients with eGFR ≥ 30 $ml \cdot min^{-1} \cdot (1.73\ m^2)^{-1}$ at baseline (%)	74.2	76.7	85.4	100	100	–	100	85.5

Albumin-to-creatinine ratio was calculated with albumin measured in milligrams and creatinine measured in grams.

SGLT2, sodium-glucose cotransporter-2; eGFR, estimated glomerular filtration rate; NT-proBNP, N-terminal pro-B-type natriuretic peptide; RCT, randomized controlled trial.

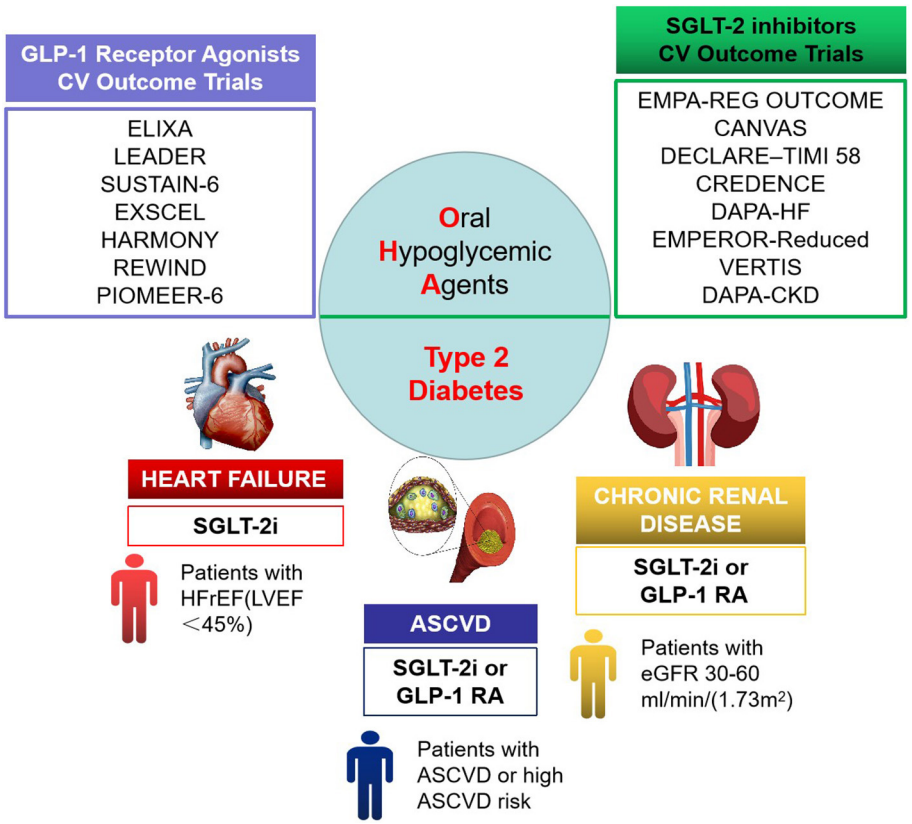

FIGURE 1 | Cardiorenal benefit of oral hypoglycemic agents in therapeutic focus of type 2 diabetes mellitus (T2DM).

of CVDs) and/or CKD, with the ESC/EASD guidelines (7) recommending them over metformin in newly diagnosed patients. The overall degree of recommendation by the American Association of Clinical Endocrinology/American College of Endocrinology (AACE/ACE) guidelines (41) is comparable with that for metformin.

Dipeptidyl peptidase-4 (DPP-4) inhibitors also occupy an increasing place in the management of T2DM. DPP-4 inhibitors may enhance homing of endothelial progenitor cells and thereby exert vascular protection (42). Available evidence suggests that DPP-4 inhibitors have a weak CV protective effect (43). However, in clinical application, it should be selected according to the actual situation. In patients with T2DM with advanced CVD or HF associated with renal function deterioration, DPP-4 inhibitors appear to be safe to use from a cardiological point of view, and SGLT2 inhibitors are contraindication (44). Of note, a study based on a large diabetic cohort of 113,051 T2DM patients showed that DPP-4 inhibitors as a second- or third-line add-on treatment provided CV benefits and posed no increased risks for HF, hypoglycemia, or death (45).

Glucagon-like peptide-1 receptor agonists (GLP-1 RAs) is viewed as the primary DPP-4 substrate capable of modulating CV function. The study found that use of GLP-1 RAs was associated with significant reductions in CV and all-cause mortality, and the researchers suggested that GLP-1 RAs should be used as a first-line treatment in patients with T2DM at higher CV risk or as a first-line treatment in patients with metformin resistance (46).

PERSPECTIVE

Exploration of Early-Stage Screening Methods

The comprehensive management of T2DM patients is important for early identification and detection of possible CV risks. An effective risk prediction model was established by Chinese scholars, and it was found that weight reduction, lowering blood pressure and blood uric acid levels, and proper control of diastolic blood pressure could significantly reduce the risk of new-onset ACS in T2DM patients in northwest China (47). The meta-analysis included 30 studies with 253,425 participants and 1,621,920 person-years of follow-up, which is about prognosis of unrecognized MI determined by electrocardiography or cardiac MRI. Unrecognized myocardial infarction (UMI) by electrocardiography (ECG) or UMI by cardiac MR (CMR) is associated with an adverse long-term prognosis similar to that of recognized MI (48). Imaging evidence indicates a high prevalence of CAD in patients with T2DM; however, there is no standard for initiating CAD screening in the T2DM population, and it has been found that routine screening for CAD using

computed tomographic coronary angiography (CTCA) should be considered for early detection of CAD in asymptomatic T2DM patients with diagnosed duration of T2DM >10.5 years and systolic blood pressure > 140 mmHg (49). A study (50) has established the triglyceride glucose index (TGI), calculated as [fasting triglycerides ($mg{\cdot}dl^{-1}$) × FPG ($mg{\cdot}dl^{-1}$)/2], to predict CV events and demonstrated that TGI may be a better predictor for the risk of CV events than FPG or glycosylated hemoglobin (HbA1c) in patients with ACS undergoing PCI. Statins have been widely used for lipid-lowering therapy in patients with CAD; however, there has been increasing clinical evidence of a correlation between statin use and NODM. A study (51) found that systolic epicardial adipose tissue thickness is an independent predictor for NODM in patients with CAD treated with high-intensity statins, which can help physicians formulate timely and appropriate monitoring or intervention plans for the early detection of NODM.

Exploration From a Genetic Perspective

Scholars have conducted useful exploration on which T2DM patients are more likely to develop MI from a genetic perspective. The common MTNR1B single-nucleotide polymorphism locus rs10830963 is strongly correlated with the risk of developing T2DM. The relationship between this genetic variation and the risk of MI in patients with T2DM has been investigated using data from a UK Biobank cohort, which investigated the relationship between rs10830963 and the incidence of MI (fatal and non-fatal) in 13,655 participants with possible T2DM over a 6.8-year follow-up period. With the use of an additive genetic model, variation in the MTNR1B gene rs10830963 was found to be positively correlated with the risk of developing MI over 6.8 years of follow-up, suggesting that rs10830963 polymorphism may be a useful genetic marker for the development of MI in patients with T2DM (52). The growing knowledge of the genetic insights between T2D and CVD is beginning to provide the potential understanding of both disorders (53). In the future, the subtle relationship between T2DM and MI can be explored further deeply in terms of genetics.

Diversity of Drug Evaluation Methods

In the clinical evaluation of the CV safety of innovative oral hypoglycemic drugs, although RCTs can better exclude the influences of confounding factors, they have poor external validity and cannot fully meet the actual clinical needs. Therefore, combining RCTs with real-world research/study (RWR/RWS) can provide more reliable and high-level clinical evidence, and real-world evidence (RWE) studies can be used as auxiliary evidence to RCTs to evaluate the efficacy and safety of drugs, which can help fill the knowledge gap between RCTs and actual clinical practice (54). RCTs provide evidence for clinical guideline recommendations, and real-world studies test the practicability of guideline recommendations, thus allowing step-by-step refinement of treatment strategies to optimize treatment and return to clinical practice. RWS includes a wider population to compensate for the poor external validity of RCTs due to stringent inclusion and exclusion criteria. A credible RWS covers a large number of patients and can reflect the routine clinical practice, which is of clinical significance for treatment and especially for safety assessment.

Moreover, SGLT2 inhibitors have been used to treat patients with T2DM to reduce the risk of CV events, including HF, and it is clear that the mechanism by which SGLT2 inhibitors reduce this risk may not be directly related to improved diabetic status and glycemic control. In addition, short-term use of SGLT2 inhibitors significantly improved volume load and symptoms in HF patients with concomitant T2DM; however, serum N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentrations, which are traditionally used to assess HF severity, did not improve significantly, suggesting that we need to explore meaningful biomarkers that can monitor and evaluate the effect SGLT2 inhibitors for HF treatment in the future (55), in order to provide scientific evidence for in-depth understanding of the many unknowns of SGLT2 inhibitors.

AUTHOR CONTRIBUTIONS

JC and YaL formed the reference collection, conducted the reference analysis, and wrote the manuscript and are considered as co-first authors. YuL and FX contributed to the topic conception, manuscript revision, and decision to submit for publication and are the co-corresponding authors. YiL contributed to reference analysis and helped in the revision 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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The U-Shape Relationship Between Glycated Hemoglobin Level and Long-Term All-Cause Mortality Among Patients With Coronary Artery Disease

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Background: Although glycated hemoglobin (HbA1c) was considered as a prognostic factor in some subgroup of coronary artery disease (CAD), the specific relationship between HbA1c and the long-term all-cause death remains controversial in patients with CAD.

Methods: The study enrolled 37,596 CAD patients and measured HbAlc at admission in Guangdong Provincial People's Hospital. The patients were divided into 4 groups according to HbAlc level (Quartile 1: HbA1c $\leq$ 5.7%; Quartile 2: 5.7% $<$ HbA1c $\leq$ 6.1%; Quartile 3: 6.1% $<$ HbA1c $\leq$ 6.7%; Quartile 4: HbA1c $>$ 6.7%). The study endpoint was all-cause death. The restricted cubic splines and cox proportional hazards models were used to investigate the association between baseline HbAlc levels and long-term all-cause mortality.

Results: The median follow-up was 4 years. The cox proportional hazards models revealed that HbAlc is an independent risk factor in the long-term all-cause mortality. We also found an approximate U-shape association between HbA1c and the risk of mortality, including increased risk of mortality when HbA1c $\leq$ 5.7% and HbA1c $>$ 6.7% [Compared with Quartile 2, Quartile 1 (HbA1c $\leq$ 5.7), aHR = 1.13, 95% CI:1.01–1.26, $P < 0.05$; Quartile 3 (6.1% $<$ HbA1c $\leq$ 6.7%), aHR = 1.04, 95% CI:0.93–1.17, $P = 0.49$; Quartile 4 (HbA1c $>$ 6.7%), aHR = 1.32, 95% CI:1.19–1.47, $P < 0.05$].

Conclusions: Our study indicated a U-shape relationship between HbA1c and long-term all-cause mortality in CAD patients.

Keywords: coronary artery disease, glycated hemoglobin, all-cause mortality, U-shape, optimal

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INTRODUCTION

Glycated hemoglobin (HbA1c), as a biomarker that reflects 2–3 months of blood glucose status, plays a pivotal role in microvascular disease and atherosclerosis (1, 2). Previous studies have shown that HbA1c was associated with cardiovascular events and all-cause mortality (3, 4).

In recent years, abnormal glucose metabolism in patients with coronary artery disease (CAD) have received widespread attention. A higher level of baseline HbA1c was an independent predictor for poor prognosis in patients with acute myocardial infarction (AMI) or stable CAD (5, 6). However, the relationship between HbA1c levels and the long-term prognosis in CAD patients is not well-defined and some studies have reported conflicting results (7). She et al. found that AMI patients with different HbA1c levels showed no difference in prognosis during 2-year follow-up (8). A meta has found that there is no significant relationship between HbA1c levels and all-cause mortality among diabetic patients after percutaneous coronary intervention (PCI) (9). Besides, recent studies have found that there is not a simple linear relationship between HbA1c and all-cause mortality (6, 10).

Therefore, we aimed to investigate the specific association between baseline HbA1c and long-term all-cause mortality in CAD patients, as well as the optimal range for the lowest risk of mortality.

METHODS

Study Design and Participants

This was a single-center, observational prospective study, which was completed in Guangdong Provincial People's Hospital (ClinicalTrials.gov NCT04407936). A total of 88,938 patients underwent coronary angiography from January 2008 to December 2018. 59,667 patients with a final diagnosis of CAD according to the 10th Revision Codes of the International Classification of Diseases (ICD-10; I20.xx–I25.xx, I50.00001 and I91.40001). A total of 37,596 CAD patients were included in the final analysis after excluding patients who lacked off HbAlc examination or follow-up information (**Supplementary Figure 1**). This research program was performed according to the Declaration of Helsinki and approved by The Ethics Committee of Guangdong Provincial People's Hospital. Baseline information such as demographic characteristics, clinical settings, laboratory examinations and medications at discharge were extracted from the electronic Clinical Management System of the Guangdong Provincial People's Hospital. Clinicians assessed whether the patients were feasible for Coronary angiography (CAG) or PCI based on the patient's condition and standard clinical practice guidelines (11).

HbAlc Measurement

The serum HbAlc was measured by an ion-exchange high performance liquid chromatography with Bio-Rad Variant Testing System (Bio-Rad Laboratories, USA) at the time of admission.

Definition and Endpoint

We calculated the estimated glomerular filtration rate (eGFR) by applying the Modification of Diet in Renal Disease (MDRD) equation (12). Diabetes mellitus (DM), and hypertension were defined using the ICD-10 code (**Supplementary Table 1**). The primary endpoint of this study was long-term all-cause death, incident events were defined as the first event occurring between the date of enrollment and the end of follow-up of December 31, 2018. Trained nurses monitored and recorded follow-up data through outpatient interviews and telephones.

Statistical Analysis

Statistical analysis for this study was performed from January 1, 2008 to December 31, 2018. We divided the patients into 4 groups according to the quartile of serum HbAlc level (Quartile 1: HbA1c $\leq$ 5.7; Quartile 2: 5.7$<$ HbA1c $\leq$ 6.1; Quartile 3: 6.1 $<$ HbA1c $\leq$ 6.7; Quartile 4: HbA1c $>$ 6.7). We reported descriptive statistics by means (SD), median [interquartile range (IQR)], or number and percentage when appropriate. We used one-way analysis of variance (ANOVA) when analyzing differences between different groups. When analyzing categorical data, we used the Pearson chi-squared test. Prognosis analysis was used by Kaplan–Meier methods and survival curves. Log-rank test to compare the survival differences between the four groups of patients.

We used cox proportional hazards regression models and restricted cubic splines to evaluate the relationship between serum HbAlc levels and all-cause mortality in CAD patients. Hazard ratios and 95% CIs are reported. Model 1 was adjusted with age $>$ 75 years and gender, and model 2 was adjusted eGFR. Model 3, as the primary results, was adjusted with the variables which were significant at $P < 0.05$ according to univariate Cox proportional hazards regression and associated with mortality according to clinical experience (included history of present illness information, drugs information). The proportional hazards assumption was tested with the use of Schoenfeld residuals. Variables with missing values $>$ 30% were not considered as candidates. We conducted subgroup analysis based on patients' characteristics and comorbidities stratified by age, gender, diabetes, AMI, PCI, renal insufficiency (eGFR $<$ 60). All data analyses were performed using R (version 3.6.3; R Core Team, Vienna, Austria). *P*-values < 0.05 were considered to represent statistical significance.

RESULT

Clinical Characteristics

We included 37,596 patients in the final analyses. Baseline clinical of the study patients are shown in **Table 1**. The mean age was 62.54 $\pm$ 10.55 years, and 27,007 (71.83%) were male. The distribution of HbA1c as follows: mean, 6.43 $\pm$ 1.33 %. Patients were divided into four groups: Quartile 1 (HbA1c $\leq$ 5.7, $n = 11{,}094$), Quartile 2 (5.7 $<$ HbA1c $\leq$ 6.1, $n = 9{,}684$), Quartile 3 (6.1 $<$ HbA1c $\leq$ 6.7, $n = 7{,}823$), Quartile 4 (HbA1c $>$ 6.7, $n = 8{,}995$). Nine thousand four hundred forty-three (27.45%) patients complicated with DM and 19,224 (55.88%)

TABLE 1 | Baseline characteristics of the patients.

Characteristic		HbA1c level Quartile				
	Overall (*n* = 37,596)	**≤5.7% (*n* = 11,094)**	**5.7–6.1% (*n* = 9,684)**	**6.1–6.7% (*n* = 7,823)**	**>6.7% (*n* = 8,995)**	***p*-value**
Demographic						
Age, year	62.54 (10.55)	60.39 (11.26)	62.86 (10.34)	64.19 (10.01)	63.43 (9.88)	<0.001
Age > 75, *n* (%)	5,150 (13.70)	1,376 (14.21)	1,220 (11.00)	1,285 (16.43)	1,269 (14.11)	<0.001
Male, *n* (%)	27,007 (71.83)	8,249 (74.36)	7,035 (72.65)	5,589 (71.44)	6,134 (68.19)	<0.001
Medical history						
AMI, *n* (%)	5,063 (14.72)	1,551 (14.91)	1,284 (14.53)	913 (12.94)	1,315 (16.22)	<0.001
Pre-MI, *n* (%)	2,043 (5.94)	500 (5.66)	526 (5.06)	453 (6.42)	564 (6.96)	<0.001
Diabetes, *n* (%)	9,443 (27.45)	640 (7.24)	400 (3.84)	1,717 (24.34)	6,686 (82.46)	<0.001
Hypertension, *n* (%)	19,224 (55.88)	4,971 (47.78)	4,704 (53.25)	4,281 (60.69)	5,268 (64.97)	<0.001
PCI, *n* (%)	19,760 (52.56)	5,151 (46.43)	4,951 (51.13)	4,184 (53.48)	5,474 (60.86)	<0.001
Laboratory test						
eGFR, ml/min/1.73 m^2	80.84 (25.62)	82.25 (25.55)	83.69 (23.77)	78.17 (24.84)	78.30 (27.85)	<0.001
WBC, 109/L	7.63 (2.42)	7.37 (2.38)	7.52 (2.38)	7.69 (2.38)	8.00 (2.50)	<0.001
HGB, g/L	133.14 (16.61)	134.24 (16.64)	134.12 (15.77)	132.61 (16.37)	131.20 (17.43)	<0.001
CHOL, mmol/L	4.50 (1.18)	4.46 (1.14)	4.53 (1.14)	4.53 (1.20)	4.50 (1.24)	<0.001
TRIG, mmol/L	1.65 (1.20)	1.48 (0.96)	1.56 (1.06)	1.68 (1.16)	1.92 (1.53)	<0.001
APOB, g/L	0.86 (0.24)	0.84 (0.23)	0.86 (0.23)	0.86 (0.24)	0.87 (0.25)	<0.001
LDLC, mmol/L	2.80 (0.95)	2.79 (0.94)	2.82 (0.95)	2.82 (0.97)	2.77 (0.97)	<0.001
HbA1c, %	6.43 (1.33)	5.39 (0.34)	5.94 (0.11)	6.40 (0.17)	8.27 (1.50)	<0.001
URIC, μmol/L	396.06 (112.37)	387.06 (107.36)	396.52 (108.09)	411.50 (112.87)	393.40 (120.94)	<0.001
ALB, g/L	36.95 (4.13)	37.46 (4.02)	36.99 (3.95)	36.79 (4.10)	36.42 (4.42)	<0.001
Medication						
ACEI/ARB, *n* (%)	23,825 (65.53)	6,255 (59.23)	6,002 (64.04)	5,249 (68.86)	6,319 (71.79)	<0.001
Beta-blockers, *n* (%)	23,008 (63.19)	6,420 (60.76)	5,797 (61.77)	4,899 (64.17)	5,892 (66.78)	<0.001
Statin, *n* (%)	32,717 (89.86)	9,200 (87.07)	8,458 (90.12)	6,910 (90.52)	8,149 (92.36)	<0.001
Death, *n* (%)	4,340 (11.54)	1,084 (9.77)	1,018 (10.51)	956 (12.22)	1,282 (14.25)	<0.001

AMI, acute myocardial infarction; PCI, percutaneous coronary intervention; eGFR, estimated glomerular filtration rate; WBC, white blood cell; HGB, hemoglobin; CHO, serum total cholesterol; TRIG, triglyceride; APOB, apolipoprotein B; LDL-C, low-density lipoprotein cholesterol; HbA1c, hemoglobin A1c; URIC, uric acid; ALB, albumin; ACEI/ARB, angiotensin-converting enzyme inhibitor/angiotensin receptor blocker.

patients complicated with hypertension. Five thousand sixty-three (14.72%) patients identified in AMI and 19,760 (52.56%) patients treated with PCI (**Table 1**).

Main Outcomes

The median follow-up period was 4.0 (2.2–5.9) years and prognostic data were fully documented during the entire follow-up period. Four thousand three hundred forty (11.54%) patients died during the follow-up. Kaplan–Meier curves analysis revealed that patients in the highest HbA1c level group (Quartile 4) had a significantly higher long-term mortality compared with those in the lower HbA1c group (Quartile1 or 2 or 3) (log-rank analysis $P < 0.01$) (**Figure 1**).

Based on the result of the univariate regression analysis, 10 variables (including gender, hypertension, AMI et al.) were significantly associated with the long-term all-cause mortality (**Supplementary Table 2**). In Cox analysis, Quartile 2 ($5.7 < \text{HbA1c} \leq 6.1\%$) was served as the reference group. The results of Cox proportional hazards regression were shown in **Figure 2**. In Cox multivariate analysis, patients in the lowest or highest HbA1c level (Quartile 1 or 4) showed a significantly higher risk of long-term mortality than other groups (Quartile 2 or 3) [Compared with Quartile 2, Quartile 1 ($\text{HbA1c} \leq 5.7$), aHR = 1.13, 95% CI:1.01–1.26, $P < 0.05$; Quartile 3 ($6.1 < \text{HbA1c} \leq 6.7\%$), aHR = 1.04, 95% CI:0.93–1.17, $P = 0.49$; Quartile 4 ($\text{HbA1c} > 6.7\%$), aHR = 1.32, 95% CI:1.19–1.47, $P < 0.05$]. The relationship between HbA1c and all-cause mortality was non-linear (non-linear $P < 0.001$). In the univariate and multivariate cox models of restricted cubic splines, we observed a U-shaped association between HbA1c and long-term all-cause mortality (**Figure 3**); low and high levels of HbA1c were associated with an increased risk of all-cause mortality. The Cox models fulfilled the proportional-hazards assumption tested by Schoenfeld residual ($P > 0.05$).

Subgroup Analysis

To assess whether the association between HbA1c and long-term all-cause mortality could be explained by patients' characteristics and comorbidities. We conducted multivariable-adjusted hazard ratios for all-cause mortality stratified by age,

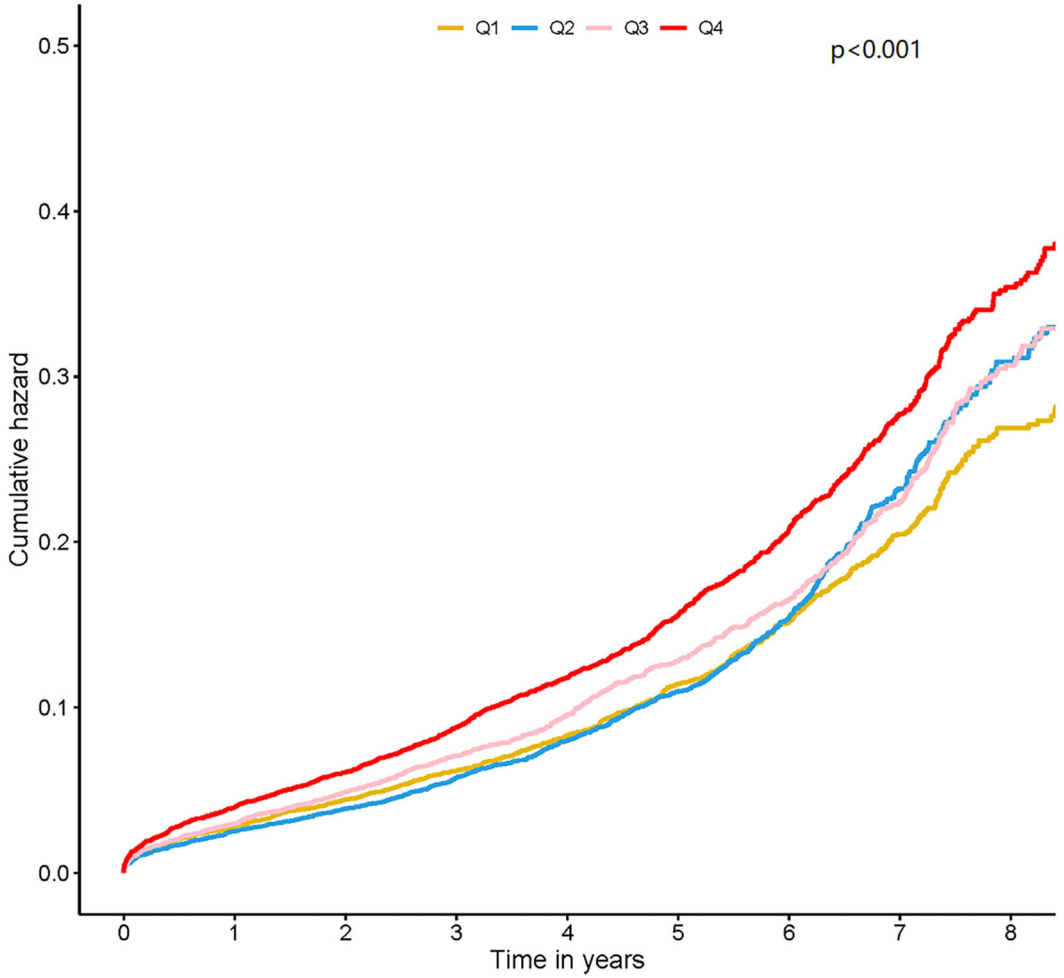

FIGURE 1 | Kaplan-Meier curves for quartile values of plasma levels of HbA1c. Quartile 1: HbA1c ≤ 5.7; Quartile 2: 5.7 < HbA1c ≤ 6.1; Quartile 3: 6.1 < HbA1c ≤ 6.7; Quartile 4: HbA1c > 6.7.

gender, PCI, diabetes, AMI and renal insufficiency (eGFR < 60) (*P* for interaction < 0.05). We observed the stable U-shape association between HbA1c and long-term mortality in most subgroups (**Figure 4**), although the association was slightly reduced in female patients (**Figure 4**). As shown in restricted cubic splines (**Supplementary Figure 2**), patients in the different subgroups showed U-shape association between HbA1c and long-term mortality.

DISCUSSION

To our knowledge, this is the largest cohort study of the value of HbA1c in CAD patients. In this study of 37,596 CAD patients with more 4 years follow-up, we found a U-shaped relationship between levels of HbA1c and the risk of all-cause mortality, with low and high levels associated with an increased risk.

The impact of abnormal glucose metabolism among patients with cardiovascular disease has drawn widespread attention (13–15). Previous studies have suggested that HbA1c is an independent risk factor for prognosis in CAD patients of different subgroups. Hong et al. found that elevated HbA1c would aggravate the severity of CAD and poor prognosis (6). Hamdi et al. demonstrated that increased HbA1c levels at admission were associated with higher rates of major adverse cardiovascular events and mortality in patients undergoing PCI (12). However, there is also some controversial finding (16, 17). She et al. found that AMI patients with different HbA1c levels showed no difference in prognosis during 2-year follow-up (8). Similarly, Lemesle et al. showed no significant effect of HbA1c on 1-year rate of major adverse cardiovascular events (including death) among 952 diabetic patients undergoing PCI (7). Most previous studies included limited number of patients with a relatively short follow-up, which may lead to controversial results. There is still a lack of discussion in large data of the CAD patients. In our research, we included large samples (more than 30,000) of CAD patients and found that HbA1c is an independent prognostic factor of mortality during more than 4 years of follow-up. Interestingly, we firstly found that

Variable	HR(95%CI)	P-value
Quartile levels(unadjusted)		
≤ 5.7	1.03(0.95-1.12)	0.506
5.7-6.1	Ref	
6.1-6.7	1.14(1.04-1.24)	0.004*
> 6.7	1.37(1.26-1.49)	<0.001*
Quartile levels(adjust for Model 1)		
≤ 5.7	1.06(0.97-1.15)	0.216
5.7-6.1	Ref	
6.1-6.7	1.13(1.03-1.23)	0.008*
> 6.7	1.40(1.29-1.52)	<0.001*
Quartile levels(adjust for Model 2)		
≤ 5.7	1.06(0.96-1.16)	0.233
5.7-6.1	Ref	
6.1-6.7	1.03(0.93-1.13)	0.606
> 6.7	1.25(1.18-1.37)	<0.001*
Quartile levels(adjust for Model 3)		
≤ 5.7	1.12(1.01-1.25)	0.028*
5.7-6.1	Ref	
6.1-6.7	1.04(0.93-1.17)	0.494
> 6.7	1.32(1.19-1.47)	<0.001*

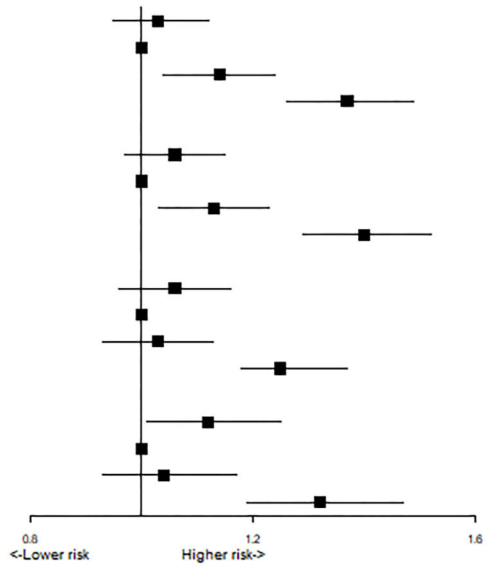

FIGURE 2 | Cox proportional hazard ratios for long-term all-cause mortality in different models. Model 1: Cox proportional hazard ratio for long-term all-cause mortality adjusted for age > 75 and gender. Model 2: Cox proportional hazard ratio for long-term all-cause mortality adjusted for eGFR. Model 3: Cox proportional hazard ratio for long-term all-cause mortality adjusted for multiple variables (age > 75, gender, AMI, HT, pre-MI, PCI, eGFR, LDLC, URIC, Statin, ACEI/ARB).

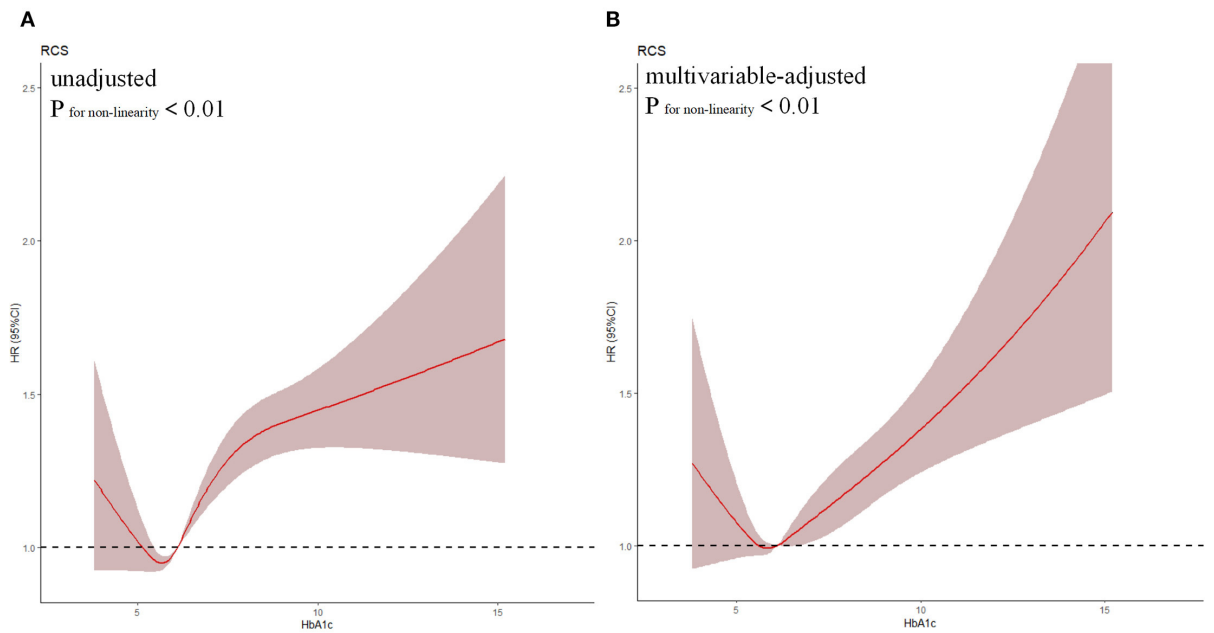

FIGURE 3 | Restricted spline curve of the HbA1c hazard ratio for mortality. **(A)** The restrict spline curve of univariate cox model; **(B)** The restrict spline curve of multivariate cox model, Adjusted for age > 75, gender, AMI, HT, pre-MI, PCI, eGFR, LDLC, URIC, Statin, ACEI/ARB.

the relationship between HbA1c and all-cause mortality was more similar to a U-shape than a linear association. Extremely low HbA1c level also leads to poor prognosis among patients with CAD.

In subgroup analysis, the U-shaped association between levels of HbA1c and long-term mortality in CAD patients is stable in most subgroups. However, the U-shape association was strong in male subgroup while slightly reduced in female subgroup.

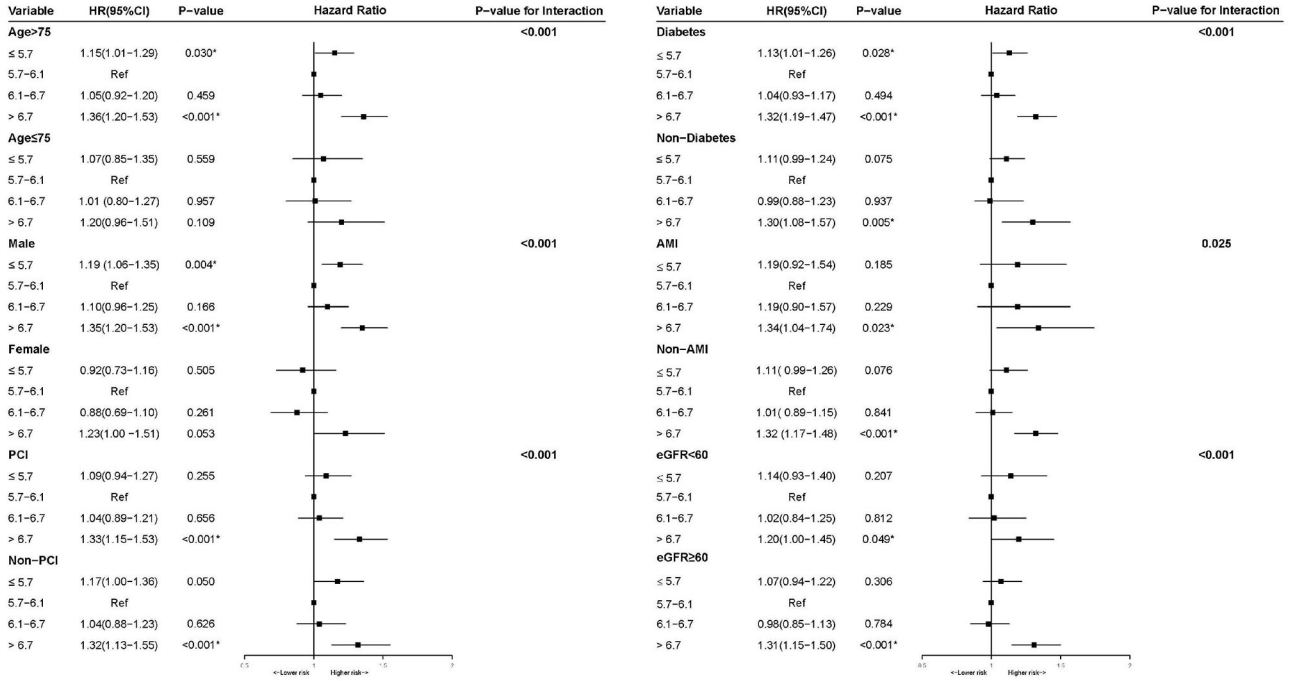

FIGURE 4 | Multivariable cox proportional hazard ratios for long-term all-cause mortality in Subgroups stratified by patients' characteristics and comorbidities.

Previous studies have found that there were sex differences in the association of HbA1c and cardiovascular disease risk (18–20). The underlying mechanisms of our finding may be related to gender differences.

According to our findings, patients with excessively high HbA1c levels need to be reduced. As a new diabetes medication, sodium-glucose cotransporter-2 (SGLT2) inhibitors has been proved to reduce the risk of cardiovascular events and mortality by the reduction of HbA1c (21). Although the guideline recommends that some populations should consider less-rigorous targets of HbA1c levels to prevent complications (including hypoglycaemic episodes), there is no clear and appropriate range for the CAD population (21). Our study seems to discover a "security zone" of HbA1c levels for management among CAD patients based on a large sample. In the future, more studies should explore whether effective HbA1c control can improve the prognosis in the CAD population.

Previous researches suggested a correlation between plasma HbA1c and inflammatory biomarkers, chemical parameters, either alone or combined. These markers or risk factors had a direct role in the progression of atherosclerotic artery disease and adverse cardiovascular events (22–24). The increases in serum biomarkers of inflammation might further promote the development of secondary cardiac arrhythmias and heart failure (25). Meanwhile, hypoglycemia induces several changes in the hemostatic parameters which include an increase in platelet aggregation, activation, degranulation as well as a rise in factor VIII and vWF levels (26, 27). These effects are exaggerated in patients and are likely to be detrimental to myocardial circulation particularly in patients with an already compromised myocardial perfusion.

LIMITATION

In this study, there are still several limitations. At first, this observational study was conducted in a single center. However, our study was derived from a southern central hospital with more than 30,000 patients from the southern cities, which can represent the information of patients with CAD in southern China. Second, as an observational study, there are uncertain risk factors (such as lifestyle, malignancy) that may cause residual confounding effects on long-term mortality. But the results in our study have adjusted for common cardiovascular prognostic factors. Thirdly, DM was defined according to the ICD code at admission. Although there are some undetected DM patients that might include in our study, this real-world study can also reflect the current situation of DM diagnosis and treatment among patients with CAD in China. Finally, only the HbA1c level at admission was included in our study and the change of value has lacked. Due to the characteristics of glycated hemoglobin, it can reflect a patient's long-term blood glucose status.

CONCLUSIONS

Our study firstly indicated a U-shape relationship between HbA1c and long-term all-cause mortality in CAD patients. For these patients, the optimal HbA1c level (5.7–6.7%) may have a better prognosis. Further study is required to explore

whether effective HbA1c control can improve the prognosis in the CAD population.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All procedures were performed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975. This study was approved by the institutional ethics board of Guangdong Province Peoples' Hospital. All patients were eligible in this study.

AUTHOR CONTRIBUTIONS

YLiu, JL, LL, JY, and JC: research idea and study design. JL, MY, QL, LL, SC, BW, YLin, GC, HH, HL, NT, and ZL: data acquisition. JL and YLiu: data analysis/interpretation. DX and MY: statistical analysis. YLiu, JC, and NT: supervision and mentorship. All authors contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.632704/full#supplementary-material

Supplementary Figure 1 | The flow of participants through the trial.

Supplementary Figure 2 | Restricted spline curve of the HbA1c hazard ratio for mortality in Subgroups stratified by patients' characteristics and comorbidities.

Supplementary Table 1 | Univariable Cox regression analysis of long-term all-cause mortality.

Supplementary Table 2 | The ICD-10 codes information of diagnoses.

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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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Seipin Deficiency Accelerates Heart Failure Due to Calcium Handling Abnormalities and Endoplasmic Reticulum Stress in Mice

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Seipin deficiency can induce hypertrophic cardiomyopathy and heart failure, which often leads to death in humans. To explore the effects and the possible mechanisms of *Seipin* deficiency in myocardial remodeling, *Seipin* knockout (SKO) mice underwent transverse aortic constriction (TAC) for 12 weeks. We found a more severe left ventricular hypertrophy and diastolic heart failure and increases in inflammatory cell infiltration, collagen deposition, and apoptotic bodies in the SKO group compared to those in the wild type (WT) group after TAC. Electron microscopy also showed a more extensive sarcoplasmic reticulum expansion, deformation of microtubules, and formation of mitochondrial lesions in the cardiomyocytes of SKO mice than in those of WT mice after TAC. Compared with the WT group, the SKO group showed increases in endoplasmic reticulum (ER) stress-, inflammation-, and fibrosis-related gene expression, while calcium ion-related factors, such as *Serca2a* and *Ryr*, were decreased in the SKO group after TAC. Increased levels of the ER stress-related protein GRP78 and decreased SERCA2a and P-RYR protein levels were detected in the SKO group compared with the WT group after TAC. Slowing of transient Ca^{2+} current decay and an increased SR Ca^{2+} content in myocytes were detected in the cardiomyocytes of SKO mice. Adipose tissue transplantation could not rescue the cardiac hypertrophy after TAC in SKO mice. In conclusion, we found that *Seipin* deficiency could promote cardiac hypertrophy and diastolic heart failure after TAC in mice. These changes may be related to the impairment of myocardial calcium handling, ER stress, inflammation, and apoptosis.

Keywords: seipin, diastolic heart failure, calcium handling, endoplasmic reticulum stress, SERCA2a

INTRODUCTION

Congenital generalized lipodystrophy (CGL) is an autosomal recessive disorder characterized by the severe loss of adipose tissue (AT), severe insulin resistance (IR), hypertriglyceridemia, fatty liver, renal injury, and cardiac hypertrophy (1). *Seipin* gene deficiency results in CGL type 2, which seems to cause the most severe lipodystrophy phenotype among CGLs in humans (2).

Several clinical studies have shown that hypertrophic cardiomyopathy is an important cause of heart failure and neonatal death in patients with CGL (3). A total of 20–25% of patients with CGL had cardiac hypertrophy, and the average age of onset was 20 years old (4). Another study found that *Seipin* gene deficiency underlying CGL2 was more likely to cause heart failure and premature death in premature infants than CGL1, and 42.9% of CGL2 patients had cardiomyopathy (5, 6). However, the exact mechanism of *Seipin* in cardiac remodeling is not clear.

SEIPIN is an intrinsic endoplasmic reticulum protein with two transmembrane structures (7). It has been reported that the SEIPIN-EGFP fusion protein colocalizes with the endoplasmic reticulum-specific protein calreticulin (4, 8, 9). Mutation or deletion of *Seipin* could result in endoplasmic reticulum stress (10, 11). Endoplasmic reticulum (ER) stress is involved in the occurrence and development of heart failure (HF) (12). Excessive or prolonged ER stress could lead to ion exchange disorders of the ER, inflammatory infiltration, and cell apoptosis (13). In this study, we investigated whether *Seipin* deficiency caused ER stress that is implicated in hypertrophic cardiomyopathy and HF. *Seipin* may regulate the distribution of intracellular calcium through the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump in adipocytes (14). Ca^{2+} plays an important role in myocardial contraction. SERCA2a is responsible for the translocation of Ca^{2+} from the cytoplasm to the ER during myocardial diastole. The patients with diastolic HF had abnormal SERCA2a function (15). Therefore, hypertrophic cardiomyopathy and HF caused by *Seipin* deficiency may be associated with a decrease in SERCA2a function. However, no evidence of cardiac hypertrophy was detected in *Seipin* knockout (SKO) mice generated by us until the age of 9 months. The mice presented only mild cardiac hypertrophy at 9–12 months of age. Therefore, we used a pressure overload model with transverse aortic constriction (TAC) to study the effect of *Seipin* on cardiac hypertrophy in SKO mice.

In this study, we found that *Seipin* gene deficiency can accelerate cardiac remodeling in animal models, leading to cardiac hypertrophy and diastolic heart failure. Further studies indicated that these changes may be related to the impairment of myocardial calcium handling, endoplasmic reticulum stress, inflammation, and apoptosis.

MATERIALS AND METHODS

Animals

The animal experiments were approved by the Institutional Animal Care Research Advisory Committee of the National Institute of Biological Science (NIBS) and the Animal Care Committee of Peking University Health Science Center, and the study was approved by the local Ethical Committee. Animals were maintained on a normal diet (10% of kilocalories were obtained from fat) and a 12-h light/12-h dark cycle with free access to water. The Principles of Laboratory Animal Care (NIH Publication, 8th Edition, 2011) were followed.

SKO and littermate wild type (WT) male mice with a C57BL/6 background at the age of 2 months were used in this study. SKO mice were generated and bred as described previously (16). Animals were divided into four groups ($n = 8$ for each group): The WT and SKO sham operation groups and the WT-and SKO-TAC operation groups. Echocardiography was performed every 2 weeks. Mice were sacrificed at 12 weeks after TAC. The heart weight to tibia length (HW/TL) ratios were determined. Samples were obtained from the heart, and some were immediately frozen in liquid nitrogen and stored at −80°C for real-time PCR, whereas some were fixed for histological studies.

To study whether AT is involved in cardiac hypertrophy in SKO mice, AT transplantation was performed 2 weeks before TAC surgery at the age of 6 weeks. The animals were divided into four groups ($n = 8$): SKO-TAC and WT-TAC mice transplanted with the AT of WT mice; SKO-TAC and WT-TAC mice underwent sham surgery. The animals were sacrificed 12 weeks after TAC, and the aforementioned parameters were analyzed.

Transverse Aortic Constriction

To investigate the impact of pressure overload-induced cardiac hypertrophy, transverse aortic constriction (TAC) surgery was performed in mice as described previously (17, 18). Briefly, mice were anesthetized with 2% isoflurane (Baxter Healthcare Corporation, New Providence, NJ, USA) and then maintained on 1.5% isoflurane. Mice were ventilated by tracheal intubation using a rodent ventilator (Alcbio Corporation, Shanghai, China) with a respiratory rate of 120 breaths/min and a tidal volume of 0.3 mL. Then, the chest was opened at the suprasternal fossa along the midsternal line, and the thymus glands were superiorly reflected. The transverse thoracic aorta between the innominate artery and the left common carotid artery was dissected, and a 6-0 silk suture was tied around the aorta, which was pressed against a 26-gauge needle. Then, the needle was removed. A sham operation involving thoracotomy and aortic dissection without constriction of the aorta was performed in both the WT and SKO sham operation groups.

Echocardiography

Cardiac hypertrophy and function were assessed by echocardiography using a high-resolution Vevo 770TM Imaging System (Visual Sonics Inc., Toronto, Canada) as described previously (19, 20).

Mice were anesthetized with 3% isoflurane (Baxter Healthcare Corp, New Providence, RI, USA), and their chests were shaved. The bodies of the mice were placed in the supine position on a movable heated platform that was maintained at 37°C. Then, the mice were anesthetized with 1.0–1.5% isoflurane to keep the heart rate stabilized at 400–500 beats per min. At the level of the papillary muscle of the heart in mice, two-dimensional parasternal long-axis views and short-axis views were obtained. The M-mode cursor was positioned perpendicular to the maximum LV dimension during end-diastole and systole. The following variables were measured digitally in the M-mode images: LV (left ventricular) internal dimensions (LVID); LV anterior wall thickness (LVAW); LV posterior wall thickness (LVPW). The values of the LV end-diastolic volume (LVEDV), ejection fraction (EF) were calculated from these parameters.

Pulsed Doppler ultrasound was used to measure the velocity of the transmitral flow of the ventricular filling. The early

diastolic mitral transmitral flow velocity (E), late diastolic mitral transmitral flow velocity (A), and the E/A ratio were used to assess diastolic function. The normal value was >1, and an inverse E/A ratio was detected in diastolic HF. The early diastolic velocity (Em) and the late diastolic velocity (Am) of the mitral valve ring and Em/Am were also determined based on the ultrasound scan. All measurements represented the average value from three continuous cardiac cycles per loop.

RNA Isolation and Quantitative Real-Time PCR

Total RNA from tissues (∼50–100 mg of each tissue) was extracted using Tri Reagent (Molecular Research Center, OH, USA), and first-strand cDNA was generated by using an RT kit (ABM, Richmond, BC, Canada). Quantitative real-time PCR was performed using the primers listed in **Table 1**. Amplifications were performed with 35 cycles using a continuous fluorescence detection system (Agilent Technologies, Santa Clara, Cal, USA) with EVA Green fluorescence reagent (ABM, Richmond, BC, Canada). Each cycle consisted of denaturation by heating for 15 s at 95°C and annealing/extension for 60 s at 60°C. All samples were quantified using the comparative CT method for relative quantification and normalized to GAPDH (21). Each sample was measured in triplicate. Melting curve analysis was used to confirm the amplification specificity. All experiments were repeated at least twice in triplicate.

TABLE 1 | Primers used in real-time polymerase chain reaction.

Genes	Sense primers	Antisense primers
Anp	GGGCTTCTTCCTCGTCTTGG	GGGGGCATGACCTCATCTTC
Bnp	TAACGCACTGAAGTTGTTGTAGG	CGCTATGTTTATTATGTTGTGGC
β-mhc	CTTCACTCCAGAAGAGAAGAACTC	CCCATGAGGTAGGCTGATTTG
Myh7	ACTGTCAACACTAAGAGGGTCA	GGATGATTTGATCTTCCAGGG
Myh6	GCCCAGTACCTCCGAAAGTC	GCCTTAACATACTCCTCCTTGTC
Il6	TTCTTGGGACTGATGCTG	CTGGCTTTGTCTTTCTTGTT
Icam1	GGACCTTAACAGTCTACAAC	AGCCGAGGACCATACA
Serca2	GAGAACGCTCACACAAAGACC	CAATTCGTTGGAGCCCCAT
Col3a1	CCTGGCTCAAATGGCTCAC	CAGGACTGCCGTTATTCCCG
Col1a2	GTAACTTCGTGCCTAGCAACA	CCTTTGTCAGAATACTGAGCAGC
Xbp1	AAGAACACGCTTGGGAATG	AGGGAGGCTGGTAAGGAA
Ryr	GCCTTATCCATGCCCAGTAA	CCAGTGGGACAGACTGGTGA
Ncx1	TGGTAGATGGCAGCAATGGA	CTTCGTCCCACCTACAGAAT
Seipin	TCAATGACCCACCAGTC	AAGGAGCCATAGAGGAAC
Bax	CCAGGATGCGTCCACCAA	AAGTAGAAGAGGGCAACCAC
Bcl2	TACCGTCGTGACTTCGCAGAG	GGCAGGCTGAGCAGGGTCTT
Grp78	ACTTGGGGACCACCTATTCCT	GTTGCCCTGATCGTTGGCTA
Gapdh	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT

Anp, Atrial natriuretic peptide; Bnp, B-type natriuretic peptide; β-mhc, Beta-Myosin Heavy Chain; Myh7, Myosin heavy chain7; Myh6, Myosin heavy chain6; Il6, Interleukin-6; Icam1, Intercellular adhesion molecule-1; Serca2, Sarco/endoplasmic reticulum Ca^{2+}-ATPase2; Col3a1, Collagen type III alpha 1 chain; Col1a2, Collagen type I alpha 2; Xbp1, X-box binding protein 1; Ryr, Ryanodine receptor; Ncx1, Na^{+}/Ca^{2+} exchanger 1; Bax, BCL-2 Associated X; Bcl2, B-cell lymphoma 2; Grp78, 78-kDa glucose-regulated protein; Gapdh, Glyceraldehyde-3-phosphate dehydrogenase.

Western Blotting

Myocardial tissues were homogenized in RIPA buffer, and the protein content was determined by a BCA protein assay (Thermo Fisher Scientific, USA) as previously described (16, 21). The antibodies that were used (Abcam, Cambridge, MA, USA) were diluted at 1:1,000 and were as follows: SERCA2a antibody (ab3625), glucose regulated protein 78 (GRP78) antibody (ab21685), ryanodine receptor-2 (RYR2) antibody (ab2868), ryanodine receptor-phospho (RYR-P) antibody (ab59225), and Na^{+}/Ca^{2+} exchanger (NCX) antibody (ab2869). The protein bands were analyzed using densitometry by an imaging system (molecular imager, ChemiDoc XRS, Bio-Rad, USA). The examined protein levels were normalized to that of GAPDH. The results are represented by the ratios of the values of the experimental groups to those of the WT control group for GRP78, RYR, and SERCA2a.

Cardiac Histological and Morphometric Analysis

Animals were anesthetized with 1% pentobarbital (45 mg/kg) and cardinally perfused with cold PBS. The hearts were cut transversally, fixed in 4% neutral formalin, and embedded in paraffin. The 3 μm-thick sections were cut from the paraffin-embedded hearts. Sections were stained with hematoxylin-eosin (HE) and Sirius Red to analyze morphology and fibrosis (16, 22). To assess the cardiomyocyte cross-sectional area, the sections were stained with wheat germ agglutinin (WGA) as previously described (23). The body weight, heart weight, and tibia length were also measured.

Electron Microscopy

The specimens from the cardiac ventricles were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in serial acetone solutions, and embedded in Epon 812 resin. The ultrathin sections were stained with uranyl acetate and lead citrate solutions and then visualized with a JEOL 1230 transmission electron microscope (JEOL, Tokyo, Japan).

Isolation of Ventricular Myocytes

Mouse LV myocytes were isolated enzymatically by a protocol described previously (24). One hour after the myocyte pellet was suspended in KB solution, the cells were initially washed with Ca^{2+}-free Tyrode's buffer to remove the KB solution, and extracellular Ca^{2+} was slowly added to bring the concentration back up to 1.8 mM. Only calcium-tolerant, quiescent, and rod-shaped cells showing clear cross striations were used for Ca^{2+} and sarcomere shortening measurements.

Transient Cytosolic Ca^{2+} and Sarcomere Shortening Measurements

Myocytes were loaded with 2 μM fura-2AM for 20 min, and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix, North America, Milton, Massachusetts, USA). After loading,

the cells were washed and resuspended in Tyrode's solution and placed in the cell chamber, in which they were stimulated for 1–4 ms; then, they were superfused at 37°C and imaged with a Fluor-40 objective. Myocytes were then exposed to light that was emitted by a 75 W xenon lamp and passed through either a 340- or 380-nm wavelength filter. The emitted fluorescence was detected at 510 nm. The background fluorescence of each myocyte was determined by moving the myocyte out of the view and recording the fluorescence of the solution alone. Cell shortening was synchronously measured *via* transient calcium currents in myocytes. Soft Edge software (IonOptix, North America, Milton, Massachusetts, USA) was used to capture and analyze the changes in sarcomere length during cell shortening and lengthening.

Caffeine-Sensitive Ca^{2+} Release

Caffeine-sensitive Ca^{2+} release was assessed as described by Bassani et al. (25) to assess the SR (sarcoplasmic reticulum) calcium content and the contributions of SERCA and NCX to diastolic calcium removal. Myocytes were perfused with Tyrode's solution and stimulated at 1 Hz. Rapid application of 20 mM caffeine was employed to induce SR Ca^{2+} release and assess the contributions of NCX and the slow transport systems (mitochondrial Ca^{2+} uniporter and sarcolemmal Ca^{2+}-ATPase). Rapid changes in the superfusate were achieved using an ALA quick switch system to rapidly change the solution bathing the cell, thus allowing us to assess the immediate effect of caffeine on cell contracture. The decrease in fluorescence (F340/380) during caffeine-induced Ca^{2+} transient currents was attributable to NCX and slow transport system mechanisms. Because slow mechanisms remove only 0.5% of Ca^{2+} in mice, the contributions of these mechanisms are negligible (26). The decline in fluorescence during stimulated twitches was attributable to both SERCA and NCX. The contribution of SERCA was therefore evaluated as the difference between the decline in fluorescence during caffeine-induced Ca^{2+} transient currents and the decline in fluorescence during electrically stimulated twitches.

AT Transplantation and Plasma Biochemical Determination

Mouse AT was transplanted as described previously (16, 21). Animals were anesthetized with 1% pentobarbital sodium (45 mg/kg). Donor subcutaneous AT was divided into 100–150-mg pieces. A total of six to eight pieces of 900-mg grafts were implanted subcutaneously in the SKO or WT mice through incisions in the shaved skin of the back. Incisions were closed using 5-0 silk sutures. The SKO and WT control mice received the same operation except for AT transplantation. After surgery, the mice were housed individually for 1 week and then at 3–4 mice/cage.

Plasma insulin, leptin, and adiponectin levels were measured by ELISA (Linco Research). For glucose tolerance tests (GTT), mice that fasted for 4 h were given i.p. glucose (2 g/kg body weight; Abbott), and blood samples were collected before (time 0) and at 15, 30, 60, and 120 min after injection for glucose measurement (16, 21).

Statistical Analysis

All data are presented as the means ± SEM. Statistical comparisons between groups were performed using the 2-way ANOVA followed by the Tukey test or the Mann-Whitney *U*-test for non-parametric analysis. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The Seipin Gene Was Expressed in the Heart

Six-month-old C57BL/6 WT mice were used to analyze *Seipin* mRNA expression in different tissues ($n = 8$). *Seipin* was abundantly expressed in testis and AT, as described previously (16, 21). We also found a low mRNA expression level of *Seipin* in the heart, which was 10% of that in the testis (**Figure 1A**).

Seipin Gene Deficiency Promoted Myocardial Hypertrophy After TAC

SKO mice showed only mild cardiac hypertrophy until the age of 9 months (HW/BW: WT: 5.18 ± 0.41; SKO: 5.97 ± 0.78; $P < 0.05$). Therefore, we established a pressure overload myocardial remodeling model to accelerate cardiac remodeling by performing TAC surgery. The velocity of blood flow in the aortic arch ligation site was detected 3 days after the surgery and was found to be increased about 3-fold compared with that in the sham group (**Figures 1B,C**; $P < 0.001$). There was no significant difference concerning the flow velocity between SKO and WT mice before or after surgery (**Figures 1B,C**). These results indicate that the TAC model was successfully established, and there was no effect of genotypes on the flow velocity at the ligation site.

The result of the whole heart images showed the enlargement of the heart in WT and SKO mice at 12 weeks after TAC, and SKO mice had a more severe phenotype (**Figure 1D**). Significantly increased HW/TL ratios were detected in WT and SKO mice after TAC than in the sham groups (**Figure 1E**; $P < 0.01$ and $P < 0.001$, respectively). The HW/TL in SKO mice were significantly higher than those in WT mice after TAC (**Figure 1E**; $P < 0.01$). The heart tissue cross-sections showed alterations similar to those noted in **Figures 1D–F**.

Seipin Deficiency Induced Severe Cardiac Hypertrophy and LV Dysfunction After TAC

Echocardiography was performed at 2, 4, 8, and 12 weeks after TAC. The LVAW in diastole (d) and LVPWd were increased in both WT and SKO mice 2 weeks after TAC and reached a peak at 8 weeks. The LVAWd and LVPWd were greater in SKO-TAC mice than in WT-TAC mice at 2 weeks (**Figures 2A–C**; $P < 0.05$ and $P < 0.01$, respectively); they were increased by 12.2–14.5% at 8 weeks and by 8.3–6.23% at 12 weeks in SKO-TAC mice compared with those in WT-TAC mice, respectively (**Figures 2B,C**). The EF% was decreased at 12 weeks in SKO-TAC mice compared with that in SKO-sham mice (**Figure 2D**;

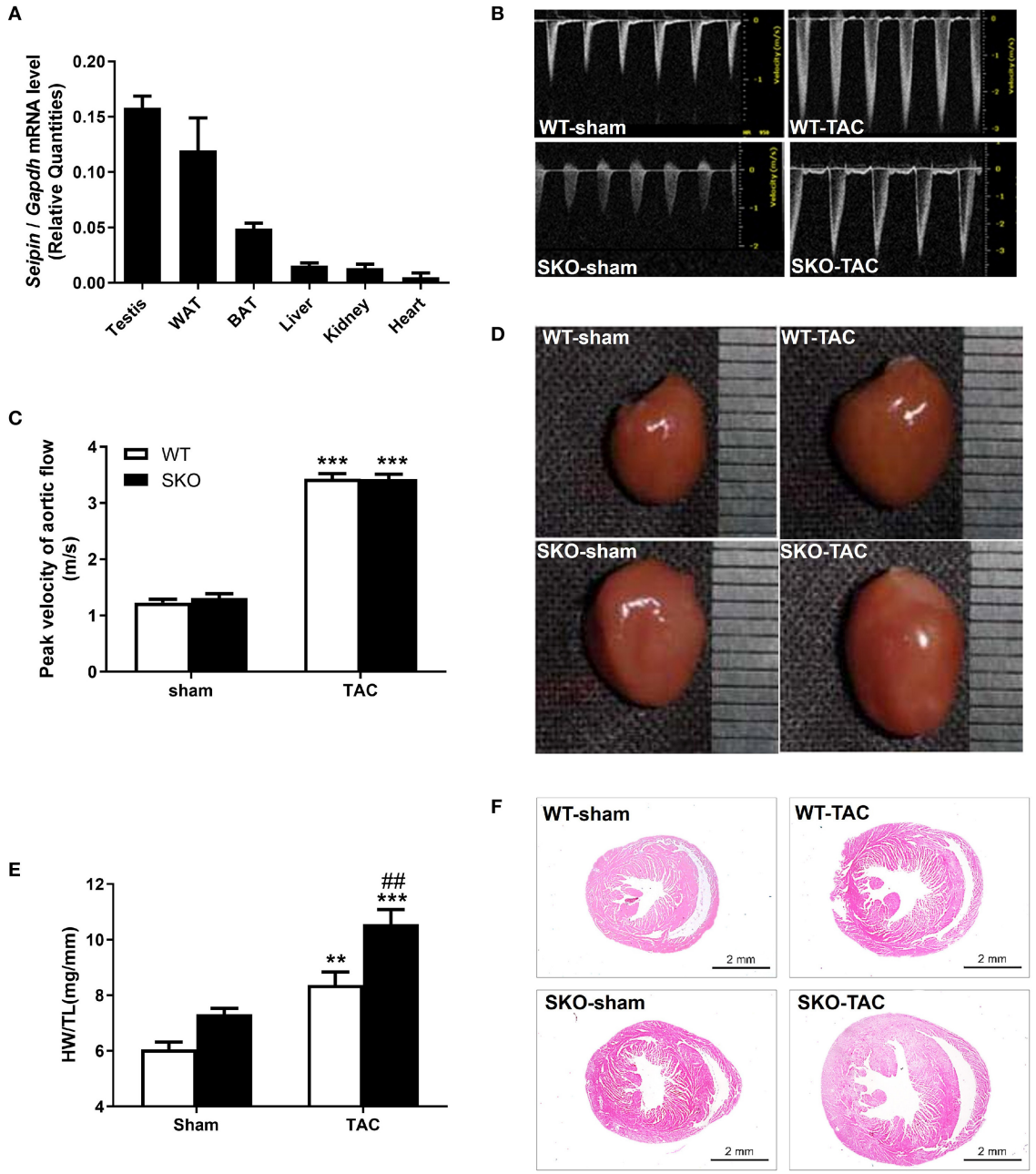

FIGURE 1 | *Seipin* mRNA expression in WT mice and *Seipin* gene deficiency promoted myocardial hypertrophy at 12 weeks after TAC. **(A)** *Seipin* mRNA expression levels in different tissues in 6-month-old C57BL/6 WT male mice; **(B)** Blood flow spectrum of the aorta; **(C)** Peak values of the blood flow velocity; **(D)** Representative photographs of the heart; **(E)** Ratio of heart weight/tibia length (HW/TL); **(F)** Hematoxylin–eosin staining of longitudinal sections of the heart. $^{**}P < 0.01$, $^{***}P < 0.001$, effect of TAC; $^{\#\#}P < 0.01$, effect of genotype. WAT, white adipose tissue; BAT, brown adipose tissue. WT, Wild-type; SKO, *Seipin* knockout; TAC, transverse aortic constriction.

$P < 0.01$). In contrast, no significant difference in the EF% was observed between SKO-TAC and WT-TAC mice (**Figure 2D**). The LVIDd and LVEDV were increased in both the SKO-TAC and WT-TAC groups compared with those in the sham groups at 12 weeks (**Figures 2E,F**), and the LVIDd in the SKO-TAC group was 7.3% higher than that in the WT-TAC group (**Figure 2E**; $P < 0.05$). Meanwhile, the LVEDV in the SKO-TAC group was 8.5% higher than that in the WT-TAC group at 12 weeks (**Figure 2F**; $P < 0.05$). The results of the evaluation of cardiac diastolic function showed that the E/A and

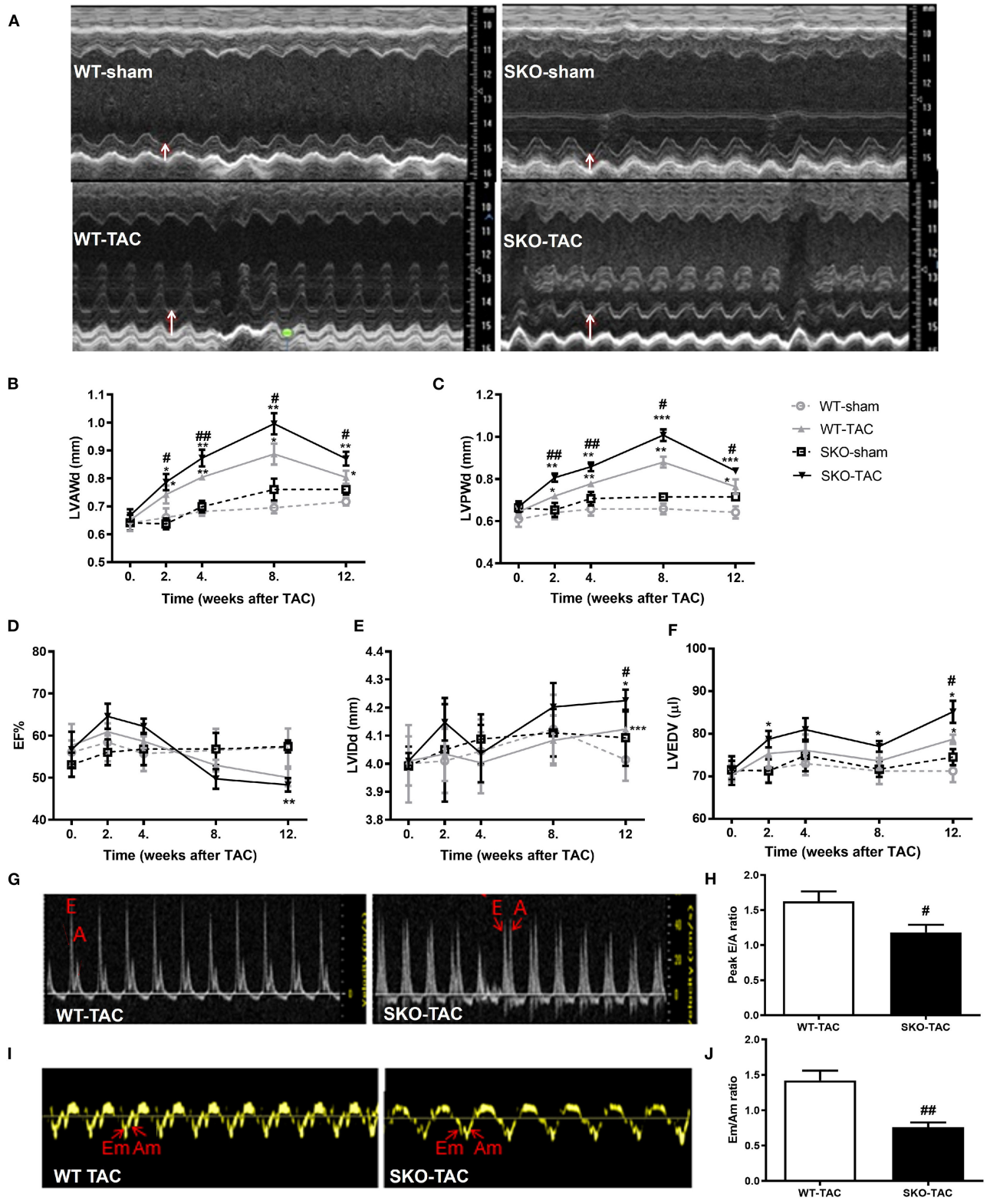

FIGURE 2 | *Seipin* deficiency induced severe cardiac hypertrophy and left ventricular dysfunction at 12 weeks after TAC by echocardiography. **(A)** Representative M-mode echocardiographs; white arrows represent the left ventricular posterior wall thickness (LVPWd); **(B)** left ventricular anterior wall thickness in diastole (LVAWd); **(C)** LVPWd; **(D)** ejection fraction (EF%); **(E)** left ventricular internal diameter in diastole (LVIDd); **(F)** left ventricular end-diastolic volume (LVEDV); **(G)** Representative transmitral Doppler flow profile; **(H)** Peak E/A ratio; **(I)** Representative Doppler images of the basal inferolateral LV wall; and **(J)** Em/Am ratio.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, effect of TAC; #$P < 0.05$, ##$P < 0.01$, effect of genotype. WT, Wild-type; SKO, *seipin* knockout; TAC, transverse aortic constriction, late diastolic mitral transmitral flow velocity (A), early diastolic mitral transmitral flow velocity (E), late diastolic velocity (Am), and early diastolic velocity (Em).

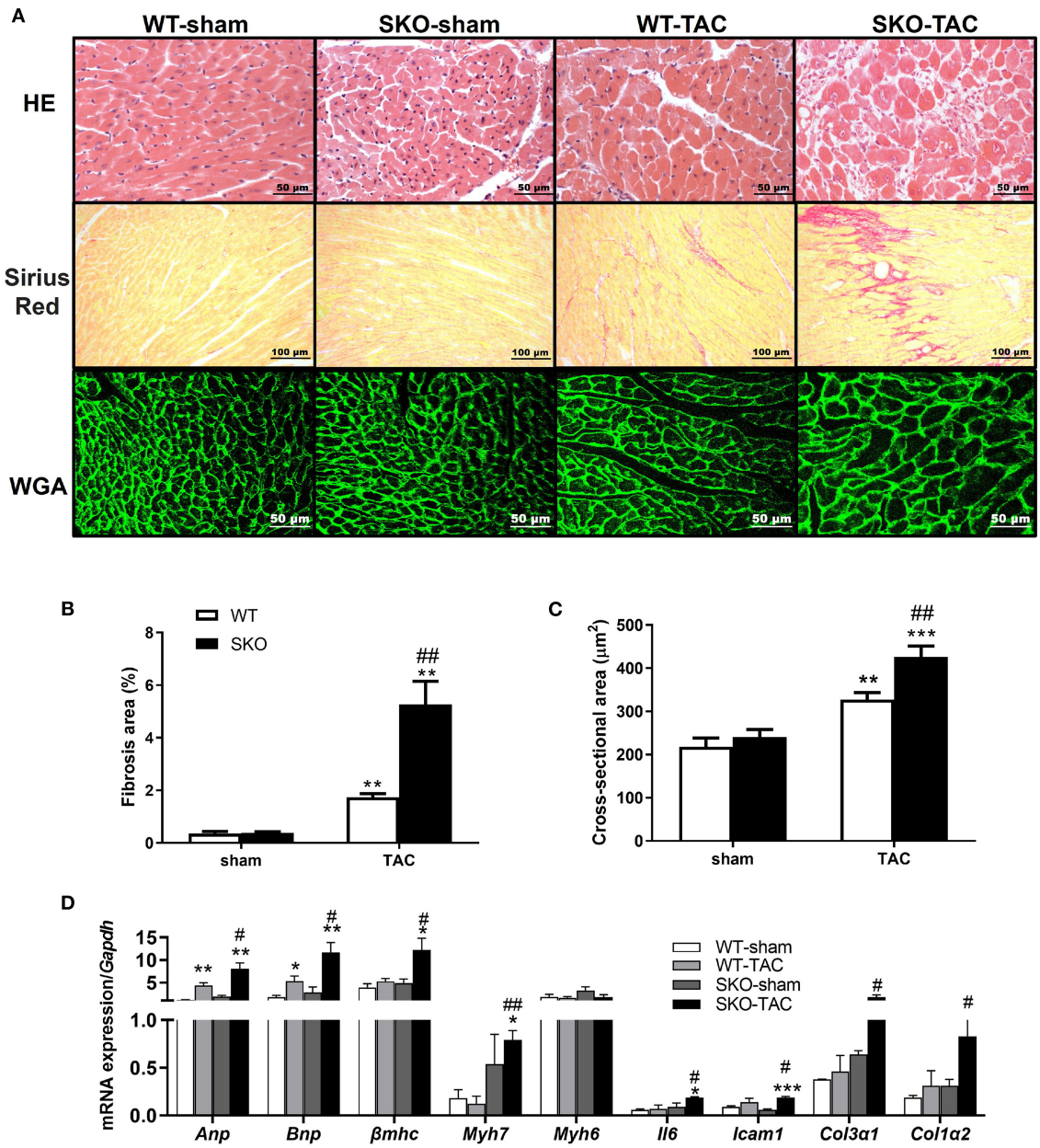

FIGURE 3 | *Seipin* deficiency accelerated cardiomyocyte hypertrophy and inflammation and fibrosis in the heart after TAC. **(A)** Hematoxylin-eosin (HE), Sirius red and wheat germ agglutinin (WGA) staining of the heart cross-section; **(B)** Percentage of fibrosis area in heart tissue by Sirius red staining; **(C)** Quantification of cardiomyocyte cross-sectional area by WGA staining; **(D)** mRNA expression levels of *Anp*, *Bnp*, *βmhc*, *Myh7*, *Myh6*, *Il6*, *Icam1*, *Col3α1*, and *Col1α2*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, effect of TAC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, effect of genotype. WT, Wild-type; SKO, *Seipin* knockout; TAC, transverse aortic constriction.

Em/Am peaks were inversed (**Figures 2G–J**); that is, the ratios of E/A and Em/Am were <1.0 in SKO-TAC mice, and were decreased significantly than in WT-TAC mice (**Figures 2H–J**; $P < 0.05$ and $P < 0.01$, respectively). This indicated that *Seipin* deficiency promoted diastolic HF after TAC in mice. Compared with WT mice, SKO mice showed a more obvious left ventricular hypertrophy and diastolic HF at 12 weeks after TAC.

Seipin Deficiency Accelerated Cardiomyocyte Hypertrophy, Inflammation, and Fibrosis in the Heart After TAC

Compared with the WT-TAC group, the SKO-TAC group showed a more extensive myocardial fiber disarray and fracture and increased inflammatory cell infiltration in the heart by HE straining (**Figure 3A**). Sirius red staining showed increased fibrosis areas in both WT (**Figures 3A,B**; $P < 0.01$) and SKO

(**Figures 3A,B**; $P < 0.01$) mice after TAC, moreover, fibrosis areas were increased in SKO-TAC mice compared with WT-TAC mice (**Figures 3A,B**; $P < 0.01$). The cross-sectional area of myocytes was measured in WGA stained LV myocardium. The mean cardiomyocyte cross-sectional areas were increased in both WT and SKO mice after TAC (**Figures 3A–C**; $P < 0.01$ and $P < 0.001$, respectively). Moreover, the cardiomyocyte cross-sectional areas were significantly greater in the SKO-TAC group than those in the WT-TAC group (**Figures 3A–C**; $P < 0.01$).

The relative expression levels of heart failure related genes, such as *Anp* and *Bnp*, were significantly increased after TAC in both the WT (**Figure 3D**; $P < 0.01$ and $P < 0.05$, respectively) and SKO (**Figure 3D**; $P < 0.001$ and $P < 0.01$, respectively) groups and were higher in the SKO-TAC group than in the WT-TAC group (**Figure 3D**; $P < 0.05$). The relative expression levels of cardiac hypertrophy-related genes, such as *βmhc* and *Myh7*, were increased in both groups after TAC and were higher in the SKO-TAC group than in the WT-TAC group (**Figure 3D**; $P < 0.05$ and $P < 0.01$, respectively). The relative expression levels of inflammatory factor genes, such as *Il6* and *Icam1*, were significantly higher in the SKO group after TAC (**Figure 3D**; $P < 0.05$ and $P < 0.001$, respectively) and were higher in the SKO-TAC group than in the WT-TAC group (**Figure 3D**; $P < 0.05$). The relative expression levels of fibrosis-related genes, such as *Col3α1* and *Col1α2*, were significantly increased in SKO-TAC mice compared with those in WT-TAC mice (**Figure 3D**; $P < 0.05$). Taken together, these results indicate that *Seipin* deficiency accelerated cardiomyocyte hypertrophy, inflammation, and fibrosis after TAC in mice.

Seipin Deficiency Aggravated Myocardial Ultrastructure Changes by Increasing Endoplasmic Reticulum Stress and Apoptosis After TAC

Electron microscopy analysis detected hypertrophy and disordered arrangement of mitochondria in WT mice at 12 weeks after TAC. In addition to the previously described changes, there was hyperplasia, partial disappearance of the ridge, obvious sarcoplasmic reticulum dilatation, and compression deformation of the transverse tube (TT), which reduced the size of the transverse tube-sarcoplasmic reticulum (TT–SR) dyadic junctions in SKO-TAC mice (**Figure 4A**).

The relative gene expression levels of the apoptotic factor *Bax* were increased in WT after TAC (**Figure 4B**; $P < 0.05$) and were higher in SKO mice than in WT mice with or without TAC (**Figure 4B**; $P < 0.01$). The gene expression levels of the anti-apoptotic factor *Bcl2* were decreased in both WT and SKO mice after TAC (**Figure 4C**; $P < 0.05$ and $P < 0.001$, respectively) and were much lower in the SKO-TAC group than in the WT-TAC group (**Figure 4C**; $P < 0.001$).

We then detected the changes in the relative expression of ER stress factors in the hearts of SKO mice caused by pathological changes of the sarcoplasmic reticulum. The gene expression levels of *Grp78* were significantly increased in SKO-TAC mice compared with those in SKO and WT-TAC mice (**Figure 4D**; $P < 0.05$). The gene expression levels of *Xbp1* were significantly increased in SKO-TAC mice compared with those in SKO and WT-TAC mice (**Figure 4E**; $P < 0.001$ and $P < 0.01$, respectively). The protein levels of GRP78 were also elevated in the SKO-TAC group compared with that in the sham operation control (**Figures 4F,G**; $P < 0.001$) and the WT-TAC group (**Figures 4F,G**; $P < 0.01$). These results showed that *Seipin* deficiency induced myocardial ER stress in mice after TAC.

Taken together, these results revealed that *Seipin* deficiency induced obvious pathological changes in the mitochondria and sarcoplasmic reticulum in cardiomyocytes after TAC, which might be associated with ER stress and apoptosis.

Seipin Deficiency Resulted in Abnormalities in Calcium Handling in Ventricular Myocytes

Ca^{2+} handling plays an important role in maintaining the function of myocytes (27). We therefore detected myocardial Ca^{2+} handling-related gene and protein expression. The gene expression levels of *Ryr* and *Serca2a* were significantly decreased in the SKO-TAC group compared with those in the SKO-sham and WT-TAC groups (**Figure 5A**; $P < 0.05$). The ratio of phospho-RYR to RYR was increased in SKO-TAC mice compared with those in SKO and WT-TAC mice (**Figures 5B,C**; $P < 0.001$ and $P < 0.01$, respectively). The protein levels of SERCA2a were considerably decreased in SKO mice after TAC (**Figures 5B,D**; $P < 0.05$) and were lower in SKO-TAC mice than in WT-TAC mice (**Figures 5B,D**; $P < 0.05$). There were no significant differences in NCX gene and protein expression levels among the four groups (**Figures 5A,B,E**).

To understand the alteration of Ca^{2+} handling and E-C coupling in ventricular myocytes, we measured Ca^{2+} transient currents and sarcomere shortening in SKO LV myocytes. We found that the Ca^{2+} transient current decline time constant (tau) was significantly lengthened in the SKO group compared with that in the WT group (**Table 2**). To understand the mechanism underlying the decrease in the capacity of Ca^{2+} decline in SKO myocytes, we assessed the caffeine-induced Ca^{2+} release peak height (phcaff) and diastolic Ca^{2+} removal by measuring the decline in fluorescence (F340/380) during caffeine-induced Ca^{2+} transient currents (taucaff) and Ca^{2+} transient currents during stimulated twitches (tautwitch) (at 1 Hz). Our results showed that taucaff was 2.690 ± 0.187 s for WT myocytes and 3.970 ± 0.189 s for SKO myocytes (**Figure 5F**; $P < 0.01$). In addition, phcaff was 0.50 ± 0.03 RU (ratio unit) for WT myocytes and 0.57 ± 0.02 RU for SKO myocytes (**Figure 5G**; $P < 0.05$). Slow Ca^{2+} transient current decay was also detected in SKO myocytes (**Figure 5H**; $P < 0.01$). The reduction in NCX function might have contributed to the slowing of Ca^{2+} transient current decay and the increase in SR Ca^{2+} content in SKO myocytes, which led to Ca^{2+} transport dysfunction in SKO ventricular myocytes (**Figures 5F–H**).

AT Transplantation Could Not Rescue Cardiac Hypertrophy After TAC in SKO Mice

Previous studies have been shown that AT transplantation can effectively ameliorate hepatic steatosis and renal injury in

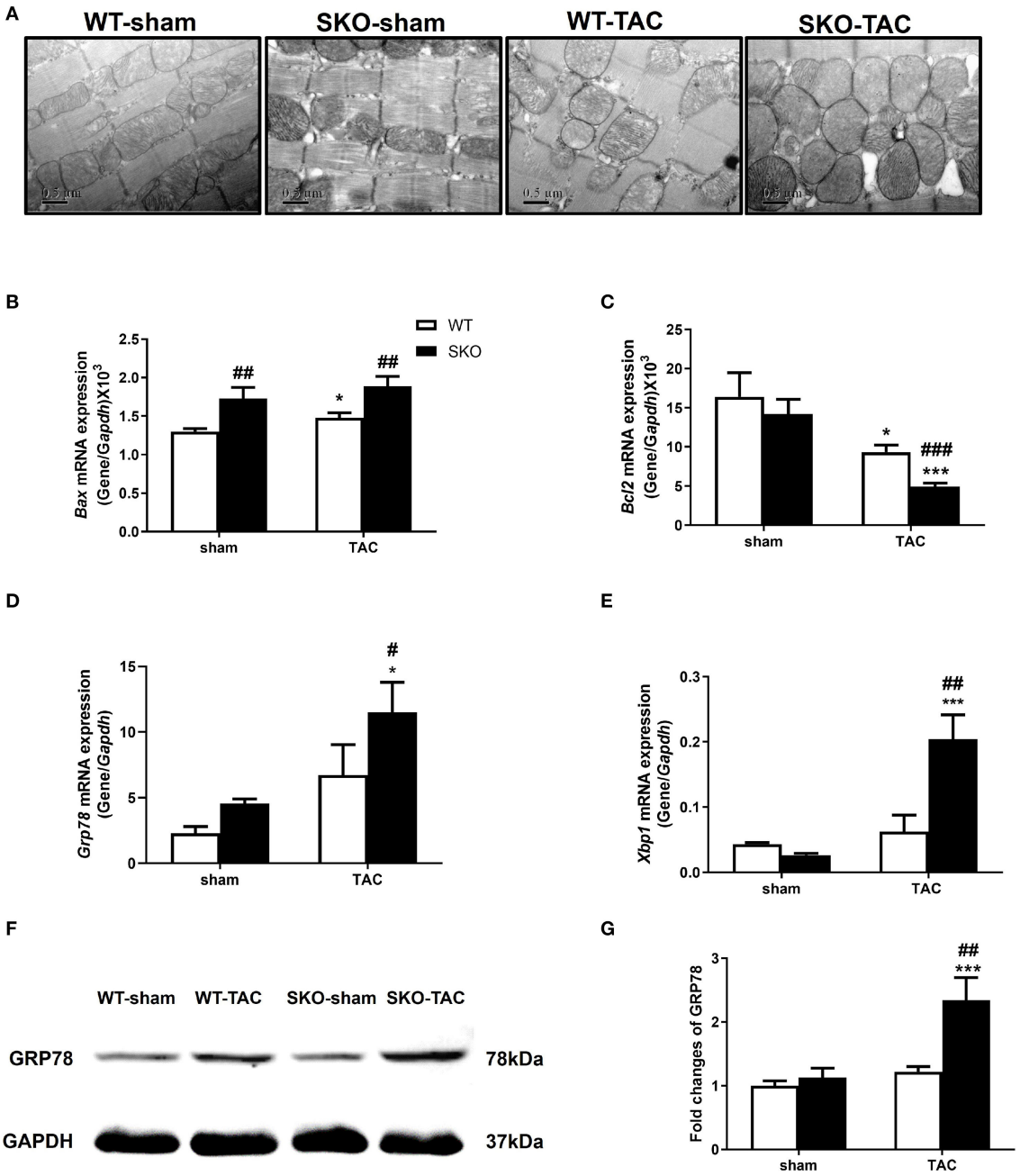

FIGURE 4 | *Seipin* deficiency aggravated myocardial ultrastructure changes and increased endoplasmic reticulum stress and apoptosis after TAC. **(A)** Representative cardiac electron microscopy images (original magnification, 40,000×); **(B–E)** mRNA expression levels of *Bax, Bcl2, Grp78, and Xbp1,* in the heart; **(F,G)** Western blot analysis of GRP78 and densitometric quantitation. The results are represented by the ratio of the values of the experimental groups to those of the WT control group. $^{*}P < 0.05$, $^{***}P < 0.001$, effect of TAC; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, effect of genotype. WT, Wild-type; SKO, *Seipin* knockout; TAC, transverse aortic constriction.

SKO mice (16, 21). Therefore, in this study, we investigated whether transplantation of normal AT can ameliorate cardiac hypertrophy in SKO mice. More severe cardiac hypertrophy was observed in SKO mice than in WT mice at 12 weeks after TAC, the HW/TL and echocardiography results were similar to those we detected previously in this study. However, AT transplantation did not rescue cardiac hypertrophy in SKO mice at 12 weeks after TAC (**Figures 6A–D**).

GTT and plasma insulin levels indicated that SKO mice showed impaired glucose tolerance and hyperinsulinemia, and AT transplantation significantly improve these two parameters (**Figures 6E,F**). Loss of AT in SKO mice induced remarkably low

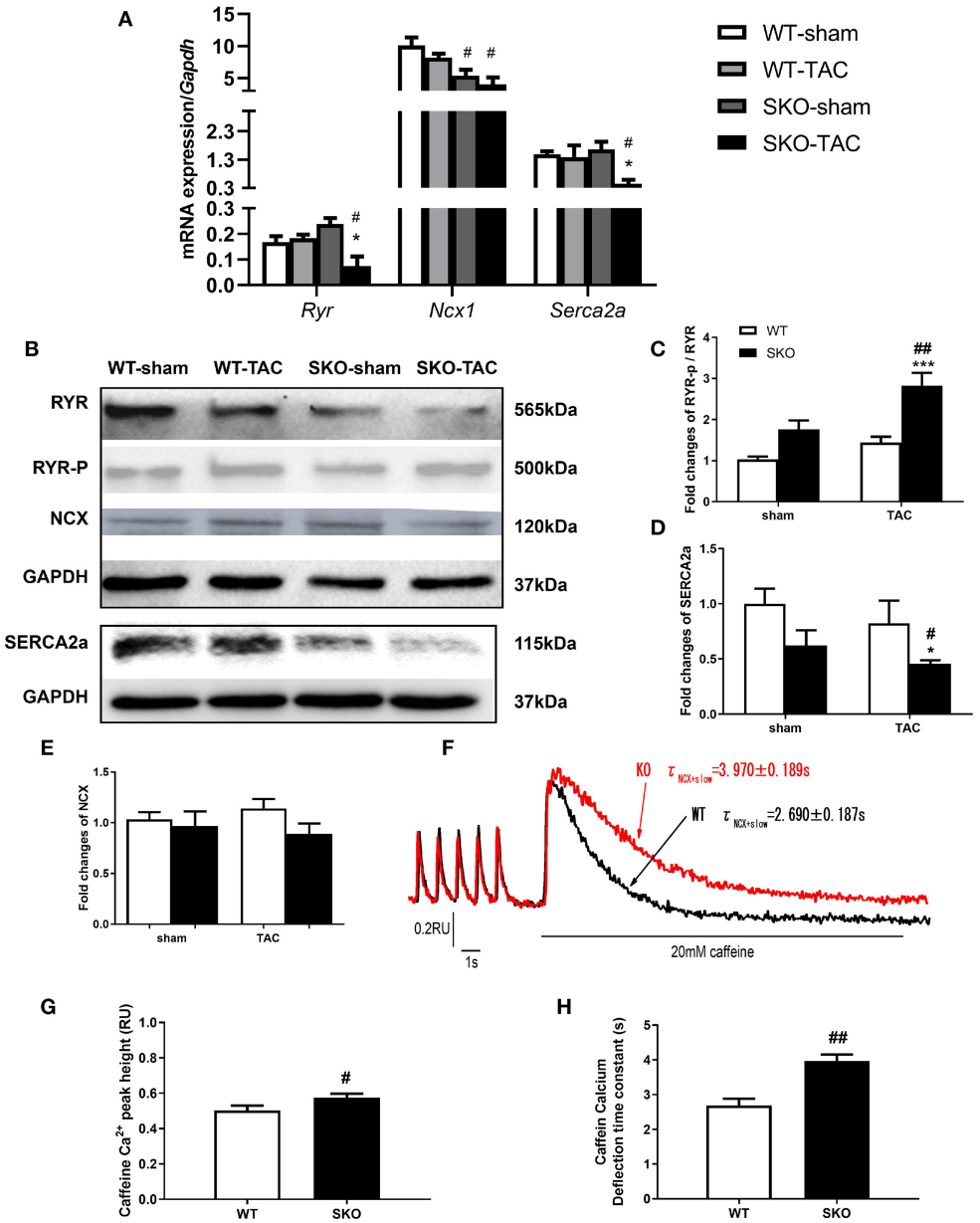

FIGURE 5 | *Seipin* deficiency induced abnormalities in the calcium handling of ventricular myocytes. **(A)** mRNA expression levels of *Ryr, Ncx1*, and *Serca2a*; **(B–E)** Western blot analysis of RYR, SERCA2a, and NCX and densitometric quantitation; **(F)** Curve plot of stimulation-induced and caffeine-induced Ca^{2+} transient current fluorescence (F340/F380, RU); **(G)** Peak height of caffeine-induced Ca^{2+} release (indicated by the SR Ca^{2+} content); **(H)** Caffeine-induced Ca^{2+} transient current deflection time constant (taucaff) (indicating the functionality of NCX in removing diastolic Ca^{2+}). The results are represented as the ratios of the values in the experimental groups to those of the WT control group for RYR and SERCA2a.*$P < 0.05$, ***$P < 0.001$, effect of TAC; #$P < 0.05$, ##$P < 0.01$, effect of genotype. WT, Wild-type; SKO, *Seipin* knockout; TAC, transverse aortic constriction.

plasma leptin levels and adiponectin, which are two important cytokines (**Figures 6G,H**; $P < 0.001$). Plasma leptin levels were rescued in SKO mice after AT transplantation (**Figure 6G**; $P < 0.001$), and plasma adiponectin levels remained unchanged after AT transplantation as described in the previous studies (**Figure 6H**).

DISCUSSION

In this study, we found that *Seipin* deficiency could promote cardiac hypertrophy and diastolic heart failure after TAC in mice. These changes may be related to the impairment of myocardial calcium handling, ER stress, inflammation, and apoptosis.

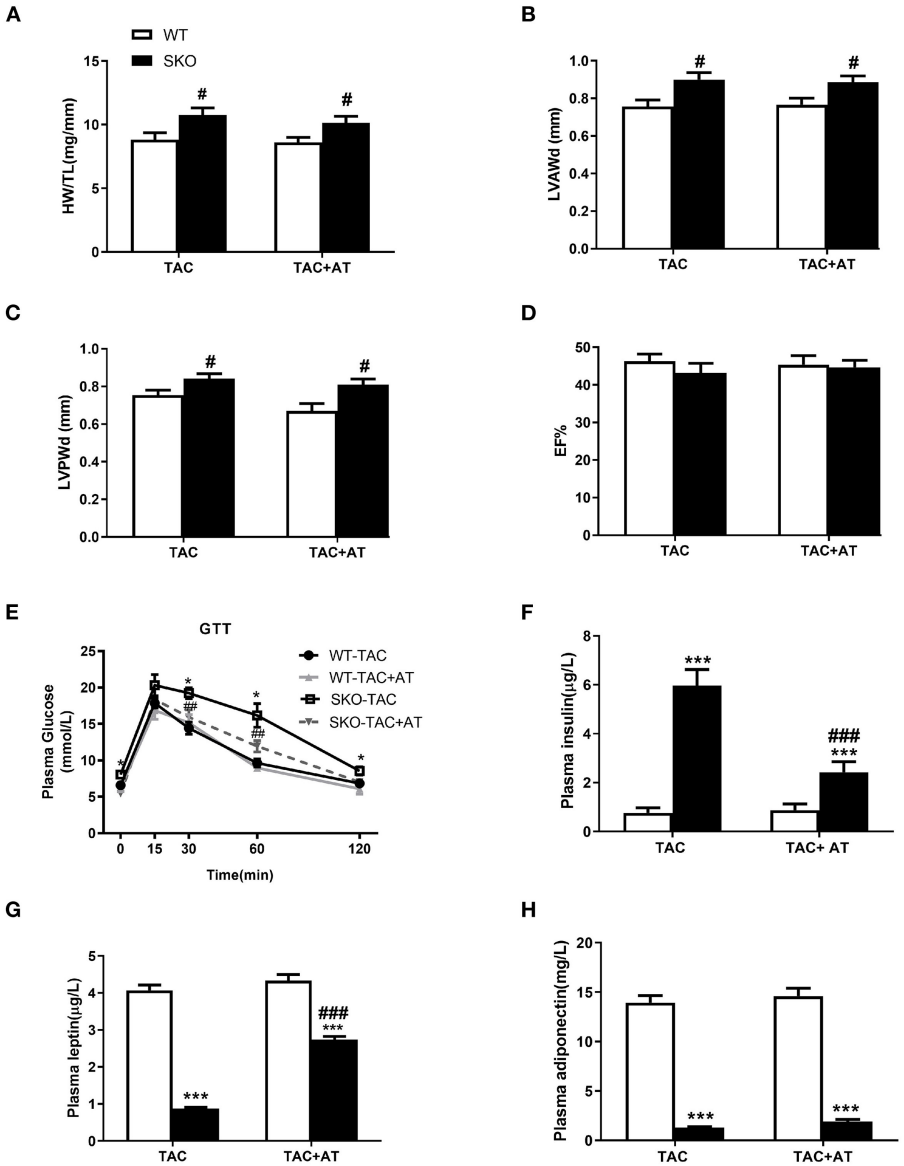

FIGURE 6 | AT transplantation did not rescue the cardiac hypertrophy after TAC in *seipin* deficient mice and improved of insulin resistance. **(A)** Ratio of heart weight/tibia length (HW/TL); **(B)** Left ventricular anterior wall thickness in diastole (LVAWd); **(C)** Left ventricular posterior wall thickness in diastole (LVPWd); **(D)** Ejection fraction (EF %); **(E)** Glucose tolerance test (GTT) results; **(F)** plasma levels of insulin; **(G)** leptin; and **(H)** adiponectin. $^{*}P < 0.05$, $^{***}P < 0.001$, effect of AT transplantation; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, effect of genotype. AT, Adipose tissue; WT, Wild-type; SKO, *Seipin* knockout; TAC, transverse aortic constriction.

Previous clinical studies on *Seipin* deficiency have mainly focused on metabolic disorders. However, an increasing number of clinical studies have shown that *Seipin* mutation or deletion could lead to cardiac hypertrophy and heart failure, which is one of the leading causes of death in patients (3). However, the exact mechanism underlying the effect of *Seipin* on cardiac remodeling is not clear.

It is well-known that an imbalance in calcium homeostasis leads to heart failure (13, 28, 29). Therefore, we examined myocardial Ca^{2+} channel-related gene expression in this study. Compared with the sham-operated group, the SKO-TAC group showed decreased *Serca2a*. A previous study showed that SEIPIN has a close relationship with SERCA2a in adipocytes in Drosophila (14). A binding site of SERCA2a was found on the circular domain of human SEIPIN. The knockdown of *Seipin* in adipocytes in Drosophila inhibits the activity of SERCA2a, leading to an impaired Ca^{2+} transport from the cytosol to the endoplasmic reticulum and activation of endoplasmic reticulum stress metabolism, finally promotes lipid storage in Drosophila fat cells (14, 30).

Reduction of *Serca2a* plays an important role in diastolic heart failure in patients (31, 32). In this study, E/A and Em/Am

TABLE 2 | Calcium transient currents and sarcomere shortening in ventricular myocytes of SKO mice; $[Ca^{2+}]i$, cytosolic Ca^{2+} concentration.

	WT ($n = 39$)	SKO ($n = 64$)
Calcium transients		
Baseline, RU	0.81 ± 0.02	0.82 ± 0.01
Departure velocity	23.73 ± 1.16	24.76 ± 0.79
Peak height, RU	0.32 ± 0.01	0.31 ± 0.01
Time to peak, ms	33.60 ± 1.08	35.23 ± 1.09
Time to 50% peak, ms	−1.77 ± 0.09	−1.78 ± 0.06
Return velocity	10.51 ± 0.25	10.69 ± 0.27
Ca_i^{2+} decay time constant, ms	233.07 ± 7.79	267.15 ± 9.60*
Sarcomere shortening		
Baseline, μm	1.72 ± 0.01	1.75 ± 0.01
Departure velocity	−1.30 ± 0.07	−1.26 ± 0.07
Fractional shortening, %	3.09 ± 0.17	3.19 ± 0.17
Time to peak, ms	100.19 ± 4.17	102.71 ± 3.15
Time to 50% peak, ms	0.55 ± 0.05	0.60 ± 0.06
Return velocity	36.16 ± 1.54	38.09 ± 1.26
Time to 50% relaxation, ms	90.66 ± 4.91	89.29 ± 4.03

WT, Wild-type; SKO, Seipin knockout. $^*P < 0.05$, *compared with the WT.*

were decreased significantly, and ventricular cavity enlargement indicated that the mice had diastolic dysfunction. These observations may be related to a decrease in the expression level of the SERCA2a (**Figures 5A,D**). In our *in vitro* experiments, it was found that the attenuation of Ca^{2+} transient currents in myocardial cells in SKO mice was slowed (**Figure 5H**), and the calcium influx decreased. A decrease in the amplitude of depleted cardiac calcium transient currents and reduced decay rates may be associated with slowing of the SERCA2a-mediated calcium transport. When SERCA2a function is inhibited, the transfer of Ca^{2+} to the endoplasmic reticulum is reduced, which may lead to a decrease in RYR (33, 34). In our study, the reduction of the *Ryr* gene expression in the hearts of SKO-TAC mice may be related to the decline in *Serca2a*.

SEIPIN is an ER membrane protein. As described previously, *Seipin* inhibits SERCA2a activity, leads to an impaired Ca^{2+} transport from cytosol to ER and actives ER stress metabolism in Drosophila fat cells (14). Another recent study has shown that the depletion of *Seipin* in the liver induces an increase in intracellular triglyceride *via* the activation of ER stress by influencing the intracellular calcium level (35). These two studies indicated that a deficiency of *Seipin* could induce ER stress due to impaired Ca^{2+} transport. In our study, we found that expanded sarcoplasmic reticulum and increased levels of ER stress related factors in the heart of SKO mice compared to WT mice after TAC (**Figures 4A,D,G**). ER stress plays an important role in heart failure. We suggested that increased ER stress induced by impaired Ca^{2+} transport aggravated heart failure in SKO mice. ER stress could also induce impaired Ca^{2+} transport. The causal relationship between the two factors needs further study.

In this study, myocardial apoptosis increased in SKO mice after TAC (**Figures 4B,C**). It has been reported that ER stress in the myocardium can cause apoptosis and inflammation of cardiomyocytes (36). Our study suggested that increased ER stress led to myocardial apoptosis in SKO mice.

Diastolic heart failure is usually related to metabolic cardiomyopathy (37). The SKO mice created by our group presented a severe loss of AT, fatty liver, and IR without hypertriglyceridemia (38). To study whether the adipokines play a role in diastolic heart failure due to *Seipin* deficiency, we transplanted AT from WT mice into SKO mice (16, 21). IR was rescued by AT transplantation as previously described (21). However, cardiac hypertrophy in SKO AT-transplanted mice was not rescued compared with that in untreated SKO mice after 3 months of TAC (**Figure 6**). Our results suggested that heart failure in SKO mice might be associated with mechanisms other than IR. Certainly we cannot rule out that the amount of transplanted fat was not sufficient to reverse heart failure, although the IR has been significantly improved with increasing plasma leptin levels. Hypoadiponectinemia in SKO mice was not improved by AT transplantation, and this result was confirmed by two previous studies (16, 39). A previous study has shown that lack of adiponectin exacerbates myocardial hypertrophy and leads to diastolic dysfunction (40). Therefore, hypoadiponectinemia might be involved in myocardial hypertrophy in the SKO mice. To clarify this problem, we could conduct replacement therapy with adiponectin in the SKO mice. Certainly, there are many additional adipokines that might also be involved in myocardial hypertrophy. The exact mechanisms require further investigation.

In a previous study, spontaneous cardiac hypertrophy and diastolic heart failure were found in 14-week-old SKO mice (41). This demonstrated that glucotoxicity with increased myocardial glucose uptake can trigger cardiac dysfunction. In our experiments, SKO mice showed no cardiac hypertrophy at 4 months, as was demonstrated in a previous study (38), and showed only mild cardiac hypertrophy at 9–10 months. These results are different from those of the Joubert et al. study (41). A review summarized the diverse phenotypes of SKO mice in the different groups in terms of body weight, food intake, plasma glucose, and organomegaly. The SKO mice created by our group had mild hyperglycemia (160 mg/dl) in fasted conditions and showed no difference after feeding (42). The exact mechanisms are unknown. No glycogen or lipid deposition in the myocardium was found in SKO-TAC mice (results not shown). In autopsies of patients with *Seipin* gene mutations or defects, the diseased myocardial tissue showed no deposition of glycogen and lipids (43, 44).

SKO mice manifest severe hepatic steatosis (21, 38), whereas liver-specific SKO mice do not exhibit hepatic steatosis (45). Interestingly, AAV-mediated *Seipin* overexpression in the liver alleviated high-fat diet-induced hepatosteatosis in mice by increasing mitochondrial activity (35). A study has shown that no heart failure was found in cardiac-specific *Seipin* gene deficient mice (46). It would be interesting to know whether *Seipin* overexpression in the heart can improve cardiac hypertrophy, and this might provide particularly valuable evidence on *Seipin* gene function in cardiac hypertrophy.

The PI3K/AKT and JAK/STAT3 signaling pathways were activated in the heart tissue of SKO mice (46, 47). Chronic activation of the p38 MAPK pathway associated with apoptotic

cell death was observed in the *Seipin*-knockdown adipocytes (48). The JNK pathway was activated in the hippocampus of the SKO mice (49). It would be interesting to know whether the p38 MAPK and CHOP/JNK pathways are involved in cardiac hypertrophy in SKO mice. The exact mechanisms should be further investigated.

In conclusion, we demonstrated that *Seipin* gene deficiency induced decreased SERCA2a and impaired Ca^{2+} transport, increased ER stress, myocardial inflammation, and apoptosis in heart, and finally increased diastolic heart failure. This experiment provides clues for studying the mechanism of heart failure caused by *Seipin* gene defects. The initial cause needs further study.

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 animal study was reviewed and approved by Institutional Animal Care Research Advisory Committee of Peking University Health Science Center.

AUTHOR CONTRIBUTIONS

XW and WH designed the project. WH supervised the project. XW, XL, HW, ZZ, LZ, and XS performed the experiments. XW and XL analyzed the data and wrote the manuscript. CY, ZL, YW, and YZ provided technical advice. XW, XX, GL, and WH revised the manuscript. All authors contributed to the article and approved the submitted version.

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

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BRIEF RESEARCH REPORT
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Different Lipid Parameters in Predicting Clinical Outcomes in Chinese Statin-Naïve Patients After Coronary Stent Implantation

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Background: Low-density lipoprotein cholesterol (LDL-C) is a critical surrogate outcome for cardiovascular disease (CVD). Recent observational studies identified different predictive lipid parameters, but these have not been fully validated in the Chinese population. This study aimed to compare the predictive value of lipid parameters for cardiovascular outcomes in Chinese statin-naïve patients who underwent percutaneous coronary intervention (PCI).

Methods: We retrospectively recruited statin-naïve patients who underwent PCI for stable angina and acute coronary syndrome at Sichuan Provincial People's Hospital between 1 January 2016 and 31 December 2017. A follow-up was conducted via outpatient visits or telephone. We divided patients into three groups based on lipid parameter tertiles. We calculated the hazard ratios (HRs) of the highest and lowest tertiles for major adverse cardiovascular events (MACEs) using multivariate Cox proportional hazards regression. We compared the association strength of lipid parameters with MACEs using the HR of non-LDL-C lipid parameters relative to LDL-C.

Results: Among 445 included patients, the highest LDL-C, LDL-C/high-density lipoprotein cholesterol (HDL-C), atherosclerosis index, and non-HDL-C level tertiles were associated with an average increase of 165% (HR 2.65, confidence interval [CI] 1.26 to 5.61; $P = 0.01$), 324% (HR 4.24, CI 1.89 to 9.52; $P < 0.001$), 152% (HR 2.52, CI 1.22 to 5.22; $P = 0.01$), and 125% (HR 2.25, CI 1.09 to 4.64; $P = 0.01$) in the hazard of composite CVD, respectively. Lipoprotein (a) levels did not show a significant association with the endpoints. Except for LDL-C/HDL-C, different lipid parameter HR ratios were <1.0; none were statistically significant.

Conclusion: Compared with non-LDL-C lipid parameters, LDL-C acts better predictive value for cardiovascular outcomes in general Chinese statin-naïve post-PCI patients.

Keywords: lipid profile, percutaneous coronary intervention, coronary artery disease, predictive factor, low-density lipoprotein

INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of death globally (1). Post-percutaneous coronary intervention (PCI) patients represent a special CVD population (2), as they require secondary prevention for coronary heart disease (3). Predicting further CVD risk in such populations is critically relevant for clinical decision making, and novel factors that are potentially associated with CVD risk are being investigated (4). Lipid profiles are the most critical factors for CVD prevention, and low-density lipoprotein cholesterol (LDL-C) is a key risk factor for CVD (5, 6). Recent studies suggest that different lipid parameters, including lipoprotein (a), are strong predictors of CVD and perform better in patients receiving statin therapy than others (7–9). Atherogenic index of plasma is easy and reflects the small dense LDL, which is feasible for prevention and control of cardiovascular diseases in a community population and a strong marker for CAD in postmenopausal women (10). However, no studies have directly compared the predictive value of different lipid parameters in the Chinese population. Most previous studies enrolled patients taking various doses of statins and patients not taking statins. Such mixed populations lead to complicated confounding that cannot be fully adjusted for using statistics. In the current study, we recruited patients who underwent PCI and who had not previously received statins or other lipid-lowering treatments. This study aimed to investigate the value of recent different lipid parameters to predict cardiovascular outcomes.

MATERIALS AND METHODS

Study Population

This study retrospectively recruited statin-naïve patients underwent PCI for stable angina, and acute coronary syndrome at Sichuan Provincial People's Hospital, Chengdu, China, between 1 January 2016 and 31 December 2017. We only included patients who had not used statins or other lipid-lowering drugs within 3 months before admission, and excluded patients with incomplete baseline or contact information for follow-up, or died not due to cardiovascular cause during initial hospitalization. Follow-up data were collected until 30 May 2020. The study was approved by the Biomedical Ethics Committee of Sichuan Provincial People's Hospital. Informed consent was obtained from all patients.

Demographic and Clinical Characteristics

We conducted a chart review to collect data including the demographic information, comorbidities, body weight, height, and blood pressure data. Body mass index (BMI) was calculated as follows: body weight (kg) / height square (m^2). We identified patients with hypertension as those who took antihypertensive drugs before admission or who were diagnosed with hypertension during hospitalization and patients with diabetes mellitus as those who took anti-diabetic agents or who were diagnosed with diabetes mellitus at hospital.

Laboratory Examination

Laboratory data were collected, including total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), LDL-C, lipoprotein (a), apolipoprotein B, apolipoprotein A-I, alanine aminotransferase, aspartate aminotransferase, uric acid, homocysteine, thyroid-stimulating hormone, total triiodothyronine, free triiodothyronine, total thyroxine, free thyroxine, and serum creatinine. The estimated glomerular filtration rate was calculated using the Chronic Kidney Disease Epidemiology Collaboration formula (11), and chronic kidney disease was defined as an estimated glomerular filtration rate of <60 mL/min/1.73 m^2. Non-HDL-C was calculated as follows: total cholesterol—HDL-C (12). Atherosclerosis index was calculated as follows: (total cholesterol—HDL-C)/HDL-C (10). The atherogenic index of plasma was defined as the base-10 logarithm of the ratio of the concentration of triglyceride to HDL-C (10). The lipoprotein combine index was defined as the ratio of the product of total cholesterol, triglyceride, and LDL-C to HDL-C (13).

Angiographic Data

Angiographic data were obtained from the image reporting system. Coronary severity was assessed using the Gensini

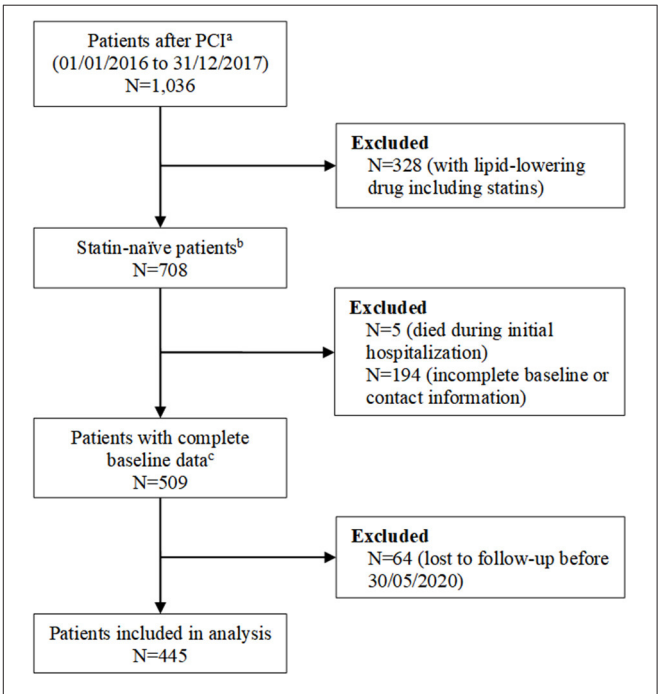

FIGURE 1 | Study flowchart. [a]Patients who were admitted for percutaneous coronary intervention (PCI) for stable angina and acute coronary syndrome at Sichuan Provincial People's Hospital, Chengdu, China, between 1 January 2016 and 31 December 2017. [b]Patients did not use statin or other lipid-lowering drugs within 3 months before admission. [c]Baseline data includes age, sex, prior medical history, comorbidities, serum creatinine, alanine aminotransferase, aspartate aminotransferase, uric acid, total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), lipoprotein **(a)**, apolipoprotein B, apolipoprotein A-I, and angiographic data.

TABLE 1 | Baseline characteristics of participants.

Characteristics	Total patients ($n = 445$)	Patients without MACE ($n = 385$)	Patients with MACE ($n = 60$)	*P* value
Age (years)	65.0 (59.0, 71.0)	65.0 (59.0, 71.0)	64.5 (55.3, 72.0)	0.59
Male, *n* (%)	321 (72.1)	280 (72.7)	41 (68.3)	0.54
BMI (kg/m^2)	24.1 ± 3.3	24.3 ± 3.2	24.9 ± 3.7	0.19
Diabetes, *n* (%)	88 (19.8)	67 (17.4)	21 (35.0)	0.003
Hypertension, *n* (%)	237 (53.3)	201 (52.2)	36 (60.0)	0.27
Gensini score	37.0 (20.0, 59.5)	34.0 (20.0, 58.0)	48.5 (28.0, 80.0)	< 0.001
TVD, *n* (%)	150 (33.7)	125 (32.5)	25 (41.7)	0.19
Stent before, *n* (%)	48 (10.8)	41 (10.7)	7 (11.7)	0.82
eGFR (ml/min/1.73 m^2)	89.9 (77.4, 98.8)	89.9 (78.0, 58.0)	88.6 (70.5, 100.2)	0.10
Cr (μmol/L)	72.6 (61.3, 86.8)	72.3 (61.2, 86.2)	76.4 (62.6, 89.5)	0.13
Ur (μmol/L)	356.0 (297.0, 432.0)	354.0 (296.5, 427.0)	367.0 (306.5, 443.8)	0.37
AST (IU/L)	34.0 (26.0, 62.0)	34.0 (26.0, 63.5)	35.0 (24.0, 50.8)	0.40
ALT (IU/L)	27.0 (19.0, 43.0)	27.0 (19.0, 43.0)	27.5 (18.3, 46.0)	0.91
TSH (mIU/L)	1.5 (1.0, 2.6)	1.6 (1.0, 2.6)	1.5 (0.8. 2.3)	0.62
TT3 (nmol/L)	1.5 ± 0.3	1.5 ± 0.3	1.4 ± 0.3	0.23
TT4 (nmol/L)	82.1 ± 17.2	82.1 ± 17.0	81.9 ± 18.3	0.93
FT3 (pmol/L)	4.0 ± 0.7	4.0 ± 0.7	3.9 ± 0.8	0.28
FT4 (pmol/L)	13.2 ± 1.9	13.16 ± 1.8	13.4 ± 2.3	0.38
Homocysteine (μmol/L)	17.4 ± 6.4	17.4 ± 6.6	17.5 ± 5.1	0.92
Total cholesterol (mmol/L)	5.1 (4.5, 5.7)	5.0 (4.4, 5.6)	5.3 (4.5, 6.1)	0.06
Triglyceride (mmol/L)	1.8 (1.3, 2.7)	1.8 (1.3, 2.6)	1.96 (1.4, 3.9)	0.002
LDL-C (mmol/L)	3.2 (2.7, 3.7)	3.2 (2.7, 3.7)	3.4 (3.0, 3.8)	0.04
HDL-C (mmol/L)	1.1 ± 0.3	1.1 (0.9, 1.3)	1.0 (0.9, 1.2)	0.06
Apolipoprotein B (g/L)	1.0 (0.8, 1.2)	1.0 (0.8, 1.2)	1.0 (0.9, 1.3)	0.43
Apolipoprotein A-I (g/L)	1.3 (1.1, 1.4)	1.3 (1.1, 1.4)	1.2 (1.1, 1.3)	0.13
Non-HDL-C (mmol/L)	3.9 (3.4, 4.5)	3.9 (3.4, 4.5)	4.2 (3.6, 5.0)	0.02
Lp(a) (mg/dl)	196.0 (84.0, 472.5)	193.0 (84.0, 495.0)	200.0 (72.8, 407.0)	0.54
LDL-C/HDL-C	3.0 (2.4, 3.6)	2.9 (2.3, 3.5)	3.4 (2.7, 4.2)	0.004
Atherogenic index of plasma	0.2 ± 0.3	0.2 ± 0.3	0.4 ± 0.4	0.001
Atherosclerosis index	3.7 (3.0, 4.5)	3.6 (2.9, 4.4)	4.2 (3.3, 5.6)	0.006
Lipoprotein combine index	28.0 (17.0, 48.0)	27.7 (15.7, 44.9)	34.9 (20.0, 96.2)	0.003
Apolipoprotein B/ apolipoprotein A-1	0.8 (0.6, 1.0)	0.8 (0.6, 1.0)	0.9 (0.7, 1.0)	0.17

BMI, body mass index; TVD, triple-vessel disease; eGFR, estimated glomerular filtration rate; Cr, creatinine; Ur, uric acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TSH, thyroid-stimulating hormone; TT3, total triiodothyronine; FT3, free triiodothyronine; TT4, total thyroxine; FT4, free thyroxine; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; Lp(a), lipoprotein (a); MACE, major adverse cardiovascular events.

score system (14). Two experienced interventional cardiologists independently calculated the Gensini score for each patient following a standardized approach and solved disagreement by discussion and re-calculation.

Triple-vessel disease was defined as angiographic stenosis of ≥50% in all three main epicardial coronary arteries, including the left anterior descending, left circumflex, and right coronary arteries.

Follow-Up and Endpoints

The investigators followed up with patients by telephone calls, home visits, or chart reviews at Sichuan Provincial People's Hospital. All data collectors were trained for data entry and extraction.

We defined major adverse cardiovascular events (MACEs) as cardiovascular death, stroke, myocardial infarction, or ischemia-driven revascularization. Only episodes of PCI and coronary artery bypass surgery for ischemic symptoms were considered as endpoints.

The follow-up duration was determined from the discharge date to the first occurrence of an endpoint. If no endpoints occurred, data were collected until the last visit. Patients without endpoints were censored by the end of the last follow-up visit.

Statistical Analysis

All statistical analyses were performed using R (R Pack 3.6.1) (15). We measured the normality of distribution using the Kolmogorov–Smirnov test. Normally distributed continuous variables were presented as mean ± standard deviation. Non-normally distributed continuous variables were presented as median and interquartile range. Categorical variables were presented as frequencies (percentages). We grouped patients into three groups based on the tertiles of each baseline lipid parameter. We compared the baseline characteristics of participants with and without MACE using student *t*-test, Mann-Whitney *U* test, or Chi-square test. We compared the risk of the first MACE in the highest tertile of each lipid parameter to its lowest tertile using multivariate Cox proportional hazards models with adjusted variables, baseline age, sex, body mass index, diabetes mellitus, hypertension, and Gensini score. We reported the hazard ratio (HR) and 95% confidence interval (CI). We used the ratio of HR (RHR) to measure the relative magnitude of the paired HR to find the largest effect size of different HRs. CIs of RHRs were calculated using the bootstrapping method. We set 5000 resamplings to generate bootstrap samples and calculated each as a bootstrap RHR. We chose the 2.5th and 97.5th percentiles as the 95% CI limits (16). A two-sided *P* value of < 0.05 was considered statistically significant.

RESULTS

Patients

As shown in **Figure 1**, we analyzed 445 patients meeting the eligibility criteria among 1,036 candidates who underwent PCI during the study period. **Table 1** shows the baseline

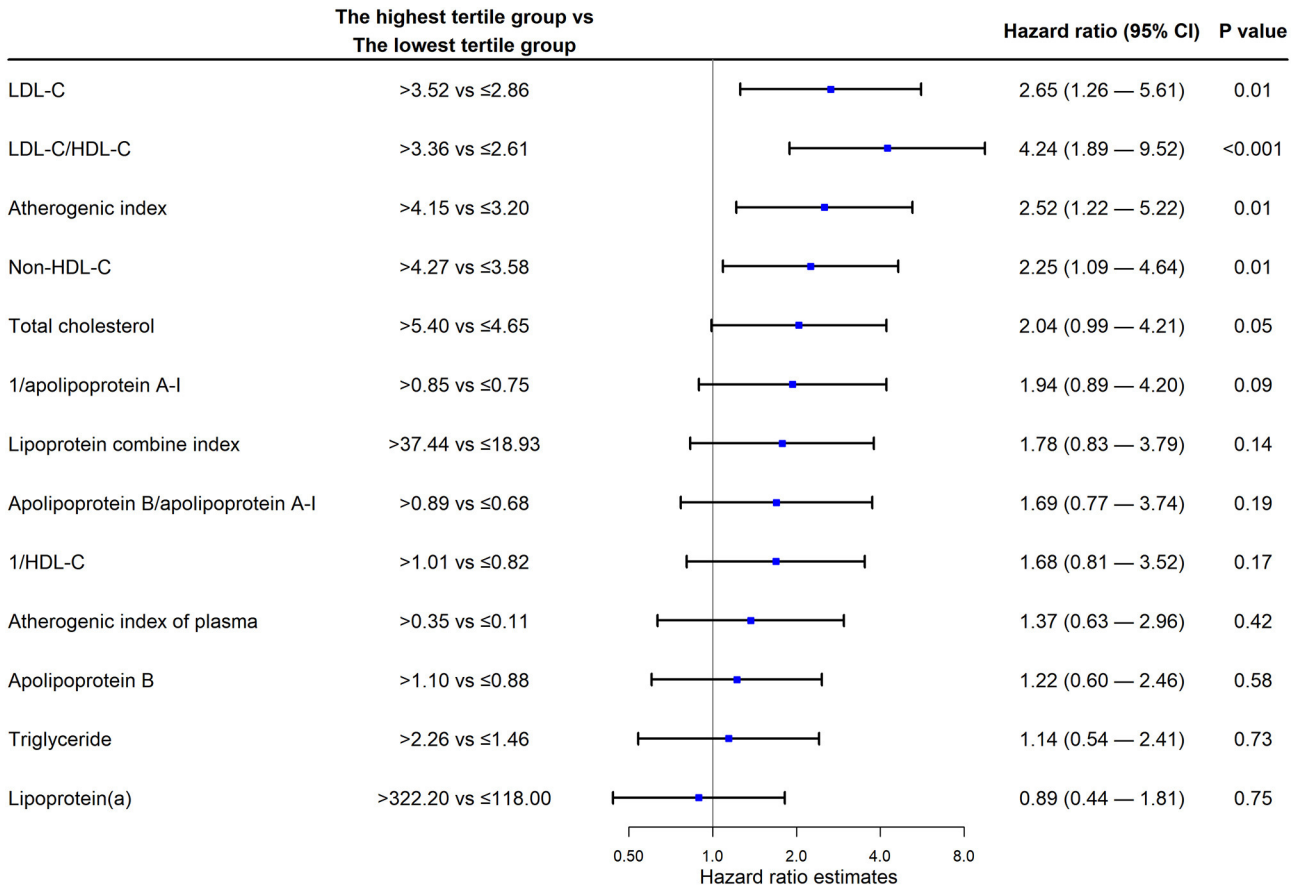

FIGURE 2 | Cox regression analysis. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CI, confidence interval.

characteristics of patients. The median age of patients was 65.0 years (P25 to P75, 59.0 to 71.0 years), 321 patients (72.1%) were male, 33.7% of patients had a triple-vessel disease, 19.8% had diabetes mellitus, and 53.3% had hypertension. When compared with patients without MACE, patients with MACE had a higher level of triglyceride, non-HDL-C, LDL-C/HDL-C, and atherogenic index of plasma, and a higher rate of diabetes. The differences of baseline characteristics of patients in different tertiles of LDL-C were shown in **Supplementary Table 1**.

Follow-Up and Endpoints

The date of the last follow-up contact with a patient was 30 May 2020. The median duration of follow-up was 36 months (interquartile range 27 to 41 months) with an incidence of 4.9 cardiovascular events per 100 person-years. MACEs occurred in 60 patients (13.5%).

As shown in **Figure 2**, the multivariate Cox proportional hazards model showed that the HR of the highest LDL-C group compared with the lowest LDL-C group was the highest (HR 2.65; 95% CI 1.26 to 5.61; $P = 0.01$). Non-HDL-C level (HR 2.25; 95% CI 1.09 to 4.64; $P = 0.01$), atherosclerosis index (HR 2.52; 95% CI 1.22 to 5.22; $P = 0.01$), and LDL-C/HDL-C ratio (HR 4.24; 95% CI 1.89 to 9.52; $P < 0.001$) were independently associated with an increased risk of MACEs (**Figure 2**). Lipoprotein (a) levels did not show an obvious association with the endpoint (HR 0.89; 95% CI 0.44 to 1.81). The HRs for the middle tertile of those parameters compared with their lowest tertile were not statistically significant (**Supplementary Figure 1**).

As shown in **Figure 3**, all RHRs of lipid parameters (except for LDL-C/HDL-C) were <1.00. The LDL-C/HDL-C ratio did not show significantly better performance compared with LDL-C (RHR 1.60; 95% CI 0.65 to 3.65).

DISCUSSION

Our study shows that baseline LDL-C level is independently associated with MACEs in statin-naïve patients after PCI. None of the different lipid parameters performed better than LDL-C in the association with cardiovascular outcomes by a tertile grouping. This is the first study to compare different lipid parameters in Chinese statin-naïve patients after PCI.

Recent longitudinal studies suggest a strong prediction effect of lipoprotein (a) in patients at risk of atherosclerotic CVD (17). European guidelines in 2019 recommended lipoprotein (a) in patients with a family history of premature CVD

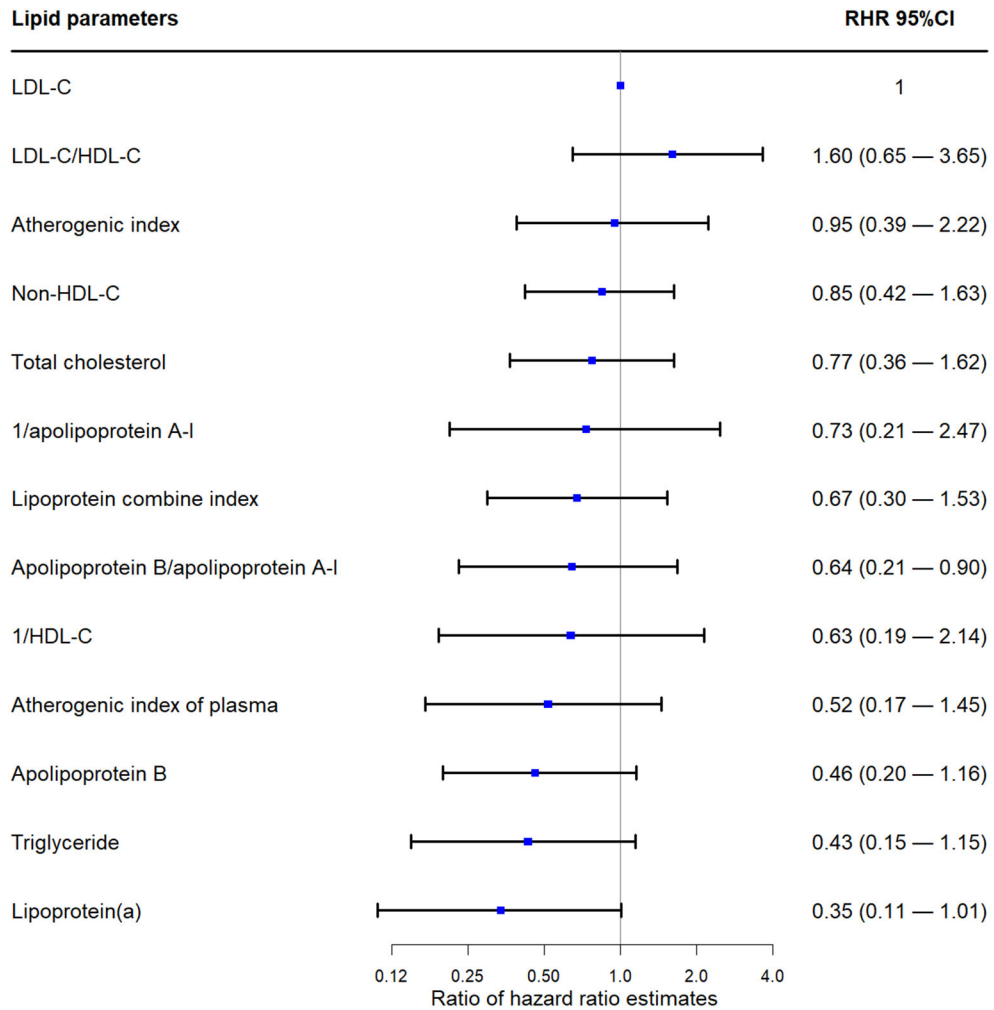

FIGURE 3 | Ratio hazard ratio of different lipid parameters. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CI, confidence interval.

and for reclassification in moderate-to-high-risk patients (12). However, our study did not show an association between baseline lipoprotein (a) level tertile and cardiovascular events in Chinese statin-naïve patients after PCI. Our findings are consistent with a previous study in China (18). This ethnic difference may be related to the absolute concentration and isoform sizes of lipoprotein (a), as Chinese people were found to have the lowest lipoprotein (a) concentration (median = 7.8 mg/dL) and the largest isoform size (median = 28) among seven major ethnic groups (19). This suggests that we need to be aware of the difference across ethnicity and the difference between randomized trials and real-world population (20). It may not be reasonable to measure lipoprotein (a) routinely in all patients undergo PCI. Further study is warranted to investigate the target population needing lipoprotein (a) measurement.

In our study, none of the lipid parameters performed better than LDL-C level, except for the LDL-C/HDL-C, despite some of those parameters being recommended in Western guidelines (21, 22). This suggests that LDL-C remains the best predictive factor for cardiovascular events in the secondary prevention of CVD. This finding is in line with LDL-C-targeting lipid-lowering trials (23). LDL-C/HDL-C is a good predictor of cardiovascular events and atherosclerosis (24, 25). However, few lipid-lowering drugs elevate HDL-C with cardiovascular benefits (26). Neither HDL-C nor LDL-C/HDL-C is a better treatment target for cardiovascular prevention than LDL-C, which is the pharmaceutical target of major lipid-lowering drugs that reduce cardiovascular events, including statins, ezetimibe, and PCSK9 inhibitors (27–29).

There are three major limitations in our study. First, this is a single-center study with a limited sample size and event rate. Further study is warranted at other cardiovascular centers to validate the results. Second, we did not measure the subtypes of LDL-C, very low-density lipoprotein cholesterol, or lipoprotein-associated phospholipase A2, which are not routinely measured in clinical practice. Finally, we could not assess the prevalence

of patients with familial hyperlipidemia, which could not be assessed based on the current data.

CONCLUSIONS

In summary, our findings indicate that LDL-C remains the best lipid parameter associated with cardiovascular events in general Chinese statin-naïve patients after PCI. External validation of our study in a larger population is warranted.

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 Biomedical Ethics Committee of Sichuan Provincial People's Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LZ, HT, and SL conceived the study. YL, MF, TH, and CW followed up the patients and was responsible for the data collection. LZ, YZ, and QS analyzed the data. QS and SL supervised the statistical analysis. LZ, ZY, HT, and SL interpreted the results. LZ, YZ, and SL drafted the manuscript. All authors read and approved the final version of this manuscript. LZ, HT and SL were responsible for the study design, data collection, and data analysis and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.638663/full#supplementary-material

Supplementary Figure 1 | Cox regression analysis comparing T2 vs. T1. LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CI, confidence interval; T1, the lowest tertile; T2, the middle tertile.

Supplementary Table 1 | Baseline characteristics of patients in different tertiles of low-density lipoprotein cholesterol. MACE, major adverse cardiovascular events; BMI, body mass index; TVD, triple-vessel disease; eGFR, estimated glomerular filtration rate; Cr, creatinine; Ur, uric acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TSH, thyroid-stimulating hormone; TT3, total triiodothyronine; FT3, free triiodothyronine; TT4, total thyroxine; FT4, free thyroxine; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; LP(a), lipoprotein (a); T1, the lowest tertile; T2, the middle tertile; T3, the highest tertile.

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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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ORIGINAL RESEARCH
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Reduced Number and Activity of Circulating Endothelial Progenitor Cells in Acute Aortic Dissection and Its Relationship With IL-6 and IL-17

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This study investigates the alteration in function and number of circulating endothelial progenitor cells (EPCs) in patients with aortic dissection (AD), compared with hypertensive patients, and its possible mechanism. Thirty-four patients with acute aortic dissection (AAD) and 20 patients with primary hypertension were involved. Flow cytometry analysis was performed to detect the number of CD34+/KDR+ cells, and acetylated low density lipoprotein (ac-LDL) and lectin fluorescent staining method was applied to test the number of cultured EPCs. In addition, EPC migration and proliferation were measured, and plasma interleukin 6 (IL-6) and interleukin 17 (IL-17) levels were investigated. The number of circulating EPCs in the AAD group was lower than that in the non-AD group, and the proliferation and migration of circulating EPCs in the AAD group were lower than that in the non-AD group. In addition, the number, proliferation, and migration of circulating EPCs were significantly inversely correlated with the aortic dissection detection risk score (ADD-RS). More importantly, increased plasma IL-6 and IL-17 level was found in the AAD group, and the two inflammatory factors were inversely associated with the function and number of circulating EPCs in the AAD group. We first demonstrated that the number and function of circulating EPCs are reduced in the AAD group, which may be partly related to upregulated plasma IL-6 and IL-17. Our study provides novel insight on the underlying mechanism and potential therapeutic target of AAD.

Keywords: acute aortic dissection, endothelial progenitor cells, endothelial injury, IL-6, IL-17

INTRODUCTION

Aortic dissection (AD) is a serious disease threatening human life, which has rare incidence rate and extremely high mortality (1, 2). The incidence of acute aortic dissection (AAD) ranges from 3 to 6 per 100,000 patient-years in the general population in the United States, which has been increasing markedly, especially in recent years (3). It is well-known believed that the changes in

the membrane structure and the function of critical cells in the aorta play an important role in the pathogenesis of AAD (4, 5). The aortic wall includes the intima, middle layer, and adventitia. The intimal rupture is one of the initial events of AD. Previous studies on the intimal layer of the aorta mainly concentrated in the pathogenesis of atherosclerotic stenosis such as coronary heart disease, and in recent years, the structure and function of the intimal layer of the aorta have been paid more attention (6, 7).

Aortic intima functions as critical factor to maintain the normal morphology and diastolic function of arteries (8). Some studies have shown that the damage and tear of vascular intima are the initial link of aortic dissection (9) and proved that the intima plays a crucial role in vascular injury and repair. Another study also have proven that vascular endothelial cell is damaged, promoting the formation of AD (10). Therefore, the repair of the intima may provide a novel way to intervene the pathological progress of aortic dissection.

Endothelial progenitor cells (EPCs) mobilized from bone marrow can directly differentiate into endothelial cells. They not only participate in the formation of early embryonic blood vessels but also participate in the regeneration of adult blood vessels (11, 12). EPCs are mainly involved in the development of the blood system and inflammatory immune response with inability for vascular regeneration, which can accelerate the repair in damaged vascular endothelium. EPCs have the characteristics of proliferation, migration, adhesion, and basement membrane matrix formation during the process of vascular repair (13, 14).

Many studies confirmed that metabolic abnormalities, smoking, and obesity can decrease circulating number and function of EPCs, reduce its ability to repair the endothelium, and cause endothelial dysfunction, which in turn give rise to the process of vascular diseases including hypertension (15–17). A systematic review summarized that 45–100% patients with acute aortic dissection have a most common comorbidity with hypertension (3). In our previous study, we found that the early stage of hypertension can cause EPC dysfunction (18, 19). Therefore, we speculate that endothelial injury may be the main cause of AAD.

Inflammation may be engaged in the development of AAD (20). During the process of AAD, inflammatory cells, such as phagocytes, can degrade elastic fibers by releasing matrix metalloproteinase (MMP), destroy the middle membrane of the aorta, and cause the thinning of the arterial wall, which leads to AD (21, 22). Interleukin 6 (IL-6) is an important proinflammatory factor, which can induce monocytes to differentiate into phagocytes (23). IL-6 was found to increase in acute aortic dissection, and when downregulating IL-6 expression, vascular macrophages production were decreased, thus delaying the occurrence of aortic dissection (24). Interleukin 17 (IL-17), a CD4+ T-helper subset, has been found to be involved in atherosclerosis and vascular dysfunction (25, 26). Previous study reported that IL-17 may function as a participant in AD pathogenesis by promoting inflammation (27). Thus, IL-6 and IL-17 may be related with AAD for its vascular inflammation.

This study is to explore the relationship between the function and number of EPC and aortic dissection detection risk score (ADD-RS) in AAD and hypertensive patients and analyze the level of IL-6 and IL-17 in the two groups. Our purpose is to find the potential molecular mechanism of AAD and a new intervention target for AAD prevention and treatment.

TABLE 1 | Clinical and biochemical characteristics in AAD and Non-AD groups.

Characteristics	AAD group ($n = 34$)	Non-AD group ($n = 20$)
Sex(M), *n* (%)	26 (76.5%)	13 (65%)
Age (years), mean(SD)	57.4 (10.4)	59.0 (10.9)
BMI (kg/cm^2), mean(SD)	25.2 (3.4)	25.2 (2.9)
Heart rate (time/min), mean (SD)	77.0 (9.6)	77.1 (8.7)
Systolic blood pressure (mmHg), mean (SD)	161.4 (19.4)	166.7 (16.7)
Diastolic blood pressure (mmHg), mean (SD)	86.8 (12.1)	88.5 (18.5)
cTn-T (ug/L), mean(SD)	0.09 (0.24)	0.04 (0.1)
ALT (mmol/L), mean (SD)	93.4 (188.6)	28.3 (12.6)
AST (mmol/L), mean (SD)	78.9 (109.8)	25.1 (6.3)*
BUN (mmol/L), mean (SD)	8.3 (7.6)	6.9 (5.2)
Cr (mmol/L), mean(SD)	164.5 (232.9)	113.0 (134.8)
GLU (mmol/L), mean (SD)	7.0 (2.3)	6.3 (2.7)
CRP (mg/L), mean (SD)	66.4 (52.8)	5.2 (5.8)*
WBC (*10^9/L), mean (SD)	12.5 (4.6)	7.9 (1.7)*
TC (mmol/L), mean (SD)	4.5 (1.0)	4.5 (1.2)
TG (mmol/L), mean (SD)	1.5 (0.6)	1.6 (0.9)
HDL (mmol/L), mean (SD)	1.0 (0.3)	1.1 (0.4)
LDL (mmol/L), mean (SD)	2.9 (0.8)	2.8 (0.8)
D-dimer (mg/L FEU), mean (SD)	15.2 (36.2)	0.4 (0.3)*
Previous history		
Smoker, *n* (%)	14 (41.2)	8 (40)
CAD, *n* (%)	4 (11.7)	3 (15)
Hypertension, *n* (%)	34 (100)	20 (100)
Hypercholesterolemia, *n* (%)	7 (20.6)	3 (15)
Diabetes, *n* (%)	6 (17.6)	3 (15)

BMI, body mass index; cTn-T, Cardiac troponin-T; AST, aspartate amino transferals; ALT, alanine transaminase; BUN, blood urea nitrogen; Cr, serum creatinine; CRP, C-reactive protein; TC, total cholesterol; WBC, white blood cell; TG, triglyceride; HDL, high density lipoprotein; LDL, low-density lipoprotein.
*Data are given as mean ± SD. *P < 0.05 vs. Non-AD group.*

MATERIALS AND METHODS

Subject Characteristics

Thirty-four patients suffering AAD, and 20 non-AD patients with essential hypertension were recruited. All patients older than 18 years admitted to the First Affiliated Hospital of Sun Yat-sen University were eligible. The definition of AD in our study was according to a previous study, which was majorly diagnosed by computed tomography (CT) scan (28). AAD was defined as any dissection that involved the aorta presenting within 14 days of symptom onset (28). The investigation protocol was approved by the Ethical Committee of the First Affiliated Hospital of Sun Yat-sen University. The basic characteristics of two groups are shown in **Table 1**.

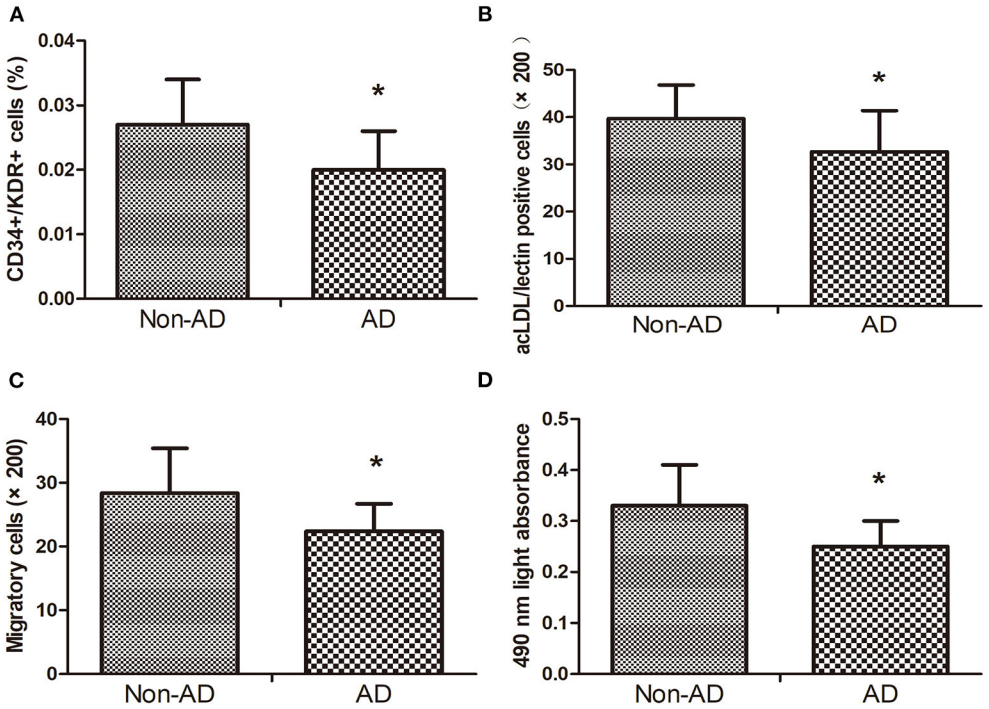

FIGURE 1 | The CD34+KDR+ positive cells **(A)** and DiI-acLDL/lectin positive cells **(B)** in AD subjects were lower than those in Non-AD subjects. The migratory **(C)** and proliferative **(D)** activities of EPCs in AD subjects decreased when compared with Non-AD subjects. Data are given as the mean ± standard deviation (SD). *$p < 0$ 05 vs. Non-AD group.

Data Collection and ADD-RS Assessment

Data collection for the assessment of the pretest probability of AAD were performed by a medical researcher in the Emergency Department. The tool applied to evaluate the pretest probability of AAD was the ADD-RS. The ADD-RS of AAD patient was evaluated according to the previous study (29).

The Evaluation of EPC Number and Activity

Circulating EPC number was detected by cell culture assay and flow cytometry analysis as previous studies (19, 30, 31). The EPC migration and proliferation assay was implemented according to previous studies (19, 30, 31).

Measurement of Plasma Levels of IL-6 and IL-17

Plasma levels of IL-6 and IL-17 were assessed as previously described (32, 33). According to the instructions of the manufacturer, the human IL-6, and IL-17 levels in plasma were determined by human IL-6 and IL-17 Quantikine ELISA kit (R&D Systems, Minneapolis, USA).

Statistical Analysis

All statistical analyses were carried out with SPSS 22.0. Continuous variables are represented by mean ± SD, and the comparison between two groups was analyzed by Student's *t*-test. Categorical variables were compared using chi-squared analysis. Pearson's coefficient (r) was used to calculate univariate correlations. If a null hypothesis could be rejected at $p < 0.05$, statistical significance was assumed.

RESULTS

Baseline Characteristics

As **Table 1** shows, the study population was predominantly male. The two groups were similar in terms of age and sex. The aspartate aminotransferase (AST), white blood cell (WBC), C-reactive protein (CRP), and D-dimer levels were significantly higher than those in the non-AD group ($p < 0.05$). There were no differences in the levels of heart rate, diastolic blood pressure, systolic blood pressure, body mass index (BMI), alanine aminotransferase (ALT), low-density lipoprotein (LDL), total cholesterol (TC), high-density lipoprotein (HDL), triglycerides (TG), blood urea nitrogen (BUN), creatinine (Cr), and cardiac troponin T (cTn-T) in the two groups ($p > 0.05$). In addition, according to the previous investigation for the International Registry of Acute Aortic Dissection (IRAD) Classification System to characterize survival after aortic dissection (34), the number of hyperacute patients is 19, the number of acute patients is 9, and the number of subacute patients is 6.

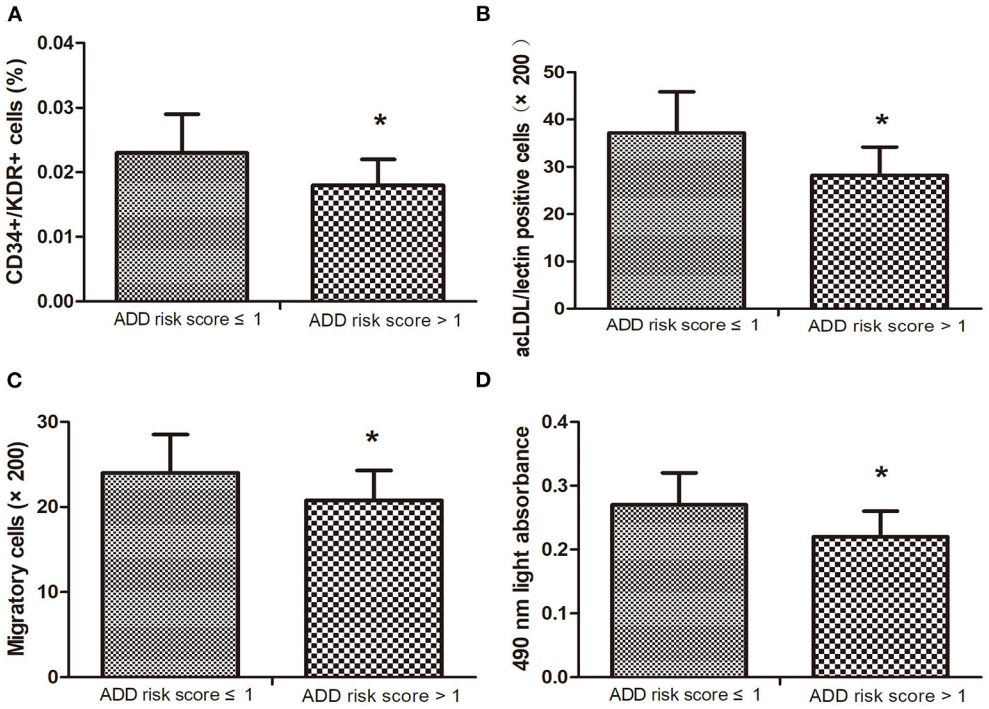

FIGURE 2 | The number of circulating endothelial progenitor cells (EPCs) **(A,B)** in high ADD-RS group were lower than that in low ADD-RS group. The migratory **(C)** and proliferative **(D)** activities in low ADD-RS group were higher than that in high ADD-RS group. Data are given as the means ± standard deviation (SD). *$p < 0\ 05$ vs. ADD risk score≤1 group.

Circulating EPC Number and Function in AD and Non-AD Groups

Compared with the non-AD group, circulating EPC number detected by CD34+/KDR+ and DiI-acLDL/lectin double-positive cells in AAD group was reduced (**Figures 1A,B**). Moreover, compared with the non-AD group, EPC migration and proliferation in the AAD group were remarkably declined (**Figures 1C,D**).

EPC Number and Function in Low and High ADD-RS

According to the previous report (29), the ADD-RS is categorized as low ADD-RS group if ADD-RS ≤ 1 (defining low clinical probability of AAD) and high ADD-RS group if ADD-RS > 1 (defining high clinical probability of AAD). In our data, the number of patients with ADD-RS ≤ 1 and ADD-RS > 1 are both 17. Interestingly, the EPC number and function were found to decrease in high ADD-RS group, compared with the low ADD-RS group (**Figures 2A–D**).

Relationship Between Characteristics of Circulating EPCs and ADD-RS

The levels of circulating CD34+/KDR+ cells ($r = -0.44$, $p < 0.05$) and DiI-acLDL/lectin double-positive cells ($r = -0.50$, $p < 0.05$) were inversely correlated with the score of ADD-RS (**Figures 3A,B**). In addition, we also found that the EPC migration and proliferation were inversely related to the score of ADD-RS ($r = -0.40$, $p < 0.05$, and $r = -0.48$, $p < 0.05$, respectively) (**Figures 3C,D**).

Plasma IL-6 and IL-17 Levels in the Two Groups

The plasma IL-6 and IL-17 level in the AAD group were higher than that in the non-AD group (**Figures 4A,B**).

Relationship Between Characteristics of Circulating EPCs and IL-6 Level

The levels of circulating CD34+/KDR+ cells ($r = -0.32$, $p < 0.05$) and DiI-acLDL/lectin double-positive cells ($r = -0.54$, $p < 0.05$) were inversely correlated with the plasma IL-6 level (**Figures 5A,B**). Additionally, the EPC migration and proliferation were inversely related to the plasma IL-6 level ($r = -0.30$, $p < 0.05$, and $r = -0.55$, $p < 0.05$, respectively) (**Figures 5C,D**).

Relationship Between Characteristics of Circulating EPCs and IL-17 Level

The levels of circulating CD34+/KDR+ cells ($r = -0.59$, $p < 0.05$) and DiI-acLDL/lectin double-positive cells ($r = -0.49$, $p < 0.05$) were inversely correlated with the plasma IL-17 level (**Figures 6A,B**). The EPC migration and proliferation were inversely related to the plasma IL-17 level ($r =$

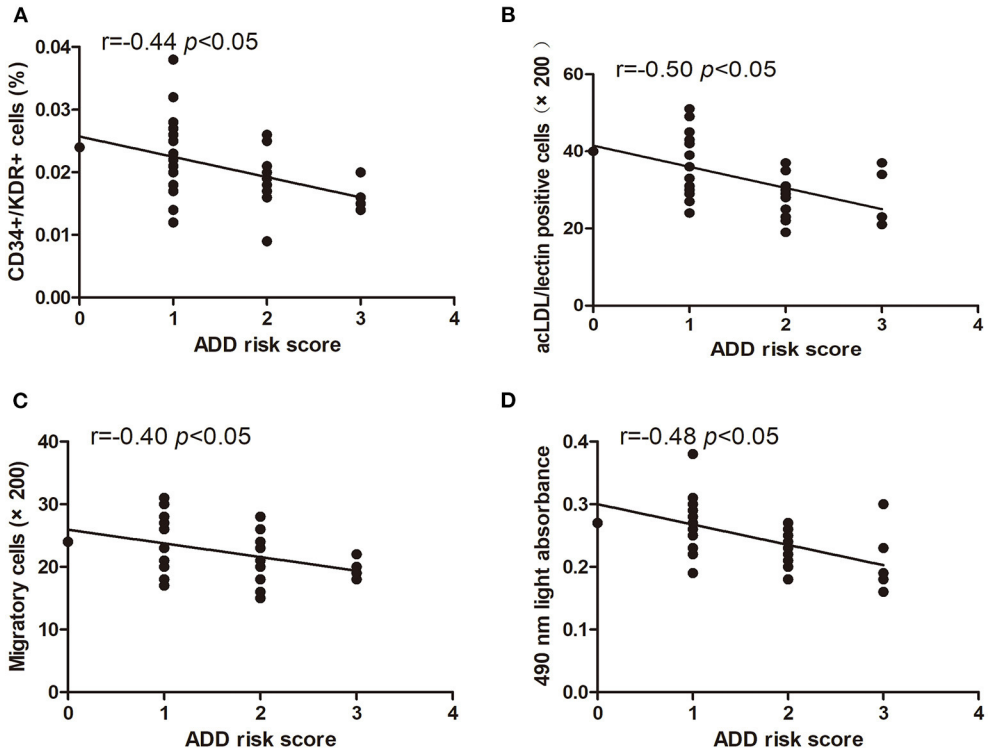

FIGURE 3 | Pearson correlation ratios were used to analyze the relationship between the number or function of EPCs and ADD-RS. The CD34+KDR+ positive cells **(A)** and DiI-acLDL/lectin positive cells **(B)** significantly correlated with ADD-RS. The migratory **(C)** and proliferative **(D)** activities of EPCs were related to ADD-RS.

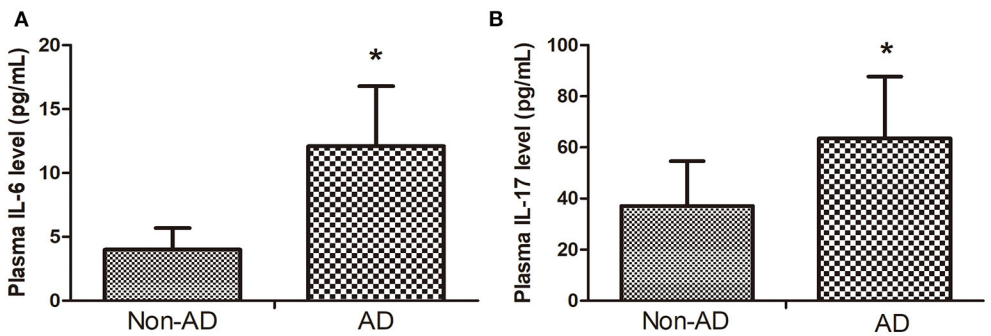

FIGURE 4 | (A) The plasma IL-6 level in AAD group was higher than that in Non-AD group. **(B)** plasma IL-17 level in AAD group was higher than that in Non-AD group. Data are given as mean ± SD. *$p < 0.05$ vs. Non-AD group.

-0.37, $p < 0.05$, and $r = -0.42$, $p < 0.05$, respectively) (**Figures 6C,D**).

DISCUSSION

Our study has first demonstrated that EPC number, migration, and proliferation in acute aortic dissection patients are significantly reduced. We further revealed that there is an inversely relationship between the circulating EPCs and the severity of AAD evaluated by ADD-RS exists. Moreover, the results indicated that the plasma IL-6 and IL-17 levels are increased in AAD patients and negatively correlated with EPC number or function. Taken together, the proinflammatory factor may be partly associated with the attenuated endogenous endothelial repair ability and participate in the process of AAD.

Numerous studies have indicated that the EPCs may predict outcomes in cardiovascular disease (35, 36). It has been proven that the levels of CD34+ cells are altered in patients with abdominal aortic aneurysms compared with patients with peripheral vascular disease, and the CD34+ cells were correlated with aneurysm diameter (37, 38). However, few studies focus on the relationship between circulating EPCs and AAD.

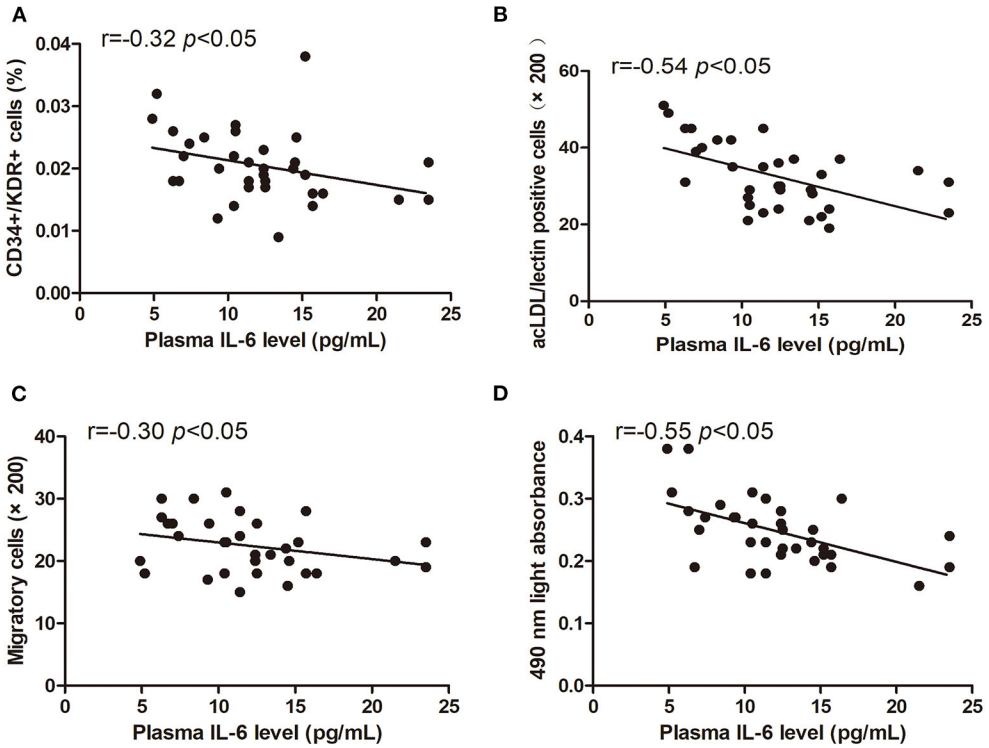

FIGURE 5 | Pearson correlation ratios were used to analyze the relationship between the number or function of EPCs and plasma IL-6 level. The CD34+KDR+ positive cells **(A)** and DiI-acLDL/lectin positive cells **(B)** significantly correlated with IL-6. The migratory **(C)** and proliferative **(D)** activities of EPCs were related to IL-6.

In the current study, circulating EPC number or function was investigated. All patients with acute aortic dissection involved in our study have hypertension. Our previous study discovered a dysfunction of circulating EPCs in hypertension (12, 19). Hereby, the hypertensive patients were recruited in the non-AD group to eliminate the influence of blood pressure on circulating EPCs. The results showed that AAD patients had decreased number and migration or proliferation of EPCs. However, our findings may be the different from that of Van Spyk E N's (37), and the possible reasons may be as follows: First, there is a difference in enrolled subjects. although commonly pathogenesis of AAD is atherosclerosis and share similar as the abdominal aortic aneurysm (AAA). However, vascular injury of AAD patients enrolled in our study may be more serious than that of AAA enrolled in the previous investigation. Second, we used CD34+KDR+ cells defined as EPCs, which is a frequently used and credible method to evaluate putative antigenic phenotypes of EPCs. However, the surface antigen CD34+ cells in Van Spyk E N's study, as a hematopoietic multilineage stem cell, are able to differentiate into myeloid, lymphoid, erythroid, and megakaryocytic cells (39). Third, in our study, EPCs were isolated from the peripheral blood mononuclear cells, and its migration and proliferation were also investigated.

ADD-RS may provide a simple and systematic method to screen the high likelihood of patients with AAD at the Emergency Department (ED) (40). On the basis of the ADD-RS, patients can be classified into two categories (ADD-RS $\leq$1, ADD-RS >1), which are adopted as diagnostic algorithms by international guidelines for AAD (29, 41). EPCs have been proven to be a predictive factor for cardiovascular diseases (42, 43). We revealed that EPC number or function were lower in high ADD-RS group than that in low ADD-RS group. Moreover, there is a remarkable inverse association between EPC number or function and ADD-RS. These results manifest that circulating EPCs may act as a surrogate indicator to evaluate the probability of AAD occurrence with the development of vascular diseases. In our study, the AAD patient in acute phase is defined according to a previous investigation (28). However, according to the previous investigation for the IRAD Classification System to characterize survival after aortic dissection (34), most patients are in the hyperacute or acute phase, and few patients are in the subacute phase. Our further study will be performed to investigate the effect of different classification of AD on the related alteration in circulating EPCs.

The development of AAD is proved to be accompanied by inflammation (44). However, it is not clear whether inflammation contributes to regulating the circulating EPCs in AAD. Previous studies have demonstrated that IL-6 and IL-17 take part in the mechanism of AAD (24, 27). Interestingly, our results found that IL-6 or IL-17 level had a negative

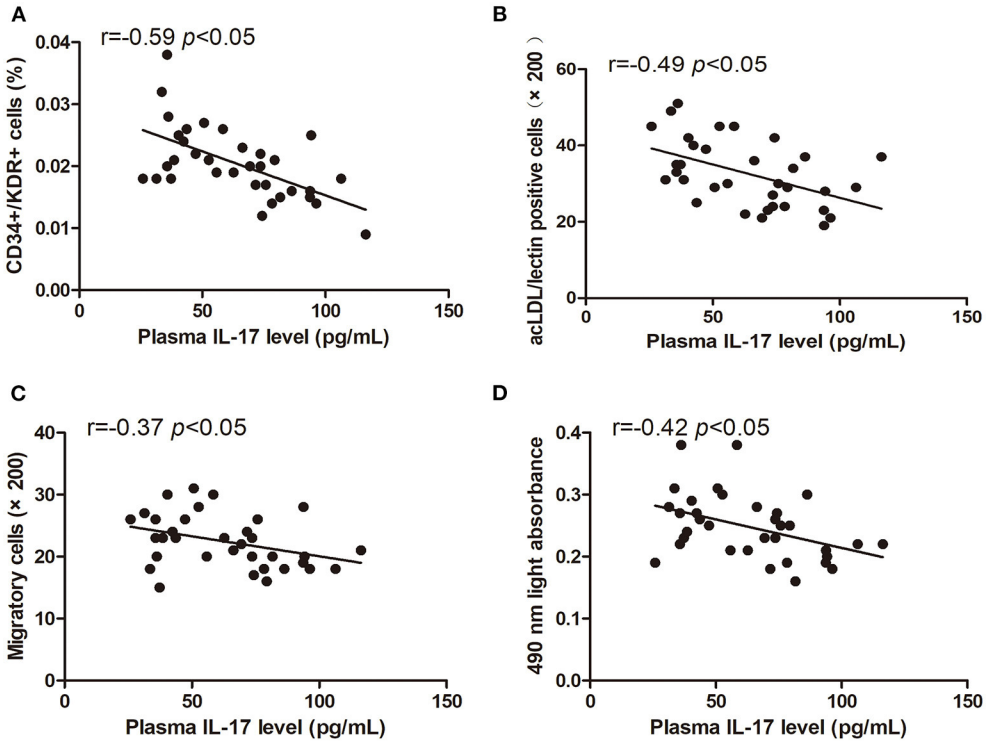

FIGURE 6 | Pearson correlation ratios were used to analyze the relationship between the number or function of EPCs and plasma IL-17 level. The CD34+KDR+ positive cells **(A)** and DiI-acLDL/lectin positive cells **(B)** significantly correlated with IL-17. The migratory **(C)** and proliferative **(D)** activities of EPCs were related to IL-17.

correlation with EPC number and migration or proliferation in AAD, indicating the possible relationship between the proinflammatory factor and the circulating EPCs in AAD. It was reported that IL-6 has an unfavorable effect on the function of EPC after myocardial infarction (44), but it is unknown whether IL-17 can regulate the circulating EPCs. As we all know, IL-17 cytokine, produced by a novel subset of CD4+ helper T cell, has a critical effect in chronic inflammatory diseases and AAD (45) and was found to activate some common downstream signaling pathway, such as tumor necrosis factor-α (TNF-α), IL-6, and IL-1(46). The current study revealed that inflammatory-mediated endothelial repair capacity incompetency may be implicated in AAD development.

The current study has some limitations. First, this study has relatively small sample size. Further large-sample research needs to be carried out to further address it. Second, our study is designed to eliminate the influence of blood pressure on EPCs, but acute inflammation or other inflammatory disease also has an effect on EPC number and function. Our further study will be performed to elucidate it. Finally, the data of EPCs before the occurrence of AAD are difficult to acquire and not presented in this study. We will further compare the quantitative and qualitative alteration in EPCs before and after AAD in animal experiment.

In summary, our findings have some clinical implications. First, circulating EPCs may be a novel surrogate biomarker for predicting the probability of AAD. Second, the improved function of EPCs can enhance endogenous endothelial repair capacity, which may be beneficial to the prevention and treatment of AAD. For example, ACEI and ARB, significantly increasing function of EPCs, may prevent the occurrence of AAD. Third, proinflammatory factor, including IL-17 and IL-6, is involved in attenuated endogenous endothelial repair ability and may be engaged in the development of AAD. It suggests that anti-inflammatory agents such as statis and PCSK9 may be a new strategy for preventing and treating AAD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the First Affiliated Hospital of Sun Yat-sen University. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HZh and ZYa designed the research. ZH, ZL, KW, ZYe, BZ, YX, JL, LZ, HZe, and GL performed the research. ZH, ZL, KW, HZh, and ZYa analyzed the data. ZH, ZL, and ZYa wrote the paper. 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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Circulating Proprotein Convertase Subtilisin/Kexin Type 9 Levels and Cardiometabolic Risk Factors: A Population-Based Cohort Study

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Aims: To evaluate the prospective association of circulating PCSK9 levels with the cardiometabolic risk profiles (high LDL-cholesterol, high triglycerides, low HDL-cholesterol, hypertension, type 2 diabetes, and metabolic syndrome).

Methods: A population-based prospective study was conducted among 7,104 Chinese individuals (age 56.2 ± 7.5 years; 32.0% men). Circulating PCSK9 levels were measured using ELISA.

Results: Circulating PCSK9 levels were higher in women than men (286.7 ± 90.1 vs. 276.1 ± 86.4 ng/ml, $p < 0.001$). And circulating PCSK9 was positively correlated with LDL-cholesterol, total cholesterol, and triglycerides both in men and women (all $p < 0.001$). The positive correlation between PCSK9 and waist circumference, fasting glucose, insulin resistance, systolic blood pressure, diastolic blood pressure and C-reactive protein (all $p < 0.01$) was observed in women only. According to Cox regression analysis, circulating PCSK9 was positively associated with incidence of high LDL-cholesterol both in men (HR 1.33, 95% CI 1.09–1.65, $p < 0.001$) and women (HR 1.36, 95% CI 1.12–1.69, $p < 0.001$). Moreover, PCSK9 was significantly associated with incident high triglycerides (HR 1.31, 95% CI 1.13–1.72, $p < 0.001$), hypertension (HR 1.28, 95% CI 1.08–1.53, $p = 0.011$), type 2 diabetes (HR 1.34, 95% CI 1.09–1.76, $p = 0.005$), and metabolic syndrome (HR 1.30, 95% CI 1.11–1.65, $p = 0.009$) per SD change in women only. No statistically significant association was observed between circulating PCSK9 and incidence of low HDL-cholesterol ($p > 0.1$).

Conclusions: Elevated circulating PCSK9 was significantly associated with cardiometabolic risk factors and independently contributed to the prediction of cardiometabolic risks in women.

Keywords: proprotein convertase subtilisin kexin type 9, dyslipidemia, hypertension, type 2 diabetes, metabolic syndrome, cardiometabolic risk factors

INTRODUCTION

Proprotein convertase subtilisin/kexin type 9 (PCSK9), the ninth member of the proprotein convertase family, is a key lipid metabolic regulator (1, 2). It is reported that PCSK9 is a serine protease produced primarily by the liver and the intestine, but only the liver releases it into circulation (3). This protease regulates cholesterol homeostasis by promoting degradation of low-density lipoprotein (LDL) receptor, the major route of clearance of circulating LDL-cholesterol, through an endosomal/lysosomal pathway (4). Subsequently, reduced LDL receptor levels resulted in impaired clearance of LDL-cholesterol, leading to hypercholesterolemia and metabolic disorders. Genetic studies have shown that gain-of-function mutations of the *PCSK9* gene caused hypercholesterolemia (5), whereas loss-of-function mutations in *PCSK9* resulted in hypocholesterolemia and reduced risk of cardiovascular disease (6, 7). On account of the involvement of PCSK9 in the degradation of LDL receptor, this crucial lipid metabolic regulator has recently emerged as new and promising pharmacological targets for the management of atherosclerotic cardiovascular disease. Indeed, treatment targeted at PCSK9 inhibition markedly reduced atherogenic lipoproteins and confers additional cardiovascular benefit beyond that achieved by lipid-lowering treatment alone (8–10).

Although the best-described impact of PCSK9 is on the levels of LDL-cholesterol, data from epidemiological studies have shown that PCSK9 is also associated with certain features of cardiometabolic profiles including high-density lipoprotein (HDL) cholesterol, triglycerides, blood pressure, and fasting glucose, as well as insulin resistance (11–13). However, to date, evidence from prospective study about the relationship between circulating PCSK9 and incidence of cardiometabolic risk factors is scarce.

To further evaluate whether PCSK9 contributes to the cluster of cardiometabolic risk factors involved in atherosclerotic cardiovascular diseases, we investigated prospectively the incidence of these metabolic abnormalities in relation to baseline PCSK9 levels in a large-scale Chinese population.

MATERIALS AND METHODS

Study Population

Subjects were recruited from the China Cardiometabolic Disease and Cancer Cohort (4C) Study, a community-based study conducted among 259,657 Chinese individuals aged 40 years and older (14, 15). The 4C study was performed in 25 communities across mainland China. The study design and methods have been described previously in detail (14). The data presented in this article are based on the subsamples from the Chongming District, Shanghai, China. From May to November 2011, a total of 9,930 subjects (40–73 years of age) participated in the baseline survey. In 2014, participants returned for a 3.1-year follow-up investigation. Individuals with the following conditions were excluded from this study: missing blood samples, the use of lipid-lowering agents (such as statins, fibrates, and natural products), tumor, infectious or systematic inflammatory diseases, significant hematologic disorders, thyroid dysfunction, severe liver and/or renal insufficiency, and PCSK9 outliers, which were defined as the extreme value (lower or upper 1% of the distribution) or repeated measurements coefficient of variation >15%, resulting in the inclusion of 7,104 participants (2,273 men and 4,831 women) in the analysis (**Figure 1**).

The study protocol was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from all participants.

Data Collection

A standardized questionnaire was used by trained physicians to collect essential information, including age, sex, lifestyle factors, educational attainment, physical activity, and previous medical history. Physical activity was evaluated based on the short form of the International Physical Activity Questionnaire by adding questions regarding the frequency and duration of moderate and vigorous activities and walking [Guidelines for data processing and analysis of the International Physical Activity Questionnaire (IPAQ)]. Current smokers or drinkers were defined as subjects who had a regular smoking or drinking status in the past 6 months. Anthropometric measurements, including height, weight, and waist circumference, were collected by certified medical staff using standard protocols. Blood pressure was measured with an automated electronic device (OMRON Model1 Plus; Omron Company, Kyoto, Japan).

Laboratory Measurements

Venous blood samples were obtained after overnight fasting for at least 10 h and were collected in tubes containing EDTA. The blood samples were centrifuged at 4°C and stored at −80°C until analysis. Circulating LDL-cholesterol, HDL-cholesterol, total cholesterol, and triglycerides, were measured with an autoanalyzer (Hitachi 7080; Tokyo, Japan). Venous plasma glucose level was determined by the glucose oxidase method (ADVIA-1650 Chemistry System, Bayer, Leverkusen, Germany). Fasting insulin was measured by RIA (Linco Research, St. Charles, MO). The circulating C-reactive protein was determined by ELISA kit (DY1707, R&D Systems, Minneapolis, MN) as recommended by the manufacturer.

PCSK9 Measurement

The circulating PCSK9 levels were measured in duplicate using a commercial ELISA kit (DY3888, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions and compared with purified human PCSK9 standards.

Definition of Cardiometabolic Risk Factors

A low HDL-cholesterol was defined as HDL-cholesterol <1.0 mmol/L in men or <1.3 mmol/L in women. A high LDL-cholesterol was defined as LDL-cholesterol ≥3.37 mmol/L. A

Abbreviations: BMI, body mass index; CI, confidence interval; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HbA_{1c}, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HR, hazard ratio; LDL, low-density lipoprotein; PCSK9, proprotein convertase subtilisin/kexin type 9; PPG, postprandial plasma glucose; SBP, systolic blood pressure; SD, standard deviation; TG, triglycerides.

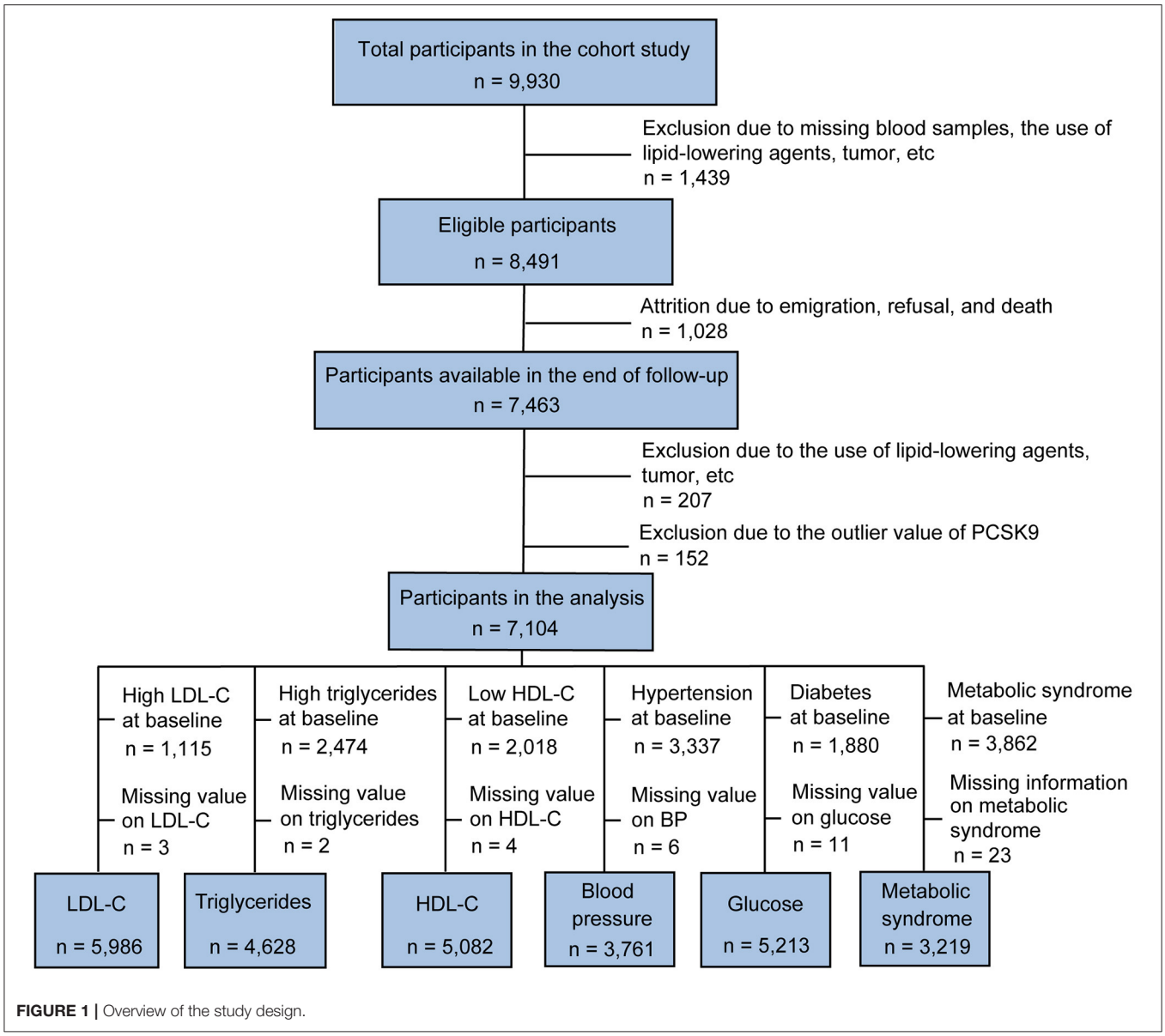

FIGURE 1 | Overview of the study design.

high triglyceride was defined as triglyceride ≥1.7 mmol/L or treatment with a lipid-lowering medication. Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or current use of antihypertensive treatment. Incident type 2 diabetes was diagnosed according to the American Diabetes Association 2010 criteria, which is defined as an FPG ≥ 7.0 mmol/L (≥126 mg/dL), 2-h PPG ≥ 11.1 mmol/L (≥ 200 mg/dL), or HbA_{1c} ≥ 6.5% (48 mmol/mol). The metabolic syndrome was defined based on the Joint Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity (16).

Statistical Analysis

The continuous variables with normal distribution are shown as means ± SD, and variables with skewed distribution are expressed as medians (interquartile range) and log-transformed to approximate normality before analysis. Categorical variables are reported as a percentage (%). The subjects were divided into two groups according to sex. For comparisons between groups, we performed an unpaired independent-samples Student *t*-test for normally distributed continuous variables and a non-parametric Mann-Whitney U test for skewed distributed variables. The Chi-squared tests were applied to compare categorical variables. The Pearson correlation analysis was used to evaluated correlation coefficients between baseline circulating PCSK9 levels and metabolic parameters. Multivariate Cox regression analysis was run to determine the potential association

TABLE 1 | Baseline characteristics of the study participants ($n = 7,104$).

	Total ($n = 7,104$)	Men ($n = 2,273$)	Women ($n = 4,831$)	*p*-value
PCSK9, ng/mL	283.3 ± 88.7	276.1 ± 86.4	286.7 ± 90.1	<0.001
Age, years	56.2 ± 7.4	57.8 ± 7.1	55.5 ± 7.7	<0.001
Current smoker, *n* (%)	963 (13.6)	750 (33.0)	213 (4.4)	<0.001
Current drinker, *n* (%)	777 (10.9)	593 (26.1)	184 (3.8)	<0.001
Physical activity				0.69
Low	5,101 (71.8)	1,618 (71.2)	3,483 (72.1)	
Middle	1,517 (21.4)	493 (21.7)	1,024 (21.2)	
High	486 (6.8)	162 (7.1)	324 (6.7)	
Educational attainment				<0.001
0–6	1,589 (22.4)	434 (19.1)	1,155 (23.9)	
7–9	3,466 (48.8)	1,123 (49.4)	2,343 (48.5)	
≥10	2,049 (28.8)	716 (31.5)	1,333 (27.6)	
BMI, kg/m^2	24.3 ± 4.7	24.4 ± 4.7	24.3 ± 4.7	0.31
WC, cm	83.4 ± 7.6	85.8 ± 7.8	82.3 ± 7.5	<0.001
SBP, mm Hg	131 ± 15	134 ± 16	129 ± 15	<0.001
DBP, mm Hg	81 ± 11	83 ± 11	80 ± 11	<0.001
FPG, mmol/L	6.3 ± 1.6	6.5 ± 1.7	6.2 ± 1.6	<0.001
PPG, mmol/L	8.8 ± 3.9	8.9 ± 4.1	8.8 ± 3.8	0.05
HOMA-IR	1.83 (1.30–2.66)	1.69 (1.16–2.52)	1.88 (1.37–2.72)	<0.001
CRP, mg/L	1..46 (0.76–2.65)	1.46 (0.78–2.70)	1.46 (0.76–2.63)	0.63
HDL-C, mmol/L	1.24 ± 0.33	1.18 ± 0.34	1.27 ± 0.32	<0.001
LDL-C, mmol/L	2.65 ± 0.76	2.58 ± 0.73	2.68 ± 0.78	<0.001
TC, mmol/L	4.70 ± 1.02	4.62 ± 0.97	4.74 ± 1.04	<0.001
Triglycerides, mmol/L	1.36 (0.96–1.98)	1.44 (0.99–2.24)	1.33 (0.95–1.89)	<0.001
Diabetes	1,880 (26.5%)	572 (25.2)	1,308 (27.1)	0.09
Metabolic syndrome	3,862 (54.4%)	1,219 (53.6)	2,643 (54.7)	0.39

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9; PPG, postprandial plasma glucose, SBP, systolic blood pressure, WC, waist circumference. Data are shown as means ± SD for normally distributed continuous variables, or median (interquartile range) for skewed distributed continuous variables.

between baseline PCSK9 levels and incidence of cardiometabolic risk factors. We also used restricted cubic splines with five knots at the 5th, 35th, 50th, 65th, and 95th centiles to flexibly model exploring the association of circulating PCSK9 levels on a continuous scale with the incidence of cardiometabolic risk factors. Hazard ratios (HRs) and 95% confidence intervals (CIs) for the relationship between PCSK9 and the incidence of cardiometabolic risk factors were generated with the Cox proportional hazards model. In order to minimize the effect of potential confounding factors, covariates were selected based on biologic interest, well-established risk factors for cardiometabolic diseases, or associated exposures and outcomes. Variables showing $p < 0.05$ in the univariable regression model were entered into the multivariable model. Multivariable adjusted models were used to explore the independent effect of PCSK9 on cardiometabolic risk factors. Data management and statistical analyses were performed with SPSS software (version 25.0) and R version 3.6.1. The significance level was set at $p < 0.05$, and *p*-values were provided for two-sided tests.

RESULTS

Baseline Characteristics of Subjects

Table 1 summarizes the baseline characteristics of 7,104 participants who were stratified according to sex. The mean age of the subjects was 56.2 ± 7.5 years, and 32.0% of the participants were males. Circulating PCSK9 levels were higher in women than men (286.7 ± 90.1 ng/mL vs. 276.1 ± 86.4 ng/mL, $p < 0.001$).

Correlation of Baseline Circulating PCSK9 Levels With Clinical Characteristics

According to the Pearson correlation analysis, circulating PCSK9 levels were significantly and positively correlated with LDL-cholesterol, total cholesterol, and triglycerides (all $p < 0.001$) both in men and women (**Table 2**). Moreover, a positive correlation was observed between circulating PCSK9 and age, BMI, waist circumference, fasting plasma glucose, systolic and diastolic blood pressure, HOMA-IR, and C-reactive protein (all $p < 0.01$) in women only.

TABLE 2 | Pearson correlation analysis between baseline circulating PCSK9 and clinical characteristics.

Variables	PCSK9					
	Total		Men		Women	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value
Age	0.080	<0.001	0.083	0.142	0.161	<0.001
BMI	0.069	<0.001	0.003	0.644	0.103	<0.001
WC	0.070	<0.001	0.029	0.235	0.124	<0.001
HDL-C	0.087	0.011	0.097	0.009	0.068	0.02
LDL-C	0.171	<0.001	0.164	<0.001	0.173	<0.001
Total cholesterol	0.249	<0.001	0.286	<0.001	0.231	<0.001
Log triglycerides	0.194	<0.001	0.183	<0.001	0.213	<0.001
FPG	0.067	<0.001	0.025	0.395	0.121	<0.001
PPG	0.047	0.052	0.032	0.271	0.093	<0.001
Log HOMA-IR	0.086	<0.001	0.006	0.765	0.153	<0.001
CRP	0.072	<0.001	0.068	0.107	0.112	<0.001
SBP	0.105	<0.001	0.065	0.083	0.132	<0.001
DBP	0.063	<0.001	0.026	0.207	0.092	<0.001

BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; PCSK9, proprotein convertase subtilisin/kexin type 9; PPG, postprandial plasma glucose, SBP, systolic blood pressure; WC, waist circumference.

Association Between Baseline Circulating PCSK9 Levels and Incidence of Cardiometabolic Risk Factors

Table 3 presents the hazard ratios (HR) for categorized cardiometabolic risk factors per 1-SD increment in baseline circulating PCSK9. Circulating PCSK9 was positively associated with incidence of high LDL-cholesterol both in men (HR 1.33, 95% CI 1.09–1.65, $p < 0.001$) and women (HR 1.36, 95% CI 1.12–1.69, $p < 0.001$) after adjustment for age, current smoking status, alcohol consumption, physical activity, educational attainment, BMI, waist circumference, C-reactive protein, fasting plasma glucose, post-loading plasma glucose, HOMA-IR, systolic blood pressure, diastolic blood pressure, and lipid profiles. Furthermore, a positive association was observed between circulating PCSK9 with incidence of high triglycerides (HR 1.31, 95% CI 1.13–1.72, $p < 0.001$), hypertension (HR 1.28, 95% CI 1.08–1.53, $p = 0.011$), type 2 diabetes (HR 1.34, 95% CI 1.09–1.76, $p = 0.005$), and metabolic syndrome (HR 1.30, 95% CI 1.11–1.65, $p = 0.009$) in women only (**Figure 2**). And the positive linear dose-response relationship was evident in the cubic spline regression model (**Figure 3**, p for non-linearity >0.1). No statistically significant association was observed between circulating PCSK9 and incidence of low HDL-C ($p > 0.1$) both in men and women.

DISCUSSION

In the present study, we found that subjects with higher circulating PCSK9 had progressively worse cardiometabolic risk profiles in women, including incident high LDL-cholesterol, elevated triglycerides, hypertension, type 2 diabetes, and metabolic syndrome. However, no significant relationship was observed between PCSK9 and cardiometabolic risk factors in males, except incidence of high LDL-cholesterol. To the best of our knowledge, this is the first prospective cohort study to investigate the association between PCSK9 and incidence of cardiometabolic risk factors.

The key and predominantly characterized activity of secreted PCSK9 is enhancing the degradation of the LDL receptor (17), a principal endocytic receptor that mediates the clearance and catabolism of LDL (18). As a consequence of reduced LDL clearance, circulating levels of LDL-cholesterol increasing, which is a well-established risk factor of cardiovascular disease. Moreover, multiple studies reported that PCSK9 is also involved in the degradation of very low-density lipoprotein receptor, apolipoprotein E receptor 2 (ApoER2), LDLR-related protein-1 (LRP1), and fatty acid transporter CD36 (19, 20). Additionally, the human evidence of the *PCSK9* (S127R) gain-of-function mutation genetic study revealed that PCSK9 dramatically increased the production rate of apoB (21). Then the effect on apoB resulted in an overproduction of very low-density lipoprotein, intermediate-density lipoprotein, and LDL. Therefore, the observed positive correlation between PCSK9 and intermediate-density lipoprotein, which is the triglyceride-rich LDL subfraction (22), raises the possibility of a significant contribution of intermediate-density lipoprotein to the positive relationship between PCSK9 and triglycerides. Furthermore, PCSK9 increases intestinal triglyceride-rich lipoprotein production and secretion through transcriptional and post-transcriptional mechanisms and then enhances triglycerides accumulation by targeting the intermediate-density lipoprotein receptor in the adipose tissue (23). No significant association was observed between PCSK9 and low HDL-cholesterol, thus, it is reasonable to speculate that PCSK9 may associate with atherosclerotic cardiovascular disease through a pathway not fully overlapping with reduced HDL-cholesterol.

The existence of a significant correlation between triglycerides and glucose metabolism has supported the investigation of a possible involvement of PCSK9 in glucose homeostasis and insulin resistance. In accordance with the previous findings (11, 13), in our female population circulating PCSK9 is positively associated with fasting glucose and insulin resistance, which involved in the initiation and progression of cardiometabolic disease. Previous study also found that high serum PCSK9 is associated with increased risk of new-onset diabetes after transplantation in renal transplant recipients (24). Notably, several studies showed that LDL-cholesterol-lowering *PCSK9* (rs11583680, rs11591147, rs2479409, and rs11206510) genetic variants were associated with higher circulating fasting glucose levels and increased risk of type 2 diabetes (25). However, other studies reported that the loss-of-function *PCSK9* (p.R46L) genetic variant was not associated with impaired glucose homeostasis in humans (26) and PCSK9 inhibition did not increase the risk of new-onset diabetes, nor did it worsen glycemia (27, 28). Further study is necessary to shed lights on the underlying mechanism of the association between PCSK9 and glucose homeostasis.

TABLE 3 | Multivariable Cox regression of cardiometabolic risk factors on per 1-SD increment in PCSK9.

	Total		Men		Women	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
High low-density lipoprotein cholesterol						
Model 1	1.43 (1.19–1.72)	<0.001	1.31 (1.14–1.63)	<0.001	1.49 (1.23–1.79)	<0.001
Model 2	1.41 (1.18–1.70)	<0.001	1.32 (1.15–1.63)	<0.001	1.47 (1.22–1.76)	<0.001
Model 3	1.39 (1.17–1.68)	<0.001	1.29 (1.12–1.61)	<0.001	1.42 (1.20–1.71)	<0.001
Model 4	1.35 (1.12–1.68)	<0.001	1.33 (1.09–1.65)	<0.001	1.36 (1.12–1.69)	<0.001
High triglycerides						
Model 1	1.21 (1.07–1.37)	<0.001	1.03 (0.80–1.31)	0.84	1.28 (1.11–1.48)	<0.001
Model 2	1.20 (1.07–1.36)	<0.001	1.05 (0.82–1.34)	0.73	1.24 (1.08–1.44)	<0.001
Model 3	1.20 (1.06–1.35)	0.002	1.06 (0.82–1.37)	0.79	1.22 (1.06–1.42)	0.003
Model 4	1.19 (1.05–1.35)	0.006	1.03 (0.79–1.35)	0.66	1.31 (1.13–1.72)	<0.001
Low high-density lipoprotein cholesterol						
Model 1	1.05 (0.90–1.22)	0.55	0.85 (0.62–1.16)	0.30	1.07 (0.89–1.28)	0.37
Model 2	1.02 (0.87–1.18)	0.85	0.84 (0.61–1.15)	0.27	1.05 (0.87–1.25)	0.33
Model 3	0.99 (0.84–1.15)	0.87	0.81 (0.58–1.13)	0.25	0.98 (0.81–1.19)	0.46
Model 4	1.00 (0.85–1.19)	0.99	0.87 (0.62–1.24)	0.34	1.01 (0.83–1.21)	0.52
Hypertension						
Model 1	1.27 (1.11–1.45)	<0.001	1.12 (0.88–1.42)	0.35	1.36 (1.16–1.60)	<0.001
Model 2	1.25 (1.09–1.43)	<0.001	1.15 (0.90–1.46)	0.28	1.29 (1.09–1.52)	0.001
Model 3	1.24 (1.08–1.42)	0.002	1.17 (0.91–1.50)	0.23	1.26 (1.06–1.49)	0.006
Model 4	1.17 (1.05–1.36)	0.019	1.12 (0.86–1.45)	0.27	1.28 (1.08–1.53)	0.011
Type 2 diabetes						
Model 1	1.20 (1.11–1.39)	0.003	0.91 (0.68–1.21)	0.52	1.35 (1.13–1.61)	<0.001
Model 2	1.18 (1.10–1.37)	0.005	0.90 (0.67–1.20)	0.48	1.29 (1.10–1.51)	<0.001
Model 3	1.17 (1.09–1.35)	0.008	0.88 (0.65–1.18)	0.30	1.25 (1.10–1.49)	0.002
Model 4	1.14 (1.07–1.33)	0.020	0.81 (0.59–1.12)	0.21	1.34 (1.09–1.76)	0.005
Metabolic syndrome						
Model 1	1.27 (1.11–1.45)	<0.001	1.18 (0.93–1.49)	0.17	1.31 (1.11–1.67)	<0.001
Model 2	1.24 (1.08–1.43)	<0.001	1.19 (0.94–1.51)	0.19	1.27 (1.09–1.57)	0.004
Model 3	1.24 (1.07–1.42)	0.002	1.17 (0.92–1.48)	0.21	1.28 (1.09–1.59)	0.003
Model 4	1.22 (1.06–1.41)	0.007	1.05 (0.80–1.37)	0.32	1.30 (1.11–1.65)	0.009

Model 1 was adjusted for age, current smoking status, alcohol consumption, physical activity, and educational attainment. Model 2 was further adjusted for BMI and waist circumference. Model 3 was further adjusted for HOMA-IR and C-reactive protein. Model 4 was further adjusted for fasting glucose, post-loading plasma glucose, systolic blood pressure, diastolic blood pressure, and lipid profiles. Subgroup variable was excluded from the model.

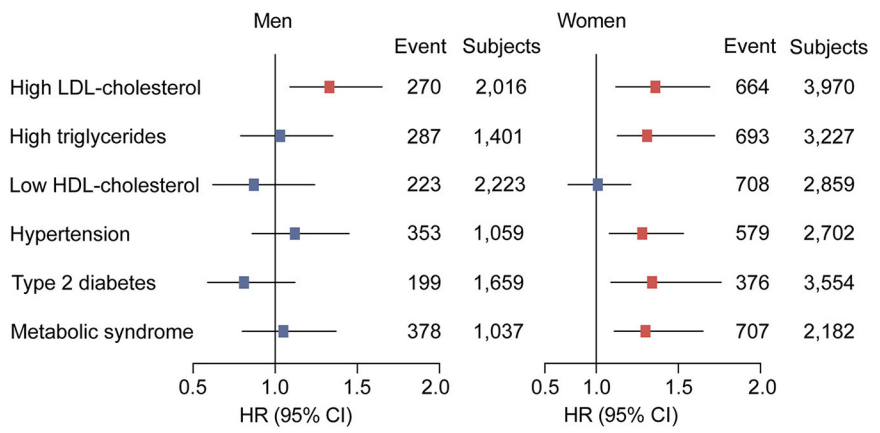

FIGURE 2 | Adjusted hazard ratios (HRs) of cardiometabolic risk factors according to per 1-SD increment in baseline circulating PCSK9 levels in men and women. Model was adjusted for age, current smoking status, alcohol consumption, physical activity, educational attainment, BMI, waist circumference, C-reactive protein, fasting plasma glucose, post-loading plasma glucose, HOMA-IR, systolic blood pressure, diastolic blood pressure, and lipid profiles. Subgroup variable was excluded from the model.

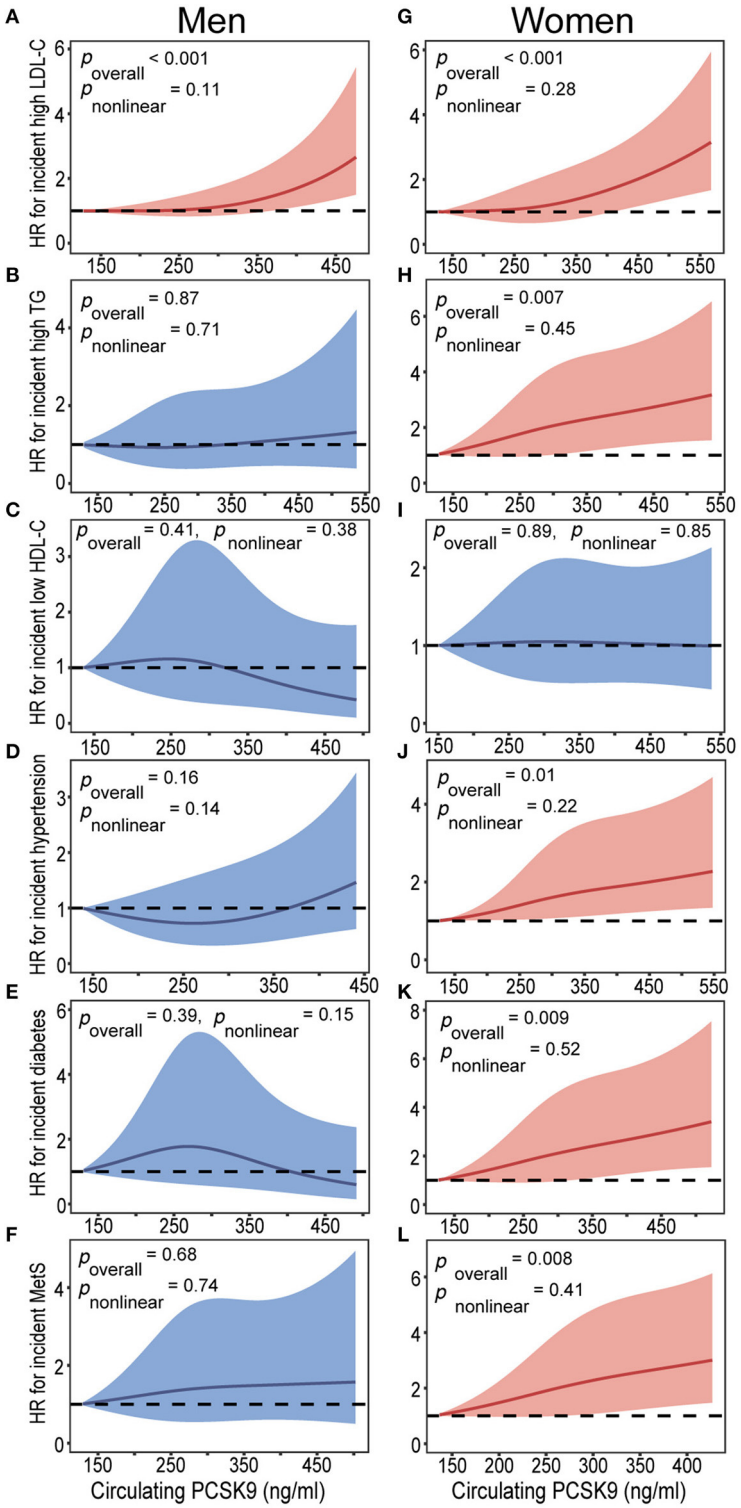

FIGURE 3 | Baseline circulating PCSK9 levels on a continuous scale and incidence of cardiometabolic risk factors in men **(A-F)** and women **(G-L)**. Cardiometabolic risk factors including high LDL-C **(A,G)**, high triglycerides **(B,H)**, low HDL-C **(C,I)**, hypertension **(D,J)**, type 2 diabetes **(E,K)**, and metabolic syndrome **(F,L)**. Hazard ratios (HRs) are indicated by solid lines and 95% confidence intervals (CIs) by shaded areas. Model was adjusted for age, current smoking status, alcohol consumption, physical activity, educational attainment, BMI, waist circumference, C-reactive protein, fasting plasma glucose, post-loading plasma glucose, HOMA-IR, systolic blood pressure, diastolic blood pressure, and lipid profiles. Subgroup variable was excluded from the model. LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; MetS, metabolic syndrome; PCSK9, proprotein convertase subtilisin/kexin type 9.

A positive correlation was observed between baseline circulating PCSK9 levels and blood pressure in females, which is in line with data obtained in other cross-sectional researches (11, 12). Genetic evidence from the Hypertension Genetic Epidemiology Network (HyperGEN) and the Reasons for Geographic And Racial Differences in Stroke study (REGARDS) studies have shown that *PCSK9* variation associated with blood pressure in African Americans (29). However, other investigation failed to identify a relationship between PCSK9 and blood pressure (30). Such an inconsistent association may be explained in part by a limited sample size and cross-sectional study design.

In line with the previous reports (11, 12, 24), we found PCSK9 was positively correlated with both BMI and waist circumference in females, suggesting that PCSK9 may be associated with obesity, especially visceral adiposity, the most prevalent features of the cardiovascular disease. Nevertheless, only a minor reduction of the cardiometabolic risk was yielded with per 1-SD increment in PCSK9 after further adjustment for BMI and waist circumference. Consequently, relationship between circulating PCSK9 and cardiometabolic risk factors in women may not be merely the link of excess adipose tissue.

The most striking finding in the present study was the positive relationship between circulating PCSK9 levels and incidence of high triglycerides, hypertension, type 2 diabetes, and metabolic syndrome only in female subjects. Consistent with the previous studies, correlations between PCSK9 and a variety of clinical characteristics, including age, BMI, LDL-cholesterol, triglycerides, glucose, blood pressure, and C-reactive protein, were either stronger or only present in females (11, 12, 31). Furthermore, evidence from small-scale research (aged 15–26 years) found that obesity and type 2 diabetes were associated with significantly higher levels of PCSK9 in America young women, but not in young men (31). In humans, many studies described circulating PCSK9 levels as being significantly higher in women than men, but differences have also been found in postmenopausal compared to premenopausal women as well as in pregnant compared to non-pregnant women. Therefore, it is reasonable to speculate a possible role for sex hormones in PCSK9 synthesis and/or metabolism. According to evidence from animal and human studies, PCSK9 is regulated by hormones such as estrogen, glucagon, insulin, growth hormone, and thyroid hormone (32). Previous studies reported that circulating PCSK9 concentration are decreased when endogenous estrogens are high in women (33), and pharmacologically elevated estrogen levels have been shown to lower PCSK9 levels both in animals and humans (34, 35). Conversely, several studies indicated that the difference in circulating PCSK9 levels between postmenopausal and premenopausal women appears to be independent of estrogen status (11), and estrogen at physiological concentrations does not affect hepatocyte PCSK9 expression in human (36). Overall, the clear molecular mechanisms underlying the sex differences remain unclear. More intensive investigations are needed to clarify the effect of gender on the association between PCSK9 and a variety of cardiometabolic abnormalities.

There are several limitations of this study. First, the use of a single baseline PCSK9 measurement to predict outcomes was a simplified and practical approach but was unable to assess the relationship between changes in PCSK9 levels and the incidence of metabolic disorders. Second, we did not record the type of diabetes among incident cases, but given that all the participants in our study were at least 40 years old at enrollment, these participants were unlikely to have type 1 diabetes because the fasting glucose was only mildly elevated and insulin levels were high normal. Third, due to the observational nature of the study, we cannot draw the causality from our findings. Thus, further studies are necessary to clarify the underlying mechanisms behind the association of PCSK9 with cardiometabolic dysregulation and to determine whether high PCSK9 level is a cause or a consequence of cardiometabolic abnormalities, or both. Forth, it is not clear whether our findings in middle-aged and elderly Chinese female individuals can be generalized to younger populations or individuals of other ethnicities. In our study, men and women are significantly different for most evaluated parameters. Therefore, we failed to assess its potential impact on differences in results between the two groups. Although we have adjusted for almost all potential confounding factors in the regression models. Further research should be undertaken among diverse groups and to shed lights on the effects of sex on associations of PCSK9 with cardiometabolic risk factors. Fifth, loss or gain of function of *PCSK9* gene mutations may have had potential impacts on the statistical results. However, due to the study design, we failed to analyze the *PCSK9* mutations status in present study.

In summary, our results indicate that subjects with higher circulating PCSK9 had progressively worse cardiometabolic risk profiles in women. These findings highlight the importance of elevated circulating PCSK9 levels in the development of atherosclerotic cardiovascular disease in female individuals. Moreover, based on our study, elevated circulating PCSK9 levels may emerge as novel target of efficacious prevention of cardiovascular disease in women.

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 Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JS, XL, and WZ drafted the manuscript and performed the experiments. ZY, QS, LQ, and GN conceived and designed the study. WZ, YN, JF, HZ, and NL recruited the subjects, processed samples, and contributed to the acquisition of data. JS, XL, WZ, YN, and ZY analyzed the

data. ZY revised the manuscript. All authors contributed to the article and approved the submitted version.

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

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Stress Induced Hyperglycemia in the Context of Acute Coronary Syndrome: Definitions, Interventions, and Underlying Mechanisms

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Elevation of glucose level in response to acute coronary syndrome (ACS) has been recognized as stress induced hyperglycemia (SIH). Plenty of clinical studies have documented that SIH occurs very common in patients hospitalized with ACS, even in those without previously known diabetes mellitus. The association between elevated blood glucose levels with adverse outcome in the ACS setting is well-established. Yet, the precise definition of SIH in the context of ACS remains controversial, bringing confusions about clinical management strategy. Several randomized trials aimed to evaluate the effect of insulin-based therapy on outcomes of ACS patients failed to demonstrate a consistent benefit of intensive glucose control. Mechanisms underlying detrimental effects of SIH on patients with ACS are undetermined, oxidative stress might play an important role in the upstream pathways leading to subsequent harmful effects on cardiovascular system. This review aims to discuss various definitions of SIH and their values in predicting adverse outcome in the context of ACS, as well as the effect of intensive glucose control on clinical outcome. Finally, a glimpse of the underlying mechanisms is briefly discussed.

Keywords: stress induced hyperglycemia, acute coronary syndrome, admission blood glucose, intensive glucose control, oxidative stress

INTRODUCTION

Stress-induced hyperglycemia (SIH) is an acute response of the bodies to many critical illnesses, including acute coronary syndromes (ACS) (1). Many observational studies have documented that hyperglycemia occurs frequently among patients hospitalized with ACS, even those without diabetes mellitus (1–3). It has been well-established that elevated glucose is associated with increased in-hospital and long-term mortality in ACS patients, especially in non-diabetic patients (4–10). Yet, hyperglycemia is not identified as an independent risk factor of ACS to date. Many questions regarding the relationship between SIH and ACS remain unclarified. For example, which glucose metrics, such as the blood glucose on admission (ABG), the average blood glucose, or the glucose variability (GV), is the most appropriate measurement of hyperglycemia and best correlated to the poor outcome; what the cut-off values that precisely define SIH are; whether there's any difference between cut-off values in the presence and absence of

recognized diabetes mellitus; whether glucose-lowering therapy can improve the prognosis of ACS patients with SIH; and finally, what the mechanisms underlying SIH in the ACS setting are. This article aims to address some of the undetermined issues based on the present available data.

DEFINING SIH IN THE CONTEXT OF ACS

Defining SIH With ABG

Despite numerous studies regarding SIH have been published, there's currently no uniform definition for SIH in the setting of ACS. Most early studies defined hyperglycemia by the first available glucose value or ABG (1, 4, 5, 8, 11–13). The most acceptable description of ABG refers to the first acquired blood glucose within 24 h of admission (1, 7). Nevertheless, the cut-point of ABG used to define hyperglycemia in patients with ACS was different from study to study. Back to 2008, the American Heart Association (AHA) Scientific Statement on Hyperglycemia and Acute Coronary Syndrome suggested using an ABG level >140 mg/dL as the definition of hyperglycemia under such circumstances (6). Evidence behind this recommendation mainly came from retrospective observational studies. In a national retrospective study of 141680 elderly acute myocardial infarction (AMI) patients, non-diabetic patients with higher ABG (range from>110 to 140 mg/dL) had increased risk of both 30-day and 1-year mortality compared with patients whose ABG was ≤110 mg/dL. In contrast, increased mortality was observed only in those with an ABG >240 mg/dL among diabetic AMI patients (4). Another study found AMI patients with a baseline glucose level <140 mg/dL had lower 30-day mortality compared to those with higher baseline glucose (14). Similarly, Buturlin et al. reported in a recent study of 4,520 ACS patients that mildly elevated ABG with glucose levels <140 mg/dL was not independently associated with increased 1-year mortality in non-diabetic patients (15). In a meta-analysis of 15 relatively small studies which discussed the association between ABG and ACS outcomes, Capes et al. indicated that among non-diabetic patients with AMI, the relative risk of in-hospital death in those with an ABG level >110 mg/dL was 3.9 compared with that of patients who were normoglycemic. On the contrary, among diabetic AMI patients, a greater risk of in-hospital death was observed only in patients whose ABG level ≥180 mg/dL (1). The phenomenon that cut-off values of ABG for predicting adverse outcome differed between diabetic and non-diabetic AMI patients was reported in similar studies (4, 8). An appropriate reason for such discrepancy might be the unawareness of different baseline glucose metabolic status between diabetic and non-diabetic patients. The ABG level is influenced by both acute physiological stress and chronic baseline glycemic levels, especially in patients with established diabetes mellitus. It's obviously that using a single cut-off value of ABG to define SIH, regardless of the previous glucose metabolic status, is not compelling. Therefore, new metrics including glycemic gap and stress hyperglycemia ratio (SHR), which eliminate the interference of chronic glycemic levels, were introduced (16–18).

Defining SIH With Glycemic GAP/SHR

The glycemic gap is calculated from the ABG minus the HbA1c derived average glucose level. A recent study showed that glycemic gap instead of ABG was associated with increased mortality (9). Similarly, SHR is calculated from ABG divided by the HbA1c derived average glucose level, which is also expressed as acute-to-chronic glycemic ratio in some articles. In a prospective study including 1,553 AMI patients, the prognostic power of glycemic ratio for in-hospital mortality was particularly evident in diabetic patients. However, among non-diabetic patients, both glycemic ratio and ABG had a similar prognostic accuracy (17). Another study of patients with ST-segment elevation myocardial infarction (STEMI) found that the glycemic ratio was closely associated with an increased risk for poor in-hospital outcome among both diabetic and non-diabetic patients (19). In contrast, ABG showed an association with poor in-hospital outcome only in non-diabetic patients. A recent randomized study evaluated the predictive value of SHR for long-term outcome in both diabetic and non-diabetic patients with STEMI. It included 6,287 STEMI patients and followed up over 5 years, and finally demonstrated that high SHR was significantly associated with worse long-term outcome in non-diabetic, instead of diabetic patients (10). Coincidently, Yang et al. reported in an AMI cohort that patients with a high SHR were at increased risk for long-term MACCE, defined as composites of all-cause death, non-fatal myocardial infarction, and non-fatal stroke (20). Again, when the same analysis was applied to diabetic patients, the risk of MACCE did not differ between patients with and without a high SHR. Hence, the predictive value of SHR was similar among diabetic and non-diabetic patients for in-hospital outcomes, but differed for long-term outcomes. The underlying mechanisms is unknown, one possible explanation might be the effect of SIH is masked by diabetes itself, given the fact that diabetes contributes to poor long-term prognosis in AMI patients (10).

Defining SIH With GV

Both ABG and SHR are derived from one blood glucose test. The nature of the metrics determines that it cannot reflect the full profile of glucose swings in the ACS setting. Patients with similar mean glucose levels can have markedly different glucose excursions. Meanwhile, glucose fluctuations can exert deleterious effects on both endothelial function and oxidative stress (21, 22). Previous studies reported increasing GV conferred a higher risk of mortality among critically ill patients, independent from mean glucose levels (23). Gong Su et al. demonstrated in an AMI cohort that GV, indicated as the mean amplitude of glycemic excursions (MAGE), was associated with increased risk of MACE instead of ABG or HbA1c (24). In a further study of 256 non-diabetic STEMI patients, high GV but not ABG was turned out to be associated with 3-month MACE (25). Subsequent studies emerged with similar conclusions that GV was a predictor of prognosis in patients with ACS regardless of the diabetic status (26–28). In addition, an elevated GV was suggested to be associated with hypoglycemia, an independent risk

factor for patients with coronary artery disease (29, 30). Yet, among several methods to quantify GV, such as standard deviation, MAGE and coefficient of variation, there is no universally accepted "gold standard." Given different methods being utilized in studies, the results should be interpreted with caution. Besides, there were a few studies focusing on other metrics, such as fasting glucose (FG). Considering the definition of FG used in these studies, it seems to be an alternative index for ABG. However, FG within 24 h of admission was reported to be associated with both increased short and long-term mortalities only in diabetic patients with ACS (31–33).

With present methodology, it seems unable to describe the complete profile of SIH in the ACS setting by utilizing a single glucose metrics. Moreover, an optimal definition of SIH should have a similar accuracy in predicting the cardiovascular outcomes among both diabetic and non-diabetic patients. Further investigations regarding how to precisely define or draw the outline of SIH are in demand.

INTERVENTIONS

Although it's widely accepted that ACS patients presenting with hyperglycemia are at increased risk for adverse outcome, it remains to be illustrated whether hyperglycemia is a direct mediator of poor outcome, or it's simply a marker indicating a greater disease severity. To address the issue, large randomized clinical trials of glucose control in hospitalized ACS patients are requisite. In contrast to plenty of clinical trials of target-driven glucose control in chronic hyperglycemia patients, a few trials exploring the optimal glycemic target for ACS patients have been performed (**Table 1**).

To our knowledge, DIGAMI was the first randomized clinical trial designed to evaluate the effect of intensive glucose control in AMI patients presenting with SIH. A total of 620 patients presenting with AMI, either had recognized diabetes mellitus or had a blood glucose level >11 mmol/L without diabetes, were enrolled (34). Patients were randomized into intervention arm with insulin-glucose infusion followed by multidose subcutaneous insulin and control arm with conventional therapy. The primary endpoint was all-cause mortality at 3 months. Patients from the insulin arm had significantly lower glucose levels compared to the control arm during the interventional period. Although there was no difference between two treatment groups for the primary outcome, reduced all-cause mortality was observed in the insulin arm at both 1- and 3.4-year follow up points (35). Nevertheless, given that over 80% of the patients had recognized diabetes mellitus and the insulin treatment lasted 3 months, it's hard to tell whether acute or chronic intensive glucose control contributed more to the reduced mortality. Although similar studies emerged subsequently, DIGAMI was the only trial demonstrating a survival benefit from intensive glucose control. The following study DIGAMI 2 was performed to compare the effects of 3 different treatment strategies in diabetic patients with AMI. Unexpectedly, no difference in the glucose control was achieved between the treatment groups, and it failed to demonstrate early and continued insulin-based intense glucose control could reduce mortality (36).

In the HI-5 study, 40% of the enrolled AMI patients were hyperglycemic without known diabetes. Patients were randomized to receive either insulin-based therapy or conventional therapy (37). There was no difference between two treatment arms in the mean 24-h blood glucose level. Despite a lower incidence of cardiac failure and reinfarction in the intervention arm within 3 months, HI-5 failed to demonstrate a reduced mortality at the in-hospital stage, 3 or 6 months. Nerenberg et al. enrolled 287 patients with AMI and hyperglycemia and randomly assigned them to either tight glucose control or usual care (38). At 24 h, patients from the tight glucose control arm had significant lower glucose levels compared to those from control arm, yet the 90-day mortality didn't differ between two arms. Besides, in a study by Marfella et al., 50 hyperglycemic patients diagnosed with AMI were randomized to intensive glycemic control (target glucose level 80–140 mg/dL) or conventional glycemic control for almost 3 days before surgery (39). Compared to the control group, patients in the intensive group had higher ejection fraction, less oxidative stress, less inflammation in peri-infarcted specimens. In their following studies, tight glucose control in hyperglycemic patients with STEMI brought benefits to both myocardial salvage and in-stent restenosis at 6 months after onset (40, 41).

Given the inconsistent results of clinical trials about glucose control in AMI patients, de Mulder et al. realized the inappropriate glucose target might be the problem. In their randomized trial BIOMArCS-2, a total of 294 patients with ACS and hyperglycemia were randomized to either intensive glucose control or conventional management (42). The target glucose levels were 85–110 mg/dL and <288 mg/dL, respectively. The primary endpoint was high-sensitive troponin *T*-value 72 h after admission. Glucose levels in the intensive arm were significantly lower than that of control arm within 36 h, but equalized by 72 h. Unexpectedly, there're no difference between the groups in the troponin *T*-values at 72 h. In contrast, a median follow-up of 5.1 years of the study reported higher rates of mortality at both 30 days and long term, suggesting intensive glucose control in the early phase of AMI resulted in persistent harmful effects (43). Compared to DIGAMI, BIOMArCS-2 had a more stringent target glucose level in the intervention arm. Although further analysis of BIOMArCS-2 didn't demonstrate an association between hypoglycemia and increased mortality, a lower glucose target might be responsible for the opposite results gained from DIGAMI and BIOMArCS-2.

Additionally, insights from the cardiovascular outcome trials of new glucose-lowering drugs, including Glucagon-Like Peptide 1 Receptor Agonists (GLP-1 RAs) and Sodium-Glucose Co-Transporter 2 (SGLT-2) inhibitors (44–46), indicated a new management strategy on hyperglycemia which focused on clinical outcomes directly instead of just glucose control itself. Despite protective effects of GLP-1 RAs and SGLT-2 inhibitors on ischemia heart proved in animal infarction models (47–51), few trials have been performed in humans in the ACS setting. A pilot study found that STEMI patients treated with exenatide at the time of PCI had improved salvage of myocardium (52).

TABLE 1 | Randomized trials designed to compare effect of intensive glycemic control with that of standard therapy in patients presenting with ACS and associated SIH.

Clinical trial (year)	Study population	Number of patients (percentage of patients without known diabetes)	Admission glycemia (mg/dL)	Intervention glycemic target (mg/dL)	Achieved glycemic target (intervention vs. control) (mg/dL)	Primary endpoint	Result
DIGAMI (1995)	AMI	620 (13%)	>198 mg/dL	126–180 mg/dL in acute phase	148 vs. 162 mg/dL at discharge*	Mortality at 3 months	NS
DIGAMI 2 (2005)	ACS	1,253 (NA)	>198 mg/dL	Group 1: 90–126 mg/dL(fasting), <180 mg/dL (non-fasting) Group 2: 126–180 mg/dL	163.8 vs. 163.8 mg/dL during first 24 h (NS) A1c ∼6.8 vs. 6.8% by the end of 2-year follow-up (NS)	All-cause mortality difference between group 1 and 2	NS
HI-5 (2006)	AMI	240 (52%)	≥140 mg/dL	<140 mg/dL	149.4 vs. 162 mg/dL during first 24 h (NS) A1C 6.9 vs. 6.8% at 3 months (NS) A1C 7.4 vs. 7.0 at 6 months (NS)	Mortality at in-hospital stage, 3 and 6 months	NS
Marfella (2009)	AMI (CABG)	50 (58%)	≥140 mg/dL	80–140 mg/dL for intervention arm 180–200 mg/dL for control arm	162.7 vs. 192.4 mg/dL*	LVEF, oxidative stress, apoptosis	↑LVEF* ↓oxidative stress and apoptosis*
Marfella (2012)	AMI (CABG)	50 (62%)	>140 mg/dL	80–140 mg/dL for intervention arm 180–200 mg/dL for control arm	160.9 vs. 193.9 mg/dL*	Myocardial regeneration	↑Myocardial regeneration*
Marfella (2012)	STEMI (pPCI)	165 (53%)	≥140 mg/dL	80–140 mg/dL for intervention arm 180–200 mg/dL for control arm	145 vs. 191 mg/dL*	ISR	↓ISR
Marfella (2013)	STEMI (pPCI)	106 (62%)	≥140 mg/dL	80–140 mg/dL for intervention arm 180–200 mg/dL for control arm	144 vs. 201 mg/dL**	Myocardial salvage	↑Myocardial salvage
RECREATE (2012)	STEMI	287 (72%)	≥144 mg/dL	90–117 mg/dL	117.5 vs. 142.9 mg/dL**	Difference in mean glucose levels at 24 h	↓Glycemia
BIOMArKS2 (2013)	ACS (pPCI)	280 (90%)	140–288 mg/dL	85–110 mg/dL	112 vs. ≈130 mg/dL**	hsTropT 72 h after admission	NS

*AMI, acute myocardial infarction; ACS, acute coronary syndrome; STEMI, ST-elevation myocardial infarction; NA, not available; NS, not significant. *p<0.05; **p<0.001; CABG, coronary artery bypass sugery; pPCI, primary percutaneous coronary intervention; LVEF, left ventricular ejection fraction; ISR, in-stent restenosis; hsTropT, high-sensitive troponin T-value.*

Similar findings were reported in ACS patients treated with liraglutide (53–55). However, patients enrolled in these studies were not required to be hyperglycemic. Empagliflozin, a SGLT-2 inhibitor, were reported to reduce LV mass and improve diastolic function in patients with ACS and diabetes (56). Nevertheless, further human studies are needed for evaluation of the cardiovascular outcome of both drugs in the presence of ACS with SIH.

So far, given limited results from clinical trials, there're no unified recommendations on the optimal glucose target and therapeutic strategy for SIH in the ACS setting. A scientific statement from AHA recommended initiation of

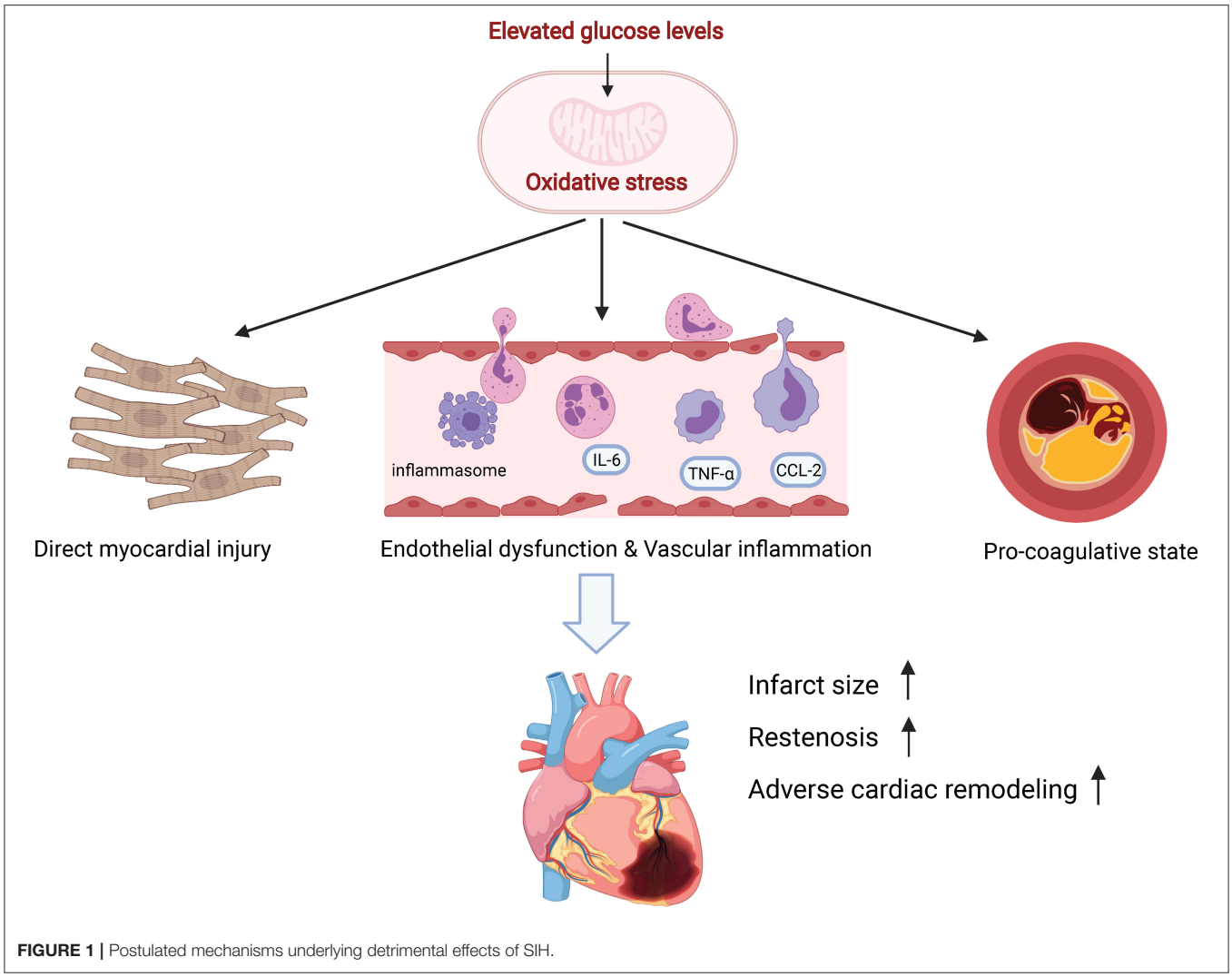

FIGURE 1 | Postulated mechanisms underlying detrimental effects of SIH.

intensive glucose control when plasma glucose was >180 mg/dL (6). In contrast, NICE recommendations suggested to manage hyperglycemia in ACS patients by keeping blood glucose levels below 198 mg/dL (57). The most recent ESC guidelines on management of non-STEMI/STEMI recommended it's reasonable to keep the blood glucose concentration <200 mg/dL (58, 59). Anyway, absolute avoiding of hypoglycemia is consistent across various statements and guidelines. As most ACS patients are hospitalized in intensive care units, intravenous insulin infusion with close blood glucose monitoring is the recommended glucose-lowering strategy.

MECHANISMS

Depending on baseline glucose metabolic status, the mechanisms underlying SIH could be very different (60). The development of SIH in patients without established diabetes mellitus in the context of ACS probably results from a combination of pancreatic β-cell dysfunction and acute insulin resistance (60, 61). Beta cell responsiveness was significantly related to ABG amongst patients with AMI (62). Furthermore, plasma proinsulin concentration and the proinsulin/insulin ratio were higher in AMI patients compared to control populations (63). These results indicated β-cell dysfunction might be prevalent among patients suffering AMI. Besides, glucose production is enhanced by upregulation of both gluconeogenesis and glycogenolysis. A complicated interplay of neurohormones and cytokines plays an important role in the development of hyperglycemia during ACS (64). In particular, excessive glucagon is the primary mediator of augmented glucogenesis. Sympathetic nervous system activation stimulates glucagon release, together with other anti-insulin hormones including cortisol and growth hormone, leading to hyperglycemia (65, 66). Cytokines, for example, tumor necrosis factor-α (TNFα), could promote gluconeogenesis *via* stimulation of glucagon production (67). Meanwhile, acute insulin resistance develops through two major pathways, including impaired post-receptor insulin signaling and downregulation of glucose transporter-4 (68). Both cytokines, such as TNFα and interleukin 1, and stimulation of β-adrenergic

receptors can inhibit post-receptor insulin signaling (69–72). Overproduction of cortisol also reduces insulin-mediated glucose uptake (73). Additionally, insulin resistance promotes lipolysis because of a catabolic state. In turn, the resultant excessive circulating free fatty acids exacerbate insulin resistance by disrupting insulin signaling and glycogen synthase (74, 75).

It's accepted that oxidative stress plays an important role in myocardial reperfusion injury as well as post-infarction remodeling (76, 77). Meanwhile, insights from both animal and human studies highlighted the role of increased oxidative stress in the pathophysiology of SIH (78–81). In turn, increased oxidative stress resulted in various tissue damaging *via* certain intracellular pathways, including the inflammatory and the non-oxidative glucose pathways (NOGPs) (60). Taking together, exacerbated oxidative stress during SIH might be a plausible mechanism responsible for additive subsequent detrimental effects in the ACS setting (**Figure 1**). First, acute hyperglycemia exerts a direct harmful effect on ischemic myocardium, probably *via* interfering with remote ischemic preconditioning (RIPerC). Kersten et al. showed that acute hyperglycemia abolished RIPerC induced cardioprotection and increased myocardial infarct size in a dose-dependent way (82). Similar finding was reported by Baranyai et al. in a rat model (83). However, some evidence suggested that chronic hyperglycemia reduced the infarct size and improved systolic function in rats after MI (84). Mechanisms underlying the cardioprotective effect of chronic hyperglycemia could be reduced cell necrosis, proinflammatory cytokines, and increased cell survival factors expression (84, 85). It seems that chronic hyperglycemia ahead of MI sets up a cellular preconditioning in response to acute rise of blood glucose. Secondly, both exacerbated vascular inflammation and endothelial cell dysfunction were implicated in the context of SIH (39, 86). Several studies showed an association of higher glucose levels with increased markers of vascular inflammation, including C-reactive protein, interleukin-6 and TNF-α (87, 88). Besides, hyperglycemia was reported to increase activation of prothrombotic factors, such as fibrinopeptide A and factor VII, and decrease plasma fibrinolytic activity (89–91). In an analysis of coronary thrombus from patients with STEMI, hyperglycemic patients showed a higher thrombus size, erythrocyte, fibrin, and macrophage levels (92). Finally, increasing studies implicated an association of SIH with post-infarct left ventricular systolic dysfunction (93, 94). Nevertheless, the underlying mechanisms need further illustration.

DISCUSSIONS

In this brief review, we discussed the definition, effects on clinical outcome, management, and pathophysiology of SIH in the context of ACS. A precise definition of SIH is helpful for designing interventional trials about glucose control in ACS patients. Only in this way, can we have high quality trials that shed lights on the nature of SIH. Therefore, we mainly focused on how to precisely define SIH. An optimal glucose metrics defining SIH should fulfill the following criteria that it correlates well with both short- and long-term outcomes regardless of the prior diabetic status. Unfortunately, a single glucose metrics seems unable to fulfill such criteria with present methodology. In the future, a combination of glucose metrics used to define SIH is reasonable and needs further investigations. We have fully understood that SIH is independently associated with adverse outcome of patients with ACS. However, it remains to be illustrated whether it's a marker of disease severity or a risk factor contributing directly to the poor clinical outcome. To address the issue, both clinical trials utilizing a unified precise definition of SIH and basic experiments revealing the underlying mechanisms are in demand. We suggest that researchers consider to set different glucose targets for patients with or without recognized diabetes mellitus in the future clinical trials targeting SIH in patients with ACS. With regards to underlying mechanisms, difference between the pathophysiological response of patients with or without previous persistent hyperglycemia should be taken into consideration.

AUTHOR CONTRIBUTIONS

ML, GC, and YF wrote the manuscript. XH revised the manuscript. All authors contributed to the article and approved the submitted version.

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

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Effects of Stress Hyperglycemia on Short-Term Prognosis of Patients Without Diabetes Mellitus in Coronary Care Unit

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Background: Diabetes mellitus (DM) has a high morbidity and mortality worldwide, and it is a risk factor for cardiovascular diseases. Non-diabetic stress hyperglycemia is common in severely ill patients, and it could affect prognosis. This study aimed to analyze the influence of different blood glucose levels on prognosis from the perspective of stress hyperglycemia by comparing them with normal blood glucose levels and those of patients with DM.

Methods: A retrospective study of 1,401 patients in coronary care unit (CCU) from the critical care database called Medical Information Mart for Intensive Care IV was performed. Patients were assigned to the following groups 1–4 based on their history of DM, random blood glucose, and HbA1c levels: normal blood glucose group, moderate stress hyperglycemia group, severe stress hyperglycemia group and DM group. The main outcome of this study was 30- and 90-day mortality rates. The associations between groups and outcomes were analyzed using Kaplan–Meier survival analysis, Cox proportional hazard regression model and competing risk regression model.

Results: A total of 1,401 patients in CCU were enrolled in this study. The Kaplan–Meier survival curve showed that group 1 had a higher survival probability than groups 3 and 4 in terms of 30- and 90-day mortalities. After controlling the potential confounders in Cox regression, groups 3 and 4 had a statistically significant higher risk of both mortalities than group 1, while no difference in mortality risk was found between groups 2 and 1. The hazard ratios [95% confidence interval (CI)] of 30- and 90-day mortality rates for group 3 were 2.77(1.39,5.54) and 2.59(1.31,5.12), respectively, while those for group 4 were 1.92(1.08,3.40) and 1.94(1.11,3.37), respectively.

Conclusions: Severe stress hyperglycemia (≥200 mg/dL) in patients without DM in CCU may increase the risk of short-term death, which is greater than the prognostic effect in patients with diabetes. Patients with normal blood glucose levels and moderate stress hyperglycemia (140 mg/dL ≤ RBG < 200 mg/dL) had no effect on short-term outcomes in patients with CCU.

Keywords: diabetes, stress hyperglycemia, CCU, short-term prognosis, insulin resistance

INTRODUCTION

Diabetes mellitus (DM) has developed into one of the most common chronic diseases worldwide, with its incidence increasing from 108 million (4.7%) in 1980 to 425 million (8.5%) in 2017 and an estimated increase to 629 million in 2045 (1). It has a high mortality rate (2), thus causing a serious burden of disease. In addition, patients with DM are several times more likely to suffer from cardiovascular diseases, especially coronary heart disease, than those without DM, and the disease progresses rapidly (3, 4). Studies showed that insulin resistance (5) is closely related to the occurrence of atherosclerosis, moreover, insulin resistance and hyperinsulinemia in type 2 DM patients are closely related to coronary heart disease (6). Up to 50% of patients with DM die due to complicated cardiovascular disease (7).

Stress hyperglycemia refers to a brief increase in blood glucose during acute physiological stress in the absence of DM, and it is very common in emergency and critically ill patients (8). The American Diabetes Association consensus on inpatient hyperglycemia defines stress hyperglycemia or hospital-related hyperglycemia as any blood glucose level of >7.8 mmol/L (140 mg/dL) without evidence of prior DM (9, 10). Stress hyperglycemia is a basic survival response when the body is stimulated by strong factors, such as infection, trauma, and surgery (11). Stress state could lead to increased secretion of various metabolic hormones, such as glucagon, epinephrine, and growth hormone, in the body's neuroendocrine system; this increase could directly or indirectly antagonize insulin, resulting in insulin deficiency, insulin resistance (12), and elevated blood sugar (13). Stress could also aggravate the decompensation of insulin secretion, and the insulin receptors of peripheral effector cells are downregulated, which makes the tissues less sensitive to insulin. However, when stress hyperglycemia exceeds 11.1 mmol/L (200 mg/dL), it is associated with poor prognosis and an increased risk of death, as evidenced by an increase in hospital mortality and an increased risk of malignant events, such as chronic heart failure or cardiogenic shock in patients with cardiovascular disease (14, 15).

Diabetes is not only a high risk factor for cardiovascular disease (16), but also a strong negative factor, with adverse effects on both short - and long-term outcomes of patients in CCU (17, 18). Stress hyperglycemia has a prognostic effect on many critical diseases (19), including AMI (17). It may also have therapeutic significance. Intervention in patients with stress hyperglycemia can reduce the possibility of complication development and mortality by maintaining blood glucose (20). However, at present, there is no unified standard for the treatment of stress hyperglycemia (21, 22). In our study, a large database, Medical Information Mart for Intensive Care (MIMIC) IV, was used to analyze the influence of different blood glucose levels on prognosis from the perspective of stress hyperglycemia by comparing them with normal blood glucose levels and those of patients with DM, so as to provide reference for clinical treatment.

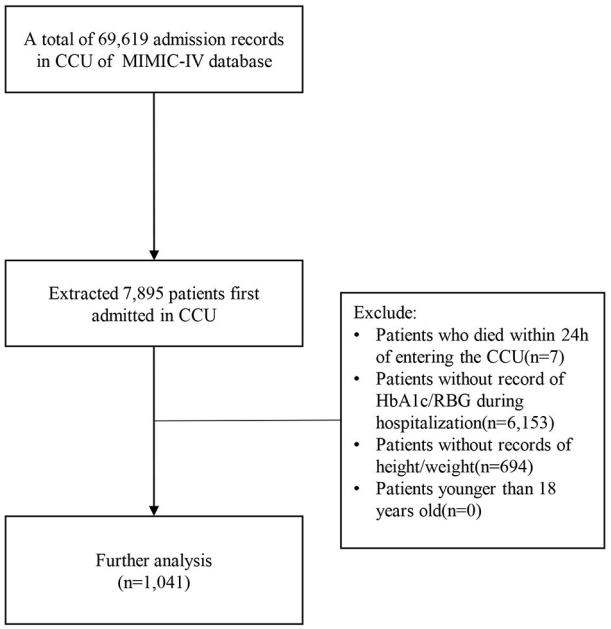

FIGURE 1 | Inclusion and exclusion flowchart of the study.

METHODS

Data Source

Data were extracted from MIMIC-IV v0.4, an openly accessible critical care database (23). MIMIC-IV contained over 250,000 electronic admission records in Beth Israel Deaconess Medical Center from 2008 to 2019 (24).

All information regarding patient identification were re-encoded and all identifiable information were hidden. Thus, obtaining an informed consent from the patients was not necessary. The author completed data research training from the Collaborative Institutional Training Initiative to obtain database permissions. All data were obtained from the official website of Physionet (https://mimic.physionet.org/).

Study Population

Among the 69,619 CCU admission records in MIMIC-IV, 7,895 patients who were first admitted to CCU were selected. Exclusion criteria were: patients who died within 24 h of entering CCU, patients who without record of HbA1c/RBG (random blood glucose levels, patient's first blood glucose measurement after entering CCU), patients without record of height/weight, patients younger than 18 years old. In the end, a total of 1,041 patients were selected for the study (**Figure 1**).

Data Extraction

Data were extracted by SQL language. The extracted variables included age, gender, BMI, vasopressor use, ventilation use, percutaneous coronary intervention (PCI), heart bypass surgery (HBS), HbA1c, RBG, acute physiology score-III (APS-III), Charlson Comorbidity Index (COMO), sepsis, ICU length of stay (LOS), hospital LOS, ICU mortality, and hospital mortality.

Outcomes

The main outcome of this study was 30-day and 90-day mortality rates. Patients with alive discharge status and those with hospital LOS of longer than 30/90 days were defined as censors for 30-/90-day status. Otherwise, they were defined as dead. Other outcomes included ICU LOS and hospital LOS. The ICU LOS was calculated by the difference between ICU discharge time (outtime) and ICU admission time (intime). The hospital LOS

TABLE 1 | Baseline characteristics of the study population.

	Group 1	Group 2	Group 3	Group 4	*p*-value
n	337	167	76	461	
Age (year)	63.0 (53.0, 75.0)	67.0 (55.5, 78.0)	67.5 (57.0, 77.3)	67.0 (57.0, 75.0)	0.030
Gender (%)					
Male	242 (71.8)	115 (68.9)	46 (60.5)	273 (59.2)	0.002
Female	95 (28.2)	52 (31.1)	30 (39.5)	188 (40.8)	
BMI	28.0 (24.1, 31.9)	28.1 (25.1, 31.8)	27.3 (22.9, 31.2)	29.6 (25.7, 34.6)	<0.001
Vasopressor (%)					
No	228 (67.7)	93 (55.7)	30 (39.5)	270 (58.6)	<0.001
Yes	109 (32.3)	74 (44.3)	46 (60.5)	191 (41.4)	
Ventilator (%)					
No	174 (51.6)	61 (36.5)	19 (25.0)	193 (41.9)	<0.001
Yes	163 (48.4)	106 (63.5)	57 (75.0)	268 (58.1)	
Percutaneous coronary intervention (%)					
No	261 (77.4)	134 (80.2)	66 (86.8)	382 (82.9)	0.136
Yes	76 (22.6)	33 (19.8)	10 (13.2)	79 (17.1)	
Heart bypass surgery (%)					
No	302 (89.6)	138 (82.6)	68 (89.5)	404 (87.6)	0.151
Yes	35 (10.4)	29 (17.4)	8 (10.5)	57 (12.4)	
Laboratory					
HbA1c (%)	5.6 (5.4, 5.9)	5.7 (5.4, 6.0)	5.7 (5.5, 6.0)	7.0 (6.3, 8.2)	<0.001
RBG(mg/dl)	112.0 (102.0, 125.0)	160.0 (147.0, 177.0)	238.0 (217.8, 292.3)	203.0 (152.5, 288.5)	<0.001
APS-III	36.0 (25.0, 50.0)	39.0 (29.0, 62.0)	52.0 (38.8, 73.3)	45.0 (35.0, 60.0)	<0.001
Charlson comorbidity index	2.0 (1.0, 3.0)	2.0 (1.0, 3.0)	2.0 (1.0, 4.0)	4.0 (2.0, 6.0)	<0.001
Sepsis (%)					
No	294 (87.2)	135 (80.8)	59 (77.6)	364 (79.0)	0.017
Yes	43 (12.8)	32 (19.2)	17 (22.4)	97 (21.0)	
Length of stay (Days)					
ICU	3.9 (1.6, 6.3)	4.1 (2.1, 8.4)	4.2 (2.3, 9.2)	4.1 (2.1, 7.8)	0.068
Hospital	8.0 (4.0, 14.0)	8.0 (5.0, 17.0)	8.0 (5.0, 12.0)	9.0 (5.0, 15.0)	0.046
Mortality					
30-day (%)	319 (94.7)	157 (94.0)	60 (78.9)	410 (88.9)	<0.001
No	18 (5.3)	10 (6.0)	16 (21.1)	51 (11.1)	
Yes					
90-day (%)	318 (94.4)	155 (92.8)	60 (78.9)	403 (87.4)	<0.001
No	19 (5.6)	12 (7.2)	16 (21.1)	58 (12.6)	
Yes					
ICU Death (%)					
No	322 (95.5)	156 (93.4)	60 (78.9)	416 (90.2)	<0.001
Yes	15 (4.5)	11 (6.6)	16 (21.1)	45 (9.8)	
Hospitalized Death (%)					
No	318 (94.4)	155 (92.8)	60 (78.9)	403 (87.4)	<0.001
Yes	19 (5.6)	12 (7.2)	16 (21.1)	58 (12.6)	

Group 1 represented the normal blood glucose group, group 2 represented the moderate stress group, group 3 represented the severe stress hyperglycemia group, and group 4 represented the diabetes group.

Charlson Comorbidity Index was calculated by Myocardial Infarct, Congestive Heart Failure, Peripheral Vascular Disease, Cerebrovascular Disease, Dementia, Chronic Pulmonary Disease, Rheumatic Disease, Peptic Ulcer Disease, Mild Liver Disease, Diabetes(uncomplicated), Diabetes(complicated), Paraplegia, Renal Disease, Malignant Cancer, Severe Liver Disease, Metastatic Solid Tumor, and Aids.

was calculated by the difference between hospital discharge (dischtime) time and admission time (admittime).

Study Cohort

All patients were divided into four groups on the basis of DM history, RBG, and HbAlc levels. Referencing the definition of stress hyperglycemia, group 1 represented the normal blood glucose group (RBG < 140 mg/dL, HbAlc < 6.5%, no history of DM), group 2 represented the moderate stress hyperglycemia group (140 mg/dL ≤ RBG < 200 mg/dL, HbAlc < 6.5%, no history of DM), group 3 represented the severe stress hyperglycemia group (RBG ≥ 200 mg/dL, HbAlc < 6.5%, no history of DM), and group 4 represented the DM group (HbAlc ≥ 6.5% or history of DM).

Statistical Analysis

The characteristics of the study population were described by median (interquartile range) for continuous variables, and

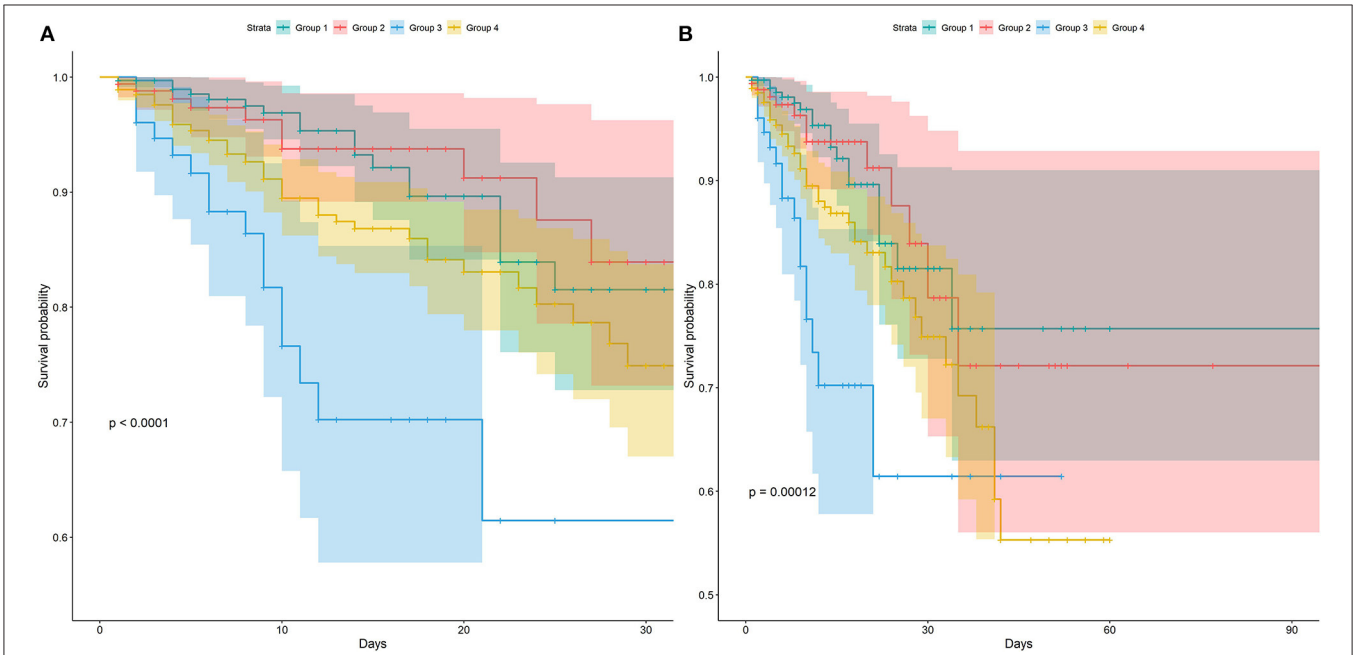

FIGURE 2 | Kaplan-Meier survival curves between groups. *P*-value calculated by Log-rank test = 0.012 showed Group 3 had lower survival probability. **(A)** Represented the survival probability in 30-day. **(B)** Represented the survival probability in 90-day.

TABLE 2 | HRs (95% CIs) for all-cause mortality and length of stay across groups.

	Group 1	Group 2		Group 3		Group 4	
		HR(95%CI)	*p*-value	HR(95%CI)	*p*-value	HR(95%CI)	*p*-value
30-day mortality							
Unadjusted	Reference	0.98(0.45,2.13)	0.969	4.04(2.06,7.93)	<0.001	1.87(1.09.3.20)	0.023
Adjusted	Reference	0.85(0.39,1.85)	0.673	2.77(1.39,5.54)	0.004	1.92(1.08,3.40)	0.027
90-day mortality							
Unadjusted	Reference	1.06(0.51,2.18)	0.882	3.79(1.95,7.38)	<0.001	1.95(1.16,3.28)	0.012
Adjusted	Reference	0.98(0.43,1.85)	0.752	2.59(1.31,5.12)	0.006	1.94(1.11,3.37)	0.020
ICU length of stay							
Unadjusted time to discharge	Reference	0.83(0.69,1.00)	0.044	0.56(0.42,0.74)	<0.001	0.79(0.68,0.91)	0.001
Adjusted time to discharge	Reference	1.10(0.91,1.34)	0.330	0.90(0.63.1.72)	0.540	1.08(0.90,1.28)	0.420
Hospital length of stay							
Unadjusted time to discharge	Reference	0.86(0.71,1.02)	0.090	0.61(0.46,0.80)	<0.001	0.74(0.64,0.85)	<0.001
Adjusted time to discharge	Reference	1.05(0.85,1.30)	0.650	1.04(0.79,1.36)	0.800	1.01(0.86,1.19)	0.910

Cox proportional hazards regression models were used to calculate hazard ratios (HRs) with 95% confidence intervals (CIs).
Model 1 was unadjusted.
Model 2 was adjusted for age, gender, BMI, COMO, PCI, HBS, vasopressor use, ventilation use, APS-III, and sepsis.

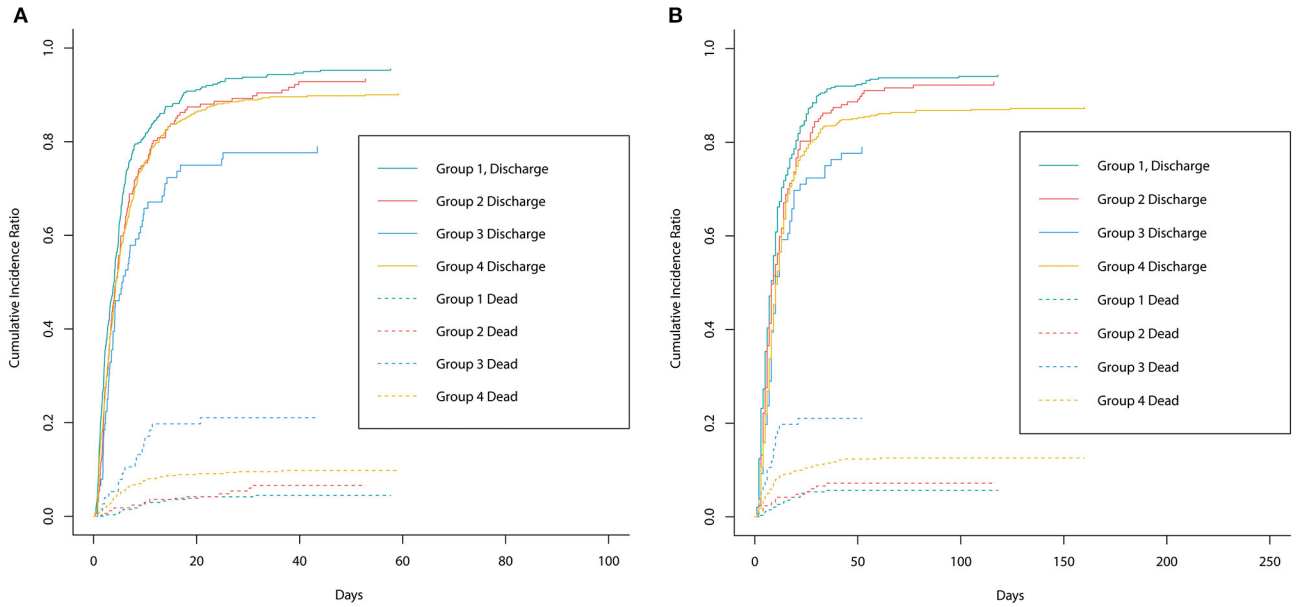

FIGURE 3 | The cumulative incidence curve between groups for ICU discharge and hospital discharge. The solid line represented the cumulative incidence ratio (CIR) of alive discharged patients while the dashed line represented the CIR of dead discharged patients (competing for risk). **(A)** Represented the cumulative incidence curve for ICU discharge between groups. **(B)** Represented the cumulative incidence curve for hospital discharge between groups.

as count (frequencies) for categorical data. Kruskal–Wallis test and Chi-squared test were conducted to analyze the difference in continuous and categorical variables among the four groups, respectively.

The associations between groups and the 30- and 90-day mortality rates were analyzed using Kaplan–Meier (KM) survival analysis and Cox proportional hazard regression model. The Log-rank test was conducted for non-parametric analysis to compare survival distributions between 4 groups (p-value < 0.05 represents a significant difference in survival between groups). Hazard ratio (HR) and 95% confidence interval (CI) were calculated via multivariable Cox regression by controlling age, gender, BMI, COMO, PCI, HBS, vasopressor use, ventilation use, APS-III, and sepsis.

We constructed a multivariable competing risk regression model to evaluate the association of patient groups and LOS in ICU/hospital (25). The included covariates are the same as the multivariate Cox regression and the time variable were ICU/hospital LOS. Since all patients will eventually be discharged, there is no censored data in this model, but patients' death in the ICU/hospital may result in a shorter LOS. Therefore, in this model, ICU and hospital LOS's failed status was defined as ICU, and hospital discharge and death are defined as a competitive risk. Therefore, for each group, HR <1 for ICU LOS represented longer ICU stay, while HR <1 for hospital LOS represented longer hospital stay compared with group 1.

Subgroup analyses were conducted for the association between groups and 30-day mortality for age (<65, ≥65), BMI (<25, 25–29.9, ≥30), PCI, myocardial infarction, congestive heart failure, APS-III (≤59, >59), and COMO (≤3, >3). The p-value of the overall interaction between the subgroup and the four cohorts was calculated from the Log-likelihood ratio test.

All statistical analyses were conducted on R software. Kaplan–Meier survival analysis and Cox proportional hazards regression models were performed using the "survival" package, and the competing risk regression models were analyzed using the "cmprsk" package. A two-side p-value <0.05 was considered statistically significant.

RESULTS

Among the 1,041 patients, 337, 167, 76, and 461 were included in groups 1–4, respectively. **Table 1** lists the baseline characteristics of patients. Age, gender, BMI, vasopressor use, ventilation use, COMO (details of the COMO are available in the **Supplementary Material**), APS-III score, 30-/90-day mortality, and ICU/hospital LOS significantly differed. Group 3 had the highest 30- and 90-day mortality rates and the longest median ICU LOS, and group 4 had the longest median hospital LOS.

In this study, 30-day mortality is 9.1% and 90-day mortality is 10.0%. The Kaplan–Meier survival curve showed no difference in survival probability between groups 1 and 2, while group 1 had a higher survival probability than groups 3 and 4 in both mortalities (**Figure 2**). After controlling the potential confounders in Cox regression, group 3 had a statistically significant higher risk of 30- and 90-day mortalities than group 1, while no difference in mortality risk was found between groups 2 and 1. The HRs (95% CI) of 30- and 90-day mortalities for group 3 were 2.77 (1.39, 5.54) and 2.59 (1.31, 5.12), respectively.

TABLE 3 | Subgroup analysis of the associations between all-cause mortality and length of stay.

	Group 1	Group 2		Group 3		Group 4		
		HR(95%CI)	*p*-value	HR(95%CI)	*p*-value	HR(95%CI)	*p*-value	*p*-interaction
Age								0.202
<65 (*n* = 493)	Reference	0.13(0.02,1.04)	0.055	1.32(0.40,4.37)	0.652	0.78(0.30,2.07)	0.622	
≥65 (*n* = 548)	Reference	1.83(0.73,4.58)	0.198	3.97(1.62,9.72)	0.003*	3.26(1.53,6.93)	0.002*	
BMI								0.223
<25 (*n* = 264)	Reference	0.95(0.65,3.61)	0.941	4.71(1.67,13.30)	0.003*	2.19(0.90,5.31)	0.080	
25–29.9 (*n* = 358)	Reference	0.52(0.12,2.16)	0.367	1.12(0.36,3.56)	0.391	1.12(0.36,3.55)	0.839	
≥30 (*n* = 418)	Reference	1.12(0.21,5.93)	0.895	2.48(0.45,13.67)	0.296	2.54(0.72,8.92)	0.147	
Percutaneous coronary intervention								0.115
No (*n* = 843)	Reference	0.56(0.23,1.37)	0.203	2.94(1.40,6.16)	0.004*	1.60(0.88,2.90)	0.121	
Yes (*n* = 198)	Reference	7.98(0.57,112.61)	0.124	2.37(0.16,34.89)	0.531	8.90(0.49,162.03)	0.149	
Myocardial infarct								0.199
No (*n* = 495)	Reference	0.29(0.06,1.32)	0.110	2.24(0.70,7.15)	0.175	1.64(0.72,3.72)	0.242	
Yes (*n* = 546)	Reference	1.39(0.44,4.36)	0.570	3.38(1.15,9.93)	0.026	2.42(0.91,6.40)	0.076*	
Congestive heart failure								0.399
No (*n* = 443)	Reference	1.01(0.28,3.72)	0.985	2.63(0.85,8.14)	0.094	1.70(0.61,4.72)	0.312	
Yes (*n* = 598)	Reference	0.89(0.32,2.43)	0.817	3.28(1.34,8.02)	0.009*	2.11(1.02,4.34)	0.043*	
APS-III								0.202
≤59 (*n* = 788)	Reference	2.06(0.41,10.33)	0.378	9.62(2.35,39.49)	0.002*	3.61(0.99,13.16)	0.051	
>59 (*n* = 253)	Reference	0.64(0.25,1.61)	0.344	1.62(0.71,3.67)	0.252	1.46(0.75,2.88)	0.269	
Charlson comorbidity index								0.401
≤3 (*n* = 668)	Reference	1.20(0.46,3.14)	0.710	3.14(1.28,7.69)	0.013*	2.00(0.89,4.51)	0.094	
>3 (*n* = 373)	Reference	0.54(0.14,2.14)	0.384	2.61(0.85,8.04)	0.094	1.93(0.83,4.49)	0.128	

*Confounders adjustment were performed as in Model 2 (**Table 2**). Cox proportional hazards regression models were used to calculate hazard ratios (HRs) with 95%. *p < 0.05.*

This finding demonstrated that compared with group 1, group 3 had 2.77 and 2.59 times of risk for 30- and 90-day mortalities, respectively. Compared to group 1, the 4 four had significantly 92 and 94% increased risk of 30- and 90-day mortality. The multivariate Cox regression results revealed that compared with the normal blood sugar group, there was no difference in the 30- and 90-day survival of the moderate stress hyperglycemia group while the death risk of severe stress hyperglycemia group and DM group was significantly increased. Besides, the severe stress hyperglycemia group presented the worst prognosis (**Table 2**).

Figure 3 shows the cumulative incidence curve between groups for ICU discharge and hospital discharge. The solid line represented the cumulative incidence ratio (CIR) of alive discharged patients, while the dashed line represented the CIR of dead discharged patients (competing for risk). **Figure 3A** shows that group 1 had the highest increasing rate of CIR for ICU discharge, which demonstrated that groups 2–4 had longer ICU LOS. **Figure 3B** shows a trend similar to that in **Figure 3A**, indicating that groups 2–4 had longer hospital LOS. The *p*-value of univariable Fine–Gray test results for ICU LOS in groups 2–4 was <0.05 and the HR was <1, thus revealing that groups 2–4 had significantly longer ICU LOS than group 1. For hospital LOS, single-factor regression results also showed that groups 3 and 4 had significantly longer hospital LOS. However, after adjusting for confounders, the difference in ICU/hospital LOS between groups was not statistically significant (**Table 2**) which indicated that the effect of stress hyperglycemia and DM on the prolongation of LOS was not significant. Therefore, stress hyperglycemia and DM may have stronger impacts on the acute prognosis of CCU patients, and other variables eventually caused the longer LOS presented in univariate analysis (e.g., BMI and comorbidities).

In the subgroup analysis (**Table 3**), although the HR (95%CI) and the corresponding *p*-value of different groups are different. However, the *p*-values of the interaction test results are all >0.05, which indicates that other factors have no significant impact on the 30-, 90-day mortality risk of stress hyperglycemia and DM.

DISCUSSION

Considering blood glucose levels are closely associated with cardiovascular disease, 1,401 patients in CCU were selected from a large critical care medical database and adjusted for a number of potential confounders, including APS-III score and COMO. Our study showed that there was no difference in 30-day and 90-day survival in the moderate stress-hyperglycemia group compared with the normal blood glucose group, while the risk of death was significantly increased in the severe stress hyperglycemia group and the DM group. We will explain them one by one, the hyperglycemic response to stress is part of the adaptive metabolic response to severe diseases, such as trauma and infection. It is involved in neuroendocrine and immune

pathways that lead to the development of insulin resistance and liver glucose production through gluconeogenesis and glycogen breakdown (26). Stress hyperglycemia forms a new glucose balance and produces a grape concentration gradient in critically ill patients, which ensures the body to absorb glucose to the maximum extent in the case of ischemia, blockage, and other microvascular blood flow obstruction. This finding is similar to that in previous research (27), which revealed that mild-to-moderate stress hyperglycemia has a protective effect in severely ill patients.

The current researches on severe stress hyperglycemia are still controversial, For example, one study (28) have shown that early post-injury blood glucose >200 mg/dL was not associated with increased infection rates and mortality in traumatic population, while another study showed that (29) that maintaining an average blood glucose level below 200 mg/dL after cardiac surgery could reduce the incidence of deep wound infection in patients with DM. Other studies have indicated that in people who don't have diabetes, short-term hyperglycemia was also associated with increased mortality after trauma and hip fracture (30, 31). Our research shows that, compared with normal blood glucose group, severe stress hyperglycemia group had 2.77 and 2.59 times of risk for 30- and 90-day mortalities, while diabetes group had 1.92 and 1.94 times of risk for both mortalities, respectively. It can be seen that, stress hyperglycemia in patients without DM causes more damage to the body than the influence of DM on the body. Research (32) has revealed that short-term hyperglycemia could reduce immunity through non-enzymatic glycosylation of circulating immunoglobulin. In addition, oxidative stress response, insulin resistance, and large amounts of catecholamine production could increase the level of free fatty acids in the body. Excessive levels of free fatty acids have toxic effects on infarcted and ischemic myocardium (33), and severe stress hyperglycemia can promote the development of patients with cardiovascular diseases (34), having a bad influence on patients in CCU. For DM patients, long-term disorder of carbohydrate, fat, and protein metabolism could cause multi-system damage, leading to chronic progressive pathological changes; functional decline; and failure of nerve, heart, blood vessels, and other tissues and organs, and they could be easily complicated with various types of infections (35). In addition, insulin resistance in diabetic patients could affect the function of vascular endothelial cells, thereby reducing the availability of nitric oxide, resulting in the increase in angiotensin and affecting blood pressure in patients in CCU (36).

Most patients in CCU are complicated with DM or insulin resistance, which affects their prognosis. Although many scholars have conducted studies on the optimal range of blood glucose control in critically ill patients, no unified standard is available as of yet (37). The present study showed that patients without DM with excessive stress blood glucose (≥200 mg/dL) had a higher risk of short-term death than patients with DM. In CCU, strengthening the control of patients' blood glucose management, developing appropriate treatment plan, improving the quality of life of patients, and reducing fatality rate are important.

This study has some limitations. First, the blood glucose level of the patients was collected for the first time after entering CCU, and whether the patients had taken blood glucose intervention measures, such as the use of insulin or hormone drugs, before entering CCU was not evaluated. Second, the types of DM were not distinguished. Finally, this study is a single-center retrospective study.

CONCLUSIONS

Severe stress hyperglycemia (≥200 mg/dL) in patients without DM in CCU may increase the risk of short-term death, which is greater than the prognostic effect in patients with diabetes. Patients with normal blood glucose levels and moderate stress hyperglycemia (140 mg/dL ≤ RBG < 200 mg/dL) had no effect on short-term outcomes in patients with CCU.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: The data were available on the MIMIC-IV website at https://mimic.physionet.org/, https://doi.org/10.13026/a3wn-hq05.

AUTHOR CONTRIBUTIONS

LZ created the study protocol, performed the statistical analyses, and wrote the first manuscript draft. ZW conceived the study and critically revised the manuscript. FX assisted with the study design and performed data collection. DH assisted with data collection and manuscript editing. SL assisted the analysis and explain of statistical methods. HY assisted with manuscript revision and data confirmation. JL contributed to data interpretation and manuscript revision. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.683932/full#supplementary-material

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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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SYSTEMATIC REVIEW
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Association Between Hemoglobin Glycation Index and Risk of Cardiovascular Disease and All Cause Mortality in Type 2 Diabetic Patients: A Meta-Analysis

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Background: The hemoglobin glycation index (HGI) has been proposed as a marker to quantify inter-individual variation in hemoglobin glycosylation. However, whether HGI is associated with an increased risk of diabetic complications independent of glycated hemoglobin (HbA1c) remains unclear. This meta-analysis aimed to determine the association between HGI and the risk of all cause mortality and composite cardiovascular disease (CVD).

Methods: PubMed, and EMBASE databases were searched for related studies up to March 31, 2021. Observational studies reported associations between HGI levels and composite CVD and all cause mortality were included for meta-analysis. A random effect model was used to calculate the hazard ratios (HRs) and 95% confidence intervals (CI) for higher HGI.

Results: A total of five studies, comprising 22,035 patients with type two diabetes mellitus were included for analysis. The median follow-up duration was 5.0 years. After adjusted for multiple conventional cardiovascular risk factors, an increased level of HGI was associated with a higher risk of composite CVD (per 1 SD increment: HR = 1.14, 95% CI = 1.04–1.26) and all cause mortality (per 1 SD increment: HR = 1.18, 95% CI = 1.05–1.32). However, when further adjusted for HbA1c, the association between HGI and risk of composite CVD (per 1 SD increment of HGI: HR = 1.01, 95% CI = 0.93–1.10) and all cause mortality (per 1 SD increment of HGI: HR = 1.03, 95% CI = 0.96–1.10) became insignificant.

Conclusions: High HGI was associated with an increased risk of composite CVD and all cause mortality after adjustment for multiple conventional cardiovascular risk factors. However, the association was mainly mediating by the level of HbA1c.

Keywords: hemoglobin glycation index, glycated hemoglobin, all-cause mortality, cardiovascular disease, risk

INTRODUCTION

Prevalence of type 2 diabetes mellitus (T2DM) has increased dramatically, with ∼422 million adults diagnosed with T2DM worldwide (1). Cardiovascular disease (CVD) is a leading comorbidity of T2DM, contributing to the significantly increased mortality in patients with T2DM (2). Even in patients with prediabetes, the risk of all cause mortality and CVD was higher than those with normoglycemia (3, 4). Therefore, detection of biomarkers or metrics for risk stratification and guiding proper treatment in T2DM is of significant importance to relieve the global burden of T2DM (5).

Glycated hemoglobin (HbA1c) is an index that reflects the mean blood glucose (MBG) for the prior 2–3 months (6). HbA1c is considered the gold standard in evaluating of glycemic control in T2DM, broadly adopted in clinical practice and trials (7, 8). However, studies have shown considerable variation in the association between measured HbA1c and MBG. Only 60–80% of the variance in HbA1c levels could be explained by the MBG levels, while the rest is attributed to ethnic and biological factors involved in hemoglobin glycation (9, 10). To quantify such variation, the hemoglobin glycation index (HGI) was introduced by Hempe et al., which is defined as the measured HbA1c minus predicted HbA1c levels (11). The predicted HbA1c levels are calculated with equations from linear regression of HbA1c and fasting blood glucose. Therefore, individuals with high HGI values have higher measured HbA1c levels than predicted HbA1 levels from their blood glucose levels.

There has been contentious about adopting the HGI as a marker of risk stratification in patients with diabetes. It had been proposed that high HGI is associated with vascular complications in patients with and without diabetes (12–15). In a large sample cross-sectional study, high HGI was independently related to composite CVD, coronary artery disease, stroke, and peripheral artery disease after adjustment for other CVD risk factors, including HbA1c levels (16). However, the cross-sectional design limits its value to explore the causal relationship between HGI and the risk of CVD. Some prospective studies showed increased HGI was associated with an increased risk for CVD (17, 18), other studies have not found a similar association (19–21), especially after adjusting HbA1c levels. Based on these inconsistencies, we performed a meta-analysis of prospective studies to evaluate the associations between HGI and the risk of all cause mortality and CVD.

METHODS

Search Strategy and Selection Criteria

According to the recommendations of the Meta-analysis of Observational Studies in Epidemiology group (22), we searched electronic databases, including PubMed, and EMBASE for studies up to March 31, 2021, using a combined MeSH heading and text search strategy with the following terms: "hemoglobin glycation index," or "HGI," and "cardiovascular disease," "cardiovascular event," "cardiocerebrovascular disease," "coronary artery disease," "coronary heart disease," "ischemic heart disease," "myocardial infarction," "stroke," "cerebral infarction," "mortality," or "death." We also reviewed reference lists of included studies to identify other potential publications. The search was restricted to human studies, while without language restriction.

We included studies for meta-analysis if they met the following criteria: (1) cohort study, nest case-control study or *post-hoc* analysis of randomized controlled trials (RCTs), evaluating the associations between HGI and risk of CVD or all cause mortality; (2) the risk of endpoints were reported in high HGI compared with low HGI patients (e.g., highest tertile vs. lowest tertile), or evaluated when HGI was defined as a continuous metric; (3) multivariable-adjusted relative risks (RRs), or hazard ratios (HRs) and 95% confidence intervals (CIs) were reported; (3) the follow up duration $\geq$1 years. We excluded studies if: (1) they were cross-sectional studies without follow-up evaluation; (2) the association between HGI and risk of endpoints was not adjusted for other cardiovascular risk factors, and (3) identical outcomes were derived from the same population.

Data Extraction and Quality Assessment

Two reviewers (JW and DL) screened the titles and abstracts of the retrieved reports, and reviewed the full copies of potentially relevant studies. Study information such as study design, ethnicity, participant's characteristics, sample size and proportion of female, follow-up duration, adjusted confounders, and endpoints assessment were recorded. Quality assessment of the included studies was conducted based on the Newcastle–Ottawa Quality Assessment Scale for cohort studies (23), in which an investigation was judged based on: selection (four items, one star each), comparability (one item, up to two stars), and exposure/outcome (three items, one star each), respectively. In this meta-analysis, we defined included studies as with good, fair, or poor quality if they were awarded $\geq$7, 4–6, or $<$ 4 stars, respectively (24, 25).

Data Synthesis and Analysis

The primary outcomes were the risk of composite cardiovascular events and all-cause mortality associated with HGI. The associations between the HGI and risk of outcomes were reported in different ways in the included studies, such as per standard-deviation (SD) increment in the continuous trait; or per tertiles or quartiles in HGI levels. To enable a consistent approach to the meta-analysis, we calculated the HRs per 1 SD increment in the level of HGI. If the included studies did not report effect measures as per 1 SD change, we converted the results according to the previously reported methods (26, 27).

Adjusted HRs and 95% CI were extracted for the meta-analysis. If a study reported multiple results based on different statistical adjusted models, results adjusted for the most number of variables were extracted for analysis. If RRs, but not HRs were reported, the RRs were considered as approximate HRs and included for meta-analysis (28). To explore whether the endpoints associated with HGI were mediated by the level of HbA1c, studies with both estimates of HRs before and after adjustment of HbA1c were also extracted for meta-analysis, respectively. I^2 statistics were used to test heterogeneity among studies, and an $I^2 > 50\%$ was considered to be with

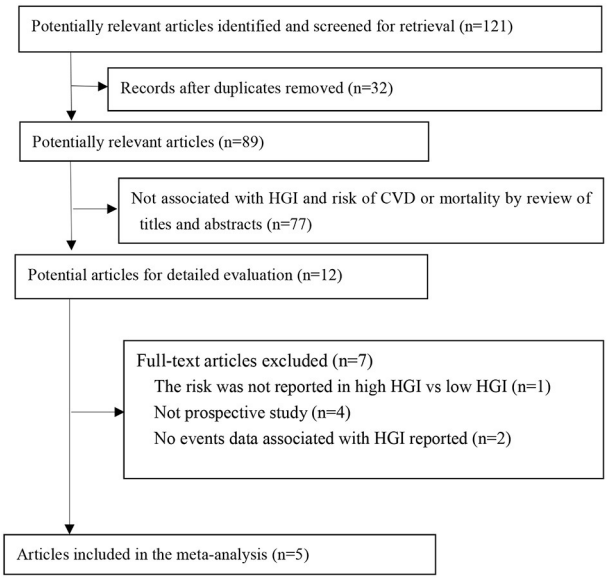

FIGURE 1 | Flow of papers through review. CVD, cardiovascular disease; HGI, hemoglobin glycation index.

significant heterogeneity. We used the inverse variance approach with a random-effects model to combine the log HRs and corresponding standard errors (SEs). We also performed a sensitivity analysis by omitting one study and recalculating the estimates of HRs at a time. Publication bias was evaluated by inspecting funnel plots for the primary outcomes in which the natural log HR was plotted against SEs, and further tested using Egger's and Begg's tests. Subgroup analyses were performed according to participant's age, follow-up duration, and baseline CVD.

Analyses were performed using Stata 12.0 (StataCorp LP, College Station, TX, USA) and RevMan 5.3 (The Cochrane Collaboration, Copenhagen, Denmark). A $P < 0.05$ was considered as with statistical significance.

RESULTS

Studies Retrieved and Characteristics

A total of 121 manuscripts were retrieved from the electronic databases. After screening of the titles and abstracts, 12 qualified for a full review, and five studies were included for meta-analysis finally (**Figure 1**) (17–21). There were 22,035 patients involved in the meta-analysis, with a median follow-up duration of 5 years.

The key characteristics of the included studies are presented in **Table 1**. Two studies were from Asia, one from the Netherlands, and two *post-hoc* analyses of RCTs enrolled patients from multiple countries worldwide. All the studies included patients with T2DM, while differed in with/without CVD. According to quality assessment criteria, 4 studies were graded as good quality, and only one as fair (Online **Supplementary File 1**).

Association Between HGI and Prognosis in Patients With T2DM Before Adjusted for Hba1c

All the included five studies provided a multi-variable adjusted risk of composite CVD associated with HGI before adjusted for HbA1c. Significant heterogeneity among all studies was observed ($I^2 = 72\%$, $P = 0.007$). Random-effects models analyses showed that per 1 SD increment of HGI was associated with a 14% higher risk of composite CVD after adjusted for multiple cardiovascular risk factors (HR = 1.14, 95% CI = 1.04–1.26, $P = 0.007$) (**Figure 2**). We did not find any evidence of publication bias by visual inspection of the funnel plot (Online **Supplementary File 2**), or using Egger's test or Begg's (all $P > 0.05$).

Three studies provided a multi-variable adjusted risk of all cause mortality associated with HGI before adjusted for HbA1c. Random-effects models analyses showed that per 1 SD increment of HGI was associated with an 18% higher risk of all cause mortality after adjusted for multiple cardiovascular risk factors, while without adjusted for HbA1c (HR = 1.18, 95% CI = 1.05–1.32, $P = 0.007$) (**Figure 3**).

Association Between HGI and Risk of All-Cause Mortality and CVD After Adjusted for HbA1c

Four studies reported the risk of composite CVD associated with HGI after adjusted for multiple cardiovascular risk factors plus HbA1c. There was significant heterogeneity among studies ($I^2 = 51\%$, $P = 0.10$). Random-effects models analyses showed that when adjusted for multiple cardiovascular risk factors and HbA1c, the association between HGI and risk of composite CVD became insignificant (per 1 SD increment of HGI: HR = 1.01, 95% CI = 0.93–1.10, $P = 0.79$) (**Figure 4**). Similarly, there was no association between HGI and risk of all cause mortality after adjustment with HbA1c (per 1 SD increment of HGI: HR = 1.03, 95% CI = 0.96–1.10, $P = 0.37$) (**Figure 5**).

Sensitivity Analyses and Subgroup Analyses

We performed several sensitivity analyses and confirmed that the results were not influenced by the use of fixed-effects models compared with random-effects models, or recalculating the HRs by omitting one study at a time.

The results of subgroup analyses for the risk of composite CVD and HGI were presented in **Table 2**. A high level of HGI was associated with an increased risk of composite CVD in patients without baseline CVD (HR = 1.22, 95% CI = 1.04–1.43), even after adjustment for HbA1c. However, there was no significant association observed between HGI and composite CVD in patients with a history of CVD (HR = 0.96, 95% CI = 0.88–1.06) (P for subgroup heterogeneity = 0.01, $I^2 = 84.5\%$). There were no significant associations for HGI and composite CVD risk among all other subgroup analyses.

TABLE 1 | Characteristics of the included studies.

References	Design	Country/Region	Patients characteristics	Sample (female %)	Age (years) (mean)	Follow-up duration (years)	Events for analysis	Risk factors adjusted
van Steen et al. (21)	*Post-hoc* analysis of RCT	Multiple countries	T2DM with acute coronary syndrome	6,458 (26.9)	60.6	2.0	All-cause mortality Composite CVD Cardiovascular mortality	Age, sex, race, SBP, DBP, BMI, hemoglobin, eGFR, LDL-C, HDL-C, TG, duration of T2DM, use of antihyperglycaemic agents, history of retinopathy and smoking Additional adjustment for HbA1c level
Jin et al. (17)	Nested case-control study	China	T2DM with stable CAD	1,282 (30)	60.0	3.0	Composite CVD	Age, sex, BMI, hypertension, family history of CAD, smoke, HDL-C, non-HDL-C, Creatinine, uric acid, hs-CRP, Gensini score
Kim et al. (18)	Prospective cohort study	Korea	T2DM without CVD	1,302 (58.1)	55.5	11.1	Composite CVD CAD Stroke	Age, sex, BMI, duration of T2DM, presence of hypertension, eGFR, treatment and use of Sulfonylurea. Additional adjustment for HbA1c level
van Steen et al. (20)	*Post-hoc* analysis of RCT	Multiple countries	T2DM with a history, or a risk factor for CVD	11,083 (42.4)	65.8	5.0	All-cause mortality Composite CVD	Age, sex, race, BMI, duration of T2DM, history of macro- and microvascular events, current drinking and smoking, use of glucose-lowering drugs, use of BP-lowering drugs, SBP, DBP, hemoglobin, eGFR, LDL-C, HDL-C, TC, TG Additional adjustment for HbA1c level
Ostergaard et al. (19)	Prospective cohort study	Netherlands	T2DM	1,910 (30)	60.0	9.6	All-cause mortality Composite CVD Cardiovascular mortality Myocardial infarction Stroke	Age, sex, BMI, insulin use, duration of T2DM, non-HDL-C, eGFR, SBP Additional adjustment for HbA1c level

BMI, body mass index; CAD, coronary artery disease; CVD, cardiovascular disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HbA1c, Glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RCT, randomized controlled trial; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglycerides.

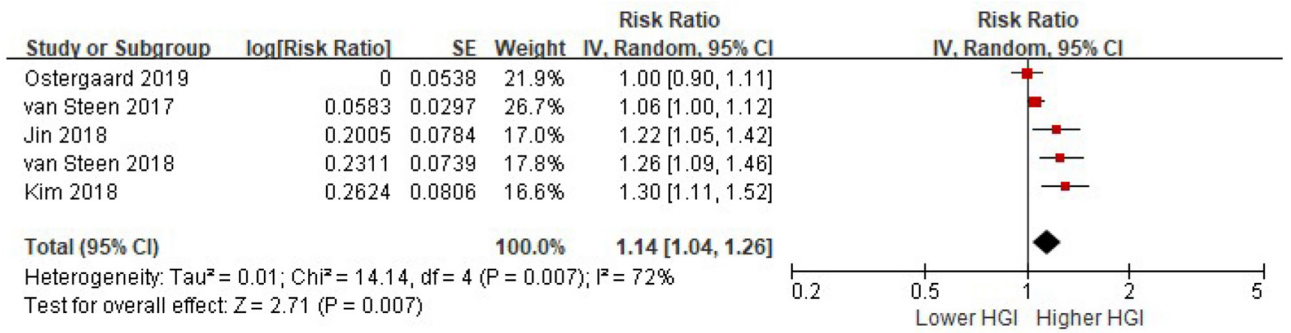

FIGURE 2 | The association between HGI and the risk of composite CVD. Data were presented as the HR and 95% CI of composite CVD for per 1 SD increment of HGI, after adjusting for multiple cardiovascular risk factors (without HbA1c). CI, confidence interval; CVD, cardiovascular disease; HGI, hemoglobin glycation index.

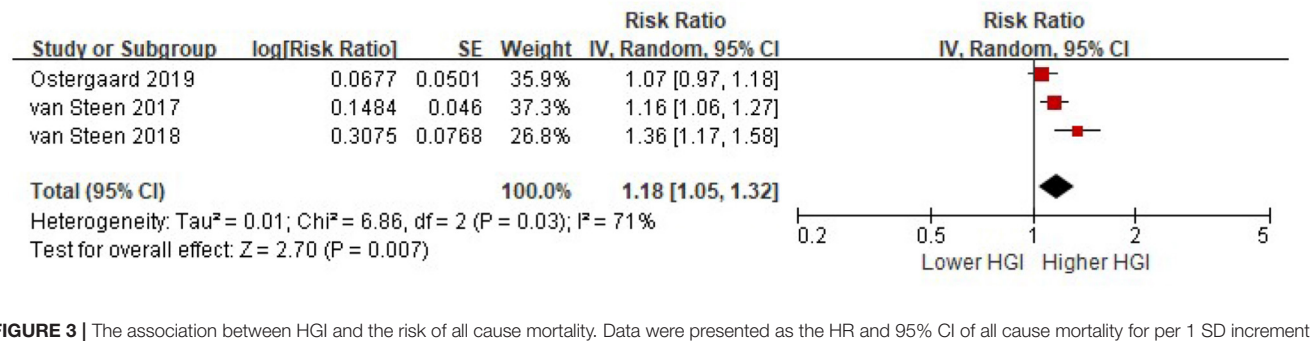

FIGURE 3 | The association between HGI and the risk of all cause mortality. Data were presented as the HR and 95% CI of all cause mortality for per 1 SD increment of HGI, after adjusting for multiple cardiovascular risk factors (without HbA1c). CI, confidence interval; HGI, hemoglobin glycation index.

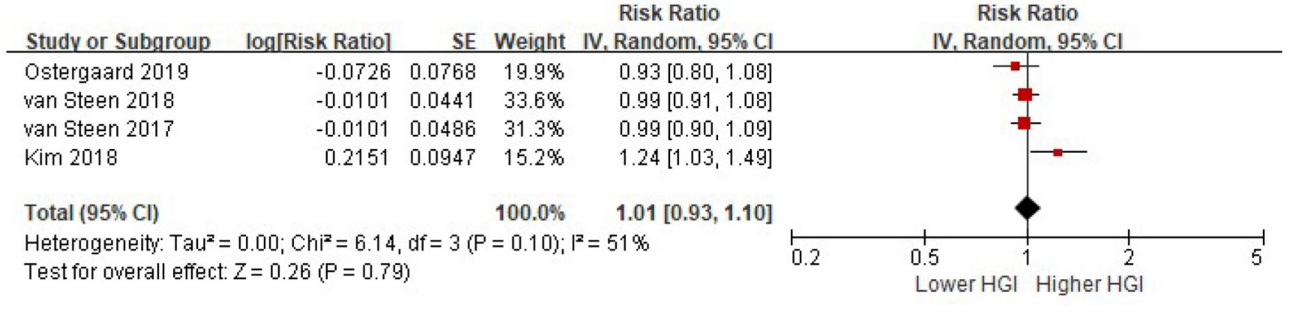

FIGURE 4 | The association between HGI and the risk of composite CVD further adjusting for HbA1c. Data were presented as the HR and 95% CI of composite CVD for per 1 SD increment of HGI, after adjusting for multiple cardiovascular risk factors and plus HbA1c. CI, confidence interval; CVD, cardiovascular disease; HGI, hemoglobin glycation index.

DISCUSSION

In this study, we included five observational studies, including more than 22,000 T2DM patients with a median follow-up duration of 5 years for meta-analysis. We found that high HGI was associated with an increased risk of composite CVD, and all cause mortality after adjustment of multiple cardiovascular risk factors. However, when further adjusted for levels of HbA1c, the association between HGI and poor prognosis was disappeared.

Although still with controversies, several mechanisms had been proposed to explain the association between HGI and the risk of CVD. First, patients with high HGI had a longer duration of diabetes and combined cardiovascular risk factors (15). Second, high HGI had been reported to be associated with subclinical target organ damage, such as coronary artery calcification (12), and carotid atherosclerosis (14). Third, high HGI is associated with activated inflammation status, which played an important role in the development of CVD

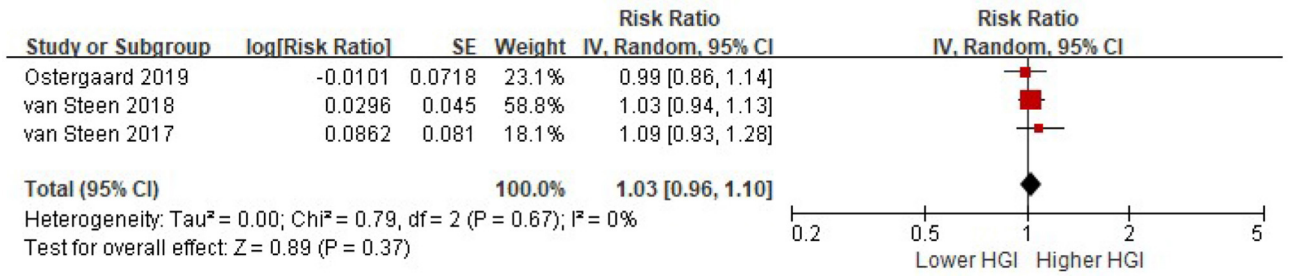

FIGURE 5 | The association between HGI and the risk of all cause mortality further adjusting for HbA1c. Data were presented as the HR and 95% CI of composite CVD for per 1 SD increment of HGI, after adjusting for multiple cardiovascular risk factors and plus HbA1c. CI, confidence interval; HGI, hemoglobin glycation index.

TABLE 2 | Subgroup analyses of the association between HGI and risk of composite CVD.

	Number of studies	Per 1 SD increment of HGI	
		HR (95% CI)[#]	*P* value*/I^2
Participants age			0.61/0%
≤60	2	1.07 [0.81, 1.42]	
>60	2	0.99 [0.93, 1.06]	
Follow-up duration			0.61/0%
≤5 years	2	0.99 [0.93, 1.06]	
>5 years	2	1.07 [0.81, 1.42]	
Baseline CVD			0.01/84.5%
Yes	2	0.96 [0.88, 1.06]	
No	2	1.22 [1.04, 1.43]	

[#]*Adjusted for multiple cardiovascular disease and level of HbA1c.*
[*]*For heterogeneity among subgroups.*
CVD, cardiovascular disease; HGI, hemoglobin glycation index; SD, standard-deviation.

(29). However, our results raised concern on these proposed mechanisms, because after adjusting for HbA1c, the association between HGI and poor prognosis was disappeared. These results indicated that the impact of HGI on CVD and all cause mortality was mainly mediating by the level of HbA1c. These finding were supported by data from the Diabetes Control and Complications Trial (DCCT), which showed that among type 1 diabetic patients, HGI was not an independent risk factor for microvascular complications, and that the effect of the HGI on risk of microvascular complications was wholly explained by the associated level of HbA1c (30).

Recently, Zhang et al. had reported a meta-analysis, which showed that for each 1-unit increment in HGI, there was a 20 and 13% increase of composite CVD and all cause mortality, respectively (31). Our analysis had several different findings compared with Zhang's report. First, we calculated the risk estimate before and after adjustingd for HbA1c, respectively, but not lumped them together, which was useful for interpreting the mediating effect of HbA1c on the association between HGI and poor prognosis. Second, we set up more strict inclusion criteria to avoid incorrect inclusion. For example, we did not include a study that reported a U-shaped association between HGI and the prognosis in patients with stroke, in which the data cannot be transformed into a continuous increment of HGI (32). We also did not include the study by Hempe et al., in which the association between HGI and risk of composite CVD or all cause mortality was not reported (33). Third, we also find that a high level of HGI was associated with an increased risk of composite CVD in patients without baseline CVD, even after adjustment for HbA1c. However, there was no significant association observed between HGI and composite CVD in patients with a history of CVD. This interesting finding raised a hypothesis that in T2DM without a history of CVD, detection of HGI may provide further information for risk stratification. However, due to the limited number of studies available, further studies are urgently needed.

Our study has several limitations. First, this is a meta-analysis based on the study level. We did not have individual participant data. Therefore, residual confounders could not be avoided. However, all the included studies had adjusted for multiple conventional cardiovascular risk factors. Second, the calculation of HGI differed among the included studies, which would cause significant heterogeneity among the studies. Third, it had been proposed that the variance in HbA1c was different in Asians and Westerns (34). However, due to the limited studies available, we did not perform subgroups analysis according to race. Further studies are needed to explore whether the associations between HGI and poor prognosis are different among different ethnicities.

CONCLUSIONS

Our results showed that although high HGI was associated with an increased risk of composite CVD and all cause mortality after adjustment for multiple conventional cardiovascular risk factors. However, the association was mainly mediating by the level of HbA1c. Considering that HbA1c can be easily evaluated in most laboratories worldwide, and the calculation of the HGI is complicated, clinical use of HGI in T2DM cannot be routinely recommended.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

J-dW, D-lL, YX, M-yC, and H-hC: research idea and study design. J-dW, D-lL, DS, and H-qH: data acquisition. J-dW and D-lL: data analysis and interpretation. J-dW and YX: statistical analysis. J-dW: supervision and mentorship. All authors contributed important intellectual content during manuscript drafting or revision and accept accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.690689/full#supplementary-material

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

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Assessment of Myocardial Dysfunction by Three-Dimensional Echocardiography Combined With Myocardial Contrast Echocardiography in Type 2 Diabetes Mellitus

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Background: We aimed to explore the value of combining real-time three-dimensional echocardiography (RT-3DE) and myocardial contrast echocardiography (MCE) in the left ventricle (LV) evaluating myocardial dysfunction in type 2 diabetes mellitus (T2DM) patients.

Patients and Methods: A total of 58 T2DM patients and 32 healthy individuals were selected for this study. T2DM patients were further divided into T2DM without microvascular complications ($n = 29$) and T2DM with microvascular complications ($n = 29$) subgroups. All participants underwent RT-3DE and MCE. The standard deviation (SD) and the maximum time difference (Dif) of the time to the minimum systolic volume (Tmsv) of the left ventricle were measured by RT-3DE. MCE was performed to obtain the perfusion measurement of each segment of the ventricular wall, including acoustic intensity (A), flow velocity (β), and A·β.

Results: There were significant differences in all Tmsv indices except for Tmsv6-Dif among the three groups (all $P < 0.05$). After heart rate correction, all Tmsv indices of the T2DM with microvascular complications group were prolonged compared with the control group (all $P < 0.05$). The parameters of A, β, and A·β for overall segments showed a gradually decreasing trend in three groups, while the differences between the three groups were statistically significant (all $P < 0.01$). For segmental evaluation of MCE, the value of A, β, and A·β in all segments showed a decreasing trend and significantly differed among the three groups (all $P < 0.05$).

Conclusions: The RT-3DE and MCE can detect subclinical myocardial dysfunction and impaired myocardial microvascular perfusion. Left ventricular dyssynchrony occurred in T2DM patients with or without microvascular complications and was related to

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left ventricular dysfunction. Myocardial perfusion was reduced in T2DM patients, presenting as diffuse damage, which was aggravated by microvascular complications in other organs.

Keywords: diabetes mellitus, myocardial dysfunction, three-dimensional echocardiography, myocardial contrast echocardiography, microvascular complications

INTRODUCTION

Type 2 diabetes mellitus (T2DM), characterized by hyperglycemia, is an independent risk factor for cardiovascular disease (1, 2). Cardiovascular consequences of diabetes are atherosclerotic epicardial coronary artery disease (CAD) and cardiomyocyte and myocardium changes. Patients with T2DM have a 2–4-fold increased risk of cardiovascular events (3). One major cause of increased mortality in T2DM is diabetic cardiomyopathy (DCM), which is defined as myocardial dysfunction that is independent of CAD and hypertension and can lead to heart failure (4, 5). Therefore, early detection of myocardial dysfunction in patients with T2DM is essential for therapeutic interventions that may prevent or reverse DCM, as the severity of cardiac disease determines prognosis.

Before clinically manifesting heart disease, patients with T2DM develop subtle changes in cardiac function such as left ventricular (LV) myocardial mechanical movement dysfunction including diastolic and systolic dysfunction (6, 7). Echocardiography is a currently available method for simultaneously evaluating myocardial morphology, motion, and perfusion. Two-dimensional echocardiography (2DE) and tissue Doppler-based techniques have shown variable results in the assessment of global LV systolic and diastolic function of T2DM (8, 9). Several parameters derived from Doppler tissue imaging (DTI) have been reported to be promising predictors of LV reverse remodeling (10). However, these indexes do not reflect the motion pattern of all LV segments, and because of the limitation of angle dependence of DTI, information regarding the apical segments cannot be evaluated. Real-time three-dimensional echocardiography (RT-3DE) is an appealing novel imaging modality that allows simultaneous assessment of the entire LV from a single full-volume data set acquired from the apex (11). In addition, this semi-automated software uses a 17-segment model of the heart to provide regional volume vs. time curves, which can be used to assess LV dyssynchrony (11, 12).

Except for LV dyssynchrony, impaired coronary microvasculature might lead to myocardial dysfunction in T2DM (13, 14). Myocardial contrast echocardiography (MCE) is a non-invasive method for assessing myocardial perfusion by observing destruction and replenishment of microbubbles into the myocardium (15). A previous study has shown that T2DM patients have impaired MCE-derived quantitative myocardial perfusion parameters compared to non-diabetic patients. Besides, various microvascular disease states are associated with risk of vascular disease, including cardiac autonomic neuropathy, retinopathy, nephropathy, and peripheral neuropathy (16–19).

Therefore, the current study aimed to quantitatively evaluate LV myocardial dyssynchrony using RT-3DE and myocardial perfusion by MCE in T2DM patients with microvascular complications and investigate the association between LV subclinical myocardial dysfunction and coronary microvascular perfusion.

PATIENTS AND METHODS

Study Population

The study was approved by the Institutional Review Board of our hospital with written informed consent obtained from all study participants. From October 2018 to May 2019, we prospectively recruited 58 patients with T2DM from in-patients attending the Department of Endocrinology at our institution. Diabetes was defined by a fasting plasma glucose of at least 7.0 mmol/L (126 mg/dl), random plasma glucose of at least 11.1 mmol/L (200 mg/dl), or the use of glucose-lowering medications, based on recommendations from the American Diabetes Association (20). Briefly, classification of T2DM was performed according to the following criteria: specific diagnostic code for T2DM (Read code C10F; ICD-10 code E11) with no contradictory code and patients with a diagnosis of diabetes at the age of 35 years or older with no insulin prescription within 1 year of diagnosis. All patients had no evidence of coronary heart disease or heart failure on the basis of history, 12-lead electrocardiograph (ECG), a normal ejection fraction on echocardiography, and metabolic exercise testing. The exclusion criteria were as follows: clinical evidence of CAD and previous myocardial infarction, primary cardiomyopathy, congenital heart disease, valvular stenosis or more than mild regurgitation, prosthetic valves or pacemakers, uncontrolled hypertension (systolic blood pressure >160 mmHg), and significant renal impairment (estimated glomerular filtration rate <30 ml/min). Importantly, all patients were initially screened for obstructive epicardial CAD (>50% of luminal stenosis) with coronary computed tomographic angiography (CCTA) or coronary angiography (CAG). Subjects were divided into two groups based on the presence or absence

Abbreviations: RT-3DE, real-time three-dimensional echocardiography; MCE, myocardial contrast echocardiography; LV, left ventricular; 2DE, two-dimensional echocardiography; T2DM, type 2 diabetes mellitus; SD, standard deviation; Dif, maximum time difference; Tmsv, minimum systolic volume; A, acoustic intensity; β, flow velocity; CAD, coronary artery disease; DCM, diabetic cardiomyopathy; DTI, Doppler tissue imaging; ECG, electrocardiograph; CCTA, coronary computed tomographic angiography; CAG, coronary angiography; eGFR, estimated glomerular filtration rate; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LA, left atrial end-systolic anteroposterior diameter; LV, left ventricular end-diastolic anteroposterior diameter; RV, right ventricular end-diastolic anteroposterior diameter; LVEF, left ventricular ejection fraction; SV, stroke volume; MI, mechanical index; ROI, region of interest; SI, signal intensity; MBV, myocardial blood volume; MBF, myocardial perfusion.

of at least one of the microvascular complications of diabetes: retinopathy, nephropathy, and peripheral neuropathy (21, 22). The diagnosis of microvascular complications should meet one of the following criteria: (1) nephropathy was defined as microalbuminuria (a moderate increase in albuminuria: 3–30 mg/mmol, 30–300 mg/g, 30–300 mg per 24 h, or reagent strip urinalysis) or an estimated glomerular filtration rate (eGFR) $<$60 ml/min per 1.73 m^2; (2) diabetic retinopathy is defined as a spectrum of retinal microvascular lesions seen on retinal examination (23); and (3) peripheral neuropathy diagnosed by clinical symptoms, physical examination, and neurophysiological examinations, excluding primary neurological diseases. A control group of 32 age-, gender-, and body mass index-matched healthy participants were recruited. All healthy volunteers had no history of cardiac disease or diabetes mellitus and underwent laboratory measurements before enrollment. The exclusion criteria were impaired fasting glucose (fasting glucose $>$6.1 mmol/L) or hypertension (blood pressure $>$140/90 mmHg).

Baseline Characteristics and Laboratory Analysis

The height and weight of all participants were recorded, and body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). The duration of diabetes was recorded as reported by the patient. Blood pressure was recorded as an average of three measurements in the right arm in a sitting position that were obtained after a 10-min resting period. Before echocardiography examination, blood samples were obtained from patients and controls for standard laboratory analysis, including plasma glucose, glycated hemoglobin, triglyceride, high-density lipoprotein cholesterol (HDL) cholesterol, and low-density lipoprotein cholesterol (LDL) cholesterol, according to standard procedures of the central clinical laboratory in our hospital.

Standard 2D, Flow, and Tissue Doppler Echo Examination

Echocardiography was performed using a Philips EPIQ 7C ultrasound scanner (Philips Medical Systems, Best, Netherlands) equipped with a X5-1 phased array probe (operated frequency range, 1.6–3.2 MHz). Scanning was performed in the left lateral decubitus position, with ECG simultaneously recorded. Aortic sinus, ascending aorta, left atrial end-systolic anteroposterior diameter (LA), left ventricular end-diastolic anteroposterior diameter (LV), right ventricular end-diastolic anteroposterior diameter (RV), left ventricular ejection fraction (LVEF), and stroke volume (SV) were obtained.

Real-Time 3D Echocardiography

The real-time 3D imaging was performed to obtain a pyramidal volume data set from the apical transducer position. Gain and compression controls and time-gain compensation settings were optimized to ensure image quality. The entire LV cavity was included within the pyramidal volume scan. Real-time 3D data sets were acquired using a wide-angle acquisition ($93^\circ \times 80^\circ$) mode, in which four wedge-shaped subvolumes ($93 \times 20^\circ$ each) were obtained from five consecutive cardiac cycles. Data were acquired from the apical four-chamber position during held end expiration. Acquisition was triggered to the R wave on the ECG of every cardiac cycle, resulting in a total acquisition time of five heartbeats.

Myocardial Contrast Echocardiography

The ultrasound contrast agent was applied by continuous infusion of SonoVue (Bracco, Milan, Italy) intravenously. SonoVue was infused initially at a rate of 1 ml/min and adjusted thereafter to minimize attenuation. Gain was optimized at the beginning of the study and held constant throughout. Focal depth and length were adjusted to cover the entire LV chamber. The focus was set at the level of the mitral annulus. A low mechanical index (MI) was used at 0.18 to guarantee that microbubbles were not destroyed. After the myocardium was filled with the contrast agent, a burst of high MI at 1.3 was triggered for five cardiac cycles to destroy contrast microbubbles within the myocardium. Then, the system was automatically converted to the low-energy real-time radiography state. The refilling of contrast microbubbles within the myocardium was recorded from the apical four-, two-, and three-chamber view. Images collected from 15 consecutive cardiac cycles after providing high-energy pulse were turned into the cineloop, which was stored in disks for offline analysis.

Image Analysis

All images were subsequently analyzed offline using QLAB analysis software (version 2.0, Philips Medical Systems, Cleveland, OH, USA) by two experienced physicians who were blinded to the clinical data. For 3D imaging, end-diastolic apical two- and four-chamber view cut planes were semiautomatically obtained from the pyramidal data set. Then, five anatomic landmarks were manually initialized, including two points to identify the mitral valve annulus and one point to identify the apex. After manual identification of these points, the program automatically identified the 3D endocardial surface using a deformable shell model. Adjustments to the automatic surface detection were performed when necessary. Thereafter, the end-systolic frame was selected by identifying the frame with the smallest LV cavity cross-sectional area in both apical views. After initialization, surface detection was then repeated on this frame to obtain end-systolic volumes. Finally, the computer algorithm automatically defined and traced the endocardial border in all frames of the cardiac cycle. "Casts" of the LV endocardium were then automatically obtained from which global LV volumes vs. time curves were derived. The LV was divided into 17 segments from apex to base, and curves depicting regional volumes over the cardiac cycle were obtained for each segment. From these regional volume curves (excluding the apical cap, segment 17), the regional ejection time, defined as the time interval between the R wave and minimal end-systolic volume (Tmsv), was automatically calculated. To assess systolic synchrony, the SD of the regional volume time curves was obtained using 16 segments (Tmsv16-SD); 12 segments, 6 basal and 6 middle segments (Tmsv12-SD); and 6 basal segments (Tmsv6-SD) in each patient. In addition to the Tmsv indices, the maximal difference of Tmsv was also calculated, generating the following additional indices of dyssynchrony: the maximal difference or range of Tmsv among 16 segments (Tmsv16-Dif); among the 6 basal and 6 middle segments (Tmsv12-Dif); and

among the 6 basal segments (Tmsv6-Dif). To eliminate the influence of heart rate difference between different subjects, the above measurements were corrected for heart rate, which are Tmsv16-SD%, Tmsv12-SD%, Tmsv6-SD%, Tmsv16-Dif%, Tmsv12-Dif%, and Tmsv6-Dif%.

For MCE, LV 16-segment model recommended by the American Society of Echocardiography was used to analyze the collected images. The regions of interest located in the first post-flash end-systolic frame were automatically copied onto subsequent selected frames and manually realigned frame by frame to maintain a central point within the ventricular wall in the entire replenishment sequence. With region of interest (ROI, 5×5 mm) placed at the center of each segment, the refilling process of contrast microbubbles within the myocardium during 15 consecutive cardiac cycles after providing high-energy pulse was analyzed. Pixel intensity of each frame image was calculated frame by frame by a computer. The mean signal intensity (SI) was measured automatically at the ROIs. The segmental SI was plotted against time (t), and the subsequent refilling curve was fitted to the following exponential function: $y(t) = A(1 - \exp - \beta t) + C$, where y is the acoustic intensity at a time of t, A is the plateau where acoustic intensity represents myocardial blood volume (MBV), and β represents the mean blood flow velocity in the intramyocardial vascular network. Myocardial perfusion (MBF) was calculated as the product of myocardial blood volume (A) and microvascular flow velocity (β) and analyzed for each coronary region.

Statistical Analysis

Statistical analyses were performed with MedCalc (version 15.2.2, MedCalc Software, Mariakerke, Belgium). All continuous data were evaluated for normality using the Kolmogorov–Smirnov test and are presented as mean ± standard deviation or median (interquartile range). Homogeneity of variance was evaluated using the Levene's test. One-way analysis of variance (one-way ANOVA) was used to compare continuous variables as clinical characteristics, routine echocardiographic parameters, 3DE parameters, and perfusion parameters among controls, and T2DM without microvascular complication, and T2DM with microvascular complication, while least significant difference test was used to analyze the difference within the groups when the *P*-value of one-way ANOVA was <0.05. To compare the difference among the groups, analysis with Bonferroni correction was applied. The Kruskal–Wallis test was used to analyze parameters that did not conform to normality or show homogeneity of variance. Binary variables were analyzed using the chi-square test. Differences were considered statistically significant for $P < 0.05$.

RESULTS

Patients and Baseline Characteristics

The demographic, clinical, and biochemical data of patients with T2DM and controls matched for age, gender, and BMI are shown in **Table 1**.

Diabetes duration, rest heart rate, glycated hemoglobin, plasma glucose, and left ventricular posterior wall thickness at end diastole (LVPWd) were significantly different among the three groups (all $P < 0.05$). Other parameters were not significantly different among the three groups ($P > 0.05$).

Mechanical Dyssynchrony

Figure 1 shows the examples obtained from three patients with RT-3DE-derived LV dyssynchrony indices and regional volume curves obtained in 16 segments. There were significant differences in all Tmsv indices except for Tmsv6-Dif among the three groups (all $P < 0.05$). Tmsv16-Dif, Tmsv16-SD, Tmsv12-Dif, Tmsv12-SD, and Tmsv6-SD of T2DM with microvascular complications group were prolonged compared with the control group (all $P < 0.05$). The Tmsv12-Dif and Tmsv12-SD of the T2DM without microvascular complications group were prolonged compared with the control group (both $P < 0.05$). The Tmsv16-Dif, Tmsv16-SD, Tmsv12-Dif, and Tmsv12-SD of the T2DM with microvascular complications group were prolonged compared with the T2DM without microvascular complications group (all $P < 0.05$).

After heart rate correction, all Tmsv indices of the T2DM with microvascular complications group were prolonged compared with the control group (all $P < 0.05$). Tmsv12-Dif% and Tmsv12-SD% of the T2DM without microvascular complications group were prolonged compared with the control group (both $P < 0.05$). All Tmsv indices of the T2DM with microvascular complications group were prolonged compared with the T2DM without microvascular complications group (all $P < 0.05$) (**Table 2**).

MCE

The parameters of quantitative MCE among the three groups were compared, and the results are shown in **Table 3**. The parameters of A, β, and A·β for overall segments showed a gradually decreasing trend in three groups, while the differences between the three groups were statistically significant ($P < 0.01$) (**Figure 2**). All parameters of T2DM with microvascular complications group were significantly lower than the control and T2DM without microvascular complications groups ($P < 0.05$). Compared with the control group, the values of β and A·β in the T2DM without microvascular complications group decreased, both of which were statistically significant ($P < 0.05$).

For segmental evaluation of MCE, the value of A, β, and A·β in all segments showed a decreasing trend and significantly differed among the three groups ($P < 0.05$). A, β, and A·β in all segments of the T2DM with microvascular complications group decreased compared with the control group ($P < 0.05$); β and A·β in all segments of the T2DM with microvascular complications group decreased compared with the T2DM without microvascular complications group ($P < 0.05$). Compared with the control group, the values of β and A·β in the basal segments, middle segments, and apical segments significantly decreased in the T2DM without microvascular complications group ($P < 0.05$).

DISCUSSION

Diabetic cardiovascular complications such as myocardial infarction and congestive heart failure are one of the main causes of death in T2DM patients (24, 25). Therefore, it is

TABLE 1 | Baseline characteristics of the study cohort.

Characteristics	Control group ($n = 32$)	T2DM without microvascular complications group ($n = 29$)	T2DM with microvascular complications group ($n = 29$)	*P*-value
Age (years)	52.5 ± 12.9	58.0 ± 13.8	59.5 ± 9.0	0.087
Gender (male/female)	16/16	12/17	13/16	0.172
Diabetes duration (years)	–	8.8 ± 8.2	12.9 ± 7.4	0.008
Rest HR (bpm)	73.4 ± 9.8	84.8 ± 16.0	80.7 ± 10.5	0.002
SBP (mmHg)	120.8 ± 12.8	126.9 ± 15.8	130.6 ± 20.3	0.079
DBP (mmHg)	73.0 ± 10.2	77.8 ± 11.5	75.7 ± 11.2	0.249
BMI (kg/m^2)	23.8 ± 3.2	23.8 ± 2.8	24.1 ± 2.9	0.872
Glycated hemoglobin (%)	5.6 ± 0.3	8.8 ± 2.3	9.0 ± 1.6	0.006
Plasma glucose (mmol/L)	4.8 ± 0.4	10.3 ± 4.5	11.9 ± 5.8	<0.001
Triglyceride (mmol/L)	1.6 ± 1.1	1.6 ± 1.3	1.8 ± 1.1	0.915
HDL (mmol/L)	1.3 ± 0.4	1.1 ± 0.3	1.1 ± 0.4	0.096
LDL (mmol/L)	3.3 ± 0.7	2.9 ± 0.9	2.9 ± 0.9	0.357
Aortic sinus (mm)	32.2 ± 3.3	33.0 ± 2.5	33.3 ± 3.5	0.418
Ascending aorta (mm)	30.8 ± 4.9	32.1 ± 3.4	32.4 ± 3.3	0.279
LA (mm)	34.3 ± 4.5	33.5 ± 2.8	35.7 ± 3.9	0.087
RV (mm)	21.1 ± 1.8	21.1 ± 1.8	21.0 ± 2.0	0.976
IVSd (mm)	9.7 ± 1.7	9.89 ± 1.17	10.3 ± 1.3	0.267
LVDd (mm)	48.0 ± 3.6	46.0 ± 4.8	45.2 ± 5.1	0.063
LVPWd (mm)	8.4 ± 1.3	8.79 ± 1.03	9.4 ± 0.8	0.003
LVEF (%)	70.7 ± 6.1	71.0 ± 5.4	70.4 ± 5.7	0.933
SV (ml)	76.1 ± 13.5	70.7 ± 20.2	66.2 ± 18.2	0.113

Data are mean ± standard deviation or numbers.

HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; LA, left atrial end-systolic anteroposterior diameter; RV, right ventricular end-diastolic anteroposterior diameter; IVSd, interventricular septal thickness; LVDd, left ventricular end-diastolic anteroposterior diameter; LVPWd, left ventricular posterior wall thickness at end-diastole; LVEF, left ventricular ejection fraction; SV, stroke volume.

important to detect early LV dysfunction and treat it properly to reduce the long-term mortality of diabetic patients. LV dyssynchrony and impaired coronary microvasculature might lead to myocardial dysfunction in T2DM patients. RT-3DE is an appealing novel imaging modality that has been recently used in quantitative evaluation of global and regional LV mechanical movement function and the severity of cardiac dyssynchrony. MCE is a non-invasive method for quantitatively assessing myocardial perfusion and coronary microvasculature. Therefore, accurate assessment of myocardial dysfunction should contain the synchronization of myocardium mechanical movement and microvascular perfusion through combining with RT-3DE and MCE.

In this study, we found that LVEF and the size of the atrium and ventricle obtained by 2D echocardiography was comparable between the control and T2DM groups, and all the measured values were within a normal range, meaning that the global change of heart did not appear. After heart rate correction, all Tmsv indices obtained through RT-3DE of the T2DM with microvascular complications group were prolonged compared with the control and T2DM without microvascular complications groups ($P < 0.05$). The T2DM with microvascular complication group had significantly longer Tmsv indices. We could find that all synchronization indicators of T2DM with microvascular complications group, except for Tmsv6-Dif, were prolonged compared with the control group ($P < 0.05$), which indicates that when the conventional cardiac function measurement is within the normal range, the left ventricle of patients with T2DM exhibits asynchronized myocardial segmental contraction. Myocardial damage in patients with long-term T2DM has been confirmed by several studies, which agrees with the results of this study. The Tmsv12-Dif, Tmsv12-SD, Tmsv12-Dif%, and Tmsv12-SD% of the T2DM without microvascular complications group were also prolonged compared with the control group ($P < 0.05$), suggesting that incoordination of left ventricular movement in T2DM patients with complications is more obvious than in patients without other organ complications. One possible reason is that long-term hyperglycemia causes endothelial cells and intima fibrosis, as well as thickening of capillary basement membrane, which gradually progress to myocardial atrophy and fibrosis. These alterations lead to changes in hemorheology, leading to long-term myocardial ischemia and hypoxia and modifications in ventricular morphology and structure, which are finally manifested in the reduction in the left ventricular systolic function. In addition to the increase in blood glucose, the decline

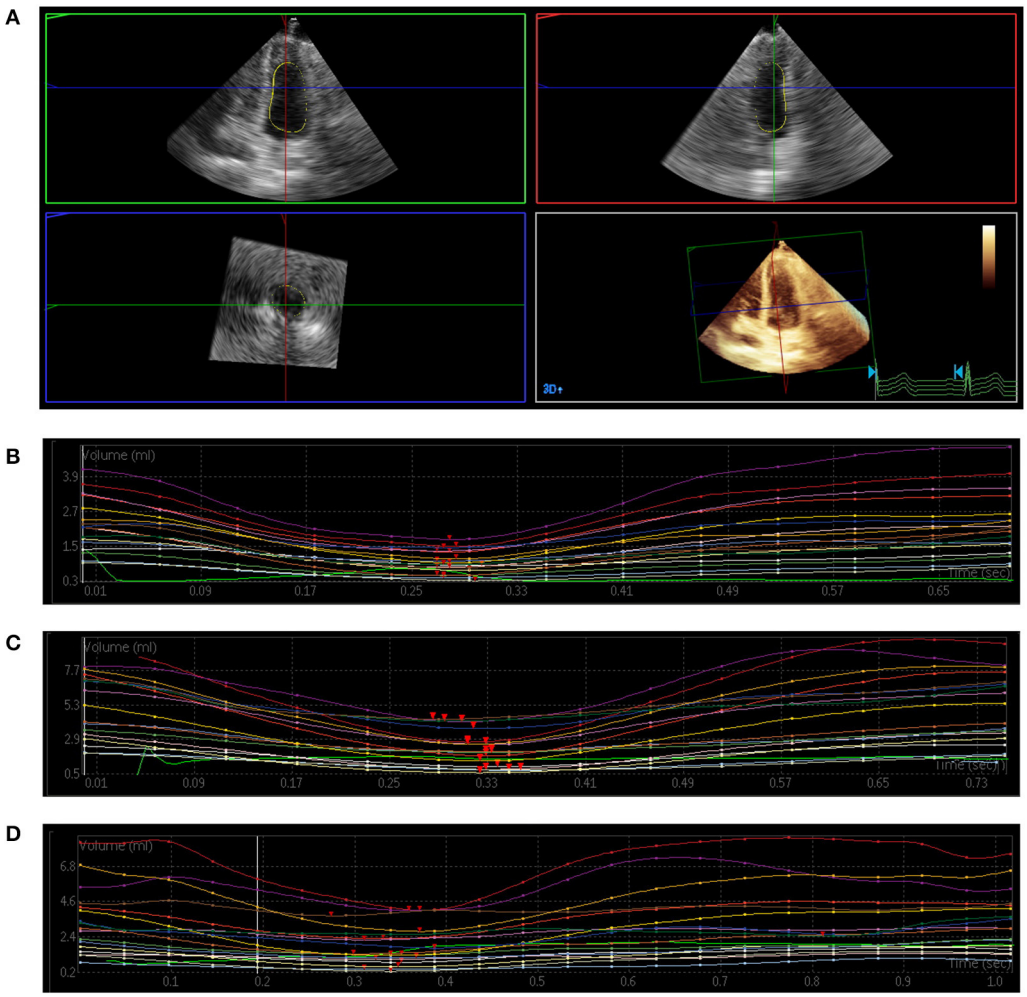

FIGURE 1 | Three-dimensional echocardiography assessment of left ventricular (LV) mechanical dyssynchrony. **(A)** Full-volume acquisition. **(B)** Patient with normal intraventricular synchrony. There is a regular time–volume curves, and the time of reaching the trough is consistent. **(C)** Patient with slightly intraventricular dyssynchrony. The time–volume curves is regular but not consistent. **(D)** Patient with severe intraventricular dyssynchrony. There is a disarray of the time–volume curves signifying LV dyssynchrony.

in renal excretion in patients with diabetic nephropathy also affects the structure and function of the left heart. Because glucose metabolism and lipid metabolites cannot be discharged in time, a large number of toxic products are introduced into the circulating blood, which is toxic to the heart, and the myocardium can be severely or permanently damaged, and thus, asynchrony of its myocardial movement becomes severe.

MCE is a technique for evaluating myocardial tissue microcirculation perfusion with a wide range of applications. It can be used for non-invasive and quantitative evaluation of myocardial microcirculation. The myocardial contrast agent does not infiltrate into the interstitial space during circulation. It can evaluate the microcirculation perfusion in real time, observe whether the myocardial blood perfusion is uniform, and detect the blood volume of the capillaries in the myocardium (26). The A, β, and A·β in the basal, middle, and apical segments of the T2DM with microvascular complications group decreased compared with the control group ($P < 0.05$); β and A·β in all segments of the T2DM with microvascular complications group decreased compared with the T2DM without microvascular complications group ($P < 0.05$). The overall myocardial contrast-enhanced ultrasound analysis showed that the A, β, and A·β of the T2DM group were lower than those of the control group, suggesting that the myocardial blood volume, blood flow velocity, and blood flow of T2DM patients were lower than those of the control group, which coincide with the results of studies using MRI to evaluate the microcirculation of T2DM. The parameters of A, β, and A·β in the T2DM with microvascular complications group were significantly lower than those in the T2DM without microvascular complications group ($P < 0.05$), suggesting that patients with T2DM have a certain degree of myocardial microcirculation disorder. With the microcirculation complications in other organs, the degree of reduction in various indicators of myocardial perfusion increased. In this study,

TABLE 2 | Comparison of parameters of 3DE in three groups.

Parameters	Control group	T2DM without microvascular complications group	T2DM with microvascular complications group	*P*-value
Tmsv16-Dif	79.3 ± 32.1	89.3 ± 30.4	126.1 ± 83.5*#	0.011
Tmsv16-Dif%	9.5 ± 4.4	10.7 ± 3.4	15.8 ± 9.5*#	0.002
Tmsv16-SD	20.7 ± 8.5	23.9 ± 7.9	33.5 ± 23.1*#	0.013
Tmsv16-SD%	2.5 ± 1.2	3.0 ± 0.9	4.3 ± 2.7*#	0.003
Tmsv12-Dif	59.2 ± 29.4	73.6 ± 24.1*	111.7 ± 84.7*#	0.004
Tmsv12-Dif%	7.2 ± 4.1	8.8 ± 2.8*	14.1 ± 9.8*#	0.001
Tmsv12-SD	17.7 ± 9.2	22.4 ± 7.5*	33.5 ± 26.4*#	0.007
Tmsv12-SD%	2.2 ± 1.4	2.8 ± 0.9*	4.2 ± 3.0*#	0.002
Tmsv6-Dif	48.4 ± 28.3	53.1 ± 25.7	64.0 ± 65.5	0.06
Tmsv6-Dif%	5.8 ± 3.7	7.3 ± 3.1	10.6 ± 8.0*#	0.010
Tmsv6-SD	18.7 ± 11.8	23.3 ± 9.8	32.5 ± 24.9*	0.022
Tmsv6-SD%	2.2 ± 1.5	2.9 ± 1.2	4.1 ± 3.2*#	0.013

Data are mean ± standard deviation.
**compared to control group, P < 0.05.*
#compared to T2DM without microvascular complications group, P < 0.05.

TABLE 3 | Comparison of MCE parameters in three groups.

Parameters	Control group	T2DM without microvascular complications group	T2DM with microvascular complications group	*P*-value
Peak Intensity (dB)				
Overall segments	22.9 ± 10.8	18.4 ± 8.5	14.0 ± 7.4*#	0.001
Basal segments	19.6 ± 9.8	16.9 ± 10.1	12.3 ± 6.8*	0.010
Middle segments	24.3 ± 12.3	19.9 ± 9.2	15.3 ± 8.2*	0.004
Apical segments	24.9 ± 11.2	18.6 ± 8.0*	14.3 ± 8.4*	<0.001
Time to peak (s)				
Overall segments	5.8 ± 1.8	8.2 ± 2.1*	9.7 ± 2.4*#	<0.001
Basal segments	5.5 ± 2.0	8.0 ± 2.2*	9.7 ± 2.6*#	<0.001
Middle segments	6.0 ± 1.8	8.2 ± 2.0*	9.5 ± 2.4*#	<0.001
Apical segments	6.0 ± 1.8	8.4 ± 2.2*	10.0 ± 2.4*#	<0.001
Wash in slope (dB/s)				
Overall segments	10.3 ± 2.5	6.6 ± 3.2*	4.0 ± 1.6*#	<0.001
Basal segments	11.1 ± 3.6	6.6 ± 4.2*	3.8 ± 1.9*#	<0.001
Middle segments	10.6 ± 3.5	7.1 ± 2.7*	4.7 ± 2.0*#	<0.001
Apical segments	9.2 ± 2.8	6.2 ± 3.7*	3.5 ± 1.9*#	<0.001
A × k				
Overall segments	250.3 ± 145.2	142.0 ± 117.2*	63.6 ± 48.9*#	<0.001
Basal segments	220.6 ± 143.1	135.2 ± 142.1*	52.5 ± 47.2*#	<0.001
Middle segments	281.5 ± 187.9	152.8 ± 113.7*	79.0 ± 59.0*#	<0.001
Apical segments	248.8 ± 144.9	138.0 ± 132.6*	59.2 ± 55.2*#	<0.001

Data are mean ± standard deviation.
**compared to control group, P < 0.05.*
#compared to T2DM without microvascular complications group, P < 0.05.

the perfusion rate and local blood flow significantly decreased, which indicates that T2DM can cause systemic diffuse diseases, except for retinopathy and kidney damage, and the heart is also damaged. This may be attributable to impaired dilation of coronary microcirculation caused by hyperglycemia, which includes a decrease in the secretion of diastolic vasoactive

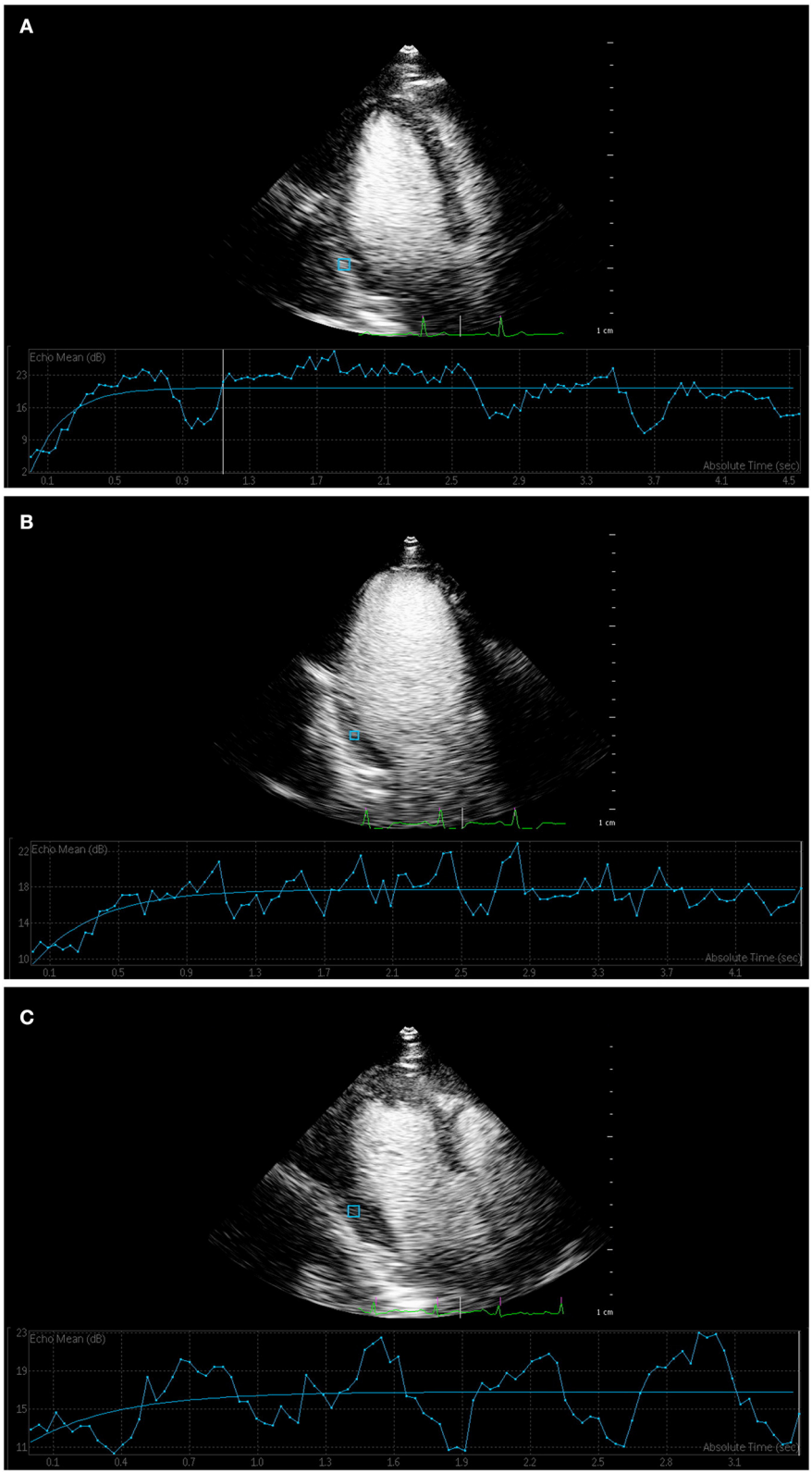

FIGURE 2 | Myocardial contrast echocardiography of myocardial perfusion. **(A)** Time–intensity curve showed that the curve of control group rose rapidly to the plateau stage, and the peak intensity was high. **(B)** Time–intensity curve of T2DM without microvascular complications group and **(C)** T2DM with microvascular complications group increased slowly, and peak intensity was lower than that of control group.

substances such as nitric oxide and increase in the secretion of vasoconstrictor substances. Pathologically, the peri-capillary basement membrane is thickened, and vascular diameter is relatively narrow in T2DM patients (27). Vascular fibrosis contributes to arterial stiffening and reduces the compliance of vascular wall, increasing the risk of cardiovascular events (28). The number of capillaries per unit volume of the myocardium is relatively reduced. The combined effects of these factors promote an increase in local myocardial vascular resistance and decrease in blood volume. Segmental MCE analysis showed that there was no statistical difference among the three segments of the same index in the same group ($P > 0.05$), and the comparison of the basal, middle, and apical segments among the three groups showed a similar trend in the overall assessment. Perfusion velocity, peak intensity, and myocardial blood flow decreased in all segments, suggesting that impairment of myocardial microcirculation is diffuse instead of limited to specific segments in T2DM patients.

Our results demonstrated that (1) Tmsv and A, β, and A·β may be used to detect early LV dysfunction including mechanical dyssynchrony and decrease in myocardial perfusion in T2DM patients with microvascular complication; (2) LV myocardium is damaged, and the coordination and synchronization of myocardial movement are poor in T2DM patients; and (3) subclinical LV diastolic and systolic function were impaired in patients with T2DM. These phenomena may be explained by the following reasons. Various pathogeneses such as metabolic disorders, cardiomyocyte apoptosis, microvascular disease, oxidative stress, and mitochondrial structural disorders may be involved in myocardial hypertrophy and compliance reduction, LV remodeling, and ventricular wall rigidity, which may lead to damage to LV systolic and/or diastolic function.

Our study had several limitations. First, the number of samples in this study is small, and no further analysis of the factors affecting the various complications of T2DM has been carried out. Second, the control healthy patients did not undergo CCTA or CAG because of the radiation dose. However, all T2DM patients were initially screened with CCTA or CAG examination. Third, the MCE and 3DE highly depended on image quality and operator proficiency. For patients with poor acoustic window conditions, such as obesity, excessive lung gas, flat chest, etc., there are certain difficulties in acquiring satisfactory images; what is more, the 3DE technology may not be able to apply in patients with large heart chambers, as the entire LV chamber is hard to cover. Meanwhile, the patients with arrhythmia and breath holding difficulties are also limited. Finally, as this was a cross-sectional study, there are inherent design limitations, and our results remain to be verified by longitudinal studies in T2DM patients. We aim to accomplish this in our future research endeavors.

Diabetic cardiomyopathy is a dangerous complication of diabetes. Active prevention and treatment can effectively reduce the high mortality of diabetes patients due to cardiovascular complications. Some studies have found that early medical treatment such as angiotensin-converting enzyme inhibitors and β-receptor blockers and synchronized treatment or strict control of the blood glucose could effectively improve left ventricular diastolic and systolic function in T2DM patients. Early insulin treatment can improve the microcirculation structure and increase myocardial blood perfusion. The MCE of A and A·β could be used as important parameters for monitoring the therapeutic intervention. RT-3DE and MCE can increase the detection rate of systolic dysfunction and decrease perfusion before myocardial damage develops into cardiomyopathy. Early evaluation of cardiomyopathy has a certain significance for the treatment and prognosis of patients, and it can also be used as an effective, economical, and simple method for regular follow-up after treatment.

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 Institutional Review Board of the First Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

F-jY and H-lH contributed conception and design of the study. WL collected data of clinical trials and drafted the manuscript together with X-zL and J-hZ. MY, HL, and C-lL revised and edited the final version of the manuscript for important intellectual content. JL and RF gave the technical support. The methodology of this study was developed by WL and X-zL. WL and X-zL contributed equally for the acquisition of data, e.g., WL contributed in imaging examination, and X-zL contributed in patient collection. WL and X-zL performed data analysis together, including imaging analysis, computational, and statistical analysis, and edited the manuscript together. 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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Congestive Heart Failure Exhibited Higher BMI With Lower Energy Intake and Lower Physical Activity Level: Data From the National Health and Examination Nutrition Survey

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Background: Despite that nutritional deficiency existed in congestive heart failure (CHF), there is a large amount of CHF patients suffering from obesity. This study aimed to identify the differences for increased BMI or obesity in CHF patients.

Methods: This cross-sectional study included adults from the National Health and Nutrition Examination Survey 2007–2016. Differences were compared between CHF participants vs. non-CHF participants, and BMI $\geq$ 30 kg/m^2 vs. BMI $<$ 30 kg/m^2 CHF participants.

Results: CHF participants were with higher BMI, lower energy and macronutrient intake, lower physical activity level and longer rest time, and lower hematocrit and hemoglobin level (all P <0.05) than non-CHF participants. The prevalence of BMI $\geq$ 30 kg/m^2 in participants with CHF was 53.48%. There was no significant difference observed in energy and macronutrient intake between CHF participants with BMI $\geq$ 30 kg/m^2 or $<$ 30 kg/m^2. The water intake ($P = 0.032$), sedentary time ($P = 0.002$), and hematocrit ($P = 0.028$) were significantly different between CHF with BMI $\geq$ 30 kg/m^2 and with $<$ 30 kg/m^2.

Conclusion: Compared with non-CHF participants, CHF participants exhibited higher BMI with lower energy and macronutrient intake, lower physical activity level, longer rest time, and hemodilution with lower hematocrit and hemoglobin level. Among CHF participants with BMI $\geq$ 30 kg/m^2, higher sedentary time and hematocrit were observed.

Keywords: congestive heart failure, body mass index, total nutrient intakes, physical activity, hemodilution, obesity

HIGHLIGHTS

- CHF participants were with higher BMI, lower energy and macronutrient intake, lower physical activity level and longer rest time, and lower hematocrit and hemoglobin level.
- Only water intake, sedentary time, and hematocrit were significantly different between CHF with BMI $\geq$ 30 kg/m^2 and with $<$ 30 kg/m^2.

- Longer resting time and an unbalanced diet may be associated with a higher prevalence of increased BMI or obesity in CHF participants.

INTRODUCTION

Congestive heart failure (CHF) is a complex clinical condition that represents the final evolution of all cardiac diseases and a global public health problem which affects an estimated 26 million worldwide (1), which has a tremendous economic impact on the public healthcare system (2). Studies revealed that CHF is associated with alterations in cardiac energy metabolism (3) and that nutrition intake may influence the evolution of the disease progress (4). A deficiency in energy and macronutrient intake among CHF patients was reported, which caused an undernourished or malnutritional condition (5–7). Obesity [body mass index (BMI) $\geq$30 kg/m^2], with up to 40% prevalence among CHF patients (8), is recognized as a major independent risk factor for the development of CHF (9) and has a paradoxical impact on the prognosis of CHF (10, 11). Studies indicated a non-linear U-shaped association between BMI and the risk of HF mortality, with a greater risk from being at the lowest group (mean BMI = 19.43 kg/m^2), rather than being at the top category (mean BMI = 30.16 kg/m^2) (12). The beneficial effects of weight loss in HF patients are still controversial, given the view that despite nutritional deficiency existed in CHF, there is a large amount of CHF patients suffering from obesity, and we still lacked studies to explain the reasons for the increased BMI in CHF patients. In this research, we used the data from the National Health and Examination Nutrition Survey (NHANES) 2007–2016 to identify the differences for increased BMI or obesity in CHF participants.

METHOD

Dataset

The NHANES data, a nationally representative multistage cross-sectional survey of the non-military and non-institutionalized population of the United States, was used as the data source (13). The data is released by the National Center for Health Statistics once every 2 years. Each survey cycle collects self-reported and directly measured information from participants who undergo a series of questionnaires in a detailed in-home interview such as health conditions, behaviors, and dietary intake, and a physical examination during mobile examination. Also, each participant provides their blood for laboratory tests. This survey included NHANES data from 2007 to 2016.

Study Population

To identify the study population, we implemented the following exclusion criteria: participants <18 years old, participants who were pregnant or breastfeeding at the time of the survey, and participants who lack information on CHF diagnosis, dietary data, examination data, laboratory data, or questionnaire data. The final sample for this study consisted of 660 CHF participants and 20,923 non-CHF participants.

Definition of CHF and Obesity

CHF was self-reported and was obtained from the medical condition files that were administered in the home by an interviewer using the Computer-Assisted Personal Interviewing system as part of the survey participant household interview. The final sample provided yes/no responses to the following question: "Has a doctor or other health professional ever told {you/SP} that {you/s/he}… had congestive HF?"

Height and weight were measured at the Mobile Examination Center (MEC) examination. BMI was calculated as weight in kilograms divided by the square of height in meters. BMI was analyzed as both a continuous variable and as a categorical variable dichotomizing into BMI $\geq$ 30 kg/m^2 (obese) and BMI $<$ 30 kg/m^2 (non-obese) (14).

Sample Demographics, Dietary Condition, and Other Covariates

We obtained information on age and sex from the NHANES self-reported demographics data. Blood pressures and heart rate (resting) were measured in the MEC examination by a physician. Averages of up to four values for systolic and diastolic blood pressures were recorded. Intakes of energy, water, and macronutrients, which consisted of protein, carbohydrate, total sugars, total fat, and cholesterol, were obtained from self-reported dietary data, and an average of 2 days was recorded. Physical activity, including work, and recreational activities, was classified as vigorous, moderate, bicycling or walking, and sedentary and was obtained by self-reporting the number of days they engaged in that activity in a typical week and the average duration they engaged in that activity. For each activity, the Metabolic Equivalent of Task (MET)-hr-week was calculated by multiplying the number of days, the mean duration, and the respective MET level (MET-hr-week = days $*$ duration $*$ MET level). The MET levels for each activity are provided as vigorous work/recreational-related activity = 8 MET, moderate work/recreational-related activity = 4 MET, and walking or bicycling for transportation = 4 MET. It was analyzed as a continuous variable for total activates MET and MET in each activity. Hematocrit (%) and hemoglobin (g/dL), which may imply hemodilution (15), were acquired from blood collected during the MEC examination using methods described by the US Department of Health and Human Services.

Statistical Analysis

Data are expressed as the mean $\pm$ standard deviation or as the number (%). Differences between groups were tested by the chi-square test for categorical variables and independent Student's *t*-test, as well as ANOVA with Tukey's *post-hoc* analysis for continuous variables, as appropriate. Since the sample size is large, a simple application of the Lyapunov or Lindeberg's Central Limit Theorem guarantees large sample convergence of the weighted mean to a standard normal distribution, ensuring that the t-statistic would have a limiting t-distribution. We also used restricted cubic splines with three knots at the 5th, 25th, 50th, 75th, and 95th centiles to flexibly model the association of sedimentary time with BMI $\geq$ 30 kg/m^2 in CHF participants and non-CHF participants, respectively. We tested for potential non-linearity by using a likelihood ratio test comparing the

model with only a linear term against the model with linear and cubic spline terms. A P value < 0.05 was considered statistically significant. All tests were two-sided. All analyses were performed using R: A Language and Environment for Statistical Computing, version 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Participants' Characteristics Between CHF and Non-CHF

The flowchart for participant selection is outlined in **Figure 1**. **Table 1** presents the clinical characteristics of CHF and non-CHF participants in NHANES 2007–2016. As noted, CHF participants were older than non-CHF participants ($P < 0.05$) with higher systolic blood pressure, lower diastolic blood pressure, and lower heart rate (all $P < 0.05$). Intake of total nutrients, including energy, protein, total carbohydrate, total sugar, total fat, and cholesterol, as well as water was much lower compared with that in non-CHF participants (all $P < 0.05$). Also, CHF participants had lower physical activity level ($P < 0.05$) and longer sedentary time ($P < 0.05$). Moreover, hemoglobin and hematocrit were lower in CHF participants ($P < 0.05$). However, BMI was higher in CHF participants than counterparts ($P < 0.05$).

Participants' Characteristics by BMI Category

As shown in **Table 2**, the proportions of BMI $\geq$ 30 kg/m^2 were 53.48% ($n = 353$) in CHF participants and 37.01% ($n = 7{,}744$) in non-CHF participants. Compared with BMI $<$ 30 kg/m^2 CHF participants, BMI $\geq$ 30 kg/m^2 CHF participants showed significantly younger age, larger amount of water intake, longer sedentary time, and higher hematocrit (all $P < 0.05$). Among non-CHF participants, those with BMI $\geq$ 30 kg/m^2 were older and had higher blood pressure and heart rate and lower energy, protein, total carbohydrate, and total sugar intake but higher cholesterol and water intake (all $P < 0.05$). Physical activity level was lower and sedentary time was longer (both $P < 0.05$) in BMI $\geq$ 30 kg/m^2 non-CHF participants. Meanwhile, hemoglobin and hematocrit were lower in BMI $\geq$ 30 kg/m^2 non-CHF participants than BMI$<$30 kg/m^2 ones (both $P < 0.05$). Restricted cubic spline showed that the risk of BMI $\geq$ 30 kg/m^2 in CHF participants was increased with the sedimentary time (odds ratio 1.08, 95% confidence interval 1.03–1.13) (**Figure 2**), and such result could be also seen in participants without CHF (**Supplementary Figure 1**).

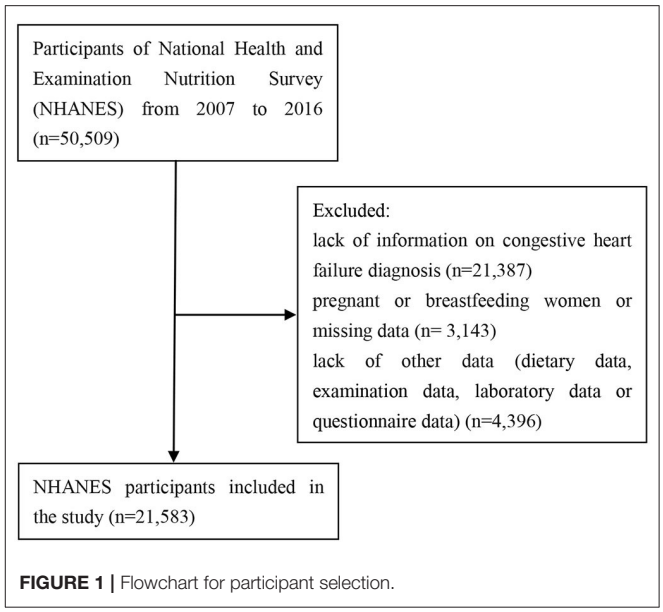

FIGURE 1 | Flowchart for participant selection.

DISCUSSION

There are two major findings in the present study. First, compared with non-CHF participants, CHF participants exhibited higher BMI with lower energy and macronutrient intake, lower physical activity level, and longer rest time and hemodilution (lower hematocrit and hemoglobin). Second, higher water intake, sedentary time, and hematocrit were observed in CHF participants with BMI $\geq$ 30 kg/m^{2}s, while energy and macronutrient intake and physical level were similar between CHF participants with BMI $\geq$ 30 kg/m^2 and $<$ 30 kg/m^2.

Excess body weight and a sedentary lifestyle are major public health problems worldwide (16). Obesity (BMI $\geq$ 30 kg/m^2) has been considered as a major independent risk factor for cardiovascular disease (17). A previous study showed that up to 40% of patients with CHF suffered from obesity (8). The interplay between obesity and CHF is complex. Despite that obesity increases the risk of CHF, studies found that CHF patients who developed obesity or overweight (25–29.9 kg/m^2) are associated with better prognosis compared with those who did not (18–20), with reductions in cardiovascular disease mortality (19 and 40%, respectively) and all-cause mortality (16 and 33%, respectively) compared to heart failure patients with normal weight (BMI 20–24.9 kg/m^2), whereas heart failure patients with BMI $<$ 20 kg/m^2 increased total and cardiovascular mortality (27 and 20%, respectively) (21). This phenomenon is termed as "obesity paradox," and several potential reasons may account for it, such as earlier presentation, different etiology, greater metabolic reserves, protective cytokines, more tolerance of cardiac medications, different cardiorespiratory fitness level, and less cachexia (22). Based on previous studies, our study termed "obesity" as "BMI $\geq$ 30 kg/m^2" to investigate the differences for increased BMI in CHF participants.

In our analysis, we recognized that CHF participants presented with higher BMI than non-CHF participants and a higher proportion of BMI $\geq$ 30 kg/m^2 among CHF participants. Meanwhile, we also noticed an inadequate energy and macronutrient intake in CHF participants, seen as significantly lower energy, protein, carbohydrate, total sugar, total fat, cholesterol, and water intake. This implied that nutritional deficiency existed in CHF participants. Malnutrition is commonly prevalent in CHF, with a reported incidence of 7.5 and 57% in severe or moderate malnutrition, respectively (23). These patients are older and frail, with negative calorie and nitrogen balance and energy availability (24) for physical

TABLE 1 | Characteristics of congestive heart failure group and non-congestive heart failure participants.

	Congestive heart failure (n = 660)	Non-congestive heart failure (n = 20,923)	P-value
Demographic and weight characteristics, mean (SD)			
Age (y)	66.46 (12.72)	48.65 (17.51)	<0.001
Sex			
Male (no., %)	360 (54.55)	10,153 (48.53)	<0.001
BMI (kg/m^2)	31.96 (8.54)	28.99 (6.80)	<0.001
SBP (mmHg)	129.82 (21.94)	123.26 (18.17)	<0.001
DBP (mmHg)	65.57 (15.03)	70.03 (12.76)	<0.001
Heart rate (beats/min)	71.02 (12.69)	72.81 (12.15)	<0.001
Total nutrient intake, mean (SD)			
Energy (kcal)	1734.71 (717.33)	2051.32 (872.86)	<0.001
Protein (gm)	69.85 (31.39)	80.65 (36.92)	<0.001
Carbohydrate (gm)	213.08 (94.19)	251.13 (111.62)	<0.001
Total sugars (gm)	93.56 (57.98)	110.98 (68.26)	<0.001
Total fat (gm)	66.36 (32.80)	76.58 (39.85)	<0.001
Cholesterol (mg)	266.10 (175.92)	286.80 (197.44)	0.0102
Water (gm)	531.16 (584.86)	646.00 (647.87)	<0.001
Physical activity, mean (SD)			
Total activity (MET-h/week)	24.54 (66.46)	57.68 (98.97)	<0.001
Vigorous activity (MET-h/week)	9.62 (42.83)	27.83 (70.54)	<0.001
Moderate activity (MET-h/week)	11.89 (28.80)	23.64 (42.33)	<0.001
Sedimentary activity (h/week)	6.81 (3.52)	5.79(3.39)	<0.001
Walk or bicycle (MET-h/week)	3.03 (14.95)	6.21 (20.27)	<0.001
Complete blood count—whole blood, mean (SD)			
Hemoglobin (g/dL)	13.48 (1.73)	14.08 (1.53)	<0.001
Hematocrit (%)	39.81 (4.82)	41.25 (4.24)	<0.001

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MET, metabolic equivalent of task; SD, standard deviation.

activity, and are triggered by multiple factors such as anorexia, malabsorption secondary to intestinal edema, high energy demand, and cytokine-induced hypercatabolism (7, 25) and worse outcomes (6, 26, 27). However, when compared with CHF participants with BMI $<$ 30 kg/m^2, participants with BMI $\geq$ 30 kg/m^2 did not show a distinct increase in energy and macronutrient intake. A constellation of researches revealed that failing myocardium utilized glucose, switching from fatty acids, as the primary energetic substrate to produce ATP (28), which may exert a protective role in preventing cardiomyocytes from oxidative radical excess and cell damage (29, 30) but a lower energy production (31). With more total fat intake and less glucose intake, cells may have the less energetic substrate to produce enough energy. This would affect the metabolism in the body and cause adipose tissue accumulation and subsequently increased body weight. This may imply that inadequate or unbalanced energy and macronutrient intake accounts for the increase in BMI or obesity among CHF participants. A daily caloric intake of about 29 kcal/kg and a daily protein intake of 1.2–1.4 g/kg were recommended for CHF patients at normal weights and a less energy intake was required (20–24 kcal/kg/day), and a reduction in dietary fat intake to about 25% of total caloric intake (0.6–0.8 g/kg/day) was required for overweight and obese CHF patients (32, 33).

Our study found lower hematocrit and hemoglobin levels in CHF participants, while CHF with BMI $\geq$ 30 kg/m^2 participants had higher hematocrit and hemoglobin concentration than those with BMI $<$ 30 kg/m^2. These results demonstrated that hemodilution and fluid retention are common in CHF, especially in those with lower BMI level (BMI$<$30 kg/m^2). This observation may be another potential reason to explain the "obesity paradox." Considerable evidence demonstrated that total blood volume and cardiac output are positively correlated with the degree of excess body weight (18, 22, 34) and further preserved or even increased skeletal muscle mass (i.e., lean mass) (35, 36), which may cause BMI to be higher, and vice versa. This may explain the phenomenon of elevated hematocrit and hemoglobin concentration in CHF with BMI $\geq$ 30 kg/m^2 participants, and fluid retention may not be the reason for the increased BMI or obesity in CHF participants. Hemodilution was common in chronic heart failure (37) and has a deleterious effect as it may impair peripheral oxygen delivery (38) and is often neglected, as compensatory mechanisms may mask signs of volume (39). Our study found lower hematocrit and hemoglobin level in CHF participants, while CHF with BMI $\geq$ 30 kg/m^2 participants had higher hematocrit and hemoglobin concentration than BMI $<$ 30 kg/m^2 ones. These results demonstrated that hemodilution and fluid retention are common in CHF, especially in those with

TABLE 2 | Characteristics of congestive heart failure group and non-congestive heart failure group (stratified by obese and non-obese).

BMI (kg/m^2)	Congestive heart failure			Non-congestive heart failure			*P-value
	BMI < 30 kg/m^2 (n = 307)	BMI ≥ 30 kg/m^2 (n = 353)	P value	BMI < 30 kg/m^2 (n = 13,179)	BMI ≥ 30 kg/m^2 (n = 7,744)	P value	
Demographic and weight characteristics, mean (SD)							
Age (y)	68.67 (12.86)	64.54 (12.30)	0.012	48.28 (18.17)	49.29 (16.30)	<0.001	<0.001
Sex							
Male (no., %)	178 (57.98)	181 (51.27)	<0.001	6,786 (51.49)	3,367 (43.48)	<0.001	<0.001
BMI (kg/m^2)	25.34 (3.03)	37.72 (7.56)	<0.001	24.95 (3.13)	35.86 (5.78)	<0.001	<0.001
SBP pressure (mmHg)	130.38 (23.38)	129.34 (20.63)	0.89	122.01 (18.53)	125.40 (17.33)	<0.001	<0.001
DBP (mmHg)	64.13 (14.56)	66.81 (15.33)	0.04	69.15 (12.45)	71.51 (13.16)	<0.001	<0.001
Heart rate (beats/min)	70.05 (12.37)	71.84 (12.92)	0.24	71.73 (11.9)	74.64 (12.23)	<0.001	<0.001
Total nutrient intakes, mean (SD)							
Energy (kcal)	1759.97 (716.89)	1712.74 (718.01)	0.90	2079.46 (894.56)	2003.43 (832.49)	<0.001	<0.001
Protein (gm)	70.93 (32.74)	68.91 (30.19)	0.90	81.30 (37.76)	79.55 (35.44)	0.005	<0.001
Carbohydrate (gm)	217.22 (95.26)	209.48 (93.24)	0.81	256.08 (114.39)	242.69 (106.21)	<0.001	<0.001
Total sugars (gm)	96.15 (59.90)	91.31 (56.23)	0.80	112.65 (70.08)	108.13 (64.96)	<0.001	<0.001
Total fat (gm)	65.65 (30.90)	66.98 (34.41)	0.97	76.48 (40.51)	76.73 (38.72)	0.97	<0.001
Cholesterol (mg)	261.71 (181.87)	269.92 (170.75)	0.95	282.87 (200.12)	293.50 (192.62)	<0.001	<0.001
Water (gm)	457.69 (484.71)	595.06 (653.72)	0.032	615.04 (621.65)	698.70 (687.04)	<0.001	<0.001
Physical activity, mean (SD)							
Total activity (MET-h/week)	28.17 (67.00)	21.38 (65.91)	0.81	60.85 (101.41)	52.28 (94.43)	<0.001	<0.001
Vigorous activity (MET-h/week)	11.36 (41.45)	8.11 (43.99)	0.93	29.53 (71.50)	24.93 (68.79)	<0.001	<0.001
Moderate activity (MET-h/week)	12.85 (29.27)	11.06 (28.41)	0.94	24.39 (42.80)	22.36 (41.49)	<0.001	<0.001
Sedimentary activity (h/week)	6.32 (3.26)	7.24 (3.69)	0.002	5.62 (3.32)	6.08 (3.48)	<0.001	<0.001
Walk or bicycle (MET-h/week)	3.97 (16.42)	2.21 (13.51)	0.68	6.93 (21.51)	4.99 (17.92)	<0.001	<0.001
Complete blood count—whole blood, mean (SD)							
Hemoglobin (g/dL)	13.34 (1.80)	13.61 (1.65)	0.121	14.12 (1.50)	14.00 (1.57)	<0.001	<0.001
Hematocrit (%)	39.31 (5.02)	40.25 (4.61)	0.028	41.32 (4.21)	41.13 (4.30)	0.009	<0.001

**P-value was analysis between four groups.*

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MET, metabolic equivalent of task; SD, standard deviation.

lower BMI level (BMI<30 kg/m^2). Previous studies indicated a higher mortality rate in patients with hemodilution than in those with hemoconcentration in acute heart failure patients (15), while fluid restriction could only improve signs and symptoms of chronic heart failure in patients in moderate to severe chronic heart failure (40) and aggressive fluid removal positively affected survival (41). In the present study, CHF with BMI ≥ 30 kg/m^2 participants had higher hematocrit and hemoglobin concentration than BMI < 30 kg/m^2 ones, indicating that obese CHF participants were less likely to develop hemodilution. This could be one explanation for the "obesity paradox." Furthermore, hemodynamic changes including increased stroke volume and increased arterial pressure may compensate the impaired peripheral oxygen delivery, leading CHF patients with increased BMI or obesity to have better prognoses. Still, we could not ignore that there was fluid retention in our CHF group, which implied an inadequate usage of diuretics and fluid management should be enhanced.

Another finding in our analysis is that we demonstrated that CHF participants had lower physical activity levels and spent a longer time in rest, especially participants with BMI ≥ 30 kg/m^2. It was revealed that sedentary time caused metabolic alterations at the muscle level and next influenced gross metabolic disturbances in the whole body (42). It has been reported that prolonged sedentary time would impair mitochondrial function by elevating oxidative stress levels (43), which decreased the mitochondrial respiration level (44) and caused insufficient ATP production for daily activity and metabolism. Emerging evidences have demonstrated a significant dose–response association between sitting time and cardiovascular disease mortality (45–48), and the relative risks associated with sedentary time were higher among participants without regular physical activity (49, 50). Meanwhile, there have been proven cardioprotective effects of regular physical activity on cardiovascular health, improving cardiac compliance, reducing arterial stiffness and ventricular afterload, and finally reducing the risk of future cardiac dysfunction and improving cardiovascular outcomes (42). Moreover, researches showed that an increasing lean mass with resistance exercise training could effectively improve muscular fitness in CHF (51, 52), which could prevent sarcopenia (53) or even cachexia in CHF patients and have better prognosis (54).

Our investigation has several limitations. We only included macronutrient in our analysis and lacked the data

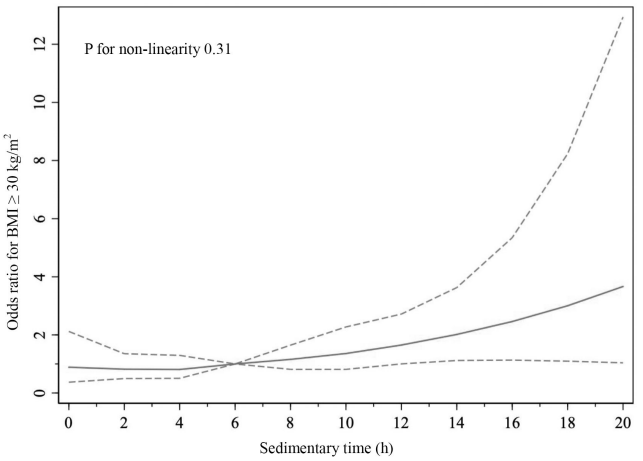

FIGURE 2 | Association of sedimentary time with BMI ≥ 30 kg/m^2 in CHF participants.

of micronutrient intake. Therefore, the present study may have underestimated the nutritional deficit and its effects on obesity among participants. Also, details regarding the etiology, subtype (HF with reduced ejection fraction vs. HF with preserved ejection fraction), severity of HF, and complications were not available. Moreover, CHF participants included in our analysis were self-reported in the NHANES survey. This may lead to possible selection, reporting, and recall bias. Additionally, we did not include socioeconomic data, and thus we may neglect the socioeconomic impacts on nutrition intake and lifestyle self-management of CHF participants. Finally, we lacked follow-up data of these participants, including BMI changes and relative outcomes, so we were unable to recognize the effects of nutrients intake and physical activity on BMI changes and prognoses in participants with CHF.

CONCLUSION

Higher water intake, sedentary time, and hematocrit were observed in BMI ≥ 30 kg/m^2 CHF participants. It seems that longer resting time and an unbalanced diet may be associated with a higher prevalence of increased BMI or obesity in CHF participants. Future research is warranted to explore the mechanisms underlying this finding and whether intentional weight loss with combination of diet, exercise, and others could be contributing to better health outcomes in BMI ≥ 30 kg/m^2 CHF participants.

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/s.

ETHICS STATEMENT

This study was reviewed and approved by the National Center for Health Statistics research ethics review board, and written informed consent was obtained from all NHANES participants.

AUTHOR CONTRIBUTIONS

TX: study concept and design, acquisition, analysis, or interpretation of data, and drafting of the manuscript. HZho: study concept and design, acquisition, analysis, or interpretation of data, and statistical analysis. ZM and HZha: acquisition, analysis, or interpretation of data, and statistical analysis. QZ and DX: critical revision of the manuscript for important intellectual content, administrative, technical, or material support, and study supervision. YZ and JZ: study concept and design, critical revision of the manuscript for important intellectual content, administrative, technical, or material support, and study supervision. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.680371/full#supplementary-material

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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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Hypofunction of Circulating Endothelial Progenitor Cells and Aggravated Severity in Elderly Male Patients With Non-ST Segment Elevation Myocardial Infarction: Its Association With Systemic Inflammation

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Background: Aging patients easily suffer from non-ST segment elevation myocardial infarction (NSTEMI). Our previous studies revealed declined function of endothelial progenitor cells (EPCs) in the elderly. However, the impact of aging on EPC function and severity in male NSTEMI patients and its possible mechanism is unclear until now.

Methods: We measured the circulating EPC function including migration, proliferation, and adhesion in aging or young male patients with NSTEMI. The GRACE and TIMI risk score were evaluated. Plasma levels of interleukin-6 (IL-6) and interleukin-17 (IL-17) were also detected in all patients.

Results: Compared with the young group, the old male patients with NSTEMI had higher GRACE score and TIMI score and decreased function of circulating EPCs. EPC function was negatively correlated with GRACE score and TIMI score. IL-6 and IL-17 level were higher in the old group than those in the young group. There was a significant negative correlation between EPC function and IL-6 or IL-17. Moreover, IL-6 and IL-17 positively correlated with GRACE and TIMI score. Age was positively related with GRACE or TIMI score and plasma level of IL-6 or IL-17, but inversely correlated with EPC function.

Conclusions: The current study firstly illustrates that the age-related decrement in EPC function is related to the severity of NSTEMI in male patients, which may be connected with systemic inflammation. These findings provide novel insights into the pathogenetic mechanism and intervention target of aging NSTEMI.

Keywords: endothelial progenitor cells, aging, GRACE risk score, non-ST segment elevation myocardial infarction, interleukin-17

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INTRODUCTION

Aging which can cause organ function decline is an important independent predictor in cardiovascular disease (1, 2). The incidence and mortality in elderly patients with acute coronary syndrome (ACS) significantly increased when compared to young patients with ACS (3). GRACE and TIMI score are pivotal protocols to evaluate the severity and prognosis of NSTEMI in the clinic (4, 5). Nevertheless, the influence of aging on GRACE and TIMI score in NSTEMI remains notx fully understood.

Circulating endothelial progenitor cells (EPCs) is an important repair approach for vascular endothelial injury (6). Our previous investigation illuminated that aging led to mitigating EPC function via the CXCR4 pathway (7). It is reported that the function of late EPCs is subdued in elderly patients with acute myocardial infarction (AMI) (8), but other investigators found that the number of circulating CD34+CD133+ progenitor cells is not related to age after AMI (9). Because there is a great distinction in pathogenesis between ST-segment elevation myocardial infarction (STEMI) and NSTEMI, the effect of aging on EPC function in NSTEMI patients and its underlying mechanism are still unclear.

Interleukin-6 (IL-6) and interleukin-17 (IL-17) are important pro-inflammatory factors. The increased IL-6 can stimulate the differentiation of Th17 CD4 + T cells during inflammation, which leads to increment of IL-17 (10). It is reported that elevated IL-6 is found in acute myocardial infarction and related to EPC hypofunction (11). Accumulating evidence indicates that IL-6 and IL-17 increase with aging (12, 13). Base on the previous studies, we hypothesized that the attenuated circulating EPC quality may be related to exacerbated severity in aging male patients with NSTEMI, which may associate with the increment in plasma IL-6 and IL-17 levels. To test these hypotheses, we evaluated EPC function in young and aging male NSTEMI patients, compared their IL-17 and IL-6 levels, and analyzed the relationship between EPC function, severity of NSTEMI and inflammatory cytokines.

METHODS

Characteristics of Patients

The NSTEMI diagnosis criteria was defined by acute chest pain, electrocardiogram and elevated cardiac Troponin T. 41 male NSTEMI patients were enrolled in the study, and their age was >18 years. All patients were classified into two groups: young group (<65 years) and aging group (≥65 years). **Table 1** showed the basic characteristics of enrolled patients. Venous blood samples were collected to evaluate EPC function, plasma IL-17, and IL-6 levels.

Calculation of GRACE Risk Score

Calculation of the GRACE risk score was performed on the basis of each individual predictive factor and was calculated online (www.outcomes.org/grace).

TABLE 1 | Clinical and biochemical characteristics in young and old-aged male NSTEMI.

Characteristics	Young group ($n = 20$)	Old-aged group ($n = 21$)
Age (years)	44.0 ± 7.4	77.2 ± 7.8#
Heart rate (time/min)	79.0 (71.3, 93.3)	75.0 (68.5, 82.5)
Systolic BP (mmHg)	131.6 ± 18.3	129.9 ± 22.7
Diastolic BP (mmHg)	80.0 (72.3, 94.5)	72.0 (68.5, 82.5)
BMI (kg/cm^2)	25.7 ± 4.0	23.8 ± 3.8
Glucose (mmol/L)	5.8 (4.9, 11.2)	6.4 (5.6, 8.0)
Cr (mmol/L)	77.5 (60.7, 95.2)	82.4 (72.3, 120.9)
TC (mmol/L)	5.3 ± 1.5	4.5 ± 1.6
TG (mmol/L)	1.9 (1.4, 4.3)	1.3 (1.0, 2.5)
HDL (mmol/L)	1.1 (0.9, 1.2)	1.2 (1.0, 1.3)
LDL (mmol/L)	3.5 ± 1.2	2.9 ± 1.2
GRACE score	110.0 (100.3, 133.3)	179.0 (162.5, 196.0)#
TIMI score	3.0 (3.0, 3.0)	4.0 (3.0, 5.0)#

BMI, body mass index; BP, blood pressure; Cr, serum creatinine; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low-density lipoprotein.
Data which are normal distribution are given as mean ± SD and those which are abnormal distribution are given as median (25, 75%). #$P < 0.05$ *vs. Young group.*

Calculation of TIMI Risk Score

The TIMI score was calculated as previous study described (4).

EPC Function Assay

EPC function including migration, adhesion, and proliferation were evaluated according to previous investigations (14–17).

Detection of IL-17 and IL-6

Plasma IL-17 and IL-6 levels have been tested by ELISA kit (R&D Systems, Minneapolis, USA) according to related studies (18, 19).

Analysis of Statistics

Analysis of statistics was performed using the program SPSS (version 23.0; SPSS Inc., Chicago, Illinois). Comparisons between two groups were done with Student's *t*-test for the data with normal distribution while Mann–Whitney *U*-test was used for the data with abnormal distribution. Pearson test was performed for variables with normal distribution and Spearman test was used for abnormal distribution variables to evaluate the correlation. Values of $P < 0.05$ were considered as statistical significance.

RESULTS

Baseline Characteristics

Baseline characteristics and laboratory findings of the young and old-aged groups were listed in **Table 1**. No significant difference of heart rate, blood pressure and BMI were found in the two groups ($P > 0.05$). The laboratory findings in plasma glucose, creatine, cholesterol, triglycerides, HDL, and LDL cholesterol were all similar between the two groups ($P > 0.05$). GRACE and TIMI score were remarkably higher in the old-aged group than the young ($P < 0.05$).

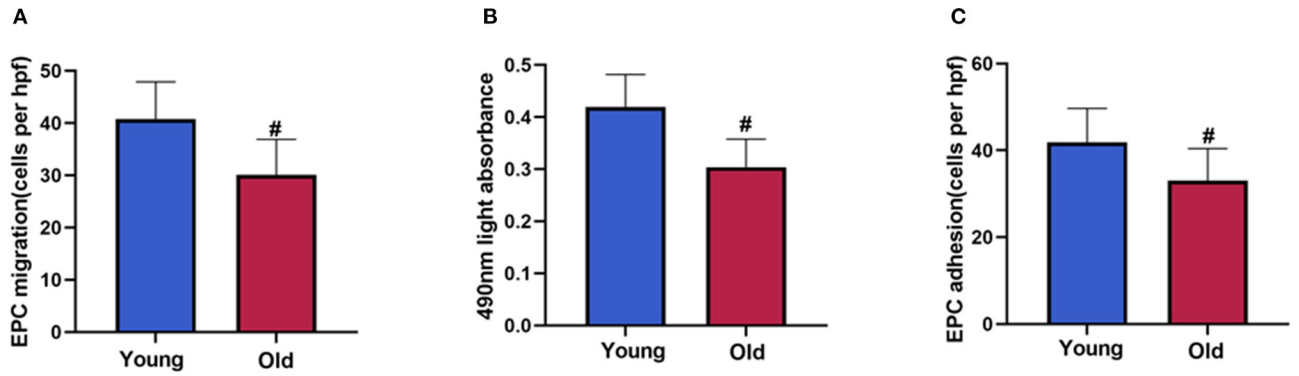

FIGURE 1 | EPC function between young and old-aged group. The circulating EPC migration **(A)**, proliferation **(B)**, adhesion **(C)** decreased in old-aged patients with NSTEMI compared with young patients. Data are given as mean ± SD. $^{\#}P < 0.05$ vs. young group.

EPC Function Between Young and Old-Aged Male Patients

Patients in the old-aged group presented decreased migration, proliferation and adhesion of circulating EPCs compared to the young group (**Figures 1A–C**, $P < 0.05$).

Correlation Between the Severity of NSTEMI and EPC Function

Significant negative correlations between GRACE score and EPC migration, proliferation or adhesion were observed in male NSTEMI patients (**Figures 2A–C**, $P < 0.05$). The similar relationships were also found between TIMI score and EPC function (**Figures 2D–F**, $P < 0.05$).

Correlation Between Inflammatory Cytokines and Circulating EPC Function or GRACE/TIMI Risk Score

As shown in **Figures 3A,B**, the plasma levels of IL-17 or IL-6 in young patients were lower than those in old-aged patients ($P < 0.05$). In addition, IL-17 and IL-6 were negatively correlated with the migration, proliferation, adhesion of circulating EPCs (**Figure 4**, $P < 0.05$). In contrast, IL-17 and IL-6 were positively correlated with GRACE and TIMI score (**Figure 5**, $P < 0.05$).

Correlation Between Age and GRACE/TIMI Risk Score, Inflammatory Cytokines, and EPC Function

Figures 6A,B showed that age was positively correlated with GRACE score or TIMI score ($P < 0.05$). Similarly, the positive relationship was found between age and IL-6 or IL-17 (**Figures 6C,D**, $P < 0.05$). On the contrary, age was negatively related to EPC migration, proliferation, and adhesion (**Figures 6E–G**, $P < 0.05$).

DISCUSSION

The present study firstly demonstrated that aging male NSTEMI patients had higher GRACE or TIMI score and lower EPC function than young. Circulating EPC function was significantly associated with the severity of NSTEMI. Moreover, aging male NSTEMI patients presented elevated IL-17 and IL-6 levels, which positively correlated with age and severity, and negatively connected with EPC function. These findings revealed the relationship between mitigated endothelial repairability and aging NSTEMI, with the latent involvement of systemic inflammation.

The GRACE and TIMI risk score have been recognized as useful indicators for evaluating disease severity and predicting adverse outcomes in NSTEMI (4, 5). Our results showed that GRACE and TIMI score of aging NSTEMI patients were higher than those of young, suggesting that age plays an important role in the prognosis of NSTEMI and it was similar with previous study which revealed the impact of age on the outcome of acute coronary syndrome (3). Interestingly, our research revealed that the correlation between age and GRACE score was stronger than that of TIMI score. Previous report showed that GRACE score was better than TIMI score in evaluating the long-term prognosis of NSTEMI (20). Together with our findings, it suggested that aging may have an important impact on the long-term prognosis of NSTEMI.

Circulating EPCs are critical for repairing endothelial damage and associated with prognosis of coronary artery diseases (21). Previous studies revealed that aging had close relationship with circulating EPC dysfunction (22, 23), and late-outgrowth EPC function significantly decreased in aging AMI (8). In the present study, we found that circulating EPC activity was significantly reduced in aging NSTEMI patients compared to young group and there was negative correlation between circulating EPC function and age. The results indicate that subdued endogenous endothelial repair capacity may be involved in the pathogenesis of aging NSTEMI.

In addition, our study found that circulating EPC function was significantly negative with GRACE or TIMI score in male patients with NSTEMI. To our knowledge, few studies had focused on the relationship between EPC function and GRACE or TIMI score. Considering the prediction value of GRACE score and TIMI score in NSTEMI (4, 5), it is reasonable to infer that circulating

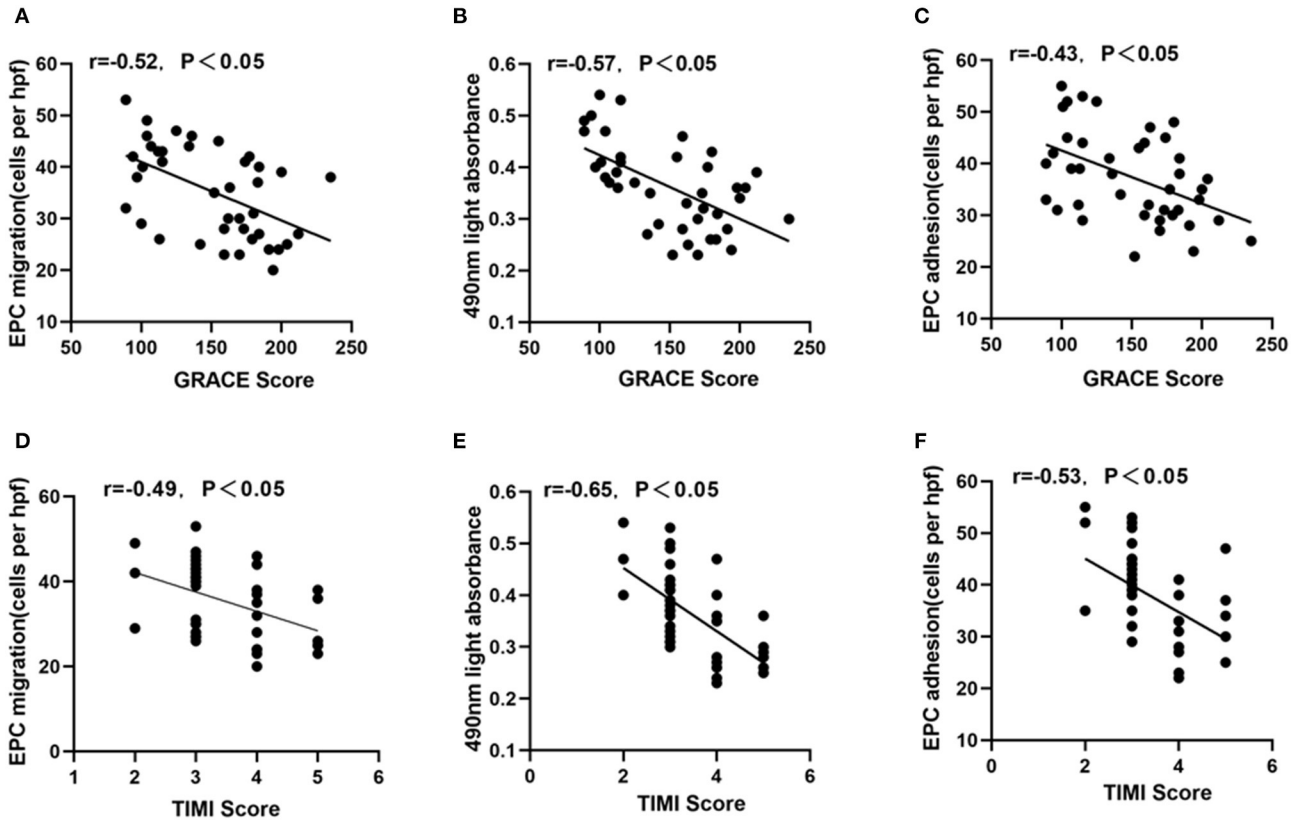

FIGURE 2 | Correlation between GRACE or TIMI score and EPC function. GRACE score was negatively correlated with EPC migration **(A)**, proliferation **(B)**, adhesion **(C)**. Similarly, TIMI score was also inversely related with EPC migration **(D)**, proliferation **(E)**, and adhesion **(F)**.

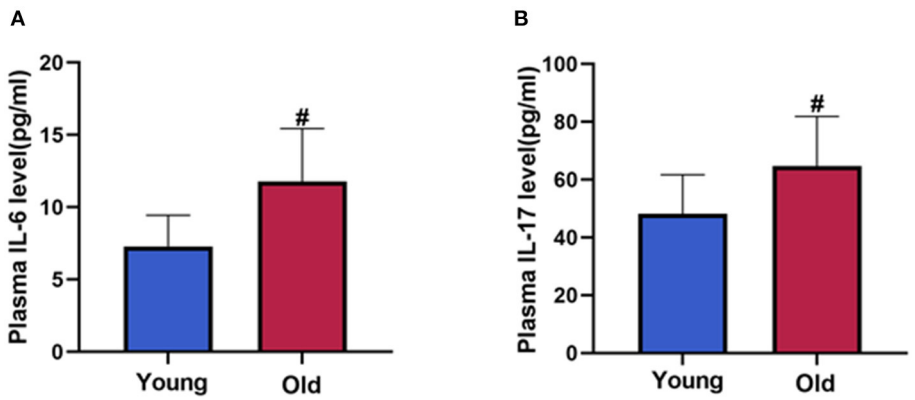

FIGURE 3 | IL-6 and IL-17 level between young and old-aged group. Patients in old-aged group had higher levels of IL-6 **(A)** and IL-17 **(B)** than young patients. Data are given as mean ± SD. $^{\#}P < 0.05$ vs. young group.

EFC function may be used as an indicator to assess the severity and prognosis of NSTEMI, and increment of circulating EPC function may be beneficial to the prognosis of NSTEMI.

Research illustrated that circulating levels of inflammatory cytokines are remarkably influenced by age (12). Consistent with previous findings, our study found that plasma levels of IL-6 and IL-17 significantly increased in aging NSTEMI and positively related with age. Moreover, it was reported that increment of pro-inflammatory cytokines might impair vascular damage, leading to EPC apoptosis and dysfunction

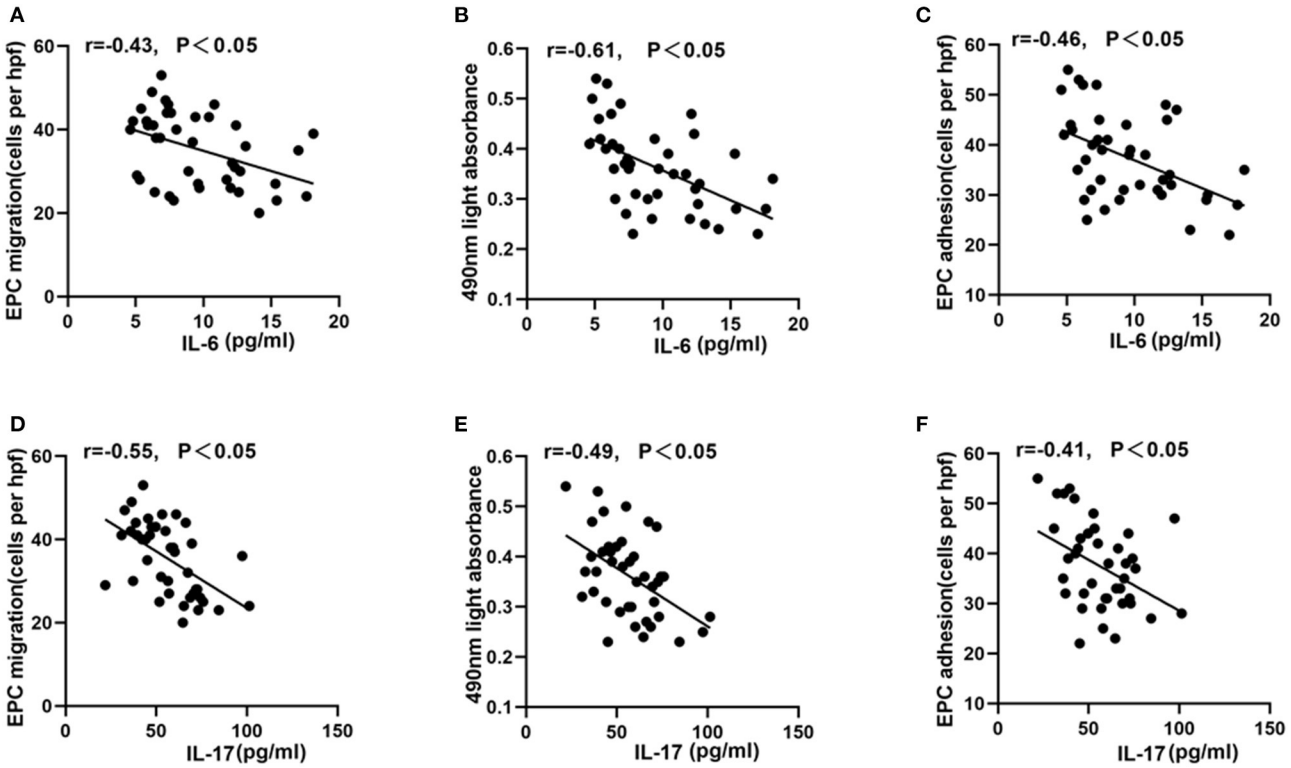

FIGURE 4 | Correlation between EPC function and IL-6 or IL-17. IL-6 **(A–C)** and IL-17 **(D–F)** were negatively correlated with EPC migration, proliferation, and adhesion.

(24, 25). In the present study, we found IL-6 and IL-17 were inversely correlated with EPC function in NSTEMI patients, suggesting the negative impact of inflammation on EPC function. Furthermore, our study showed that IL-6 and IL-17 were positively related to the severity of NSTEMI. A prior study pointed out that IL-6 was a positive feedback factor, which caused the aging mitochondrial dysfunction in coronary artery diseases (26). Systemic inflammation has been confirmed as an important risk factor in the pathogenesis of NSTEMI (27), while anti-inflammatory treatment is beneficial for the prognosis of coronary artery diseases (28). Thus, the current findings indicated that systemic inflammation may be the potential mechanism underlying hypofunction of circulating EPCs, which may be associated with adverse outcomes of aging NSTEMI.

The findings presented in this study have important clinical implications. First, circulating EPC function impaired in aging NSTEMI and inversely correlated with GRACE or TIMI score, implying that circulating EPC function may be an important biological indicator to provide prognostic information concerning aging NSTEMI. Interventions which may enhance EPC function may be helpful to improve the prognosis of NSTEMI, especially in aging male patients. Second, systemic inflammation may be partly responsible for the hypofunction of circulating EPCs and worse the prognosis of aging NSTEMI. Anti-inflammation treatment, such as IL-6 receptor antagonist tocilizumab or atorvastatin, may be beneficial to aging NSTEMI (28, 29).

Some limitations should be acknowledged. First, the present study does not follow up the cardiovascular outcomes in patients with NSTEMI. However, previous research had revealed the relationships between cardiovascular outcomes and GRACE or TIMI score (20), and our study illuminated the correlation between GRACE or TIMI score and EPC function which suggested the possible value of EPCs in predicting outcomes of NSTEMI. Further efforts will be made to verify the potential predictive value of circulating EPCs in NSTEMI. Second, we did not confirm the direct influence of systemic inflammation on EPC dysfunction. The *in vitro* experiment will be performed to discover the exact underlying mechanism.

CONCLUSION

Our findings firstly uncovered that circulating EPC function decrement enhanced severity in aging male patients with NSTEMI, and systemic inflammation may be related to the alteration. The present study may provide us new insights into the

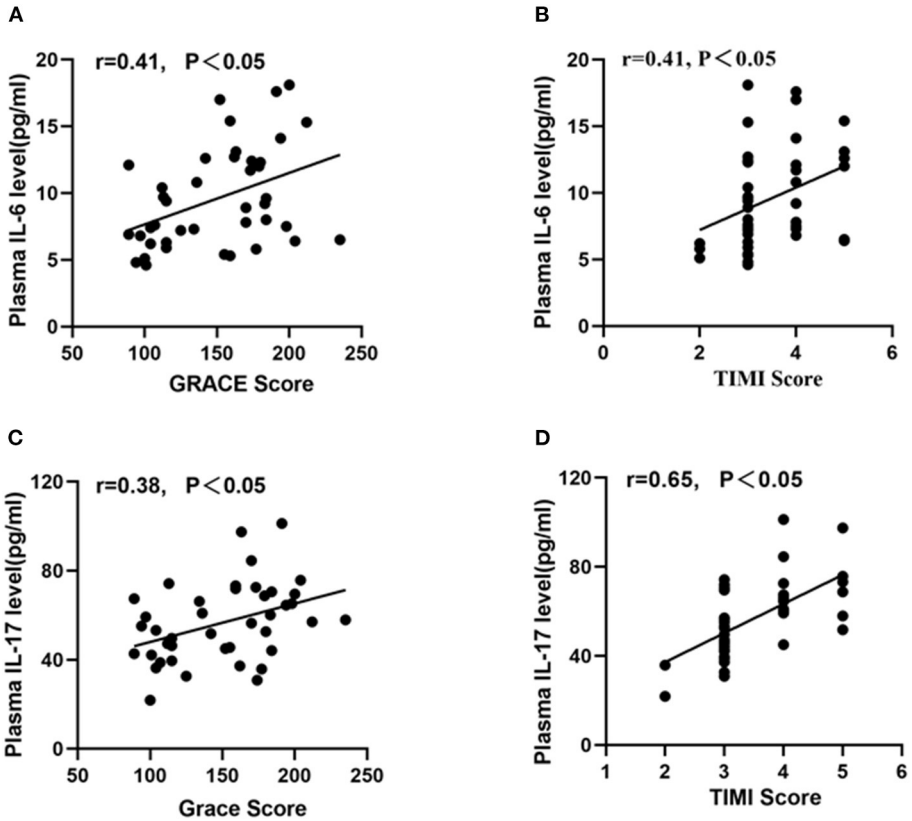

FIGURE 5 | Correlation between inflammatory cytokines and GRACE or TIMI score. Both IL-6 **(A,B)** and IL-17 **(C,D)** were positively correlated with GRACE score and TIMI score.

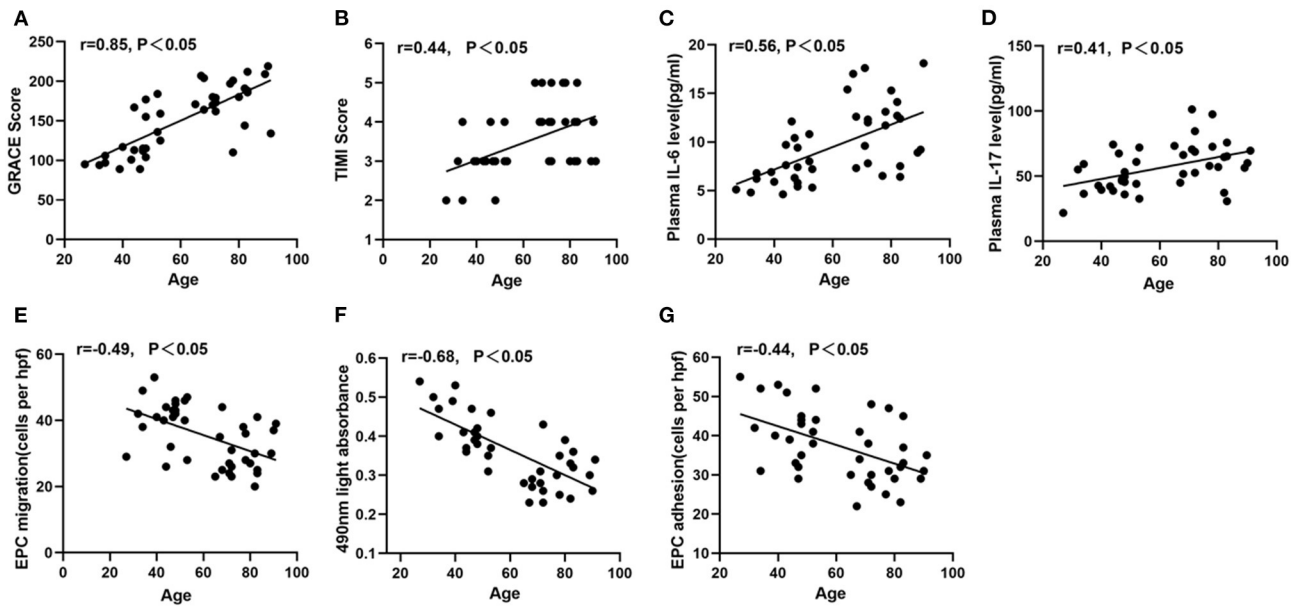

FIGURE 6 | Correlation between age and GRACE or TIMI score or inflammatory cytokines or circulating EPC function. Age was positively correlated with GRACE score **(A)**, TIMI score **(B)**, IL-6 **(C)**, IL-17 **(D)**. Age was negatively correlated with EPC migration **(E)**, proliferation **(F)**, and adhesion **(G)**.

underlying mechanism and therapeutic potentials for aging NSTEMI.

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/s.

ETHICS STATEMENT

The study was approved by the Ethics Committee of our hospital. The patients provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZY designed the study. LZ, CZ, and CT did the experiments, analyzed the data, plotted the figures, and wrote the manuscript. YZ, BZ, ZL, and GL performed the experiments. 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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ORIGINAL RESEARCH
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Triglyceride–Glucose Index and Extracellular Volume Fraction in Patients With Heart Failure

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Background: The triglyceride–glucose (TyG) index had been proposed as a reliable surrogate marker of insulin resistance. We aimed to evaluate the association between TyG index and myocardial fibrosis, which was quantified by extracellular volume (ECV) fraction using cardiovascular magnetic resonance (CMR) examination, and their prognostic value in patients with heart failure (HF).

Methods: In this retrospective cohort study, 103 hospitalized HF patients were included. ECV fraction was calculated using CMR measurements and T1 mapping. TyG index was calculated using fasting triglyceride and blood glucose. The primary outcome events were defined as all-cause mortality and HF hospitalization during follow-up.

Results: During the median follow-up of 12.3 months, 39 patients (37.9%) experienced primary outcome events and had higher levels of TyG index, N-terminal pro–B-type natriuretic peptide (NT-proBNP), and ECV fraction compared with those without events. Multivariate linear regression analysis showed that the TyG index was the significant factor determined for ECV fraction ($r_{partial} = 0.36$, $P = 0.01$). In multivariate Cox regression analysis, presence of diabetes [hazard ratio (HR) = 1.28, 95% confidence interval (CI) = 1.01–1.62], higher TyG index (HR = 2.01, 95% CI = 1.03–4.01), ECV fraction (HR = 1.73, 95% CI = 1.04–2.88), and NT-proBNP (HR = 2.13, 95% CI = 1.08–4.20) were independent risk factors for the primary outcome events.

Conclusions: TyG index is a novel biomarker of myocardial fibrosis in HF patients and can be considered as a useful risk stratification metric in the management of HF.

Keywords: triglyceride-glucose index, heart failure, insulin resistance, biomarkers, myocardial fibrosis

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INTRODUCTION

Epidemiological studies have shown that heart failure (HF) is a growing global public health burden, with prevalence up to 1 to 2% in the adult population (1). Detection of novel biomarkers and mechanisms would be of significant clinical importance for the prevention and treatment of HF (2, 3). It had been documented that metabolic disorders, including obesity, insulin resistance, and diabetes mellitus (DM) play an important role in the development and progression of HF (4). The triglyceride–glucose (TyG) index, a metric derived from fasting triglyceride and blood glucose levels, had been proposed as a reliable surrogate marker of insulin resistance (5). Previous studies had reported that a higher TyG index was associated with an increased risk of cardiovascular disease

(CVD) (6, 7), chronic kidney disease (8), and diabetic retinopathy (9). It had also been reported that there is a positive correlation between the TyG index and the prognosis in patients with HF and type 2 DM (10). However, the underlying mechanisms were unexplored.

Cardiovascular magnetic resonance (CMR) imaging has emerged as a novel, non-invasive diagnostic tool to assess myocardial fibrosis (11), a key pathological process in HF (12). The importance of myocardial fibrosis to predict the prognosis in different cohorts of patients had been reported (13); however, there are limited clinical data on the interaction between insulin resistance and myocardial fibrosis in patients with HF.

This study investigated the association between TyG index and myocardial fibrosis among patients with advanced HF. We also determined the possibility that TyG index and myocardial fibrosis could serve as new biomarkers for the prognosis in patients with HF.

METHODS

Study Design and Population

We retrospective reviewed hospitalized HF patients (aged ≥18 years) with CMR examination from January 2019 to December 2020. Patients with acute myocardial infarction, history of malignancy, sepsis, severe renal function failure [estimated glomerular filtration rate (eGFR) <30 mL/min per 1.73 m^2 or under renal replacement therapy], severe anemia (Hb < 60 g/L), autoimmune disease, heart transplantation, or severe hepatic disease or without data for calculating TyG index were excluded.

The study complied with the principles of the Declaration of Helsinki and was approved by the committee of the institutional review board at Shunde Hospital, Southern Medical University, China (no. 20200320). Because of the retrospective design of the current study, the patient informed consent form was waived by the institutional review board.

Baseline Characteristics, TyG Index Detection, and CMR Examination

We collected baseline characteristics, clinical data, and biochemistry tests from the hospital medical records. Hypertension was defined as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg or received antihypertensive treatment according to the Chinese guideline for the diagnostic and management of hypertension (14). DM was defined as fasting blood glucose (FBG) ≥7.0 mmol/L and/or hemoglobin $A1_c$ ($HbA1_c$) ≥6.5% or received antihyperglycemic medications (15). We calculated the eGFR according to the Modification of Diet in Renal Disease equation adapted for Chinese patients (16).

Fasting venous blood was collected, and biochemistry tests were performed in the second morning after the patients were admitted. Hemoglobin, FBG, HbA_{1c}, total cholesterol (TC), triglycerides, low-density lipoprotein-C (LDL-C), high-density lipoprotein cholesterol, serum creatinine, high-sensitivity C-reactive protein (hs-CRP), and N-terminal pro–B-type natriuretic peptide (NT-proBNP) levels were detected. LDL-C was calculated using the Friedewald equation (17, 18). TyG index was calculated using the following: Ln [fasting triglycerides (mg/dL) × FBG (mg/dL)/2] (5).

Two experienced radiologists performed CMR measurements and T1 mapping during the hospitalization of the patients. Quantification of extracellular volume (ECV) fraction by T1-mapping technique in CMR imaging was used to assess myocardial fibrosis. The detailed procedure had been reported in the previous study (19). Briefly, all patients were examined in the supine position using a 3.0-T scanner (Skrya; Siemens Medical Solutions, Erlangen, Germany). A total dose of 0.1 mmol/kg gadobutrol (Gadavist, Bayer Healthcare Leverkusen, Germany) was injected at a rate of 2.0 to 3.0 mL/s; 10–15 min after contrast injection, short- and long-axis two-dimensional inversion recovery late gadolinium enhancement images were acquired to evaluate focal myocardial fibrosis. Pre- and post-contrast myocardial T1 were measured in six regions of interest in the myocardium (anterior, anterolateral, inferolateral, inferior, inferoseptal, anteroseptal) and in the left ventricular blood pool. We calculated the ECV fraction using the following formula (20), in which R1 represents 1/T1, myo pre and myo post represent the precontrast and postcontrast myocardial T1 values, respectively, and blood pre and blood post represent the precontrast and postcontrast blood pool T1 values, respectively.

$$ECV\ \text{fraction} = \left(1 - hematocrit\right) \times \frac{R1\ myo\ post - R1\ myo\ pre}{R1\ blood\ post - R1\ blood\ pre}$$

Follow-Up and Endpoint Ascertainment

The primary outcomes in the current study were defined as composite endpoints of all-cause death or HF rehospitalization (21). Patients were followed by reviewing the electronic medical record and/or telephone interview with the participants (or the family members if patients were deceased). The follow-up duration was up to February 28, 2021.

Statistical Analysis

All the included patients were categorized as with or without primary outcome events during follow-up. Categorical variables are presented as numbers and percentages. Continuous variables are presented as mean and standard deviation (SD), or median and interquartile range. Baseline characteristics of patients with or without primary outcome events were compared using the χ^2 test for categorical variables, Wilcoxon rank-sum test for non–normally distributed continuous variables, and two-tailed *t*-test for normally distributed continuous variables.

Non-Gaussian data, including NT-proBNP level, left ventricular ejection fraction, eGFR, hs-CRP, ECV fraction, and cholesterol level, were log_2-transformed. We used the Pearson product–moment correlation coefficient (*r*) as a measure of linear association between covariates and ECV fraction and performed multivariate linear regression analysis to identify factors associated with levels of ECV fraction.

Patients were divided into three groups according to tertiles of TyG index, ECV fraction, and NT-proBNP levels. Kaplan–Meier curves were employed to evaluate the study endpoints over time, and the log–rank test was used to assess differences in

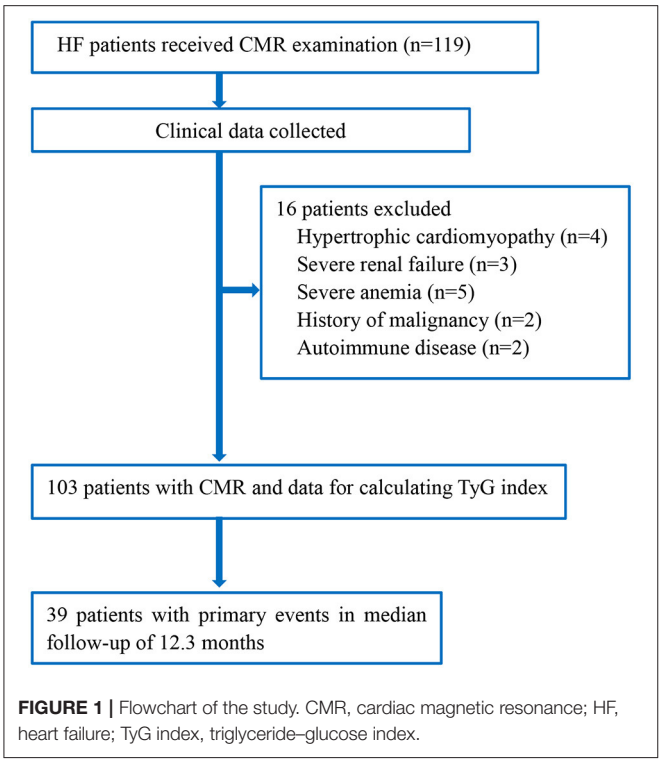

FIGURE 1 | Flowchart of the study. CMR, cardiac magnetic resonance; HF, heart failure; TyG index, triglyceride–glucose index.

outcome events among different groups. Predictors of outcome were analyzed through univariate and multivariate adjusted Cox regression analyses, and the hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) were presented. Risk factors with $P < 0.10$ in univariable analysis were included in the multivariable Cox regression model. Finally, receiver operating characteristic (ROC) curves and the area under the curve (AUC) were used to identify the potential relationships among TyG index, ECV fraction, and NT-proBNP levels and the primary outcomes. We used the method proposed by Delong et al. for the comparisons of the difference between two AUCs (22).

Statistical analyses were performed using SPSS version 20.0 (IBM Corp, Armonk, NY, USA). All *P*-values were two-tailed, and a $P < 0.05$ was considered statistically significant.

RESULTS

Baseline Characteristics of the Included HF Patients

We screened 119 HF patients who had received CMR examination; 16 were excluded because of predefined exclusion criteria (**Figure 1**). Finally, 103 patients (mean age = 58.3 years) were included in the analysis. The demographic and clinical characteristics of the included patients with and without events are presented in **Table 1**. Compared with patients without primary adverse outcomes during follow-up, those with events had higher levels of TyG index, NT-proBNP, and ECV fraction (all $P < 0.001$).

Associations of Clinical and Laboratory Variables With ECV Fraction

Pearson correlation analysis showed that Log_2(TyG index) ($r = 0.34$, $P = 0.01$), Log_2(hs-CRP) ($r = 0.32$, $P = 0.02$), and $Log_2(HbA_{1c})$ ($r = 0.28$, $P = 0.04$) were positively associated with ECV fraction (**Table 2**). However, in multivariate linear regression analysis, TyG index was the only significant factor determined for ECV fraction ($r_{partial} = 0.36$, $P = 0.01$). The R^2 value of SFRP2 for ECV fraction was 0.13, which indicated that 13.0% of the total ECV fraction variation can be attributed to the TyG index.

Prognostic Value of Markers in HF Patients

During a mean follow-up period of 12.3 months (interquartile range = 9.2–16.9 months), 39 patients (37.9%) in the cohort experienced the composite primary outcome. Kaplan–Meier survival analysis showed that higher levels of TyG index (log–rank test for trend: $P < 0.001$), ECV fraction (log–rank test for trend: $P < 0.001$), and NT-proBNP (log–rank test for trend: $P = 0.002$) were significant predictors of composite primary outcomes (**Figure 2**). In multivariate Cox regression models, presence of diabetes (HR = 1.28, 95% CI = 1.01–1.62), higher TyG index (HR = 2.01, 95% CI = 1.03–4.01), ECV fraction (HR = 1.73, 95% CI = 1.04–2.88), and NT-proBNP (HR = 2.13, 95% CI = 1.08–4.20) were independent risk factors for the primary outcomes (**Table 3**).

ROC analysis showed that levels of TyG index (AUC = 0.709, 95% CI = 0.611–0.794), ECV fraction (AUC 0.715, 95% CI = 0.618–0.80), and NT-proBNP (AUC 0.741, 95% CI = 0.646–0.823) had a significant predictive role on the primary outcomes (**Figure 3**). Pairwise comparisons of ROC curves showed that there were no significant differences in AUC among the three markers (all $P > 0.63$).

DISCUSSION

To the best of our knowledge, this is the first study to report that the TyG index, a surrogate marker of insulin resistance, is positively associated with myocardial fibrosis detected by CMR. We also found that a higher TyG index was associated with poorer prognosis in HF patients after adjusting for other risk factors, including DM.

Myocardial fibrosis is a key pathological process and represents a potential therapeutic target for HF (23). The gold standard for evaluating myocardial fibrosis is myocardial biopsy. However, the invasive nature limits its clinical use. In the past decade, studies have demonstrated myocardial fibrosis estimated by CMR can provide important prognostic value (13, 23). In this study, we also found that a higher ECV fraction was associated with an increased adverse outcome in patients with HF, further supporting the notion that ECV fraction could be used as a non-invasive marker to evaluate myocardial fibrosis and risk stratification. Furthermore, we found that the TyG index was the only significant factor determined for ECV fraction, which provided the message that insulin resistance is an important risk factor for myocardial

TABLE 1 | Baseline characteristics of the included HF patients.

	All HF patients ($n = 103$)	With events ($n = 39$)	Without events ($n = 64$)	*P*-value
Clinical characteristics and comorbidities				
Age (years)	58.3 (8.9)	57.9 (9.0)	58.6 (9.0)	0.71
Male [n (%)]	71 (68.9)	27 (69.2)	44 (68.7)	0.87
Smoking [*n* (%)]	34 (33.0)	13 (33.3)	21 (32.8)	0.87
Hypertension [*n* (%)]	71 (68.9)	31 (79.5)	41 (62.5)	0.11
Diabetes [*n* (%)]	45 (43.7)	15 (38.5)	30 (46.9)	0.53
Atrial fibrillation/flutter [n (%)]	47 (45.6)	17 (43.6)	30 (46.9)	0.90
LVEF	48.9 (38.9, 58.7)	46.7 (38.9, 58.6)	51.5 (41.2, 58.8)	0.58
Causes of heart failure				0.92
Ischemic heart disease	68 (66.0)	25 (64.1)	43 (67.2)	
Non-ischemic heart disease	35 (34.0)	14 (35.9)	21 (32.8)	
Current medication				
ACEI/ARBs [*n* (%)]	71 (68.9)	28 (71.8)	43 (67.2)	0.79
Aldosterone antagonist [*n* (%)]	73 (70.9)	27 (69.2)	46 (71.9)	0.95
CCB [*n* (%)]	33 (32.0)	13 (33.3)	20 (31.2)	0.99
β-Blockers [*n* (%)]	48 (46.6)	19 (48.7)	29 (45.3)	0.89
Loop diuretics/HCT [*n* (%)]	81 (78.6)	28 (71.8)	53 (82.8)	0.28
Digoxin [*n* (%)]	55 (53.4)	21 (53.8)	34 (53.1)	0.89
Statins [*n* (%)]	87 (84.5)	34 (87.2)	53 (82.8)	0.75
Antithrombotics [*n* (%)]	77 (74.8)	27 (69.2)	50 (78.1)	0.44
Physical examination				
Heart rate (beats/min)	92.2 (18.2)	92.9 (16.2)	91.7 (19.4)	0.74
Systolic BP (mm Hg)	147.6 (23.6)	146.3 (23.5)	148.4 (23.8)	0.67
Diastolic BP (mm Hg)	81.7 (16.3)	82.5 (16.4)	81.2 (16.4)	0.92
BMI (kg/m^2)	25.5 (5.0)	25.8 (4.5)	25.3 (5.3)	0.25
Laboratory indices				
Hemoglobin (g/L)	115.5 (18.7)	115.8 (18.2)	115.2 (19.1)	0.88
ALT (IU/L)	34.5 (32.6, 44.7)	34.5 (32.5, 47.8)	34.9 (33.2, 44.7)	0.55
eGFR (mL/min per 1.73 m^2)	51.8 (45.2, 74.7)	50.4 (43.6, 74.5)	52.8 (45.3, 74.5)	0.66
FPG (mmol/L)	8.3 (6.3, 10.5)	8.5 (6.6, 10.8)	7.7 (5.8, 10.3)	0.09
HbA_{1c}	6.3 (5.7, 7.4)	6.4 (5.9, 7.4)	5.9 (5.6, 7.6)	0.09
TC (mmol/L)	5.0 (3.8, 5.5)	4.9 (3.9, 5.3)	5.1 (3.9, 5.7)	0.70
LDL-C (mmol/L)	2.7 (2.1, 3.0)	2.6 (2.1, 2.9)	2.9 (2.4, 3.1)	0.42
HDL-C (mmol/L)	1.0 (0.9, 1.2)	0.9 (0.8, 1.1)	1.1 (0.9, 1.2)	0.45
Triglyceride (mmol/L)	2.4 (1.9, 3.5)	2.5 (1.9, 3.6)	2.2 (1.7, 3.4)	0.18
hs-CRP (mg/L)	7.7 (2.1, 10.2)	8.5 (4.0, 14.7)	5.8 (1.6, 8.9)	0.07
Sodium (mmol/L)	133.9 (11.5)	135.4 (11.9)	133.0 (11.3)	0.30
Potassium (mmol/L)	4.2 (0.9)	4.1 (1.0)	4.3 (0.8)	0.049
NT-proBNP (ng/L)	5,723.0 (3,259.6, 8,292.9)	7,856.4 (5,802.7, 8,859.5)	4,013.7 (2,450.5, 655.2)	<0.001
ECV fraction (%)	36.5 (33.4, 39.6)	39.6 (36.2, 41.1)	35.8 (32.4, 37.9)	<0.001
TyG index	10.0 (0.82)	10.7 (0.81)	8.6 (0.78)	<0.001

Continuous variables are presented as median (interquartile range) or mean (standard deviation). Categorical variables are expressed as number (percentages).
ACEI/ARB, angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers; ALT, alanine aminotransferase; BMI, body mass index; BP, blood pressure; CCB, calcium-channel blocker; ECV, extracellular volume; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA_{1c}, glycated hemoglobin; HCT, hydrochlorothiazide; HDL-C, high-density lipoprotein cholesterol; HF, heart failure; hs-CRP, high sensitive C-reactive protein; LDL-C, low-density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro–B-type natriuretic peptide; TyG index, triglyceride–glucose index.

fibrosis. Our study was supported by another retrospective cohort study, which showed a positive correlation between the TyG index and the prognosis of patients with HF and type 2 DM (10). Several mechanisms involve the interaction among insulin resistance, myocardial fibrosis, and worse prognosis in HF. First, insulin resistance is associated with low-degree

TABLE 2 | Association between clinical variables and ECV fraction in HF patients.

Variables	ρ	*P*-value
Age	−0.11	0.35
Sex	0.17	0.27
Smoking	0.05	0.77
Hypertension	0.18	0.25
Systolic blood pressure	−0.15	0.61
Ischaemic etiology	0.25	0.10
Atrial fibrillation/flutter	0.05	0.71
Log_2 (LVEF)	−0.15	0.23
Heart rate	0.04	0.79
Body mass index	0.19	0.68
Log_2 (ALT)	−0.04	0.65
Log_2 (eGFR)	0.25	0.08
Log_2 (Fasting plasma glucose)	0.26	0.07
Log_2 (HbA_{1c})	**0.28**	**0.04**
Hemoglobin	0.06	0.82
Log_2 (TC)	−0.20	0.34
Log_2 (HDL-C)	0.08	0.63
Log_2 (LDL-C)	0.15	0.42
Log_2 (Triglyceride)	0.28	0.07
Log_2 (hs-CRP)	**0.32**	**0.02**
Sodium	−0.06	0.67
Potassium	−0.20	0.17
Log_2 (NT-proBNP)	0.05	0.73
TyG index	**0.34**	**0.01**

ALT, alanine aminotransferase; ECV, extracellular volume; eGFR, estimated glomerular filtration rate; HbA_{1c}, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HF, heart failure; hs-CRP, high sensitive C-reactive protein; LDL-C, low-density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro–B-type natriuretic peptide; TyG index, triglyceride–glucose index.
Bold values indicates data with statistical significance ($P < 0.05$).

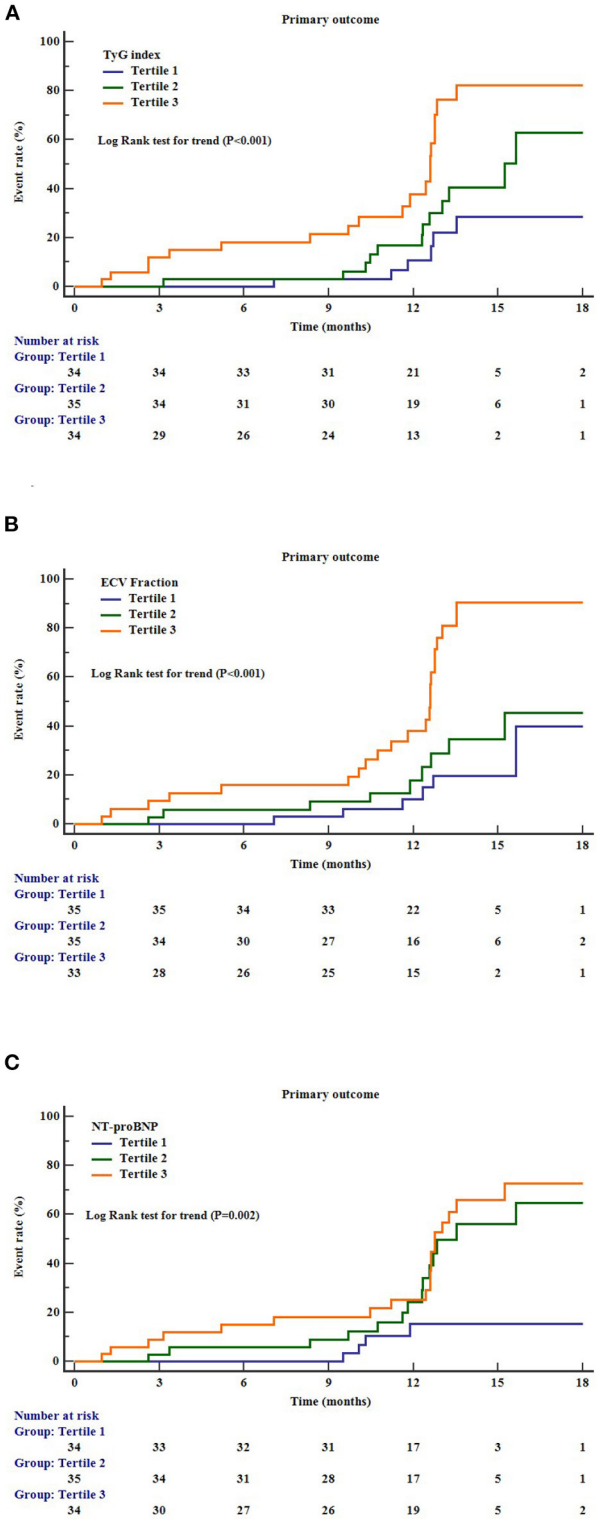

FIGURE 2 | Cumulative incidence curves for the primary outcomes according to the different markers. **(A)** TyG index, triglyceride–glucose index; **(B)** ECV, extracellular volume; **(C)** NT-proBNP, N-terminal pro–B-type natriuretic peptide.

inflammation, which plays an important role in the pathogenesis of cardiomyocyte apoptosis and myocardial fibrosis (24). Second, insulin resistance has been associated with the increased sympathetic nervous system and renin–angiotensin–aldosterone system activity; both of them were involved in myocardial fibrosis and cardiac dysfunction (25, 26). Third, insulin resistance is related to the deposition of extracellular matrix deposition (27) and intramyocardial lipids, (28) resulting in subsequent myocardial fibrosis.

The homeostatic model assessment of insulin resistance (HOMA-IR) is another marker broadly used for defining insulin resistance. Because of the retrospective design, HOMA-IR data were not available in the current study. However, several studies aimed to compare the predictive effect of the TyG index and HOMA-IR for cardiovascular risk. In a cross-sectional study, the TyG index was still significantly associated with coronary artery stenosis in patients with type 2 DM (29). It had been reported that the TyG index was independently associated with arterial stiffness and 10-year CVD risk in a Chinese cohort, while in the same cohort, the association of the HOMA-IR and the

TABLE 3 | The association between risk factors and primary outcome in HF patients.

Variables	Univariate		Multivariate	
	HR (95%CI)	*P*-value	HR (95%CI)	*P* value
Age (each 10 years)	1.10 (0.98, 1.23)	0.09		
Sex (female vs. male)	1.08 (0.14, 8.33)	0.94		
Diabetes (yes vs. no)	1.32 (1.04, 1.68)	0.02	1.28 (1.01, 1.62)	0.04
Hypertension (yes vs. no)	1.12 (0.35, 3.58)	0.85		
Smoking (yes vs. no)	1.75 (0.68, 4.50)	0.25		
BMI (each 1 kg/m^2)	1.08 (0.55, 2.12)	0.82		
HbA_{1c} (each doubling)	1.85 (1.02, 3.36)	0.04		
Triglyceride (each doubling)	1.35 (0.75, 2.47)	0.31		
LVEF (each doubling)	0.85 (0.45, 1.61)	0.62		
eGFR (each doubling)	0.66 (0.17, 2.56)	0.55		
NT-proBNP (each doubling)	3.35 (1.36, 8.25)	0.008	2.13 (1.08, 4.20)	0.03
hs-CRP (each doubling)	1.32 (0.45, 3.87)	0.61		
TyG index (per SD increment)	2.78 (1.06, 7.29)	0.04	2.01 (1.01, 4.01)	0.047
ECV fraction (each doubling)	1.98 (1.10, 3.56)	0.02	1.73 (1.04, 2.88)	0.03

BMI, body mass index; CI, confidence interval; ECV, extracellular volume; eGFR, estimated glomerular filtration rate; HbA_{1c}, glycated hemoglobin; HR, hazard ratio; LVEF, left ventricular ejection fraction; hs-CRP, high sensitive C-reactive protein; NT-proBNP, N-terminal pro–B-type natriuretic peptide; TyG index, triglyceride–glucose index.

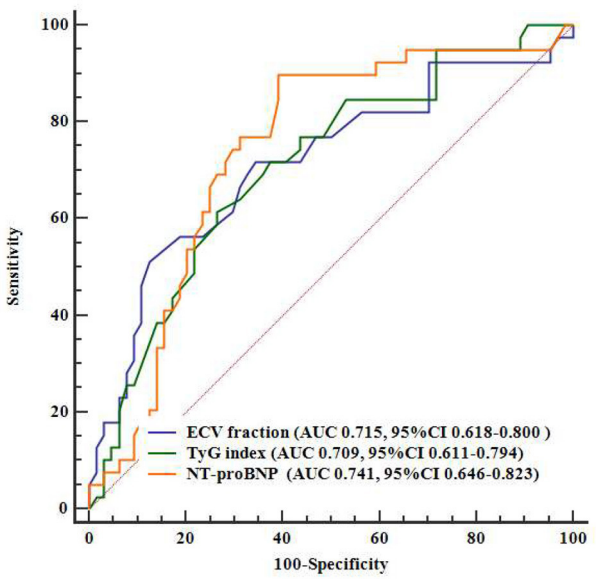

FIGURE 3 | Comparisons between AUCs on ROCs. AUC, area under the curve; ECV, extracellular volume; NT-proBNP, N-terminal pro–B-type natriuretic peptide; ROC, receiver operating characteristics; TyG index, triglyceride–glucose index.

10-year CVD risk was absent when adjusting for multiple risk factors (6). Other studies also showed that the TyG index was more independently associated with increased arterial stiffness and coronary artery calcification than HOMA-IR in Korean (30, 31). Nowadays, the detection of metrics for calculating the TyG index (including fasting triglycerides and FBG) is convenient and affordable worldwide. However, the calculation for HOMA-IR was based on fasting insulin, which was much expensive than triglycerides and not available in most clinical laboratories. Therefore, we proposed that the TyG index could be considered as a more convenient marker of insulin resistance and regarded as a useful predictor of adverse prognosis in HF.

Some limitations to this study should be noted. First, the retrospective design of the current research would cause recall bias, and residual confounders could not be totally avoided. Prospective cohort studies would be useful to further ascertain the association between TyG index and myocardial fibrosis. Second, our study had a relatively small sample size, which limits us to further perform analysis based on patients with and without DM. However, in the multivariable Cox regression analysis, the association between TyG index and primary outcome events was still significant after adjusting for DM, which indicated that the higher TyG index was associated with a worse prognosis of HF independent of DM. Third, the TyG index was available only at baseline, not during follow-up. Thus, any changes in the TyG index that may have occurred in response to treatment of HF are unknown and require further exploration. Finally, the ECV fraction is a surrogate marker, not the gold standard of myocardial fibrosis. Therefore, the association between TyG index and ECV fraction is only an indirect evidence of myocardial fibrosis.

CONCLUSIONS

TyG index is a novel biomarker of myocardial fibrosis in HF patients. We also demonstrated that a higher TyG index was significantly associated with a worse prognosis in HF, which can be considered as a useful risk stratification metric in the management of HF.

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 Institutional review board at Shunde Hospital, Southern Medical University, China. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

SY, YD, and HC: conception, design, and administrative support. SY, YD, ZL, and RZ: data analysis and interpretation. SY, YD, ZL, and XL: manuscript writing. All authors provision of study materials or patients, collection and assembly of data, and final approval of 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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Primary Percutaneous Coronary Intervention in Patients With Type 2 Diabetes With Late/Very Late Stent Thrombosis and *de novo* Lesions: A Single-Center Observational Cohort Study of Clinical Outcomes and Influencing Factors

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Background: This study compared differences in the risk factors and clinical outcomes of primary percutaneous coronary intervention (PCI) in type 2 diabetes mellitus (DM) and non-DM patients with *de novo* lesions (DNLs) and late or very late stent thrombosis (LST/VLST).

Methods: We used angiography to screen 4,151 patients with acute coronary syndrome for DNL and LST/VLST lesions. Overall, 3,941 patients were included in the analysis and were allocated to the DM ($n = 1,286$) or non-DM ($n = 2,665$) group at admission. The primary endpoint was a composite of major adverse cardiovascular events (MACEs), defined as death, myocardial infarction, revascularization, and ischemic stroke, within a median follow-up period of 698 days.

Results: In the group with a total white blood cell count $>10 \times 10^9$/L ($P = 0.004$), a neutral granular cell count $>7 \times 10^9$/L ($P = 0.030$), and neutrophil–lymphocyte ratio >1.5 ($P = 0.041$), revascularization was better for DNL than for LST/VLST lesions. Among DM patients with DNLs, each unit increase in age was associated with a 53.6% increase in the risk of MACEs [hazard ratio (HR): 1.536, 95% confidence interval (CI), 1.300–1.815, $P < 0.0001$]. Older age (≥65 years) was associated with a significantly greater risk of MACEs ($P < 0.0001$). Furthermore, each standard deviation (SD) increase in the level of peak white blood cell counts was associated with a 50.1% increase in the risk of MACEs (HR, 1.501; 95% CI, 1.208–1.864; $P = 0.0002$). When stratifying the DM population with DNLs according to the D-dimer baseline and peak levels <0.5 vs. ≥0.5 mg/L, the high

D-dimer group at baseline had a 2.066-fold higher risk of MACEs ($P < 0.0001$), and the high peak level D-dimer group had a 1.877-fold higher risk of MACEs ($P = 0.001$) compared to the low-level groups. Among DM patients with LST/VLST, each unit increase in age was associated with a 75.9% increase in the risk of MACEs (HR: 1.759, 95% CI, 1.052–2.940, $P = 0.032$). Furthermore, for each SD increase in the peak D-dimer level, the risk of MACEs increased by 59.7% (HR, 1.597; 95% CI, 1.110–2.295; $P = 0.041$).

Conclusion: Following successful primary PCI, the measurement of baseline and peak D-dimer values may help identify individuals at high cardiovascular risk. This suggests a potential benefit of lowering D-dimer levels among T2DM patients with DNL. Furthermore, age and the peak D-dimer values may facilitate the risk stratification of T2DM patients with LST/VLST.

Keywords: *de novo* lesions, late or very late stent thrombosis, diabetes mellitus, percutaneous coronary intervention, metabolic

INTRODUCTION

Patients with type 2 diabetes mellitus (DM), a pro-inflammatory disease (1), exhibit an enhanced inflammatory reaction at the site of implantation of stents. Compared with non-DM patients, DM patients who have undergone stent implantation often present neointimal hyperplasia and diffusely diseased vessels, along with deleterious local phenomena (2), various healing responses, and arterial remodeling (3). However, the long-term prognosis of DM patients with *de novo* lesions (DNLs) and very late stent thrombosis (LST/VLST) who have undergone primary percutaneous coronary intervention (PCI) remains unknown.

This retrospective, single-center, all-comer trial aimed to compare the differences in the long-term prognosis of DM and non-DM patients who underwent PCI for DNLs and for LST/VLST.

METHODS

Study Population and Design

This retrospective observational study adhered to the Strengthening the Reporting of Observational Studies in Epidemiology statement. This study was conducted according to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Fuwai Hospital. All study subjects provided written informed consent.

The study was conducted on patients who had undergone primary PCI at Fuwai Hospital (National Center for Cardiovascular Diseases, Peking Union Medical College and Chinese Academy of Medical Sciences) in Beijing, China, between January 2010 and June 2017. From among 4,151 patients admitted for acute myocardial infarction (MI), 3,941 patients were included in this study (**Figure 1**) and were divided into a DM group ($n = 1{,}286$) and a non-DM group ($n = 2{,}655$). The types of coronary lesions, including DNL ($n = 3{,}661$) and LST/VLST ($n = 280$), were identified angiographically. Patients who were lost to follow-up, whose coronary angiography parameters were not available, or who refused participation were excluded from the analysis.

By using coronary angiography, stent thrombosis (ST) was defined as when a thrombus originated in a segment 5 mm distal or proximal to the stent, or in the stent, in patients with acute coronary syndrome (ACS) (4). The Academic Research Consortium (ARC) defined late ST as ST that occurred between 30 days and 1 year, and VLST as ST that occurred >1 year, after stent implantation (4). Three independent and blinded interventional cardiologists with >5 years' experience in interventional cardiology screened all patients with a history of stent implantation. Anonymized angiographic data for each patient were allocated to two of the three cardiologists at random. The cardiologists analyzed the data independently and blindly; disagreements were resolved by discussion to consensus among all three cardiologists.

Clinical Outcomes

Clinical outcomes were obtained during follow-up via a telephone call or were confirmed from health records, as approved by the Review Board of Fuwai Hospital. The primary endpoints were major adverse cardiovascular events (MACEs), all-cause death, cardiac-related death, recurrent MI, revascularization, and ischemic stroke. A MACE was a composite of all-cause death, recurrent MI, and ischemic stroke. The physicians in charge of the follow-up identified and extracted primary endpoints from hospital records, laboratory reports, and clinical notes in the event of death.

Statistical Analyses

Time-to-event variables are presented as Kaplan–Meier (K-M) curves, in R (https://www.r-project.org/), and MACE incidences in subgroups were compared using the log-rank test. Baseline patient characteristics were compared between patients with DNL and LST/LVST and among DM and non-DM patients.

Continuous variables are presented as the means ± standard errors and categorical data are presented as counts and percentages. Differences between continuous variables were compared using independent *t*-tests, and those between categorical variables were compared using the χ^2-test or Fisher's exact test to assess the interaction between lesion

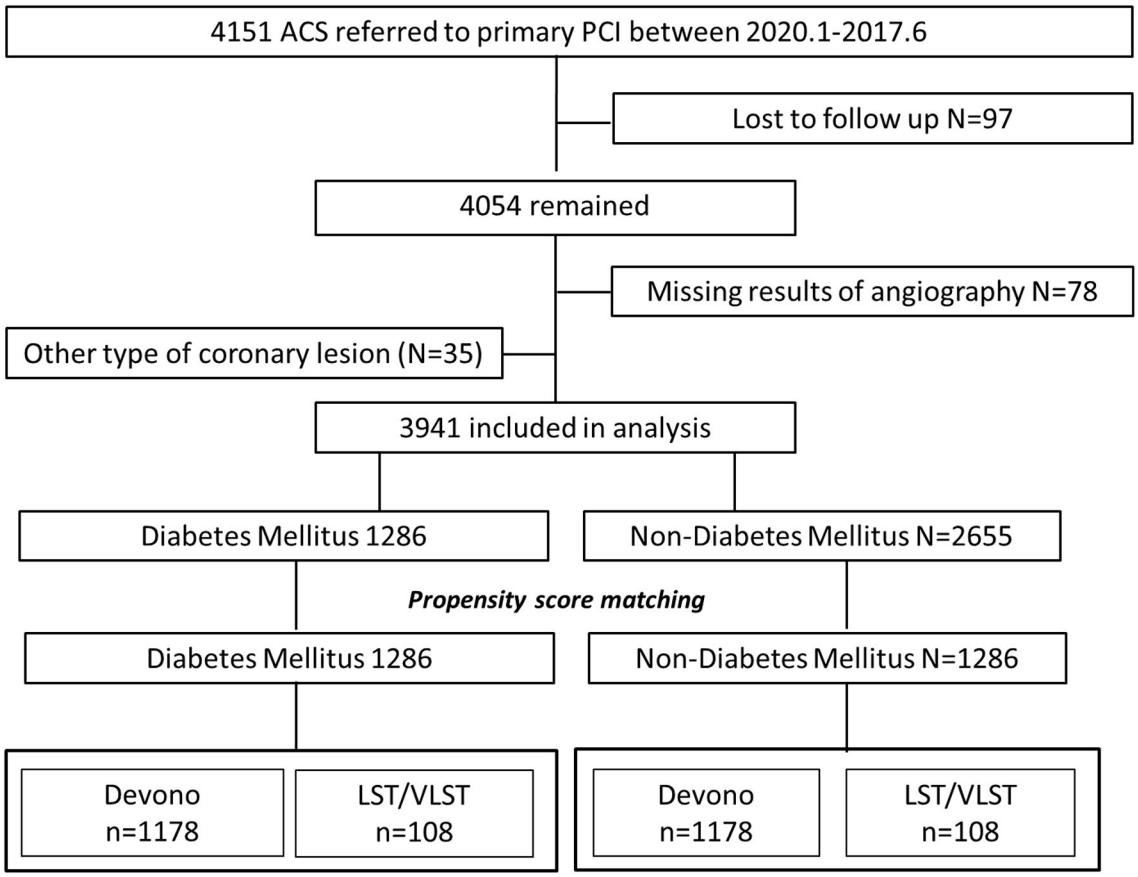

FIGURE 1 | Flow chart. ACS, acute coronary syndrome; LST, late stent thrombosis; VLST, very late stent thrombosis.

types and baseline clinical, laboratory index, or angiographic characteristics. We conducted the subgroup analysis by stratification into WBC, other cell counts, D-dimer levels, etc. to access the association between the MACEs and various parameters.

Propensity score matching (PSM) is an increasingly utilized statistical method in non-randomized and observational research in order to make estimation of the influence of the treatment on endpoints accurately (5). It removes the confounding bias factors from the observational cohorts by matching already treated subjects with observational subjects to reduce the unwanted influences of covariates, help account for such imbalances, and allow for proper measurement of the intended variable (6). PSM was employed to adjust for potential confounders and to minimize the impact of selection bias on the comparison between the DM and non-DM groups. We used the one-to-one nearest-neighbor matching for the PSM of patients in different groups, with a caliper width equal to 0.2 of the standard deviation (SD). The procedure yielded 1,178 matched pairs among DNL patients and 108 matched pairs among LST/VLST patients.

The event-free survival rates among groups were calculated by the Kaplan–Meier analysis using method of propensity match and compared by the log-rank test. The confounder factors of models include age, gender, history of hypertension, history of PCI, history of coronary artery bypass graft, history of chronic kidney disease, Killip classification, high-sensitivity C-reactive protein, and estimated glomerular filtration rate. The Mantel–Cox method was used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for comparisons of clinical outcomes, including MACEs and all-cause death, between groups, and the log-rank test was used to calculate corresponding *P*-values. We conducted two-sided analyses to allow conventional interpretation of results, and a $P < 0.05$ was considered statistically significant. Missing data were handled by single imputation.

Most of the statistical analyses were conducted using R version I 386 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria). Other analyses were performed using SPSS Statistics version 20.0 (SPSS, Inc., Chicago, IL).

RESULTS

Table 1 presents the baseline demographic data, indicators of serum inflammation, lipids, angiographic features, and procedural characteristics of the entire study population. In total, 3,941 patients were divided into the DM group (1,178 DNL and

TABLE 1 | Baseline characteristics of entire population.

Variables	*De novo* lesion patients			Propensity-matched *de novo* lesion patients			LST/VLST patients			Propensity-matched LST/VLST patients		
	DM *De novo* lesion (*N* = 1,178)	Non-DM *De novo* lesion (*N* = 2,483)	P_1	DM *De novo* lesion (*N* = 1,178)	Non-DM *De novo* lesion (*N* = 1,178)	P_1'	DM LST/VLST (*N* = 108)	Non-DM LST/VLST (*N* = 172)	P2	DM LST/VLST (*N* = 108)	Non-DM LST/VLST (*N* = 108)	P2′
Age (years)	60.5±11.3	58.0 ± 12.3	<0.001*	60.5 ± 11.3	66.6 ± 11.1	<0.001*	62.4 ± 9.8	59.46 ± 8.8	0.018*	62.4 ± 9.8	61.5 ± 12.5	0.529
Male [% (*n*)]	861 (73.1%)	2,027 (81.6%)	<0.001*	861 (73.1%)	723 (61.4%)	<0.001*	76 (70.4%)	142 (82.6%)	0.013*	76 (70.4%)	78 (72.2%)	0.764
Heart rate (beats/min)	78.7±15.4	76.9 ± 15.3	0.177	78.7 ± 15.4	77.0 ± 16.0	0.008*	77.9 ± 14.8	75.99 ± 1.18	0.859	77.9 ± 14.8	76.5 ± 14.2	0.506
SBP (mmHg)	125.7±17.7	123.5 ± 18.1	0.001*	125.7 ± 17.7	124.4 ± 18.8	0.106	127.5 ± 18.7	124.98 ± 1.64	0.979	127.5 ± 18.7	125.5 ± 20.0	0.441
DBP (mmHg)	73.8±12.4	74.4 ± 12.9	0.185	73.8 ± 12.4	72.8 ± 7.4	0.049*	74.6 ± 12.2	72.53 ± 1.43	0.630	74.6 ± 12.2	76.4 ± 12.7	0.299
EF at admission	53.4±7.9	54.0 ± 7.3	0.033	53.4 ± 7.9	53.5 ± 7.4	0.712	52.3 ± 8.5	51.40 ± 0.63	0.392	52.3 ± 8.5	50.6 ± 8.3	0.156
Risk factors												
Hypertension [% (*n*)]	769 (65.3%)	1,149 (58.4%)	<0.001*	769 (65.3%)	762 (64.7%)	0.762	74 (68.5%)	107 (62.2%)	0.282	74 (68.5%)	69 (63.9%)	0.472
Hyperlipidemia [% (*n*)]	997 (94.0%)	2,031 (91.3%)	0.008*	997 (94.0%)	940 (89.7%)	<0.001*	91 (95.8%)	143 (91.7%)	0.158	91 (95.8%)	87 (88.8%)	0.069
Smoking [% (*n*)]	622 (58.3%)	1,553 (69.3%)	<0.001*	622 (58.3%)	574 (54.0%)	0.046	58 (61.1%)	103 (64.8%)	0.321	58 (61.1%)	53 (53.5%)	0.290
Previous PCI [% (*n*)]	122 (10.4%)	164 (6.6%)	<0.001*	122 (10.4%)	86 (7.3%)	0.009	97 (89.8%)	139 (80.8%)	0.030*	97 (89.8%)	84 (77.8%)	0.016
Previous CABG [% (*n*)]	24 (2.0%)	17 (0.7%)	0.001*	24 (2.0%)	9 (0.8%)	0.009	1 (0.9%)	1 (0.6%)	0.624	1 (0.9%)	1 (0.9%)	1.000
CKD [% (*n*)]	107 (9.1%)	170 (6.8%)	0.019*	107 (9.1%)	118 (10.0%)	0.441	11 (10.2%)	12 (7.0%)	0.231	11 (10.2%)	11 (10.2%)	1.000
Comorbidities												
Malignancy	12 (1.0%)	34 (1.4%)	0.374	12 (1.0%)	27 (2.3%)	0.015	1 (0.9%)	2 (1.2%)	0.851	1 (0.9%)	2 (1.9%)	0.561
Arrhythmology	284 (24.1%)	625 (25.2%)	0.487	284 (24.1%)	359 (30.5%)	<0.001	21 (19.4%)	48 (27.9%)	0.110	21 (19.4%)	36 (33.3%)	0.021
Alimentary ulcer	64 (5.4%)	157 (6.3%)	0.291	64 (5.4%)	80 (6.8%)	0.169	–	–	–	–	–	–
Hypoproteinemia	17 (1.4%)	18 (0.7%)	0.037	17 (1.4%)	14 (1.2%)	0.588	2 (1.9%)	0 (0.0%)	0.073	2 (1.9%)	0 (0.0%)	0.155
Pulmonary disease	81 (6.9%)	147 (5.9%)	0.264	81 (6.9%)	104 (8.8%)	0.078	–	–	–	–	–	–
Gastritis	95 (8.1%)	216 (8.7%)	0.520	95 (8.1%)	115 (9.8%)	0.148	4 (3.7%)	15 (8.7%)	0.104	4 (3.7%)	10 (9.3%)	0.097
Reflux esophagitis	259 (22.0%)	527 (21.2%)	0.600	259 (22.0%)	245 (20.8%)	0.482	0 (0.0%)	1 (0.6%)	1.000	0 (0.0%)	1 (0.9%)	0.316
Cardiomyopathy	9 (0.8%)	8 (0.3%)	0.066	9 (0.8%)	4 (0.3%)	0.164	1 (0.9%)	2 (1.2%)	1.000	1 (0.9%)	2 (1.9%)	0.561
Respiratory failure	13 (1.1%)	12 (0.5%)	0.033	13 (1.1%)	9 (0.8%)	0.392	1 (0.9%)	0 (0.0%)	0.386	1 (0.9%)	0 (0.0%)	0.316
Laboratory examinations												
HDL (mg/dl)	1.87 ± 1.37	1.62 ± 0.02	<0.001*	1.87 ± 1.37	1.49 ± 0.93	<0.001*	1.80 ± 1.31	1.57 ± 0.85	0.082	1.80 ± 1.31	1.58±0.98	0.163
LDL (mg/dl)	2.69 ± 0.90	2.81 ± 0.02	<0.001*	2.69 ± 0.90	2.75 ± 0.90	0.158	2.29 ± 0.91	2.45 ± 1.05	0.199	2.29 ± 0.91	2.44±0.95	0.244
Triglycerides (mg/dl)	1.02 ± 0.27	1.06 ± 0.01	0.027*	1.02 ± 0.27	1.10 ± 0.29	0.001*	1.06 ± 0.40	1.05 ± 0.32	0.816	1.06 ± 0.40	1.09±0.30	0.576
LPA (g/L)	252.87 ± 235	269.58 ± 4.95	0.052	252.87 ± 235.70	284.70 ± 250.69	0.002	264.45 ± 242.23	328.38 ± 312	0.071	264.45 ± 242.23	319.63 ± 298.18	0.137
hs-CRP	7.88 ± 5.01	7.54±0.10	0.054	7.88 ± 5.01	7.90 ± 5.03	0.913	7.27 ± 4.71	6.53 ± 4.75	0.205	7.27 ± 4.71	6.78 ± 4.83	0.451
D-dimer of baseline	0.74 ± 1.83	0.53 ± 0.04	0.533	0.74 ± 1.83	0.77 ± 1.50	0.645	0.96 ± 2.21	0.77 ± 2.11	0.484	0.96 ± 2.21	0.99 ± 2.59	0.937
Peak level of D-dimer	1.14 ± 2.57	0.91 ± 0.05	0.524	1.14 ± 2.57	1.28 ± 2.38	0.247	1.64 ± 0.64	1.13 ± 2.63	0.238	1.64 ± 0.64	1.46 ± 3.22	0.747
Crea	82.69 ± 28.29	81.30 ± 0.45	0.138	82.69 ± 28.29	81.39 ± 26.30	0.249	82.53 ± 25.90	81.93 ± 22.21	0.836	82.53 ± 25.90	82.76 ± 24.65	0.946
eGFR (MDRD)	87.89 ± 65.59	91.78 ± 1.83	0.191	87.89 ± 65.59	83.70 ± 64.67	0.119	81.90 ± 22.42	91.51 ± 98.04	0.318	81.90 ± 22.42	82.27 ± 22.22	0.904
Peak level of TnI	8.44 ± 16.94	4.09 ± 0.28	0.177	8.44 ± 16.94	9.10 ± 15.98	0.585	6.37 ± 4.31	16.44 ± 26.99	0.078	6.37 ± 4.31	17.78 ± 26.90	0.048
Glycemia	3.53 ± 2.72	3.02 ± 1.62	<0.001*	3.53 ± 2.72	3.06 ± 1.66	<0.001*	4.05 ± 3.34	3.05 ± 1.63	0.002	4.05 ± 3.34	3.11 ± 1.76	0.015

(Continued)

TABLE 1 | Continued

Variables	*De novo* lesion patients			Propensity-matched *de novo* lesion patients			LST/VLST patients			Propensity-matched LST/VLST patients		
	DM *De novo* lesion (*N* = 1,178)	Non-DM *De novo* lesion (*N* = 2,483)	P_1	DM *De novo* lesion (*N* = 1,178)	Non-DM *De novo* lesion (*N* = 1,178)	P_1'	DM LST/VLST (*N* = 108)	Non-DM LST/VLST (*N* = 172)	P2	DM LST/VLST (*N* = 108)	Non-DM LST/VLST (*N* = 108)	P2′
Discharge medication regimen												
Statin [% (*n*)]	1,112 (94.4%)	2,316 (93.3%)	0.218	1,112 (94.4%)	1,122 (95.2%)	0.353	102 (94.4%)	159 (92.4%)	0.349	102 (94.4%)	100 (92.6%)	0.580
Aspirin [% (*n*)]	1,160 (98.5%)	2,467 (99.4%)	0.015*	1,160 (98.5%)	1,168 (99.2%)	0.128	106 (98.1%)	170 (98.8%)	0.500	106 (98.1%)	106 (98.1%)	1.000
Clopidogrel [% (*n*)]	924 (78.4%)	1,912 (77.0%)	0.352	924 (78.4%)	920 (78.1%)	0.842	75 (69.4%)	121 (70.3%)	0.488	75 (69.4%)	81 (75.0%)	0.362
Ticagrelor [% (*n*)]	244 (20.9%)	553 (22.4%)	0.303	244 (20.9%)	553 (22.4%)	0.290	31 (29.2%)	50 (29.1%)	0.540	31 (29.2%)	27 (25.0%)	0.485
ACEI [% (*n*)]	721 (61.2%)	1,547 (62.3%)	0.536	721 (61.2%)	709 (60.2%)	0.613	61 (56.5%)	105 (61.0%)	0.263	61 (56.5%)	64 (59.3%)	0.679
ARB [% (*n*)]	124 (10.5%)	195 (7.9%)	0.008*	124 (10.5%)	97 (8.2%)	0.056	13 (12.0%)	18 (10.5%)	0.412	13 (12.0%)	12 (11.1%)	0.832
ACEI/ARB [% (*n*)]	843 (71.6%)	1,741 (70.1%)	0.393	843 (71.6%)	805 (68.3%)	0.088	74 (68.5%)	123 (71.5%)	0.344	74 (68.5%)	76 (70.4%)	0.768
Beta-Blockers [% (*n*)]	1,040 (88.3%)	2,150 (86.6%)	0.154	1,040 (88.3%)	1,011 (85.8%)	0.075	89 (82.4%)	161 (93.6%)	0.003*	89 (82.4%)	98 (90.7%)	0.072
Diuretic [% (*n*)]	365 (31.0%)	674 (27.1%)	0.017*	365 (31.0%)	366 (31.1%)	0.964	35 (32.4%)	66 (38.4%)	0.189	35 (32.4%)	46 (42.6%)	0.122
Spironolactone [% (*n*)]	246 (20.9%)	530 (21.3%)	0.762	246 (20.9%)	267 (22.7%)	0.295	26 (24.1%)	56 (32.6%)	0.083	26 (24.1%)	39 (36.1%)	0.054
P2Y12 inhibitors	1,167 (99.1%)	2,465 (99.3%)	0.550	1,167 (99.1%)	1,166 (99.0%)	0.834	106 (98.1%)	171 (99.4%)	0.331	106 (98.1%)	108 (100.0%)	0.155
Lesion and procedural characteristics												
Lesion length, mm	28.53 ± 16.33	27.01 ± 15.16	<0.001*	28.53 ± 16.33	27.99 ± 16.44	0.366	26.01 ± 15.80	27.72 ± 15.31	0.369	26.01 ± 15.80	27.2 ± 16.0	0.596
Lesion diameter, mm	3.05 ± 0.52	3.12 ± 0.51	0.006	3.05 ± 0.52	3.08 ± 0.53	0.055	3.03 ± 0.42	3.06 ± 0.49	0.657	3.03 ± 0.42	3.0 ± 0.4	0.881
Degree of lesion stenosis	97.00 ± 0.16	97.22 ± 0.11	0.249	97.02 ± 5.28	97.16 ± 5.97	0.425	98.34 ± 4.19	98.40 ± 4.15	0.431	98.34 ± 4.19	98.86 ± 2.89	0.93
Bifurcation lesion	405 (34.4%)	862 (34.7%)	0.853	405 (34.4%)	413 (35.1%)	0.729	27 (25.0%)	49 (28.5%)	0.310	27 (25.0%)		0.178
TIMI after PCI			0.112			0.585			0.748			0.441
0	25 (2.1%)	28 (1.1%)	–	25 (2.1%)	17 (1.4%)	–	5 (4.6%)	5 (2.9%)	-	5 (4.6%)	2 (1.9%)	
1	5 (0.4%)	9 (0.4%)	–	5 (0.4%)	6 (0.5%)	–	0 (0.0%)	1 (0.6%)	-	0 (0.0%)	0 (0.0%)	
2	23 (2.0%)	42 (1.7%)	–	23 (2.0%)	27 (2.3%)	–	1 (0.9%)	2 (1.2%)	-	1 (0.9%)	2 (1.9%)	
3	1,125 (95.5%)	2,404 (96.8%)	–	1,125 (95.5%)	1,128 (95.8%)	–	102 (94.4%)	164 (95.3%)	-	102 (94.4%)	104 (96.3%)	
PTCA	1,031 (87.5%)	2,176 (87.6%)	0.915	1,031 (87.5%)	1,047 (88.9%)	0.307	100 (92.6%)	158 (91.9%)	0.509	100 (92.6%)	103 (95.4%)	0.391
Thrombus aspiration	456 (38.7%)	1,085 (43.7%)	0.005*	456 (38.7%)	489 (41.5%)	0.165	39 (36.1%)	76 (44.2%)	0.113	39 (36.1%)	45 (41.7%)	0.402
Stent implantation	1,043 (88.5%)	2,264 (91.2%)	0.014*	1,043 (88.5%)	1,063 (90.2%)	0.181	61 (56.5%)	118 (68.6%)	0.027*	61 (56.5%)	75 (69.4%)	0.049
IABP	120 (10.2%)	230 (9.3%)	0.400	120 (10.2%)	136 (11.5%)	0.290	11 (10.2%)	21 (12.2%)	0.377	11 (10.2%)	11 (10.2%)	1.000
Multi-vessel disease	958 (81.3%)	1,780 (71.7%)	<0.001*	958 (81.3%)	903 (76.7%)	0.005	86 (79.6%)	130 (75.58%)	0.432	86 (79.6%)	79 (73.1%)	0.262
Type of ACC			0.087			0.010			0.580			0.590
A	25 (2.1%)	42 (1.7%)	–	25 (2.1%)	13 (1.1%)	–	2 (1.9%)	4 (2.33%)	–	2 (1.9%)	3 (2.8%)	–
B1	90 (7.6%)	147 (5.9%)	–	90 (7.6%)	59 (5.0%)	–	5 (4.6%)	7 (4.07%)	–	5 (4.6%)	4 (3.7%)	–
B2	132 (11.2%)	323 (13.0%)	–	132 (11.2%)	144 (12.2%)	–	11 (10.2%)	10 (5.81%)	–	11 (10.2%)	6 (5.6%)	–
C	931 (79.0%)	1,971 (79.4%)	–	931 (79.0%)	962 (81.7%)	–	90 (83.3%)	151 (87.79%)	–	90 (83.3%)	95 (88.0%)	–
Site of lesion			0.050			0.092			0.097			0.006
LAD	170 (14.4%)	385 (15.5%)	–	170 (14.4%)	148 (12.6%)	–	23 (21.3%)	20 (11.6%)	–	23 (21.3%)	7 (6.5%)	–

(Continued)

TABLE 1 | Continued

Variables	*De novo* lesion patients			Propensity-matched *de novo* lesion patients			LST/VLST patients			Propensity-matched LST/VLST patients		
	DM *De novo* lesion (*N* = 1,178)	Non-DM *De novo* lesion (*N* = 2,483)	P_1	DM *De novo* lesion (*N* = 1,178)	Non-DM *De novo* lesion (*N* = 1,178)	P_1'	DM LST/VLST (*N* = 108)	Non-DM LST/VLST (*N* = 172)	P2	DM LST/VLST (*N* = 108)	Non-DM LST/VLST (*N* = 108)	P2′
RCA	476 (40.4%)	940 (37.9%)	–	476 (40.4%)	474 (40.2%)	–	35 (32.4%)	49 (28.5%)	–	35 (32.4%)	31 (28.7%)	–
LAD	493 (41.9%)	1,091 (43.9%)	–	493 (41.9%)	511 (43.4%)	–	47 (43.5%)	99 (57.6%)	–	47 (43.5%)	68 (63.0%)	–
LM	29 (2.5%)	61 (2.5%)	–	29 (2.5%)	42 (3.6%)	–	3 (2.8%)	3 (1.7%)	–	3 (2.8%)	1 (0.9%)	–
Vein graft	10 (0.8%)	6 (0.2%)	–	10 (0.8%)	3 (0.3%)	–	0 (0.0%)	1 (0.6%)	–	0 (0.0%)	1 (0.9%)	–
Endpoint events												
MACE	141 (12.0%)	209 (8.4%)	0.001*	141 (12.0%)	138 (11.7%)	0.848	15 (13.9%)	30 (17.4%)	0.269	15 (13.9%)	22 (20.4%)	0.206
All caused mortality	82 (7.0%)	106 (4.3%)	0.001*	82 (7.0%)	85 (7.2%)	0.810	6 (5.6%)	17 (9.9%)	0.144	6 (5.6%)	13 (12.0%)	0.093
Cardiovascular death	50 (4.2%)	71 (2.9%)	0.037*	50 (4.2%)	56 (4.8%)	0.551	5 (4.6%)	11 (6.4%)	0.386	5 (4.6%)	9 (8.3%)	0.269
Recurrence MI	43 (3.7%)	70 (2.8%)	0.184	43 (3.7%)	70 (2.8%)	0.176	6 (5.6%)	10 (5.8%)	0.576	6 (5.6%)	7 (6.5%)	0.775
Ischemic stroke	21 (1.8%)	42 (1.7%)	0.892	21 (1.8%)	42 (1.7%)	0.845	5 (4.6%)	3 (1.7%)	0.149	5 (4.6%)	2 (1.9%)	0.249

*DM, diabetes mellitus; LST, late stent thrombosis; VLST, very late stent thrombosis; SBP, systolic blood pressure; DBP, diabetes blood pressure; EF, ejection fraction; PCI, percutaneous coronary intervention; CABG, coronary artery bypass graft; AF, atrial fibrillation; CKD, chronic kidney disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LPA, lipase activator; hs-CRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; TnI, troponin; TIMI, thrombolysis in myocardial infarction; PTCA, percutaneous transluminal coronary angioplasty; IABP, intra-aortic balloon pump; MACE, major adverse cardiovascular event; MI, myocardial infarction. *P < 0.05.*

108 LST/VLST lesions) and non-DM group (2,483 DNL and 172 LST/VLST lesions). The mean ages of the patients were 60.51 ± 0.33 and 62.44 ± 0.95 years in the DNL and LST/VLST groups among the DM patients, respectively. Compared with the non-DM patients, subgroups of patients with LST/VLST among the DM patients were older (62.44 ± 0.95 years vs. 59.46 ± 0.80 years, $P = 0.018$), had a smaller proportion of men (70.4% years vs. 82.6%, $P = 0.013$), and underwent PCI (89.8 vs. 80.8%, $P = 0.003$). After PSM, these baseline differences were almost balanced between the two groups.

The findings of revascularization were consistent across the stratified subgroup analyses, including variables representing serum inflammation, lipids, and thrombus levels (**Figure 2**). In particular, in the subgroup with a total white blood cell (WBC) count >10 × 10^9/L ($P = 0.004$, $P_{interaction} = 0.233$), a neutral granular cell count >7 × 10^9/L ($P = 0.030$, $P_{interaction} = 0.847$), neutrophil–lymphocyte ratio (NLR) >1.5 ($P = 0.041$, $P_{interaction} = 0.662$), peak D-dimer level <0.5 mg/L ($P = 0.042$, $P_{interaction} = 0.001$), and not currently smoking ($P = 0.012$, $P_{interaction} = 0.028$), DNLs were more likely to be revascularized than LST/VLST lesions at a median follow-up of 698 days.

Table 2 describes the associations of MACEs, stratified according to the immunity index, and lipid and inflammatory marker levels during treatment. Among the DM patients with DNLs, each unit increase in age was associated with a 53.6% increase in the risk of MACEs (HR: 1.536, 95% CI, 1.300–1.815, $P < 0.0001$). Older age (≥65 years) was associated with a significantly greater risk of MACEs (HR, 2.115; 95% CI, 1.519–2.944; $P < 0.0001$). Furthermore, each SD increase in the peak WBC level was associated with a 50.1% increase in the risk of MACEs (HR, 1.501; 95% CI, 1.208–1.864; $P = 0.0002$). When stratifying the DM population with DNLs according to the D-dimer levels (baseline and peak level) <0.5 vs. ≥0.5 mg/L, the group with the higher D-dimer level at baseline had a 2.066-fold higher risk of MACEs ($P < 0.0001$). Patients with a higher peak D-dimer level had a 1.877-fold higher risk of MACEs ($P = 0.001$) than those with lower levels. Among the DM patients with LST/VLST lesions, each unit increase in age was associated with a 75.9% increase in the risk of MACEs (HR: 1.759, 95% CI, 1.052–2.940, $P = 0.032$). Furthermore, each SD increase in the peak D-dimer level was associated with a 59.7% increase in the risk of MACEs (HR, 1.597; 95% CI, 1.110–2.295; $P = 0.041$). Each SD increase in the level of lipoprotein A was associated with a 58.1% decrease in the risk of MACEs (HR, 0.419; 95% CI, 0.179–0.979; $P = 0.045$).

Multivariate Cox regression of the DNL and LST/VLST patients with DM is summarized in **Table 3**. Age >65 years was the only independent predictor for the composite endpoint among the DM patients with DNL, and no predictor of the composite endpoint was identified in this subgroup.

Table 4 and **Figure 3** present the cumulative incidence of clinical outcomes by KM analysis at 2 years after PCI. Among the patients with DNL, the cumulative incidences of MACEs (8.42 vs. 11.97%, log rank = 0.0002), all-cause death (4.27 vs. 6.91%, log rank = 0.00032), cardiac-related death (2.86 vs. 4.24%, log rank = 0.021), and revascularization (1.34 vs. 1.49%,

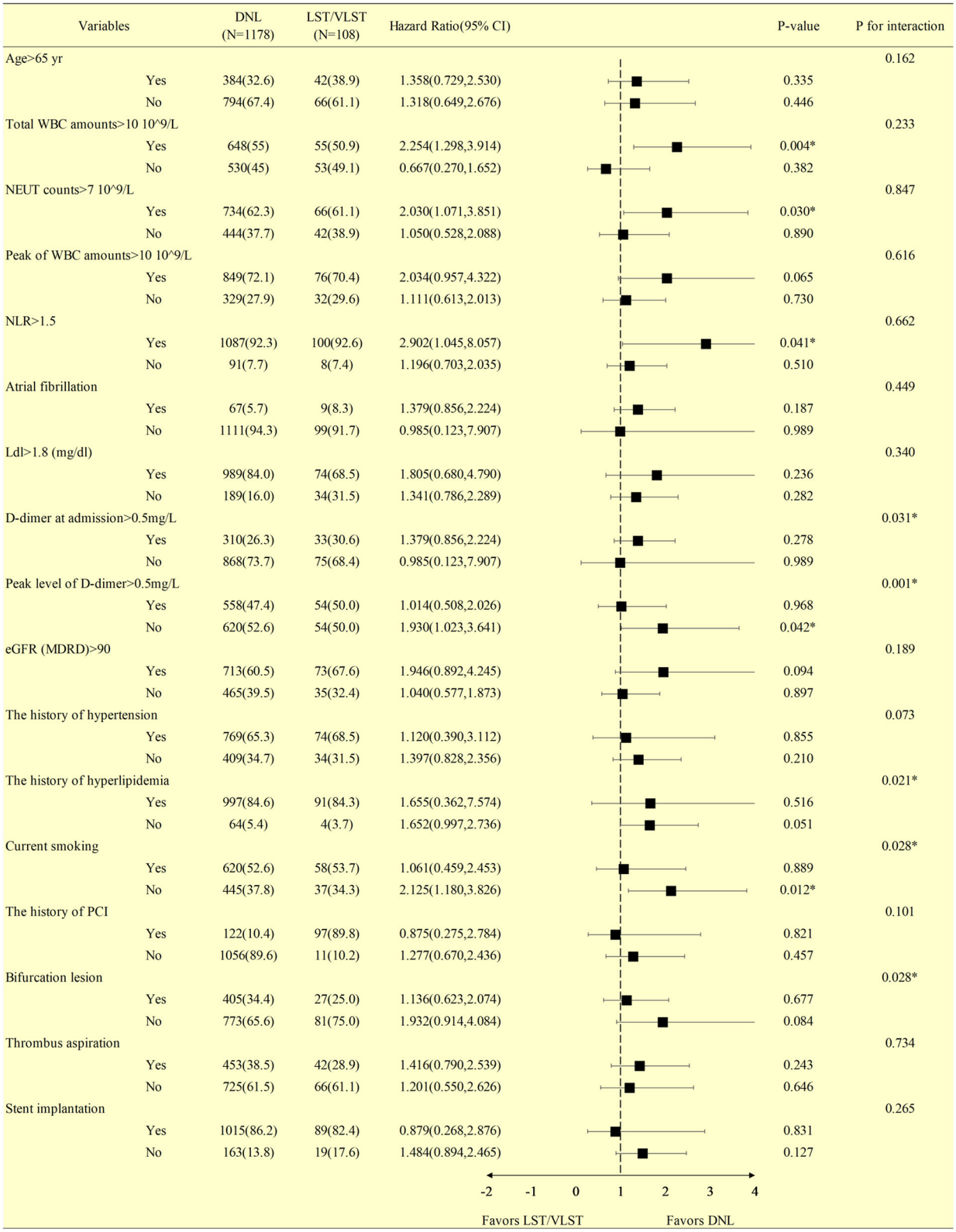

FIGURE 2 | Stratified analysis of the revascularization at a median follow-up of 698 days in patients with DNL or LST/VLST lesion. Values are *n* (%). The primary endpoint is revascularization. *Interaction is for risk ratio −2 to 1 year and risk ratio 1–4 years for LST/VLST and DNL. DNL, *de novo* lesion; LST, late stent thrombosis; VLST, very late stent thrombosis; CI, confidence interval; WBC, white blood cell; NEUT, neutral granular cell counts; NLR, neutrophil–lymphocyte ratio; LDL, low-density lipoprotein; eGFR, estimated glomerular filtration rate; PCI, percutaneous coronary intervention.

TABLE 2 | Association between MACE and different subgroups of variables among patients with DM.

Subgroups	DNL patients with DM		LST/VLST patients with DM	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age				
Age per SD	1.536 (1.300, 1.815)	<0.0001*	1.759 (1.052, 2.940)	0.032*
<65 yr	1 [reference]		1 [reference]	
≥65 yr	2.115 (1.519, 2.944)	<0.0001*	2.217 (0.789, 6.232)	0.131
Total WBC amounts				
Total WBC per SD	1.197 (0.936, 1.530)	0.153	1.202 (0.727, 1.988)	0.474
$<10 \times 10^9$/L	1 [reference]		1 [reference]	
$\geq 10 \times 10^9$/L	1.306 (0.761, 2.241)	0.333	0.897 (0.240, 3.348)	0.871
Peak of WBC amounts				
Peak of WBC per SD	1.501 (1.208, 1.864)	0.0002*	1.023 (0.978, 1.176)	0.752
$<10 \times 10^9$/L	1 [reference]		1 [reference]	
$\geq 10 \times 10^9$/L	1.128 (0.575, 2.211)	0.726	0.867 (0.202, 3.722)	0.848
NLR				
NLR per SD	1.289 (1.035, 1.603)	0.023*	0.920 (0.463, 1.828)	0.812
<1.5	1 [reference]		1 [reference]	
≥1.5	1.128 (0.575, 2.211)	0.726	0.419 (0.050, 3.517)	0.423
Hs-CRP				
Hs-CRP per SD	1.021 (0.864, 1.207)	0.806	0.912 (0.533, 1.561)	0.738
<Median	1 [reference]		1 [reference]	
≥Median	1.040 (0.744, 1.453)	0.819	0.511 (0.179, 1.454)	0.208
Lpa				
Lpa per SD	0.908 (0.714, 1.153)	0.428	0.419 (0.179, 0.979)	0.045*
<Median	1 [reference]		1 [reference]	
≥Median	1.092 (0.784, 1.521)	0.602	0.312 (0.099, 0.986)	0.047*
TG				
TG per SD	0.952 (0.806, 1.125)	0.056	1.292 (0.917, 1.819)	0.143
<Median	1 [reference]		1 [reference]	
≥Median	1.108 (0.795, 1.546)	0.544	1.123 (0.396, 3.183)	0.827
Ldl				
Ldl per SD	1.046 (0.880, 1.243)	0.609	0.661 (0.343, 1.275)	0.217
<1.8 (mg/dl)	1 [reference]		1 [reference]	
≥1.8 (mg/dl)	1.176 (0.708, 1.923)	0.532	0.647 (0.233, 1.802)	0.405
D-dimer at admission				
D-dimer per SD	1.150 (1.050, 1.260)	0.0026*	1.139 (0.828, 1.566)	0.425
<0.5	1 [reference]		1 [reference]	
≥0.5	2.066 (1.448, 2.948)	<0.0001*	2.089 (0.635, 6.871)	0.225
Peak value of D-dimer				
D-dimer per SD	1.103 (0.993, 1.226)	0.067	1.597 (1.11, 2.295)	0.041*
<0.5	1 [reference]		1 [reference]	
≥0.5	1.877 (1.291, 2.728)	0.001*	2.742 (0.646, 11.635)	0.172
eGFR (MDRD)				
eGFR per SD	0.906 (0.732, 1.122)	0.365	0.681 (0.421, 1.103)	0.118
<90	1 [reference]		1 [reference]	
≥90	0.681 (0.473, 0.980)	0.359*	0.314 (0.071, 1.394)	0.128

*SD, standard deviation; HR, hazard ratio; CI, confidence interval; DNL, de novo lesion; LST, late stent thrombosis; VLST, very late stent thrombosis; DM, diabetes mellitus; MACE, major adverse cardiovascular events; yr, year; WBC, white blood cell; NLR, neutrophil–lymphocyte ratio; LDL, low-density lipoprotein; LPA, lipase activator; hs-CRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; *P < 0.05.*

log rank = 0.029) were lower in the non-DM group than in the DM group.

Table 5 presents the cumulative incidence of clinical outcomes by KM analysis at a median follow-up of 698 days after PSM. Among the patients with DNL, the cumulative incidences of MACEs (crude HR, 1.497, 95% CI, 1.209–1.853, log rank $P < 0.001$), all-cause death (crude HR, 1.687, 95% CI, 1.264–2.251, log rank $P < 0.001$), and cardiac-related death (crude HR,

TABLE 3 | Multivariate Cox regression analysis of patients with divided into DNL and LST/VLST.

Variables	DNL patients with DM (*N* = 1,177)					LST/VLST patients with DM (*N* = 108)				
	Coefficient	HR [exp(coef)]	95% CI upper	95% CI lower	*P*-value	Coefficient	HR [exp(coef)]	95% CI upper	95% CI lower	*P*-value
Male	−0.2879	0.7498	0.4899	1.1476	0.1848	1.4519	4.2712	0.3730	48.9158	0.2432
Age >65 yr	0.4245	1.5289	1.0016	2.3338	0.0492*	0.0897	1.0938	0.1260	9.4931	0.9352
History of hypertension	0.2176	1.2431	0.8039	1.9223	0.3279	0.8767	2.4029	0.2104	27.4361	0.4804
History of hyperlipidemia	−0.0493	0.9519	0.4572	1.9820	0.8952	−0.1430	0.8667	0.0288	26.1243	0.9344
LDL-C ≥median	0.0417	1.0426	0.5927	1.8340	0.8850	−0.2628	0.7689	0.1151	5.1358	0.7862
Hs-CRP ≥median	−0.2108	0.8100	0.5456	1.2025	0.2958	−1.2242	0.2940	0.0320	2.7025	0.2794
eGFR (MDRD) ≥90	−0.0398	0.9610	0.6163	1.4984	0.8605	−2.1170	0.1204	0.0079	1.8250	0.1269
D-dimer at admission ≥0.5	0.2899	1.3363	0.7108	2.5123	0.3681	18.3358	Inf.	0.0000	Inf.	0.9985
Peak value of D-dimer ≥0.5	0.2484	1.2819	0.6880	2.3884	0.4341	−16.6900	Inf.	0.0000	Inf.	0.9986
Lpa ≥median	0.2343	1.2640	0.8548	1.8693	0.2405	−1.6927	0.1840	0.0254	1.3350	0.0941
TG ≥median	0.0968	1.1017	0.7478	1.6229	0.6242	−0.1185	0.8882	0.1324	5.9576	0.9029

*DNL, de novo lesion; LST, late stent thrombosis; VLST, very late stent thrombosis; DM, diabetes mellitus; yr, year; TG, triglyceride; LDL, low-density lipoprotein; LPA, lipase activator; hs-CRP, high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; MDRD, modification of diet in renal disease equation; HR indicates hazard ratio; CI, confidence interval; *P < 0.05.*

TABLE 4 | The cumulative incidence of clinical outcomes by Kaplan–Meier analysis at median 698 follow-up days among all enrolled patients.

Endpoints	DNL *N* = 2,661			LST/VLST *N* = 280		
	DM (*N* = 1,178)	Non-DM (*N* = 2,483)	Log rank	DM (*N* = 108)	Non-DM (*N* = 172)	Log rank
MACE	141 (11.97%)	209 (8.42%)	0.0002*	15 (12.89%)	30 (17.44%)	0.94
All-cause death	82 (6.91%)	106 (4.27%)	0.00032*	6 (5.56%)	17 (9.88%)	0.44
Cardiac-caused death	50 (4.24%)	71 (2.86%)	0.021*	5 (4.63%)	11 (4.40%)	0.87
Recurrence MI	43 (3.65%)	70 (2.82%)	0.12	6 (5.56%)	10 (5.81%)	0.79
Revascularization	175 (1.49%)	332 (1.34%)	0.029*	21 (19.44%)	27 (15.70%)	0.085
Ischemic stroke	21 (1.78%)	42 (1.69%)	0.67	5 (4.63%)	3 (1.74%)	0.088

LST, late stent thrombosis; VLST, very late stent thrombosis; DM, diabetes mellitus; MACE, major adverse cardiovascular events; MI, myocardial infarction. P < 0.05.*

1.529, 95% CI, 1.065–2.196, log rank $P = 0.021$) were lower in the non-DM group than in the DM group. After adjusting for age, sex, history of hypertension, history of PCI, history of CABG, history of CKD, Killip classification, high-sensitivity C-reactive protein, and estimated glomerular filtration rate, the cumulative incidences of MACEs (adjusted HR, 1.269, 95% CI, 1.023–1.576, log rank $P = 0.031$) and all-cause death (crude HR, 1.355, 95% CI, 1.011–1.815, log rank $P = 0.042$) were lower in the non-DM group than in the DM group after PSM.

The results of the time-to-event analysis for the primary endpoint of MACEs, all-cause death, cardiac-related death, recurrent MI, revascularization, and ischemic stroke at follow-up between the DNL and LST/VLST groups among the DM patients are summarized in **Table 6**.

DISCUSSION

Main Findings

In this study, which involved 3,941 real-world consecutive patients who had undergone primary PCI in China, we obtained the following major findings. The cumulative incidences of MACEs, all-cause death, cardiac-related death, and revascularizations were lower among patients with DNL in the non-DM group than in those in the DM group. Age, WBC count, and baseline and peak D-dimer levels were associated with increased MACEs. In particular, after successful primary PCI, the measurement of baseline and peak D-dimer levels in patients may help identify individuals at higher cardiovascular risk. Additionally, our finding suggests a potential benefit of lowering D-dimer levels among DM patients with DNL. Age and peak D-dimer levels may facilitate the risk stratification of DM patients with LST/VLST.

Impact of Inflammatory Cells on DNLs and LST/VLST Lesions Among DM Patients

Previous studies showed that an elevated WBC count was correlated with myocardial perfusion disorders (7) and an increased death risk during the first 6 months following MI among patients with ACS (8). Furthermore, a WBC count >10,000/L indicated an increased mortality risk among acute MI

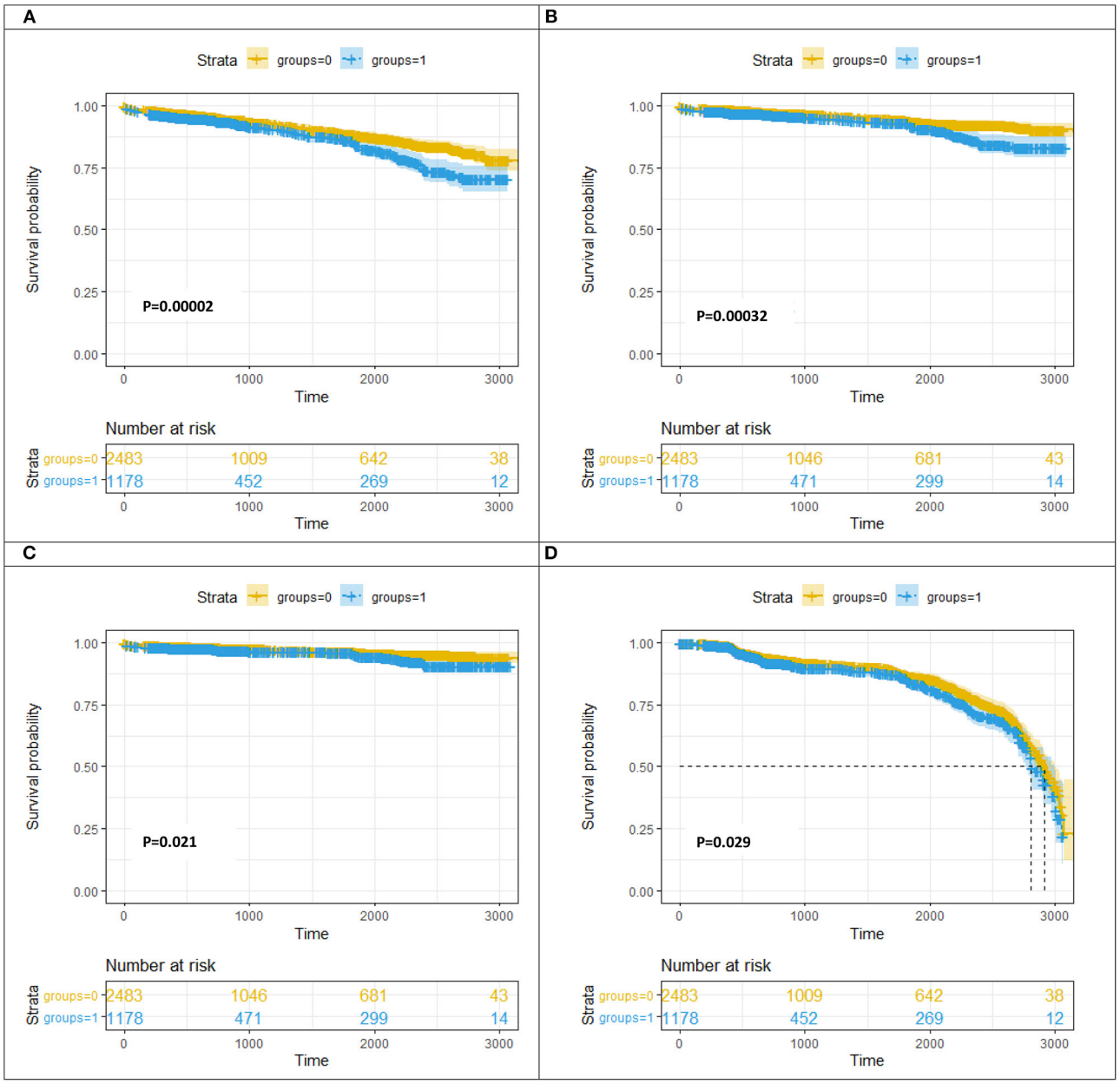

FIGURE 3 | Kaplan–Meier curve analysis for MACE **(A)**/all-cause death **(B)**/cardiac-caused death **(C)**/revascularization **(D)** at follow-up between the DM group and the non-DM group. DNL, *de novo* lesion; LST, late stent thrombosis; VLST, very late stent thrombosis groups = 0, non-diabetes mellitus group; groups = 1, diabetes mellitus group.

patients (9). It has been reported that increased leukocyte counts on admission are associated with congestive heart failure, shock, and the development of worse microvascular injury in patients with ACS (10).

The main mechanism of post-angioplasty restenosis is the binding of leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) to platelet P-selectin, which causes marked neointimal proliferation and thrombo-inflammation, leading to luminal loss (11). Consequently, in our study, we observed that each SD increase in the level of peak WBC counts was associated with a 50.1% increase in the risk of MACEs (HR, 1.501; 95% CI, 1.208–1.864; $P = 0.0002$) among DM patients with DNLs.

The NLR, one of the best-assessed hematological biomarkers, is measured by dividing the neutrophil count by the lymphocyte count; it provides diagnostic and prognostic information in ACS (12). In patients with ST-segment elevation MI (STEMI), a high pre-procedural NLR (>4.9) enables a clinician to predict in-hospital mortality with 70% accuracy and 65% specificity

TABLE 5 | Primary outcomes by propensity score matching before and after fully adjustment.

Endpoints	Propensity-matched DNL patients				Propensity-matched LST/VLST			
	DM (*N* = 1,178) vs. non-DM (*N* = 1,178)				DM (*N* = 108) vs. non-DM (*N* = 108)			
	Crude HR (95% CI)	*P*-value	Adjusted HR (95% CI)	Adjusted *P*-value	Crude HR (95% CI)	*P*-value	Adjusted HR (95% CI)	Adjusted *P*-value
MACE	1.497 (1.209, 1.853)	<0.001*	1.269 (1.023, 1.576)	0.031*	0.858 (0.442, 1.668)	0.652	0.629 (0.290, 1.363)	0.240
All-cause death	1.687 (1.264, 2.251)	<0.001*	1.355 (1.011, 1.815)	0.042*	0.578 (0.217, 1.544)	0.274	0.201 (0.049, 0.829)	0.027*
Cardiac-caused death	1.529 (1.065, 2.196)	0.021*	0.861 (0.588, 1.261)	0.442	0.723 (0.236, 2.217)	0.571	0.121 (0.018, 0.836)	0.032*
Recurrence MI	1.348 (0.922, 1.971)	0.123	2.398 (0.539, 10.675)	0.251	1.025 (0.344, 3.054)	0.965	2.335 (0.593, 9.198)	0.225
Revascularization	1.009 (0.828, 1.230)	0.929	1.018 (0.832, 1.247)	0.860	0.462 (0.210, 1.018)	0.055	0.503 (0.187, 1.352)	0.173
Ischemic stroke	1.120 (0.663, 1.892)	0.671	1.006 (0.592, 1.709)	0.982	3.233 (0.627, 16.677)	0.161	2.290 (0.429, 12.214)	0.332

*HR indicates hazard ratio; DNL, de novo lesion; LST, late stent thrombosis; VLST, very late stent thrombosis; DM, diabetes mellitus; MACE, major adverse cardiovascular events; MI, myocardial infarction. *P < 0.05. Adjusted P values are HRs (95% confidence intervals) from models adjusted for age, gender, history of hypertension, history of percutaneous coronary intervention, history of coronary artery bypass graft, history of chronic kidney disease, Killip classification, high-sensitivity-C reaction protein, and estimated glomerular filtration rate.*

TABLE 6 | The cumulative incidence of clinical outcomes by Kaplan–Meier analysis at 698 median follow-up days among DM subjects.

Endpoints	DNL *N* = 1,178	LST/VLST *N* = 108	Log rank
MACE	141 (11.97%)	15 (12.89%)	0.250
All-cause death	82 (6.91%)	6 (5.56%)	0.736
Cardiac-caused death	50 (4.24%)	5 (4.63%)	0.720
Recurrence MI	43 (3.65%)	6 (5.56%)	0.320
Revascularization	175 (1.49%)	21 (19.44%)	0.0002*
Ischemic stroke	21 (1.78%)	5 (4.63%)	0.033*

*LST, late stent thrombosis; VLST, very late stent thrombosis; DM, diabetes mellitus; MACE, major adverse cardiovascular events; MI, myocardial infarction. *P < 0.05.*

and is associated with both LST and VLST (13). Among patients undergoing angiography or cardiac revascularization, NLR is related to the progression of coronary atherosclerosis (14) and plays the role of a predictor of cardiovascular events and all-cause mortality (15). In the present study, we observed that each SD increase in the peak NLR level was associated with a 28.9% increase in the risk of MACEs (HR, 1.289; 95% CI, 1.035–1.603; $P = 0.023$), which was consistent with a previous report (16) on type 2 DM patients with DNL undergoing primary PCI. However, no significant difference was observed in the subgroup of patients with LST/VLST lesions. Furthermore, Soehnlein et al. (17) examined the contribution of neutrophils to the process of arterial healing after injury and found that neutrophil-derived cathelicidin limited neointima formation and promoted re-endothelialization after stent implantation. However, the potential causal mechanisms are currently still obscure and require further investigation.

Impact of D-Dimer on DNL and LST/VLST Lesions Among DM Patients

D-dimer, a marker of hypercoagulability, fibrin formation, and thrombin generation, has been implicated in angiogenesis and metastatic spread (18). Increased D-dimer levels were reported to be an independent risk factor for in-hospital MACEs and a long-term risk of cardiovascular disease-related mortality in STEMI patients undergoing primary PCI (19, 20). Over the 6 years of the trial, the D-dimer level was one of several biomarkers that predicted cardiovascular disease events, and the associations remained strong more than 10 years after the initial D-dimer reading (21). The long-term risk indicated by D-dimer levels is related to many pathogenic pathways, including inflammatory and atherosclerotic processes. However, the role of D-dimer levels in predicting outcomes ≥5 years in LST/VLST patients with type 2 DM who have undergone primary PCI has not been as clearly defined. In the present study, DM patients with DNL with high D-dimer levels at baseline had a 2.066-fold increase in the risk of MACEs ($P < 0.0001$) and those with a high peak D-dimer level had a 1.877-fold increase in the risk of MACEs ($P = 0.001$), compared with those with low D-dimer levels. Furthermore, each SD increase in the peak D-dimer value was associated with a 59.7% increase in the risk of MACEs (HR, 1.597; $P = 0.041$) in DM patients with LST/VLST.

DM and DNL

This study showed that DM patients have significantly higher incidences of MACEs (11.97 vs. 8.42%, $P = 0.0002$), all-cause death (6.91 vs. 4.27%, $P = 0.00032$), cardiac-related death (4.24 vs. 2.86%, $P = 0.021$), and revascularization (1.49 vs. 1.34%, $P = 0.029$) than non-DM patients among patients with DNL. Owing to microvascular dysfunction, thrombus burden, unstable plaque, and diffuse distribution of atherosclerotic lesions, DM patients with ACS generally had higher incidences of LST/VLST (22, 23). Inflammation and accumulation of reactive oxygen species and metabolic cytokines are primary mechanisms of vascular remodeling and progression of adverse myocardial diseases resulting from glycemic variability and hyperglycemia (24–28). Furthermore, a previous study has reported that insulin resistance is higher in patients with cardiovascular disease (29). Various biomarkers are proposed to play a role in the stratification of ACS. Cyr61, which predicts primary endpoints

in patients with ACS, is involved in cell adhesion, proliferation, migration, and inflammation (30). Indeed, glycemic variability has previously been shown as an outcome predictor of patients with ACS undergoing PCI (31). Furthermore, the risk for repeat revascularization has been related to DM severity, with insulin-dependent DM having the highest risk factor for repeat revascularization (32). Elevated glucose level is markedly related to sympathetic stimulation, and catecholamine can stimulate glucose release and control hyperglycemia (33). Mechanistically, an increase in the incidence of MACE, mortality, and stroke among DM patients might be a result of direct glucotoxic effects, which lead to the attenuation of endothelium-dependent vasodilatation and myocardial perfusion damage (34). Furthermore, hyperglycemia can cause conformational changes in platelet glycoproteins and affect platelet function and intraplatelet signaling pathways; as a result, more solid coronary clots are formed (35, 36).

DM and LST/VLST

In the implantation of first-generation DES, the incidence of LST/VLST was correlated with incomplete stent apposition and delayed endothelial coverage, thereby leading to chronic inflammation (37). However, second-generation DES, which is characterized with durable, biodegradable, and biocompatible polymers, is not resistant to LST/VLST (38). The mechanisms of thrombosed stent segments are fibrin deposition and chronic inflammation leading to strut malapposition, delayed healing, and heart remodeling, which are distinct from early ST (37, 39). Previous studies (40, 41) have identified DM as an important clinically independent predictor of poor outcome in ST in the real world of mixed use of bare-metal stents (BMS) and DES. Longer lesion length, smaller vessel size, a higher rate of residual dissections, increasing thrombus burden, and bifurcation lesions might be the underlying reasons for a predisposition of DM patients to ST (42, 43). This study highlights that total WBC count ($P = 0.021$) and neutral granular cell count ($P = 0.018$) were independent risk factors of LST/VLST among DM patients. This is consistent with the severe inflammation status of DM patients with LST/VLST. In addition, this study found a significant increase in the incidence of ischemic stroke in patients with LST/VLST compared with those with DNL (log rank = 0.033). A previous study has reported that neovascularization, fibrin accumulation, and thrombus burden are accompanied by inflammation, which correlated with the early healing of thrombus (44). Occasional accumulation of macrophages, giant cells, and lymphocytes is a main characteristic of the inflammatory response after percutaneous coronary stenting (45). Presence of peristrut eosinophilic material in the plasma might be a marker of endothelial cell leakage. Therefore, it is necessary to compare the effects of hypercholesterolemia using a healthy model.

DM and In-stent Thrombosis

DM patients have a 2- to 4-fold higher risk of developing in-stent restenosis (ISR) after PCI than non-DM patients and thus deserve additional attention. Although new-generation DES have greatly decreased neointimal proliferation, ISR and late stent failure are common complications and crucial after coronary stenting. A recent study (46) confirmed that a higher hemoglobin A1c (HbA1c) variability in type 2 DM patients was more likely to cause higher incidences of neointimal hyperplasia and ISR and hypothesized that post-prandial glucose variability might be more important than fasting glucose in the development of ISR. Compelling evidence from a notable study (47) has confirmed a significantly increased rate of ISR in DM patients undergoing PCI irrespective of specific treatment modalities, such as BMS, DES, and balloon angioplasty. Another study reported that endothelial dysfunction and impaired bioavailability of endothelium-derived nitric oxide play a critical role in the pathogenesis of post-PCI restenosis (48). The possible mechanisms of glycemic and HbA1c variabilities that affect the progression of ISR in DM patients remain unclear. Previous studies concluded that hyperglycemia (24, 25), insulin resistance (26), and glycemic variability (27) result in adverse vascular and myocardial remodeling directly and indirectly by stimulating the production of inflammatory factors, metabolic cytokines, and reactive oxygen species. This is consistent with our finding that the prognosis of DNL outperformed ISR, especially in the subgroup with total WBC count $>10 \times 10^9$/L, neutral granular cell count $>7 \times 10^9$/L, and NLR >1.5. Furthermore, accumulating evidence confirmed that delayed re-endothelialization (49) and endothelial dysfunction (50) play major roles in the development of ISR and are significant predictors of ISR after stent implantation. Among patients with restenosis of the stent, insulin resistance was an established and acknowledged contributory element. The higher incidence of MACEs was correlated with endothelial dysfunction and dysregulated glucose homeostasis, which play a significant role in restenosis (49). Therefore, delayed re-endothelialization and endothelial dysfunction are potential mechanisms in the progression of ISR under the setting of high glycemic variability (50). Previous studies have reported (51) that endothelial vasomotor function in the systemic artery tree is significantly related to the pathobiological process of ISR by suppressing the proliferation of smooth muscle and inhibiting intimal hyperplasia. Endothelial vasomotor function has been shown to reflect nitric oxide-mediated dilation (52). Furthermore, asymmetric dimethylarginine has been shown to be correlated with the pathogenesis of atherosclerosis and endothelial dysfunction (51). A previous study (53) revealed that serum soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) level, which is considerably affected by DM, is a predictive biomarker of ISR and an important mediator of migration, cellular inflammation, vascular smooth muscle cell proliferation, and sTREM-1 concentration. A high ISR rate may be related to dyslipidemia in DM, mainly due to increased remnant-like particle cholesterol, which is identified as lipoproteins rich in triglycerides, and in the fasting state, very low density lipoproteins are major components (54). Estimated GFR <60 ml/min·m^2, a pre-clinical sign of end-stage renal disease, was a strong independent predictor of documented poor clinical outcome among patients with acute MI undergoing successful PCI (55). Due to lipid metabolic disturbance (56), raised inflammation and oxidative stress (57),

elevated the level of serum of homocysteine (58), coagulation and endothelial dysfunction (59), jeopardized homeostasis of calcium phosphate (60), renal insufficiency lead to poor clinical outcome. Levey et al. (61) suggested that the most reliable method to estimate GFR was MDRD equation, which takes gender and age into consideration. In the present study, we have used the equation of MDRD to translate into a reliable assessment of kidney function.

Limitations

study had some limitations. First, we retrospectively collected the clinical data on definite ST in patients who underwent primary PCI, as reported by site investigators in this study. Furthermore, the trial was conducted in a single center in China. Therefore, we cannot exclude geographical variations in PCI practice outside China or in higher-volume centers. Third, we did not enroll patients with ST in terms of probability, possibility, or secondary to chance, which may have led to an underestimation of the actual ST incidence. However, ST was an endpoint pre-specified according to the definitions of ARC (26). All ST events were adjudicated independently by a blinded clinical events committee according to established criteria, and the incidence of definite ST continued to diverge between the two investigated devices for up to 5 years, which would render a chance finding unlikely. Thus, it is necessary to evaluate demographic covariates and the longitudinal management of therapeutic options carefully. Furthermore, the use of ticagrelor/prasugrel was lower than that of clopidogrel, which could have influenced the clinical outcomes and should therefore be considered a study limitation.

CONCLUSION

In conclusion, the study found that DM patients with DNLs have a higher incidence of composite clinical outcomes than their non-DM counterparts. Furthermore, compared with patients with DNL, patients with LST/VLST lesions had more long-term composite clinical outcome events. Thus, LST/VLST lesions are critical problems after coronary stenting, particularly among DM patients. Stronger antithrombotic therapy may help to reduce the incidence of ST and improve clinical outcomes after PCI in patients with type 2 DM.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was conducted according to the principles which were outlined in the declaration of Helsinki and has been approved by the Ethics Committee of Fuwai Hospital. All study subjects have provided written informed consent. Written informed consent for publication was obtained from all participants.

AUTHOR CONTRIBUTIONS

HY, XZ, JZ, YT, RC, YW, CL, PZ, ZS, JLi, YC, LS, and HZ: substantial contributions to conception and design, data acquisition, or data analysis and interpretation. HY, XZ, JLa, XY, JZ, RC, YW, YT, CL, PZ, ZS, JLi, YC, LS, and HZ: drafting the article or critically revising it for important intellectual content, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. All authors contributed to the article and approved the submitted version.

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Prognostic Impact of Metabolic Syndrome in Patients With Heart Failure: A Meta-Analysis of Observational Studies

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Background: The metabolic syndrome (MS) is significantly associated with the risk of incident heart failure (HF). However, there are still great controversies about the impact of MS on the prognosis in patients with established HF. This meta-analysis aimed to ascertain the effect of MS on the prognosis in patients with HF.

Methods: We searched multiple electronic databases, including PubMed, Opengrey, EMBASE, and Cochran Library, for potential studies up to February 15, 2021. Observational studies that reported the impact of MS on the prognosis in patients with established HF were included for meta-analysis.

Results: Ten studies comprising 18,590 patients with HF were included for meta-analysis. The median follow-up duration of the included studies was 2.4 years. Compared with HF patients without MS, the risk of all-cause mortality and cardiovascular mortality was not increased in HF with MS (HR = 1.04, 95% CI = 0.88–1.23 for all-cause mortality; HR = 1.66, 95% CI = 0.56–4.88 for cardiovascular mortality, respectively). However, there was a significant increase in composited cardiovascular events in the HF patients with MS compared with those without MS (HR = 1.73, 95% CI = 1.23–2.45).

Conclusions: In patients with established HF, the presence of MS did not show an association on the risk of all-cause mortality or cardiovascular mortality, while it may increase the risk of composite cardiovascular events.

Keywords: heart failure, metabolic syndrome, all-cause mortality, cardiovasclar disease, prognosis

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INTRODUCTION

Metabolic syndrome (MS) is a cluster of cardiovascular risk factors characterized by insulin resistance, central obesity, elevated blood pressure, and dyslipidemia (1, 2). Epidemiological studies have shown that MS and its components are highly prevalent and significantly associated with the development of diabetes and cardiovascular disease (CVD) (2). Heart failure (HF) is a growing global public health burden, attributed to significantly increased mortality worldwide (3). Detection for the pathophysiological characteristics and novel treatment targets would be important for management of HF (4–6). It is well-documented that MS is an independent risk factor in the development of HF (7). Individuals with MS were associated with a 2-fold risk of HF incidence compared with those without MS (8). However, there are still great controversies about the impact of MS on the prognosis in patients with established HF. Some studies had reported that HF patients

with MS had a significantly reduced risk of in-hospital mortality (9), as well as long-term all-cause mortality (10, 11), compared with HF patients without MS. However, such association was not documented in other studies (12, 13), raising concern of whether such "epidemiologic paradox" was true in the HF–MS relationship.

Given these inconsistent results and controversies, we performed a meta-analysis to ascertain the impact of MS on the prognosis in patients with HF, focusing on all-cause mortality, cardiovascular mortality, and composite cardiovascular events.

METHODS

Search Strategy and Selection Criteria

This study was performed under the recommendations of the Meta-analysis of Observational Studies in Epidemiology group (14). We searched multiple electronic databases, including PubMed, Medline, EMBASE, and Cochran Library, for potential related studies up to February 15, 2021. Items including "metabolic syndrome," "syndrome X," "insulin resistance syndrome," "heart failure," "myocardial failure," "cardiac failure," "cardiac dysfunction," or "myocardial dysfunction" were searched using a combined MeSH terms and text word search strategy. No language restriction was set in the search strategy. However, we restricted the search to human studies by using the "Humans" filters. The reference lists of included studies were reviewed to identify other potential associated research.

Inclusion criteria of studies for meta-analysis were the following: (1) observational studies (cohort studies, nest case–control studies, or *post-hoc* analysis of randomized controlled trials) with a follow-up duration ≥6 months; (2) the prognoses in HF patients were reported in those with MS, compared with those without MS. Exclusion criteria were the following: (1) cross-sectional studies without follow-up evaluation; (2) duplicated publications derived from the same observational study. When duplicated publications were derived from the same observational study, we only included the latest published article for analysis.

Data Extraction and Quality Assessment

Two researchers (ZH and WC) screened the titles and abstracts of all the retrieved items and reviewed the full manuscripts of potentially relevant studies independently. Key information of the included studies, such as authors, study design, race, definition and prevalence of MS, sample size, sex proportion, age, follow-up duration, adjusted confounders, and outcome events were recorded. We also contacted the corresponding authors of the original studies for any additional data if required.

We evaluated the quality of the included studies according to the Newcastle–Ottawa Quality Assessment Scale (NOS) for cohort studies (15). The NOS judged the quality based on selection, comparability, and exposure/outcome, with the highest score up to 9. In the present analysis, included studies were graded in quality as good, fair, or poor if they were awarded ≥7, 4–6, or <4 scores, respectively (16, 17).

Data Synthesis and Analysis

The primary outcome in our study was the risk of all-cause mortality in HF patients with MS compared with those without MS. The secondary outcomes included the risk of cardiovascular mortality and composite cardiovascular events, respectively. Hazard ratios (HRs) and 95% confidence intervals (CIs) adjusted for the maximal number of confounders were extracted for analysis. If relative risks (RRs) were reported, they were considered as approximate estimate for HRs and used for meta-analysis (18). In situations where outcomes were presented as odds ratios (ORs), data were converted to RRs for analysis, according to previously published method (19). I^2 statistics were used to test heterogeneity among studies, and an $I^2 > 50\%$ was considered to be with significant heterogeneity. We used the inverse variance approach to combine the log HRs and corresponding standard errors (SE). A random-effects model was used for meta-analysis if there was significant heterogeneity. Otherwise, a fixed-effects model was used. Sensitivity analyses were performed by omitting one study and recalculating the estimates of HRs at a time or interchange using random-effects models and fixed-effects models for the meta-analysis. Publication bias was evaluated by inspecting funnel plots for the primary outcome in which the lnHR was plotted against SE and also tested by using Egger's and Begg's tests. Subgroup analyses were performed according to participant's age, follow-up duration, race, the severity of HF, adjusted confounders, and definition of MS.

Analyses were performed using RevMan 5.3 (The Cochrane Collaboration, Copenhagen, Denmark) and Stata 12.0 (StataCorp LP, College Station, TX, USA) for Egger's and Begg's tests. A $p < 0.05$ was set for statistical significance.

RESULTS

Studies Retrieved and Characteristics

A total of 3,356 items were detected from the online electronic databases. After screening the titles and abstracts, we found that 27 articles were qualified for a full review. Finally, we included 10 studies comprising 18,590 patients with HF for meta-analysis (**Figure 1**) (10–13, 20–25). The median follow-up duration of the included studies was 2.4 years.

In the 10 studies, 8 were from the United States and Europe; 2 were from Asia. The key messages of the included studies are presented in **Table 1**. All studies included advanced HF patients, except one study that included patients with asymptomatic HF (stage A/B HF). Five, four, and one study defined MS according to the Third Adult Treatment Panel Report of the National Cholesterol Education Program (NCEP-ATP III), the International Diabetes Federation guideline (IDF), and the Japanese Committee for the Diagnostic Criteria of MS, respectively. Two studies were published as meeting abstracts, and full articles were not available. Therefore, quality assessment was only performed in the eight studies

Abbreviations: CIs, confidence intervals; CVD, cardiovascular disease; HF, heart failure; HRs, hazard ratios; IDF, International Diabetes Federation; MS, metabolic syndrome; NCEP-ATP III, Third Adult Treatment Panel Report of the National Cholesterol Education Program; NOS, Newcastle–Ottawa Quality Assessment Scale; RRs, relative risks; SE, standard errors.

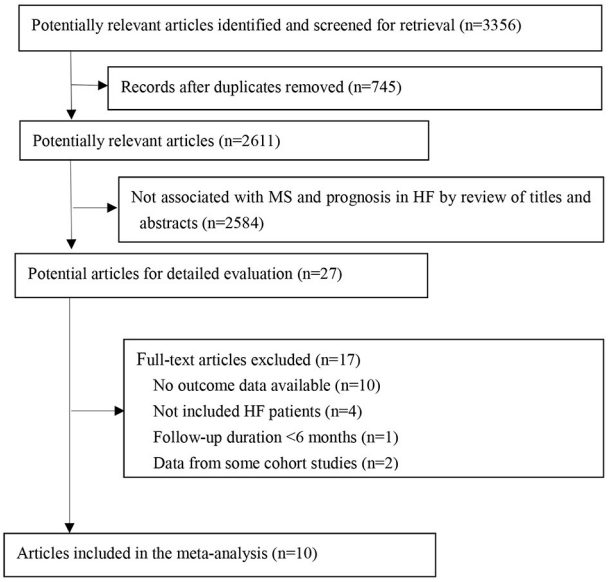

FIGURE 1 | Flow diagram of study selection. HF, heart failure; MS, metabolic syndrome.

with full published articles. According to NOS criteria, six studies were graded as good quality and two as fair quality (**Supplementary Table 1**).

Association Between MS and Prognosis in Patients With HF

There were eight studies that provided data for analysis of the association between MS and all-cause mortality in patients with HF. Due to the significant heterogeneity among the included studies (I^2 = 73%, p < 0.001), a random-effects model was used to combined the pooled estimates. Compared with patients without MS, the risk of all-cause mortality was not increased in HF patients with MS (HR = 1.04, 95% CI = 0.88–1.23, p = 0.62) (**Figure 2**). No evidence of publication bias was found by visual inspection of the funnel plot (**Supplementary Figure 1**) or using Egger's test or Begg's (all $p > 0.05$).

Three studies provided the risk of cardiovascular mortality in HF patients with and without MS, and significant heterogeneity was observed among the studies ($I^2 = 86\%$, $p < 0.001$). Random-effects models meta-analysis showed that there was no significant difference in cardiovascular mortality between the groups (HF with MS vs. HF without HF, HR = 1.66, 95% CI = 0.56–4.88, $p = 0.36$) (**Figure 3**).

Although significant heterogeneity existed among three studies that reported composited cardiovascular events (I^2 = 64%, p = 0.06), pooled estimates showed that there was a substantial increase in composited cardiovascular events in HF patients with MS, compared with those without HF (HR = 1.73, 95% CI = 1.23–2.45, $p = 0.002$) (**Figure 4**).

Subgroup Analyses and Sensitivity Analyses

The subgroup analyses for the relative risk of all-cause mortality in HF patients with MS are presented in **Table 2**. Patients with HF and MS did not show an association with all-cause mortality in all the subgroup analyses. Furthermore, there was no significant heterogeneity observed among subgroup comparisons (all $p > 0.15$).

Several sensitivity analyses confirmed that there was no significant association between MS status and risk of all-cause mortality, using fixed-effects models instead of random-effects models or recalculating the estimated risk by omitting one study at a time.

DISCUSSION

To the best of our knowledge, this is the first meta-analysis exploring the impact of MS on the prognosis in patients with established HF. Our data showed that MS is not associated with the risk of all-cause mortality or cardiovascular mortality in HF patients, while an increased risk of composite cardiovascular events was observed. Therefore, this study challenges the "epidemiologic paradox" in the relationship between HF and MS.

Clinical Implications

Observational studies had reported that in patients with established HF, risk factors that contribute to the development of CVD and HF, including hypertension, obesity, and hypercholesterolemia, might have a protective effect on prognosis. This phenomenon was termed as "reverse epidemiology" or "risk factor paradox" (26). However, the nature of observational studies could not exclude "reverse causation" in such observed associations; i.e., it may not be that obesity, hypertension, and hypercholesterolemia can play a protective role, while low body mass index (BMI), blood pressure, or serum cholesterol are "markers" of a severe pathological condition in HF, closely linked to cachexia, frailty, and death (7). In our study, the presence of MS was not associated with a favorable prognosis in the HF population, and no evidence of a "metabolic syndrome survival paradox" was found. Our results were supported by the study of Ozcan et al. (25). which showed that the risk of all-cause mortality was not statistically different in HF patients with and without MS. However, in HF patients with "reverse metabolic syndrome" (i.e., low BMI, blood pressure, and total cholesterol than normal level), the risk of all-cause mortality was increased in metabolically healthy patients. In this view, we should not recommend that MS is a protective factor; rather we should advocate that in HF patients with "reverse metabolic syndrome," targeting catabolic syndrome by nutritional support and anti-inflammatory treatment should be considered in future studies.

Actually, we found that in HF with MS, the risk of composite cardiovascular events was increased compared with those without MS. This finding further supports that MS is an independent risk of atherosclerotic cardiovascular events (2), even in patients with established HF. Besides the effect

TABLE 1 | Characteristics of the included studies.

References	Design	Region	Patients characteristics	Sample (female %)	Age (years) (mean)	Follow-up duration (years)	Definition and prevalence of MS	Events for analysis	Risk factors adjusted
Hassan et al. (10)	Retrospective cohort study	US	Hospitalized HF	886 (20.1)	64.9	2.4	NCEP-ATP III (68.3%)	All-cause mortality	Age, sex, race, LVEF, hemoglobin, eGFR, TC, LDL-C, AF, QRS duration on electrocardiogram, health care site, ACEI/ARB
Tamariz et al. (12)	Prospective cohort study	US	HFrEF	865 (37.0)	55.1	2.6	NCEP-ATP III (40%)	All-cause mortality	Demographics, use of ACEI, β-blocker, hematocrit, creatinine, educational level, and LVEF.
Ahmed et al. (20)[#]	Retrospective cohort study	US	HF received ICD	171 (NA)	71.0	2.3	NCEP-ATP III (25.1%)	CVD mortality	Age, sex
Bajraktari et al. (21)[#]	Prospective cohort study	Sweden	Congestive HF	188 (NA)	62.0	1.5	NCEP-ATP III (44.1%)	Composite CVD	Multivariate adjusted, but confounder were not reported
Perrone-Filardi et al. (11)	*post-hoc* analysis of RCT	Italy	Symptomatic HF (NYHA II–IV)	6,648 (21.8)	67.2	3.9	IDF (18.2%)	All-cause mortality CVD mortality	Age, HR, LVEF, previous MI, CABG, PAD, percentage of patients with pacemaker, ICD, COPD, ischemic HF, ARB, diuretics, and CCB.
Carrubba et al. (13)	Prospective cohort study	Italy	Stage A/B HF (asymptomatic HF)	1,920 (43.8)	60.0	0.9	IDF (13.4%)	All-cause mortality Composite CVD CVD mortality	Left ventricular dysfunction, age, gender, history of MI.
Tadaki et al. (22)	Prospective cohort study	Japan	Stage C/D CHF.	4,762 (32.0)	68.8	3.2	Japanese criteria (41.3%)	All-cause mortality Composite CVD	Age, sex, BMI, BNP, LVEF, SBP, DBP and HR.
Vest et al. (23)	Retrospective cohort study	US	HFrEF	1,953 (26.0)	55	5.1	NCEP-ATP III (37%)	All-cause mortality	Age, sex, LVAD, transplantation, and factors unbalanced between the +MS and –MS subgroups
Welnicki et al. (24)	Retrospective cohort study	Poland	Stage C/D CHF without AF	893 (31.0)	63.6	1.0	IDF (13.0%)	All-cause mortality	Unadjusted
Cetin et al. (25)	Retrospective cohort study	Turkey	HFrEF	304 (17.8)	47.4	1.3	IDF (36.2%)	All-cause mortality	Unadjusted

ACEI, angiotensin-converting enzyme inhibitor; AF, atrial fibrillation; ARB, angiotensin receptor blockers, BMI, body mass index; BNP, B-type natriuretic peptide; CCB, calcium channel blockers; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HF, heart failure; HFrEF, HF with reduced ejection fraction; HR, heart rate; ICD, Implantable cardioverter-defibrillator; IDF, International Diabetes Federation; LDL-C, low-density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; MI, myocardial infarction; MS, Metabolic Syndrome; NA, not available; NCEP-ATP III: the Third Adult Treatment Panel Report of the National Cholesterol Education Program; PAD, peripheral artery disease; RCT, randomized controlled trial; SBP, systolic blood pressure; TC, total cholesterol.

[#]Meeting abstract.

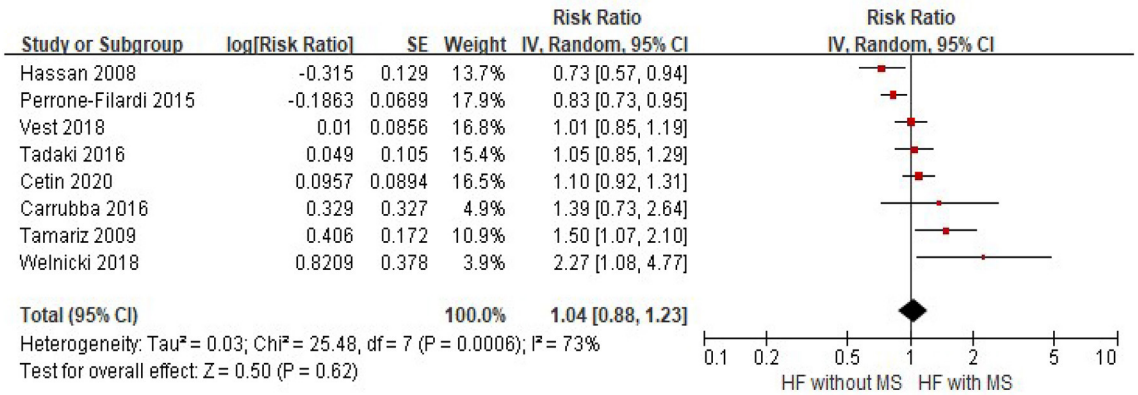

FIGURE 2 | The association of MS and the risk of all-cause mortality in HF patients. CI, confidence interval; HF, heart failure; MS, metabolic syndrome.

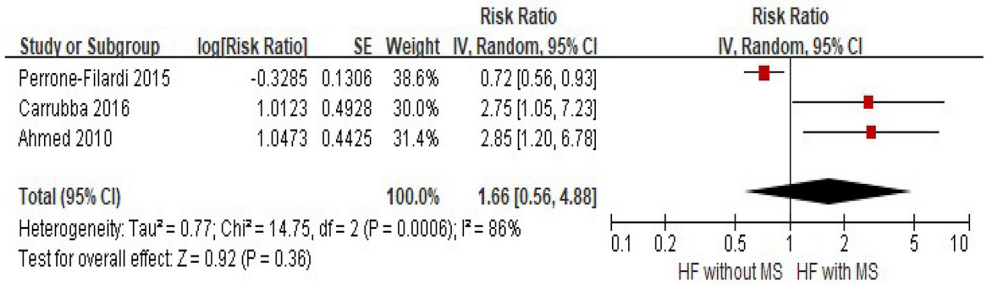

FIGURE 3 | The association of MS and the risk of cardiovascular mortality in HF patients. CI, confidence interval; HF, heart failure; MS, metabolic syndrome.

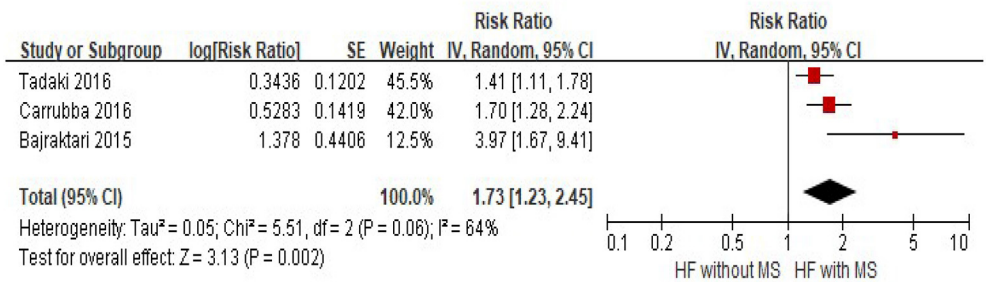

FIGURE 4 | The association of MS and the risk of composite cardiovascular events in HF patients. CVD, cardiovascular disease; CI, confidence interval; HF, heart failure; MS, Metabolic Syndrome.

of the cluster of atherosclerotic risk factors, activation of the sympathetic nervous system and renin–angiotensin system may also be involved in this process. It had been proposed that in HF patients with MS, activation of the sympathetic nervous system and renin–angiotensin system was higher than those without MS (27, 28). Our study also found that the prevalence of MS was up to 13.0–68.3%, based on different definitions. Considering the high prevalence and association with increased risk of composite cardiovascular events, proper management of this population would be of important clinical effect. During the past decade, the treatment of HF had made great progress, and many patients with severe HF can survive for a long time. In this situation, prevention of CVD in established HF with MS should be considered.

Limitations

Several limitations of the current study should be mentioned. First, the definitions of MS were different in the included studies. However, our subgroup analysis showed that the risk of all-cause mortality was not increased in HF with MS; neither was

TABLE 2 | Subgroup analyses of the association between MS and risk of all-cause mortality in HF patients.

	Number of studies	HF + MS vs. HF + non-MS	
		HR (95% CI)[#]	*p*-value*
Participants' age			0.20
≤60 years	4	1.14 (0.97, 1.33)	
>60 years	4	0.94 (0.73, 1.20)	
Follow-up duration			0.15
≤3 years	6	1.14 (0.90, 1.43)	
>3 years	2	0.91 (0.75, 1.10)	
Race			0.86
Asian	2	1.08 (0.94, 1.23)	
Non-Asian	6	1.05 (0.83, 1.33)	
Severity of HF			0.37
Asymptomatic HF	1	1.39 (0.73, 2.64)	
Symptomatic HF	7	1.03 (0.87, 1.22)	
Adjusted confounders			0.30
Unadjusted	2	1.44 (0.72, 2.86)	
Multivariable adjusted	6	0.99 (0.83, 1.18)	
Definition of MS			0.70
NCEP-ATP III	3	1.11 (0.82, 1.50)	
IDF	4	1.02 (0.72, 1.43)	

**For heterogeneity among subgroups.*

HF, heart failure; HR, hazard ratio; IDF, International Diabetes Federation; MS, Metabolic Syndrome; NCEP-ATP III, the Third Adult Treatment Panel Report of the National Cholesterol Education Program.

defined according to the IDF nor NCEP-ATP III criteria. Second, the definitions of HF among the included studies were with significant heterogeneity. The etiologies of HF are also unknown in the studies. However, we did not observe a difference in the subgroup analysis according to the severity of HF. Even in patients with advanced HF, the risk of all-cause mortality was not associated with the presence of MS. Third, MS is a cluster of cardiovascular risk factors, including obesity, elevated blood pressure, elevated blood glucose, and dyslipidemia. Due to limited data, we did not evaluate the effect of the individual components of MS on the prognosis in HF. Fourth, although we reported that the risk of composite cardiovascular events was increased in HF with MS, only three studies were available for the meta-analysis of this outcome. Further studies are needed to explore the association between MS and the risk of CVD in patients with established HF.

CONCLUSIONS

In patients with established HF, the presence of MS did not show a protective effect on the risk of all-cause mortality or cardiovascular mortality, while it may increase the risk of composite cardiovascular events.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Z-MH and W-RC: research idea, study design, and data acquisition. Q-WS and Z-WH: data analysis/interpretation and statistical analysis. W-RC: supervision and mentorship. All authors contributed important intellectual content during manuscript drafting or revision and accept accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.704446/full#supplementary-material

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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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Association Between Fasting Hyperglycemia and New-Onset Atrial Fibrillation in Patients With Acute Myocardial Infarction and the Impact on Short- and Long-Term Prognosis

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Background: The relationship between fasting hyperglycemia (FHG) and new-onset atrial fibrillation (AF) in patients with acute myocardial infarction (AMI) is unclear, and whether their co-occurrence is associated with a worse in-hospital and long-term prognosis than FHG or AF alone is unknown.

Objective: To explore the correlation between FHG and new-onset AF in patients with AMI, and their impact on in-hospital and long-term all-cause mortality.

Methods: We performed a retrospective cohort study comprising 563 AMI patients. The patients were divided into the FHG group and the NFHG group. The incidence of new-onset AF during hospitalization was compared between the two groups and sub-groups under different Killip grades. Logistic regression was used to assess the association between FHG and new-onset AF. In-hospital mortality and long-term all-cause mortality were compared among patients with FHG, AF, and with both FHG and AF according to 10 years of follow-up information.

Results: New-onset AF occurred more frequently in the FHG group than in the NFHG group (21.6 vs. 9.2%, $p < 0.001$). This trend was observed for Killip grade I (16.6 vs. 6.5%, $p = 0.002$) and Grade II (17.1 vs. 6.9%, $p = 0.005$), but not for Killip grade III–IV (40 vs. 33.3%, $p = 0.761$). Logistic regression showed FHG independently correlated with new-onset AF (OR, 2.56; 95% CI, 1.53–4.30; $P < 0.001$), and 1 mmol/L increased in fasting glucose was associated with a 5% higher rate of new-onset AF, after adjustment for traditional AF risk factors. AMI patients complicated with both fasting hyperglycemia and AF showed the highest in-hospital mortality and long-term all-cause mortality during an average of 11.2 years of follow-up. Multivariate Cox regression showed FHG combined with AF independently correlated with long-term all-cause mortality after adjustment for other traditional risk factors (OR = 3.13, 95% CI 1.64–5.96, $p = 0.001$), compared with the group with neither FHG nor new-onset AF.

Conclusion: FHG was an independent risk factor for new-onset AF in patients with AMI. AMI patients complicated with both FHG and new-onset AF showed worse in-hospital and long-term all-cause mortality than with FHG or AF alone.

Keywords: ST-segment elevation myocardial infarction, atrial fibrillation, fasting hyperglycemia, prognosis, mortality

BACKGROUND

Hyperglycemia frequently complicates the clinical course of patients hospitalized with acute myocardial infarction (AMI) (1), and is associated with a worse prognosis regardless of diabetic status (2–4). Previous reports have shown that hyperglycemia causes oxidative stress, induces apoptosis, and activates coagulation, which exacerbates damage in the setting of ischemia (5–8). Atrial fibrillation (AF) is the most common supraventricular tachyarrhythmia in the general population (9, 10). It is also frequently observed after AMI, with a reported incidence of 5–23% (9, 11–13). AF is also a well-established predictor of poor short- and long-term prognosis in patients with AMI (9).

Although both hyperglycemia and AF are common in AMI patients, and extensive studies have indicated their value in predicting prognosis, little data are available concerning the correlation between hyperglycemia and new-onset AF. Whether hyperglycemia contributes to the prevalence of new-onset AF in this setting is unknown. Moreover, whether hyperglycemia combined with AF shows a worse prognosis than hyperglycemia or AF alone is unclear.

Therefore, we performed a retrospective cohort study with 10 years of follow-up data to explore the correlation between hyperglycemia and new-onset AF in patients with AMI, and their impact on in-hospital and long-term prognosis.

MATERIALS AND METHODS

Study Population

We retrospectively enrolled patients who were admitted to our institute and diagnosed with AMI [including ST-segment elevation myocardial infarction (STEMI), and non-ST-segment elevation myocardial infarction (NSTEMI)] from 1 January 2007 to 1 January 2009. The ethics committee on clinical scientific research and laboratory animal of Zhongshan People's Hospital approved the study (review number: K2019-057). The exclusion criteria were patients (1) with previous known AF or atrial flutter, (2) with unavailable hyperglycemia data, (3) complicated with pulmonary failure defined as a PO_2 level <60 mmHg or a PCO_2 level more than 50 mmHg according to blood gas analysis, (4) complicated with liver damage defined as an ALT level 3 times higher than the normal upper limit, (5) complicated with severe renal failure defined as a serum creatinine (CR) level >265 μmol/L (6) complicated with pancreatic system diseases or heart valve disease (moderate to severe valve regurgitation or stenosis), (7) complicated with hyperthyroidism, and (8) complicated with carcinoma.

Abbreviations: AMI, acute myocardial infarction; AF, atrial fibrillation; FHG, fasting hyperglycemia; NFHG, non- fasting hyperglycemia; FG, fasting glucose; DM, diabetes mellitus.

Methods

Baseline Data Collection

Data on age, sex, previous history of diabetes, hypertension, cerebral stroke, and smoking habits were collected according to inpatient records. Other data, such as the peak value of creatine kinase MB isoenzyme (CK-MB), CR, fasting glucose (FG) level, glycosylated hemoglobin level, left atrial diameter, and left ventricular ejection fraction (LVEF) according to echocardiography, Killip grades, medication treatment during hospitalization, whether the percutaneous coronary intervention (PCI) was performed and new-onset AF complications, were also collected.

Correlation Between Hyperglycemia and New-Onset AF

All the enrolled patients were divided into a fasting hyperglycemia group (FHG group, FG $\geq$ 7 mmol/L), and a no fasting hyperglycemia group (NFHG group, FG < 7 mmol/L). The incidence of new-onset AF during hospitalization was compared between the two groups as well as sub-groups under different Killip grades.

In-Hospital Mortality

The patients were divided into four groups according to whether they had AF, hyperglycemia or both: FHG–AF– (have neither hyperglycemia nor AF), FHG–AF+ (have AF only), FHG+AF– (have hyperglycemia only), and FHG+AF+ (have both hyperglycemia and AF). In-hospital mortality among the four groups was compared.

Ten Years of Follow-Up

We choose all-cause mortality as the endpoint of the study. All patients who survived and were discharged were followed up until December 31, 2019. We collected follow-up information either by outpatient medical records or through routine telephone follow-up records every year. Death events were recorded during this period. We compared the influence of FHG and new-onset AF, and their co-occurrence on all-cause mortality.

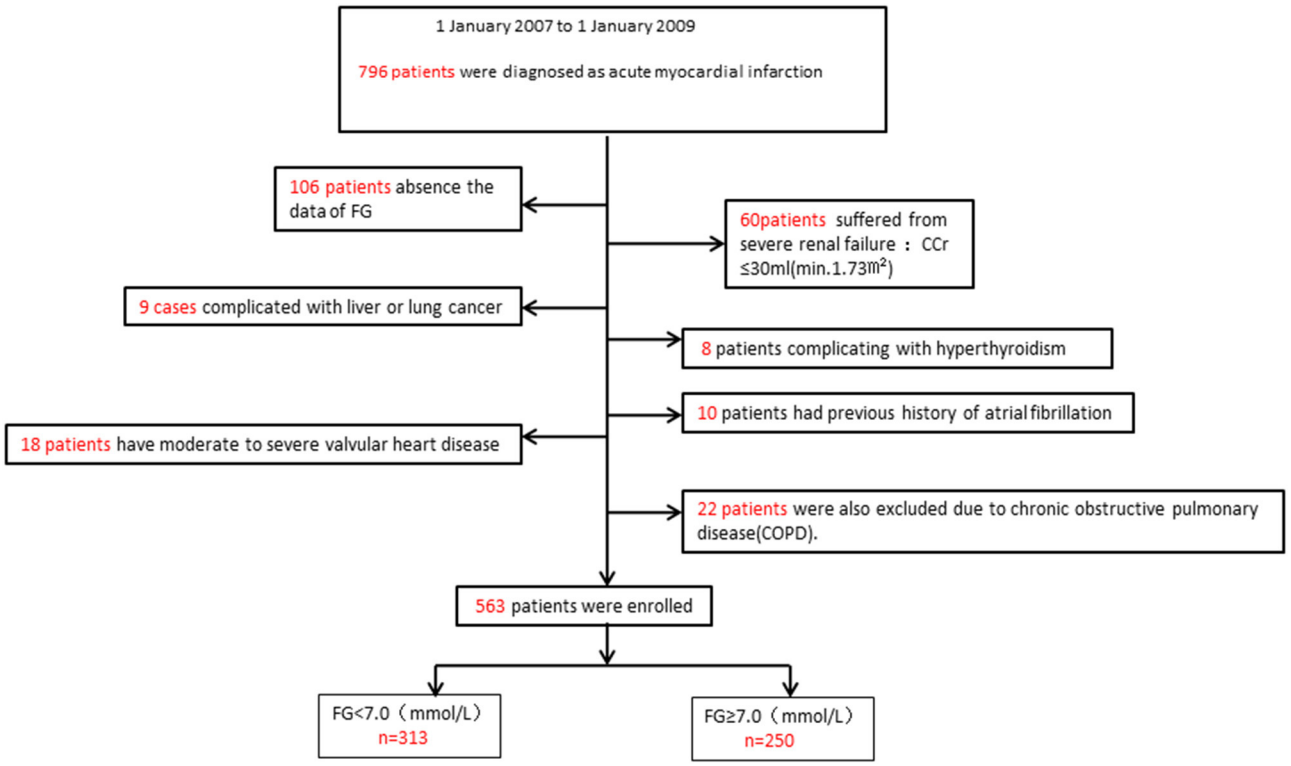

FIGURE 1 | Flow chart of participants. FG, fasting glucose; Ccr, creatinine clearance rate.

Definition of Exposure and Outcomes

Definition of New-Onset AF

AF was diagnosed as the absence of P waves, coarse or fine fibrillatory waves, and irregular RR intervals. New-onset AF was defined as electrocardiographic evidence of AF during hospitalization, and those patients were absent of persistent or paroxysmal AF or atrial flutter previously.

Definition of AMI

AMI was defined as patients with typical angina pectoris and ST-elevation at the J-point with the cutoff point of C 0.1 mV in at least two contiguous leads of the electrocardiogram (ECG) in standard lead I through AVF. An ST-elevation of 0.2 mV in V1 through V6 was defined as STEMI, and patients with increased cardiac biomarkers (mainly creatinine phosphokinase, CPK) were defined as NSTEMI.

Definition of Hyperglycemia

In our study, we chose the FG concentration as the evaluation index, and samples were measured upon hospital admission with at least 8 h of overnight fasting. A cutoff value of 7 mmol/L (126 mg/dl) was used to define fasting hyperglycemia.

Statistical Analysis

Statistical analysis was conducted with Statistical Package for the Social Sciences (SPSS) 17.0 software (SPSS Inc., Chicago, IL, United States). Numerical variables are represented as the mean ± standard deviation or median, and categorical variables as percentages or rates. To test differences between the groups, the Student's *t*-test was used for numerical variables with a Gaussian distribution, and the Mann–Whitney *U*-test was employed if there was a non-Gaussian distribution. Categorical variables were analyzed with the chi-squared and Fisher's exact tests. Logistic regressions were used to assess the relationship between FG and new-onset AF. We primarily modeled FG as a continuous variable, and then categorized FG into hyperglycemia group (FG $\geq$ 7.0 mmol/L) and normal group (FG $<$ 7.0). The initial model was adjusted for age and gender. A second model was additionally adjusted for smoking, hypertension, previous MI, previous stroke, and creatine. Kaplan-Meier analysis was used to compare long-term all-cause mortality under different FHG and new-onset AF status. Cox regression analysis was performed to identify risk factors for all-cause mortality. A $p < 0.05$ was regarded as statistically significant.

RESULTS

Enrollment and Baseline Characteristics of the Study Population

From 1 January 2007 to 1 January 2009, a total of 796 patients diagnosed with AMI were admitted to our institute. Five hundred sixty-three patients were finally enrolled in this study. Two hundred fifty patients were assigned to the FHG group (FG $\geq$

TABLE 1 | Baseline characteristics of patients in NFHG group vs. FHG group.

	NFHG group ($n = 313$)	FHG group ($n = 250$)	*P*-value
General Characteristics			
Age, years	62.04 ± 13.15	63.29 ± 13.25	0.306
Gender, male,%	265 (84)	183 (73)	0.003*
Previous History			
Myocardial infarction, *n*, %	3 (0.9)	7 (2.8)	0.89
Angina pectoris, *n*, %	5 (1.6)	8 (3.2)	0.26
Cerebral stroke, *n*, %	19 (3.1)	11 (4.4)	0.45
Diabetes mellitus, *n*, %	26 (8.3)	45 (18)	0.001*
Smokers, *n*, %	178 (56.9)	108 (43.2)	0.003*
Hypertension, *n*, %	137 (43.8)	114 (45.6)	0.608
Biochemistry and Image Results			
Peak value of CK-MB, U/L	129.9 ± 79.4	146.0 ± 39.8	<0.001*
Killip grade I, *n*, %	217 (69.3)	141 (56.4)	<0.001*
Left ventricular ejection fraction	0.52 ± 0.01	0.50 ± 0.01	0.001*
Left atrial diameter, mm	35.5 ± 6.6	34.8 ± 8.6	0.201
Serum potassium, mmol/L	3.83 ± 0.38	3.80 ± 0.43	0.075
Medication During Hospitalization			
Aspirin, *n*, %	295 (94.2)	225 (90)	0.078
Clopidogrel, *n*, %	270 (86.2)	208 (83.2)	0.334
Statins, *n*, %	300 (95.8)	230 (92)	0.070
Low molecular weight heparin, *n*, %	248 (79.2.3)	195 (78)	0.756
Warfarin, *n*, %	38 (12.1)	50 (20)	0.014*
β blocker, *n*, %	137 (43.8)	114 (45.6)	0.391
ACEI, *n*, %	304 (97.1)	219 (87.6)	<0.001*
GPIIb/IIIa, *n* %	54 (17.3)	59 (23.6)	0.057
Diuretics, *n*, %	177 (56.5)	199 (79.6)	<0.001*
Reperfusion Therapy			
Acute anterior myocardial infarction, *n*, %	110 (35.1)	80 (32)	0.531
Patients which PCI were performed, *n*, %	247 (68)	178 (70)	0.076
Triple vessels or left main leision	117 (37.4)	84 (33.6)	0.427
Rate of new onset atrial fibrillation	29 (9.2)	54 (21.6)	<0.001*

*NFHG, no fasting hyperglycemia; FHG, fasting hyperglycemia; ACEI, renin angiotensin converting enzyme inhibitor; PCI, percutaneous coronary intervention; *P < 0.05.*

TABLE 2 | Sub-group analysis.

	Killip grade I		Killip grade II		Killip grade III–IV	
	NFHG $n = 216$	FHG $n = 140$	NFHG $n = 82$	FHG $n = 70$	NFHG $n = 15$	FHG $n = 40$
Gender, male, %	186 (86.1)	101 (72.1)*	65 (79.3)	53 (75.7)	14 (93.3)	29 (72.5)
Age, year, $\bar{x} \pm s$	61.8 ± 12.8	63.2 ± 13.2	64.2 ± 12.2	65.0 ± 12	65.7 ± 4.1	64.0 ± 2.4
Left ejection fraction $\bar{x} \pm s$	0.54 ± 0.11	0.51 ± 0.13	0.50 ± 0.11	0.46 ± 0.12	0.46 ± 0.11	0.45 ± 0.13
Serum potassium $\bar{x} \pm s$	3.82 ± 0.40	3.80 ± 0.42	3.82 ± 0.36	3.82 ± 0.46	3.79 ± 0.46	3.78 ± 0.42
Left atrial diameter $\bar{x} \pm s$	34.8 ± 5.8	34.1 ± 8.3	37.1 ± 8.1	36.2 ± 8.0	34.8 ± 5.8	34.1 ± 8.3
Diabetes, *n* %	16 (7.4)	28 (20.0)	8 (9.8)	8 (11.4)	2 (13.3)	9 (22.5)
Hypertension, *n*%	94 (43.5)	64 (45.7)	38 (46.3)	33 (47.1)	5 (33.3)	17 (42.5)
Rate of new onset fibrillation, *n*, %	14 (6.5)	23 (16.4)*	10 (12.2)	15 (21.4)§	5 (33.3)	16 (40)

*Baseline characteristic and new onset AF comparation between NFHG group and FHG group under different killip level. *p < 0.01; §p < 0.001.*

TABLE 3 | Association between fasting blood glucose and new onset atrial fibrillation.

FBG	Incident rate	Model 1		Model 2		Model 3	
		OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Continuous variables, per mmol/L	83/563	1.07 (1.02, 1.12)	0.006	1.06 (1.01, 1.11)	0.016	1.05 (1.00, 1.10)	0.044
≥7 mmol/L	55/250	2.88 (1.75, 4.74)	<0.001	2.79 (1.68, 4.63)	<0.001	2.56 (1.53, 4.30)	<0.001
<7 mmol/L	28/313	Reference		Reference		Reference	

Model 1, adjusted for age, gender; Model 2, further adjusted for smoking, hypertension, previous MI, previous stroke, creatine; Model 3, further adjusted for previous DM, ejection fraction.

7.0 mmol/L), and 313 patients were assigned to the NFHG group (FG < 7.0 mmol/L). The patient flowchart is shown in **Figure 1**.

Of the 563 patients enrolled, 448 (79.6%) were male, and the average age was 62.59 ± 13.20 years. AMI patients were treated according to guidelines recommendation. Eighty-three patients (14.7%) were complicated with new-onset AF. Fifty-two cases of which were paroxysmal AF while the other 31 were persistent AF. All 52 cases with paroxysmal AF successfully recovered sinus rhythm spontaneously. Twenty-five of the other 31 persistent AF were also converted to sinus rhythm either by direct-current, or by pharmacological cardioversion. Individualized antithrombotic therapy was also given to patients according to the guidelines recommendation and clinical evaluation. Participants in the FHG group were more likely to be female, non-smoker, lower LVEF, higher Killip classification, and previous DM (**Table 1**).

Correlation Between Fasting Hyperglycemia and New-Onset AF in Patients With AMI

New-onset AF occurred more often in the FHG group than in the NFHG group (21.6 vs. 9.2%, $p < 0.001$), **Table 1**. The FHG group showed worse pump function than the NFHG group. We performed subgroup analysis according to different Killip classifications to further clarify the relationship between hyperglycemia and AF. In subgroup Killip grade I, the FHG group had a higher rate of new-onset AF than the NFH group (16.6 vs. 6.5%, $p = 0.002$), and a similar trend was observed in subgroup Killip grade II (17.1 vs. 6.9%, $p = 0.005$), but not for Killip grade III–IV (40 vs. 33.3%, $p = 0.761$). **Table 2** shows the comparison of baseline variables and incidence of AF among groups.

We performed a multivariate logistic regression analysis in model 1 to model 3 by subsequently adjusting confounding risk factors to explore whether FHG was independently correlated with new-onset AF. In the model that measured FG as a continuous variable, an increase of 1 mmol/L in FG was associated with a 5% higher rate of new-onset AF [(odds ratio, 1.05; 95% CI, 1.00–1.10); $P = 0.044$], after adjusting for all co-variables (**Table 3**). Results were similar when we categorized individuals into the FHG and NFHG groups in both unadjusted and adjusted models ($P < 0.001$). In the final model, the odds ratio for the FHG group was 2.56 (95% CI, 1.53–4.30; $p < 0.001$), taking the NFHG group as reference (**Table 3**).

Incidence of New-Onset AF Among Stress Hyperglycemia, Newly Diagnosed DM, and Previously Diagnosed DM

According to the levels of fasting blood glucose, 2 h postprandial blood glucose (determined by the oral glucose tolerance test) and glycosylated hemoglobin (HbA1c), patients were divided into the following groups: previously diagnosed with diabetes mellitus (previous DM), newly diagnosed with diabetes mellitus (N-DM) and stress hyperglycemia (SHG). The previous DM, N-DM, and SHG patients had a higher rate of new-onset AF than patients in the normal FG group, while there was no significant difference among the previous DM, N-DM, and SHG groups (**Figure 2**).

In-Hospital and Long-Term Mortality

Either hyperglycemia or new-onset AF contributed to the increased in-hospital mortality in patients with myocardial infarction. Compared with the other patients, those complicated with both FHG, and new-onset AF exhibited the highest in-hospital mortality (**Figure 3**). Multivariate logistic regression analysis showed that both new-onset AF and FHG were independent risk factors for in-hospital mortality after adjusting other traditional risk factors (**Table 4**).

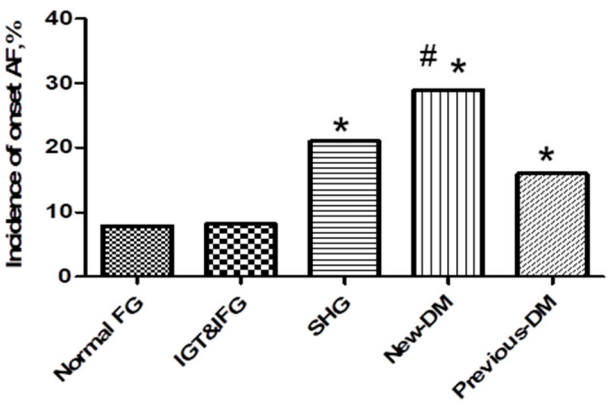

FIGURE 2 | Incidence of new-onset atrial fibrillation among stress hyperglycemia (SHG group), newly diagnosed diabetes (New-DM group), and previous diabetes (previous-DM group). Previous-DM patients, newly diagnosed DM patients and SHG patients had a higher rate of new-onset fibrillation compared with patients with normal FG group (16 vs. 29 vs. 21 vs. 7.8%,*$P < 0.05$), while there is no statistic difference among the three groups (16 vs. 29 vs. 21%, $^{\#}p = 0.152$).

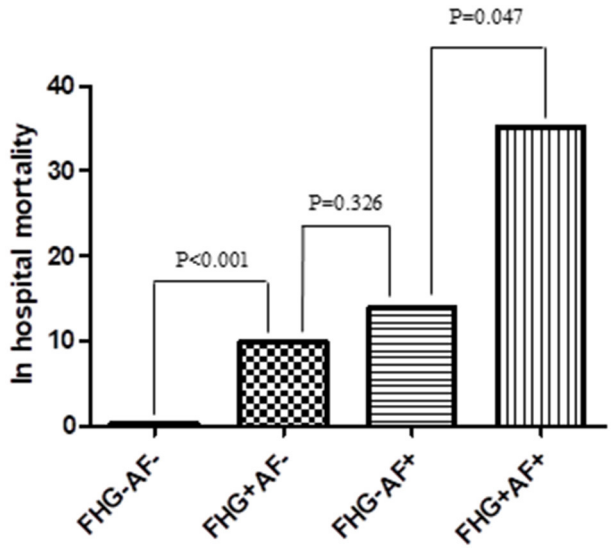

FIGURE 3 | Comparison of in hospital mortality among groups. FHG+AF– group showed higher rate of in hospital mortality compared with FHG–AF– group (9.7 vs. 0.3%, $P < 0.001$). In hospital mortality was higher in FHG+AF+ group compared with FHG–AF+ group (35.2 vs. 13.8%, $P = 0.047$), while there was no difference between FHG+AF– and FHG–AF+ group (9.7 vs. 13.8%, $P = 0.326$). FHG–AF–, patients with neither fasting hyperglycemia nor new-onset atrial fibrillation; FHG+AF– patients with fasting hyperglycemia but without new-onset atrial fibrillation; FG–AF+, patients with new-onset atrial fibrillation but without fasting hyperglycemia; FG+AF+, patients with both fasting hyperglycemia and new-onset atrial fibrillation.

A total of 520 patients were discharged except for 43 deaths during hospitalization. Forty-two other cases were lost with follow-up information and 94 deaths were recorded during an average of 11.2 ± 0.68 years of follow-up. Kaplan-Meier analysis showed that patients complicated with either FHG or AF showed a higher cumulative rates of all-cause mortality than those with neither AF nor FHG. Patients complicated with both FHG and AF showed the highest long-term all-cause mortality (P for log-rank test = 0.002, **Figure 4**). Multivariate Cox regression showed that AMI patients with both FHG and AF (FHG+AF+) were independently correlated with long term all-cause mortality after adjusting other confounding risk factors (odds ratio, 3.13; 95% CI 1.64–5.96, $P = 0.001$, **Table 5**).

DISCUSSION

To the best of our knowledge, this is the first study to systematically explore the relationship between FHG and new-onset AF, and their combined impact on prognosis in patients with AMI with a long-term follow-up. The main findings of the article are as follows: (i) fasting hyperglycemia was independently associated with new-onset AF after adjusted other traditional risk factors in patients with AMI. (ii) AMI patients complicated with both FHG and new-onset AF showed worse in-hospital mortality and long-term all-cause mortality than FHG or AF alone.

AF is the most common super-ventricular tachyarrhythmia in the general population (9, 10). It is also frequently complicating the clinical course after an acute myocardial infarction with a reported incidence of 5–23% (9, 11–13). In this study, we found that 81 of 549 (14.8%) AMI patients complicated with new-onset AF. Our result was consistent with previous reports (9, 11–13). Several clinical variables such as age, congestive heart failure, kidney disease, hypertension, diabetes, and pulmonary disease have been reported as risk factors for new-onset AF (12, 14–18). However, whether FHG was an independent risk factor for new-onset AF in AMI patients was not established. Several large sample size prospective epidemiologic studies based on nationwide or community databases have showed that, in the general population, FG was independently correlated with AF (19–21). However, whether FG remained an independent risk factor for new-onset AF in AMI has not been reported. In this study, we found new-onset AF occurred more often in the FHG group than in the NFHG group, and this association was consistent in several subgroup analyses under different Killip grades. To further clarify the relationship, we subsequently adjusted confounding risk factors such as age, gender, smoking, hypertension, previous MI, previous stroke, creatine, previous DM and ejection fraction, and found that FG remained an independent risk factor for AF. We then categorized individuals into FHG group and normal group, the results were similar.

AMI patients complicated with hyperglycemia contained three clinical status: prior or new diagnosed diabetes, prior or newly diagnosed pre-diabetes and stress hyperglycemia (2). In our study, we compared the rate of new-onset AF among (SHG), newly diagnosed diabetes (new-DM), previously diagnosed diabetes (previous-DM), prior or newly diagnosed pre-diabetes group. We found that DM, N-DM, and SHG patients had a higher rate of new-onset AF than the normal FG-group or pre-diabetes group, while there was no significant difference among the DM, N-DM, and SHG groups. After adjustment for previous diabetes and other confounding risk factors, FHG independently correlated with new-onset AF (**Table 3**). Our results may be valuable in reminding clinicians to pay more attention to the short-term fluctuation in fasting blood glucose after AMI.

The exact mechanism by which hyperglycemia is closely related to an increased incidence of new-onset AF in AMI is still unclear, and several reasons may explain this. First, in AMI, the strong excitation of the sympathetic adrenal medulla releases a large amount of catecholamines, glucagon hormones and growth hormones, which can reduce glycogen synthesis and glucose utilization in myocardial cells (22). In addition, hyperglycemia leads to platelet aggregation, fibrinolysis inhibition, and collateral circulation disturbance, which increases the imbalance of myocardial energy supply and demand (6, 23, 24). This affects the polarization of cardiac myocytes and is associated with various arrhythmias, including AF (25). Second, the hyperosmotic state of blood caused by hyperglycemia has an important impact on the electrophysiological activity of atrial myocytes in circumferential pulmonary veins, which may also be one of the important reasons for the increased incidence of AF (26). Evidence from animal experiment showed that hyperglycemia can lead to spatial heterogeneity (nerve remodeling) of autonomic innervation of atrial myocytes, which was also a potential mechanism of increased incidence of AF (27). Finally, part of AMI patients complicated with FHG is due to

TABLE 4 | Logistic regression analysis for risk factors attributing to in-hospital mortality in the study.

Variable	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age, years	1.019 (0.994–1.045)	0.132		
Gender, male	0.633 (0.314–1.276)	0.201		
History of HBP	2.362 (1.228–4.547)	0.01		
History of DM	1.658 (0.736–3.735)	0.222		
Killip grade II–IV	3.161 (1.660–6.019)	<0.001*	1.446 (0.647–3.232)	0.369
Peak value of CK-MB	1.000 (0.999–1.001)	0.938		
With left main or tripple vessels lesions	0.520 (0.251–1.079)	0.079		
Left ejection fraction	0.007 (0.001–1.067)	<0.001*	0.031 (0.002–0.533)	0.017*
Left atrium diameter	0.989 (0.948–1.031)	0.594		
Complicated with FHG	11.130 (4.130–28.741)	<0.001*	8.134 (2.664–24.841)	<0.001*
With new onset AF	6.580 (3.400–12.736)	<0.001*	6.612 (2.878–15.191)	<0.001*
PCI performed	0.348 (0.184–0.658)	0.001*	0.583 (0.257–1.322)	0.196
Use of ACEI/ARB	0.097 (0.045–0.208)	<0.001*	0.342 (0.114–1.024)	0.055
Use of β-blocker	0.213 (0.108–0.420)	<0.001	0.226 (0.081–0.628)	0.044*
Use of diuretics	0.890 (0.502–1.581)	0.692		

*DM, diabetic mellitus; HBP, hypertensive blood pressure; CK-MB, creatine kinase-MB isoenzyme; FHG, fasting hyperglycemia; AF, atrial fibrillation; PCI, Percutaneous coronary intervention; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; *P < 0.05.*

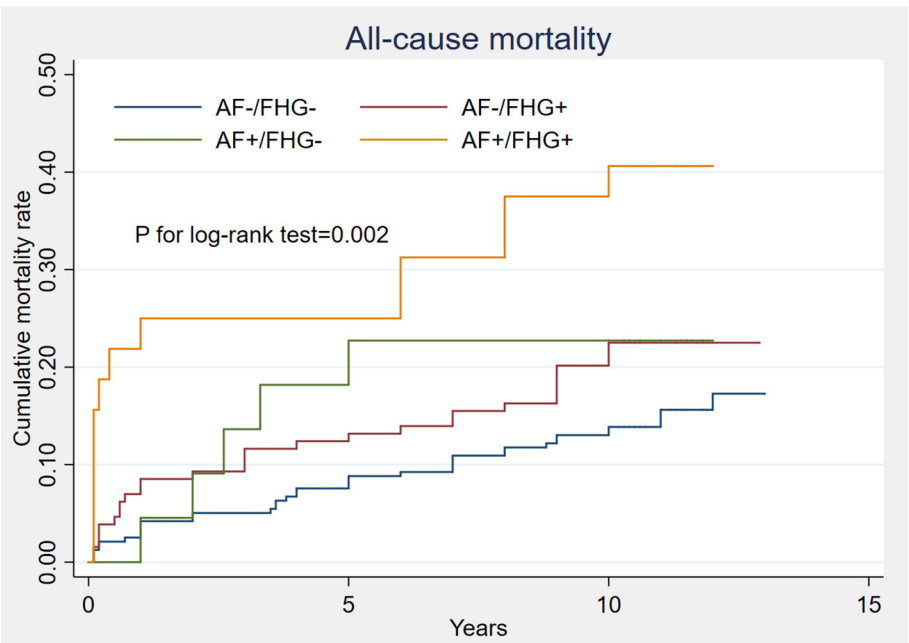

FIGURE 4 | Comparison of long term all-cause mortality under different FHG and new-onset AF status. Kaplan-Meier analysis showed that patients complicated with either FHG (AF–/FHG+) or AF (AF+/FHG–) had obviously higher cumulative rates of all-cause mortality than those with neither AF nor FHG (AF–/FHG–). Patients complicated with both FHG and AF (AF+/FHG+) showed the worst long-term all-cause mortality, *P* for log-rank test was 0.002.

DM and DM is associated with an increased risk of subsequent AF (28, 29). Other FHG patients may be with a higher incidence of metabolic syndrome and non-alcoholic fatty liver disease (NAFLD), which is also associated with the risk of AF (30).

There was sufficient clinical evidence to show that either hyperglycemia or new-onset AF were correlated with poor prognosis in patients with AMI (2–5, 9–11); pre-diabetes was also reported as an important risk factor for all-cause mortality (31). However, there are no data concerning whether hyperglycemia combined with new-onset AF adds more weight to a poor prognosis in AMI patients. In this study, we found AMI patients with either FHG or AF showed higher all-cause mortality, and

TABLE 5 | Association between AF/FHG status and all-cause mortality.

AF/FH status	Incident rate	Model 1		Model 2		Model 3	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
AF–/FHG–	40/266	Reference		Reference		Reference	
AF–/FHG+	33/148	1.47 (0.90, 2.42)	0.123	1.46 (0.88, 2.40)	0.142	1.41 (0.85, 2.35)	0.184
AF+/FHG–	6/27	1.51 (0.59, 3.83)	0.390	1.61 (0.63, 4.12)	0.317	1.44 (0.56, 3.70)	0.453
AF+/FHG+	15/37	3.76 (1.99, 7.09)	<0.001	3.45 (1.82, 6.54)	<0.001	3.13 (1.64, 5.96)	0.001

AF, atrial fibrillation; FHG, fasting hyperglycemia.
Model 1, adjusted for age, gender; Model 2, further adjusted for smoking, hypertension, previous myocardial infraction, previous stroke; Model 3, further adjusted for previous DM, ejection fraction.

patients with both FHG and AF increased three times risk of all-cause mortality after adjusting other confounding risk factors. This may guide our clinical practice: if FHG can be controlled to a certain level, it may decrease the incidence of new-onset AF and long-term all-cause mortality. Larger sample sizes and prospective clinical trials are needed to clarify this problem in the future.

LIMITATIONS

There are several limitations of this study. First, it is a single center retrospective cohort study and it has the defect of missing follow-up. Further prospective studies with larger sample sizes should be conducted to explore the relationship between FG and new-onset AF in patients with AMI. Second, as an observational study, we could not exclude residual confounders, despite being adjusted for potential covariates as much as possible. Finally, we record new-onset AF only by ECG, which may underestimate the incidence of AF in the real world. However, the incidence of new-onset AF in our study is similar to other previous studies (9, 11–13).

CONCLUSIONS

FHG was an independent risk factor for new-onset AF in patients with AMI. AMI patients complicated with FHG and new-onset AF showed worse in-hospital and long-term all-cause mortality than those with FHG or AF alone.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee on Clinical scientific research and laboratory animal of Zhongshan People's Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ML wrote and edited the manuscript. YG, KG, ZW, YL, and JL collected the research data for the article. LF, JD, XH, and YY reviewed the manuscript and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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

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Adropin Alleviates Myocardial Fibrosis in Diabetic Cardiomyopathy Rats: A Preliminary Study

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Aim: Adropin (ADR) is a novel regulatory polypeptide and has important effects on energy metabolism in the heart. However, it is still unclear whether ADR can relieve ventricular remodeling in DCM. Therefore, this study was conducted to assess the effect of ADR on myocardial fibrosis in DCM rats.

Materials and Methods: Twenty Wistar rats were randomly assigned into four groups: healthy control group (CON), DCM model group (DCM), DCM model treated with ADR group (ADR) and DCM model treated with perindopril group (PER). Collagen volume fraction (CVF) and perivascular collagen area (PVCA) were calculated. Diastolic function was assessed by echocardiography. The mitochondrial membrane potential assay was conducted by Rhodamine 123 staining. The protein expression levels of Col I, Col III, Mitofusin-1, Mitofusin-2 and Drp1 were evaluated using western blot.

Results: Compared to CON group, CVF, PVCA and the relative protein expression of Col I, Col III and Drp1 increased in DCM group. And the relative expression of Mitofusin-1 and Mitofusin-2 proteins decreased. During our investigations, CVF, PVCA and the relative protein expression of Col I, Col III and Drp1 decreased in ADR treated rats compared to DCM group. The diastolic function was elevated in ADR group. The fluorescence of Rhodamine 123 and the expression of Mitofusin-1 and Mitofusin-2 also increased in ADR group.

Conclusion: Our study demonstrated that ADR could alleviate myocardial fibrosis and improve diastolic function in DCM rats. ADR may be a putative candidate for the treatment of DCM.

Keywords: myocardial fibrosis, adropin, mitochondrial dynamics, mitofusin, diabetic cardiomyopathy

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INTRODUCTION

Diabetic cardiomyopathy (DCM) is a type of cardiovascular damage and recognized as a specific entity in patients with type 2 diabetes mellitus, excluding coronary atherosclerotic heart disease, hypertrophic cardiomyopathy, hypertensive heart disease and other heart diseases (1, 2). Metabolic disturbances, myocardial fibrosis, small vessel disease and autonomic dysfunction are associated with the development of DCM. The number of deaths caused by diabetes mellitus and its complications is as high as 1.5 to 5 million each year. In addition, more than half of diabetes mellitus related fatal or disabling events are caused by DCM (3).

Mitochondria play a pivotal role in cellular energy transduction. One-third of the heart's volume is composed of mitochondria alone as they are essential organelles in the production of adenosine triphosphate from the oxidation of fatty acid and glucose (4). Mitochondrial dysfunction may contribute to the development of DCM by inducing insulin resistance in skeletal muscle, adipose tissue and pancreatic β-cells (5).

Recent studies have found that Adropin (ADR) is closely related to cardiovascular disease (6–8). ADR, a regulatory polypeptide involved in energy homeostasis, is composed of 76 amino acids and encoded by genes related to energy balance. It is mainly expressed in tissues such as the heart, liver and brain. Besides, it is also expressed in coronary arteries and umbilical veins (9, 10).

ADR can regulate lipid metabolism and insulin resistance. ADR-treated mouse hearts showed improved cardiac efficiency and enhanced insulin signaling compared to control hearts. ADR-treatment protocols *in vivo* and *ex vivo* induced a reduction in the inhibitory phosphorylation of pyruvate dehydrogenase and the protein levels of the responsible kinase pyruvate dehydrogenase kinase 4. These results indicated that ADR has important effects on energy metabolism in the heart (11). ADR can also reduce the risk of atherosclerosis and myocardial ischemia/reperfusion injury by activating the RISK signal transduction pathway (12, 13). Therefore, ADR may be a putative candidate for the treatment of cardiac disease associated with impaired insulin sensitivity (7, 11, 14).

However, it is still unclear whether ADR can relieve ventricular remodeling by regulating mitochondrial dynamics in DCM. Therefore, the present study was conducted to assess the effect of ADR myocardial fibrosis in DCM rats.

MATERIALS AND METHODS

Subjects

Twenty Wistar rats were purchased from Model Animal Research Center of Nanjing University (SCXK Su. 2015-0001). All rats were 8 weeks of age at the beginning of the study. The rats were housed individually under room temperatures with 12: 12 h light/dark cycle and free access to food and water for the duration of the study. All experimental procedures were approved by Ethics Committee of North Sichuan Medical College and complied with the guidelines for the Care and Use of Laboratory Animals.

Experimental Design

The rats were randomly assigned into four groups: healthy control group (CON), DCM model group (DCM), DCM model treated with ADR group (ADR) and DCM model treated with perindopril group (PER). In CON group, rats were given normal saline gavage (1 ml/100 g/d) for 4 weeks without modeling operation. In DCM group, rats were given normal saline gavage (1 ml/100 g/d) for 4 weeks after modeling operation. In ADR group, rats were given ADR gavage (5 μg/100 g/d) for 4 weeks after modeling operation (15). In PER group, rats were given PER gavage (200 μg/100 g/d) for 4 weeks after modeling operation (16). After the intervention, all rats were sacrificed with cervical dislocation.

DCM Rat Modeling

One-time intraperitoneal injection of 1% streptozotocin at a dose of 70 mg/kg was used to destroy the function of pancreatic β cells. The rats' tail vein blood samples were taken on the third and seven day after the injection to test the fasting blood glucose. If both the blood glucose were more than 16.7 mmol/L, and the rats had polydipsia, polyphagia and polyuria, a rat model of DCM was established (17, 18).

Echocardiography

Two-dimensional and Doppler echocardiography was performed to assess diastolic function using a GE Vivid 7 echocardiograph with a 10-MHz phased array probe. Ratio of peak velocity of early (E) to late atrial (A) mitral flow (E/A) was detected. Circumferential Strain rate in early (SRe) and late (SRa) diastole were obtained from parasternal short-axis gray-scale images.

Left Ventricular Weight/Body Weight Ratio

Body weights were measured before the rats were sacrificed. The left ventricle was freed immediately after the heart was taken. An electronic balance was used to weigh the left ventricular mass. LVW/BW ratio (mg/g) = left ventricular weight (mg)/body weight (g) (19).

Collagen Volume Fraction and Perivascular Collagen Area

Masson staining was used to evaluate the severity of myocardial fibrosis (20). Based on Masson staining sections of myocardial tissues, CVF and PVCA were calculated by Image Pro Plus software version 6.0. Masson tri-color staining kit were purchased from KeyGEN Biotech (Nanjing, China).

Immunohistochemistry of Collagen I

Histological determinations of cardiac fibrosis were performed in 5 μm thick sections of paraffin-embedded myocardial tissue. The immunochemistry was performed following the protocol. For each immunochemistry and staining, serial sections were done. The most representative image was shown. Image J software was used to quantitatively calculate the expression of Col I in myocardial tissue.

Rhodamine 123

The mitochondrial membrane potential assay in myocardial tissue was conducted by Rhodamine 123 staining. Disruption of mitochondrial potential has been shown to be featured with the decrease in Rhodamine 123 retention and fluorescence (21). The microtubes containing 1 mL of mitochondrial solution mixed with 10 μl of rhodamine 123 dye solution were incubate at 37°C for 1 h in the dark. After washing three times with PBS, the fluorescence intensity was measured with a microplate reader.

Western Blot

The protein expression levels of Col I and Col III in myocardial tissue were evaluated using western blot, with

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein as the reference. The relative proteins of mitochondrial dynamics such as Mitofusin-1, Mitofusin-2 and Drp1 in myocardial tissue were also detected using western blot, with voltage-dependent anion channel (VDAC) protein as the reference. About 100 mg of myocardial tissue were cut into small pieces and put into a pre-cooled EP tube. Total protein concentration was determined according to the instructions of the BCA kit. The supernatants were collected. Then they were separated by SDS-polyacrylamide gels and transferred to a PVDF membrane.

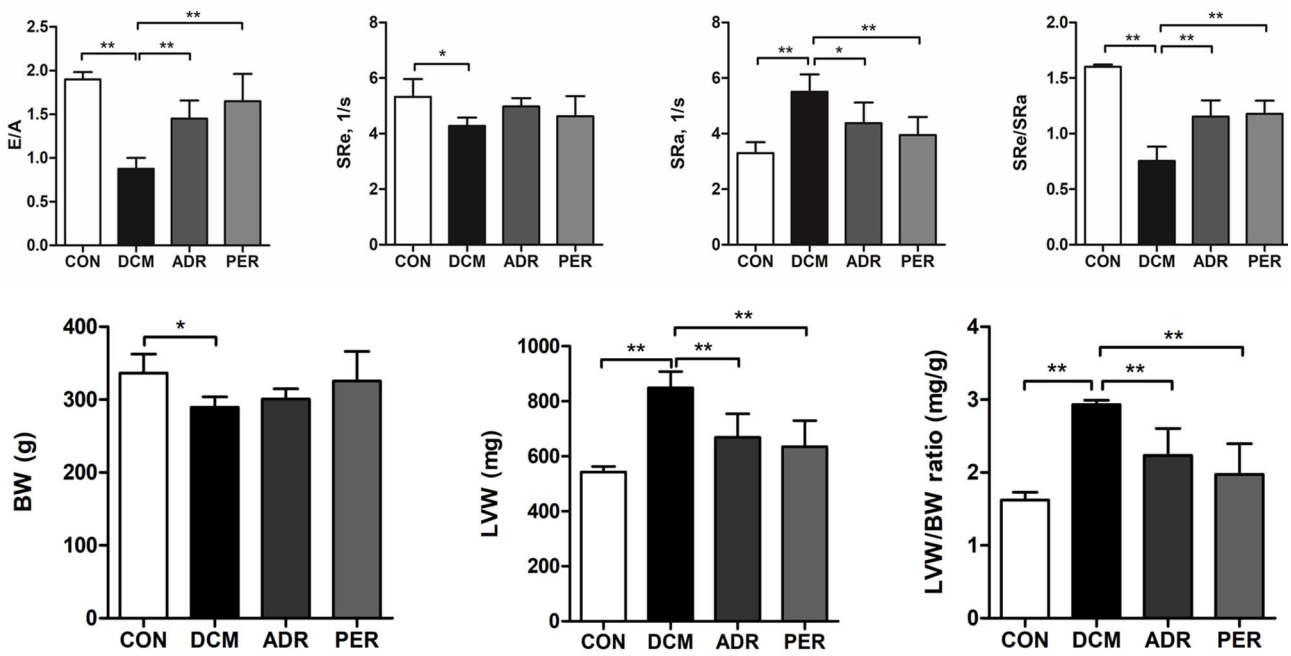

FIGURE 1 | Effects of ADR on diastolic function and LVW/BW ratio in DCM rats. E/A ratio and SRe/SRa ratio dercreased in DCM group compared with CON group. Compared to DCM group, E/A ratio and SRe/SRa ratio increased significantly in ADR group. Compared to CON group, LVW/BW ratio increased in DCM group. ADR can decrease LVW/BW ratio significantly. It also decreased in PER group, compared with DCM group. CON, control; DCM, diabetic cardiomyopathy; ADR, adropin; PER, perindopril; E/A, ratio of peak velocity of early (E) to late atrial (A) mitral flow. SRe and SRa, strain rate in early and late diastole, respectively. LVW, left ventricular weight; BW, body weight. Values are expressed as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.001$.

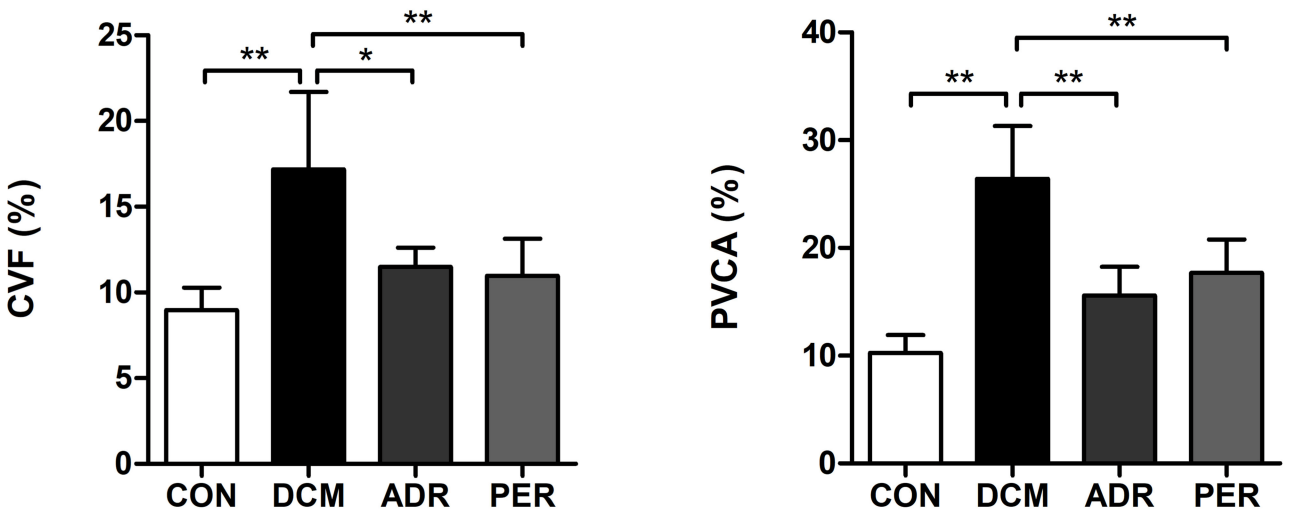

FIGURE 2 | Effects of ADR on CVF and PVCA of myocardial tissue in DCM rats. Compared to DCM group, CVF and PVCA decreased in ADR and PER group. CON, control; DCM, diabetic cardiomyopathy; ADR, adropin; PER, perindopril; CVF, collagen volume fraction; PVCA, perivascular collagen volume area. Values are expressed as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

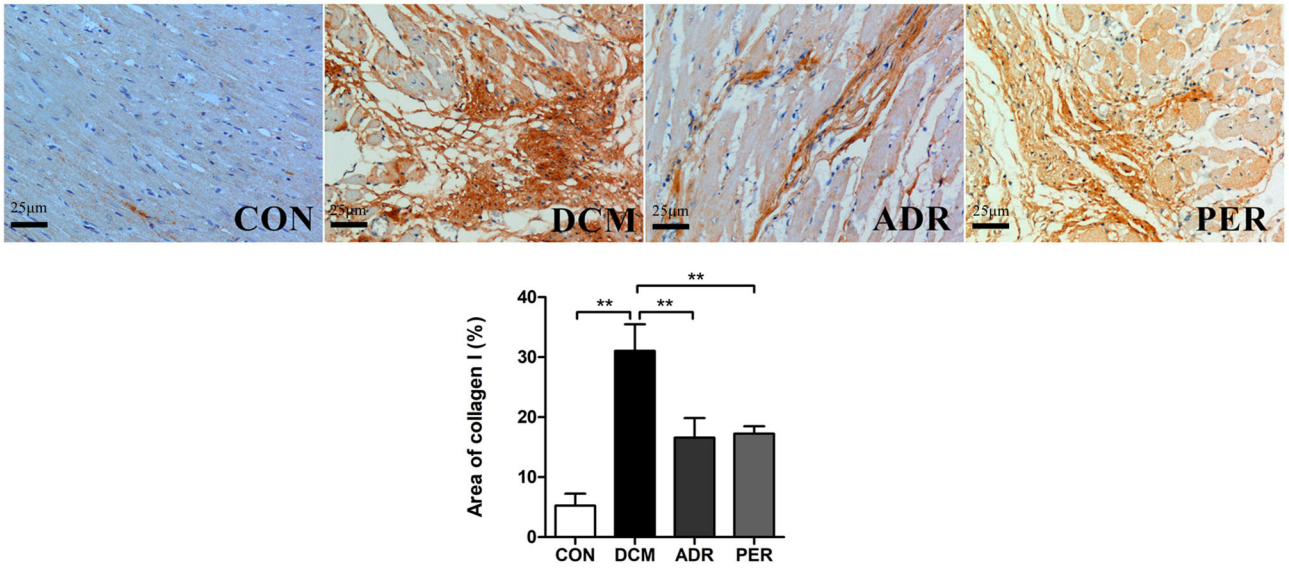

FIGURE 3 | Immunohistochemistry of Col I in myocardial tissues in DCM rats (x 200). Compared to DCM group, the color of collagen fibers in ADR group and PER group becomes lighter and the expression of Col I reduced remarkably. CON, control; DCM, diabetic cardiomyopathy; ADR, adropin; PER, perindopril. Values are expressed as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$.

The membranes were blocked with 5% skimmed milk powder TBST solution for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. The remaining liquid on the membrane was washed with TBST. Images were captured with Tanon 6600 luminescence imaging workstation (Tanon Science & Technology Co., Ltd. Shanghai, China).

Statistical Analysis

Data were presented as group mean and its standard error (mean ± SEM). One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) *post hoc* test was used for the statistical analysis of the difference between multiple groups. All statistical analyses were performed in SPSS 20.0 (IBM Corp., Armonk, USA).

RESULTS

Effects of ADR on Diastolic Function and LVW/BW Ratio

As shown in **Figure 1**, E/A ratio and SRe/SRa ratio were used to assess the diastolic function of DCM rats. LVW/BW ratio was used to assess the severity of cardiac remodeling in DCM rats. E/A ratio (1.90 ± 0.09 vs. 0.89 ± 0.12, $P < 0.001$) and SRe/SRa ratio (1.60 ± 0.02 vs. 0.75 ± 0.13, $P < 0.001$) dercreased in DCM group compared with CON group. Compared to DCM group, E/A ratio (1.43 ± 0.18 vs. 0.89 ± 0.12, $P = 0.001$) and SRe/SRa ratio (1.15 ± 0.15 vs. 0.75 ± 0.13, $P < 0.001$) increased significantly in ADR group. LVW (543.26 ± 19.44 mg vs. 849.05 ± 58.91 mg, $P < 0.001$) and LVW/BW ratio (1.62 ± 0.11 mg/g vs. 2.93 ± 0.06 mg/g, $P < 0.001$) increased in DCM group compared with CON group. Compared to DCM group, LVW/BW ratio decreased significantly in ADR (2.23 ± 0.37 mg/g vs. 2.93 ± 0.06 mg/g, $P = 0.005$) and PER group (1.98 ± 0.42 mg/g vs. 2.93 ± 0.06 mg/g, $P = 0.001$).

Effects of ADR on CVF and PVCA of Myocardial Tissue

Using Masson staining sections and Image Pro Plus version 6.0 software, CVF and PVCA of myocardial tissue were calculated (**Figure 2**). Compared to CON group, CVF (8.97 ± 1.31% vs. 17.18 ± 4.53%, $P = 0.001$) and PVCA (10.24 ± 1.68% vs. 26.41 ± 4.92%, $P < 0.001$) in DCM group increased dramatically. CVF and PVCA in ADR group (11.49 ± 1.12% vs. 17.18 ± 4.53%, $P = 0.001$; 15.58 ± 2.67% vs. 26.41 ± 4.92%, $P < 0.001$) and PER group (10.98 ± 2.16% vs. 17.18 ± 4.53%, $P = 0.001$; 17.67 ± 3.12% vs. 26.41± 4.92%, $P < 0.001$) decreased significantly in the comparison with DCM group.

Effects of ADR on Immunohistochemistry of Col I in Myocardial Tissue

The immunohistochemical staining of Col I in myocardial tissue was used to evaluate the severity of cardiac fibrosis (**Figure 3**). Collagen fibers from greenish yellow to orange red and red color as the severity of the cardiac fibrosis increased. In CON group, a small amount of Col I was scattered and the myocardial tissues arranged neatly. The Col I expression increased significantly in DCM group, showing strong positive staining in the form of flake. Quantitative calculation of the Col I expression in myocardial tissue was also made via Image J software. The Col I volume fractions in CON, DCM, ADR and PER group were 5.26 ± 1.98%, 31.06 ± 4.42%, 16.57 ± 3.29%, and 17.25 ± 1.24%, respectively. Compared with the DCM group, the color

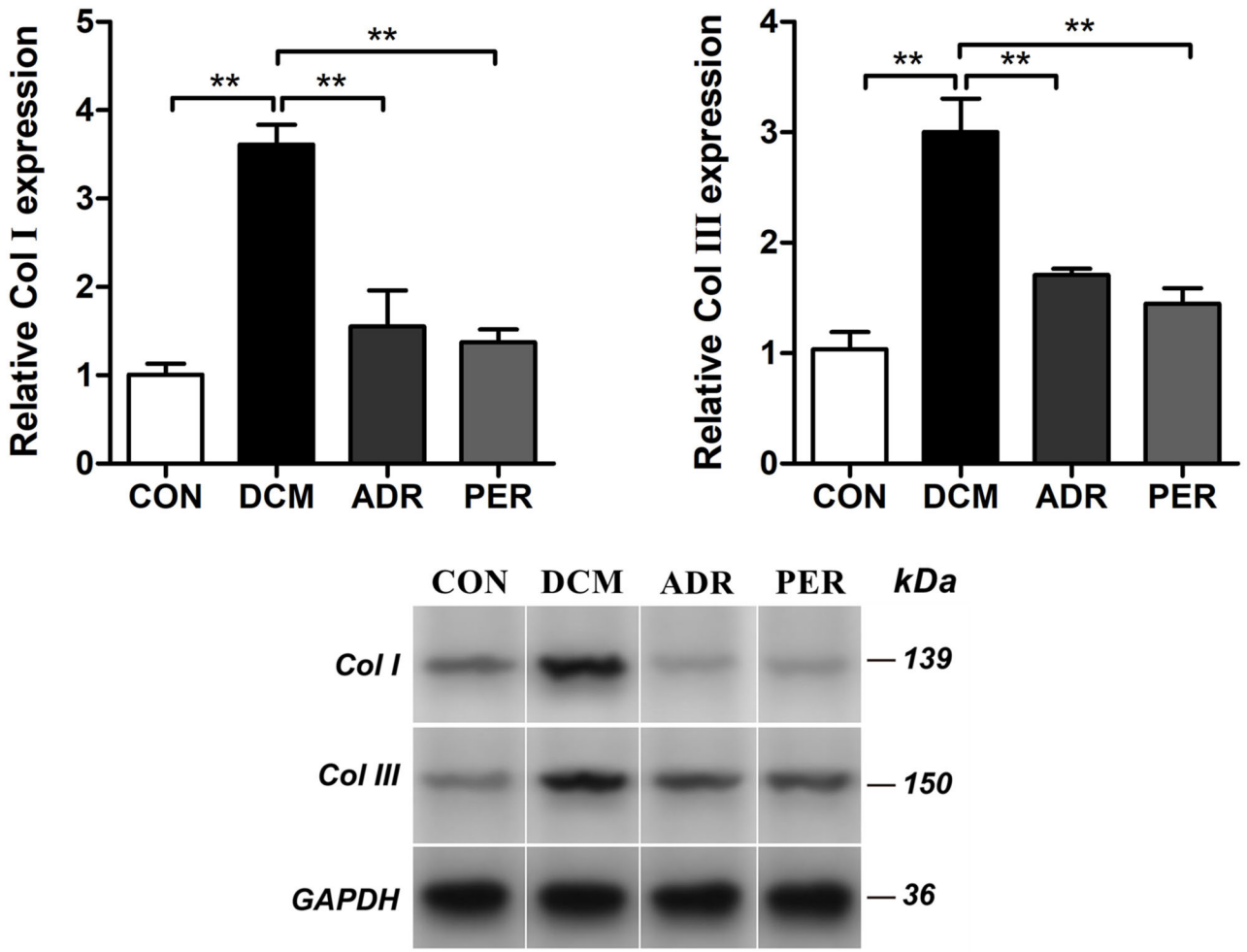

FIGURE 4 | Effects of ADR on relative expression of Col I and Col III in myocardial tissue in DCM rats. Compared to DCM group, the relative expression of Col I and Col III proteins decreased in ADR and PER group. CON, control; DCM, diabetic cardiomyopathy; ADR, adropin; PER, perindopril; Col, collagen. Values are expressed as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$.

of collagen fibers in ADR group and PER group becomes lighter and the expression of Col I reduced remarkably ($P < 0.001$).

Effects of ADR on Relative Expression of Col I and Col III Proteins

The expression of Col I and Col III proteins in myocardial tissue were detected with Western Blot (**Figure 4**). Compared to CON group, the relative expression of Col I (1.01 ± 0.13 vs. 3.61 ± 0.23, $P < 0.001$) and Col III (1.03 ± 0.15 vs. 3.00 ± 0.30, $P < 0.001$) increased dramatically in DCM group. The relative expression of Col I decreased in ADR (1.55 ± 0.41 vs. 3.61 ± 0.23, $P < 0.001$) and PER (1.37 ± 0.15 vs. 3.61 ± 0.23, $P < 0.001$) group in the comparison with DCM group. Besides, compared to DCM group, the relative expression of Col III also reduced in ADR (1.71 ± 0.06 vs. 3.00 ± 0.30, $P < 0.001$) and PER (1.45 ± 0.14 vs. 3.00 ± 0.30, $P < 0.001$) group.

Effects of ADR on the Fluorescence of Rhodamine 123

Alteration in the mitochondrial transmembrane potential in myocardial tissue was determined by Rhodamine 123 staining. Disruption of the mitochondrial membrane followed by the loss in the mitochondrial membrane potential is a key step in the mitochondrial apoptotic pathway. As shown in **Figure 5**, the fluorescence of Rhodamine 123 in DCM group (58.97 ± 4.19 vs. 36.28 ± 1.04, $P < 0.001$) decreased significantly in the comparison with CON group. However, the fluorescence of Rhodamine 123 in ADR (48.10 ± 3.58 vs. 36.28 ± 1.04, $P < 0.001$) and PER group (48.19 ± 3.85 vs. 36.28 ± 1.04, $P < 0.001$) increased in the comparison with DCM group.

Effects of ADR on the Relative Expression of Proteins in Mitochondrial Dynamics

The relative proteins of mitochondrial dynamics in myocardial tissue were detected with Western Blot (**Figure 6**). Compared to

CON group, the relative expression of Mitofusin-1 (1.00 ± 0.12 vs. 0.36 ± 0.08, $P < 0.001$) and Mitofusin-2 (1.00 ± 0.13 vs. 0.41 ± 0.04, $P < 0.001$) proteins decreased dramatically. The relative expression of Mitofusin-1 and Mitofusin-2 proteins increased in ADR (0.83 ± 0.14 vs. 0.36 ± 0.08, $P = 0.001$; 0.85 ± 0.11 vs. 0.41 ± 0.04, $P = 0.001$) and PER (0.86 ± 0.07 vs. 0.36 ± 0.08, $P < 0.001$; 0.82 ± 0.12 vs. 0.41 ± 0.04, $P = 0.001$) group, respectively, in comparison with DCM group. Compared to CON group, the relative expression of Drp1 protein (1.00 ± 0.11 vs. 2.89 ± 0.52, $P < 0.001$) increased significantly. However, it decreased in ADR (1.42 ± 0.36 vs. 2.89 ± 0.52, $P = 0.001$) or PER (1.58 ± 0.33 vs. 2.89 ± 0.52, $P = 0.002$) group in comparison with DCM group.

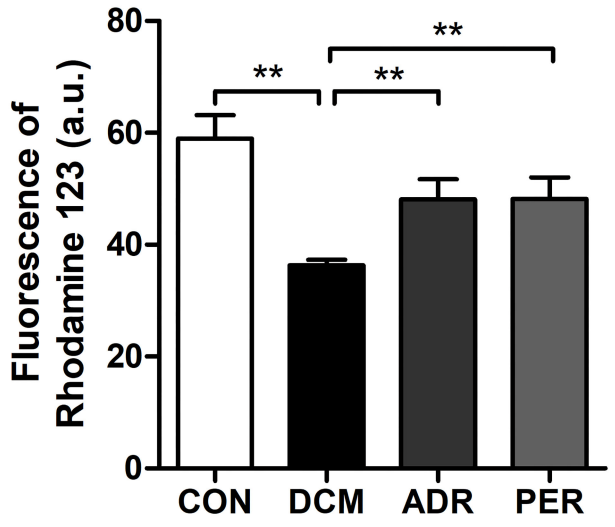

FIGURE 5 | Effects of ADR on the fluorescence of Rhodamine 123 in DCM rats. Compared to DCM group, the fluorescence of Rhodamine 123 increased in ADR and PER group. CON, control; DCM, diabetic cardiomyopathy; ADR, adropin; PER, perindopril. Values are expressed as mean ± standard deviation. *$P < 0.05$, **$P < 0.01$.

DISCUSSION

DCM is one of the most important complications of diabetes mellitus. Mitochondrial dysfunction is an important factor in the occurrence of DCM. The imbalance of mitochondrial dynamics is generally considered the cause of DCM. In the diabetic condition, total adenosine triphosphate was generated from fatty acid oxidation instead of glucose, which generates more reactive oxygen species and disrupts the oxidative phosphorylation process (4). It may cause calcium overload, induce the opening of mitochondrial permeability transition pore and lead to mitochondrial dysfunction (22).

In the present study, we found that ADR reduced LVW/BW ratio, E/A ratio, SRe/SRa ratio, CVF, PVCA and the relative expression of Col I and Col III proteins, indicating that ADR can alleviate myocardial fibrosis and improve diastolic function in DCM rats. Until now there are no published articles discussing the effect of ADR on myocardial fibrosis and cardiac remodeling in DCM. However, ADR was proven to protect against liver fibrosis in a previous study. The authors measured the serum ADR levels, liver injury and oxidative stress in diet-induced non-alcoholic steatohepatitis mice. ADR knockout mice and palmitate treated primary hepatic cells were used to investigate the influence of ADR on liver injury. The results indicated that the treatment with ADR alleviated hepatocyte injury by upregulating the expression of Gclc, Gclm, and Gpx1 in a manner dependent on Nrf2 transcriptional activity and by increasing the glutathione levels (23). Therefore, the present study is the first article to report that ADR may reduce myocardial fibrosis in DCM rats. Moreover, based on the previous related researches on other disease models (12, 23), ADR seems to be effective in improving organ injury and fibrosis.

Moreover, our study also explored the role of mitochondrial dynamics in the process of ADR protecting against myocardial fibrosis in DCM rats. Mitochondria is extremely important for energy supply of heart. As we know, the heart requires large amounts of high-energy phosphates and accounts for ∼8% of the total adenosine triphosphate consumption of the body. The majority of adenosine triphosphate is regenerated in the mitochondria via oxidative phosphorylation (5, 24). Mitochondria are highly dynamic organelles undergoing coordinated cycles of fission and fusion, which referred as mitochondrial dynamics (25). In mammalian cells, the primary regulators of mitochondrial fusion are dynamin-related mitofusins (mitofusin-1 and mitofusin-2) and optic atrophy protein 1 (OPA1). On the other side, mitochondrial fission 1 protein and the dynamin-related protein 1 (DRP1) are involved in mitochondrial fission. The balance of mitochondrial fusion and fission has been recognized as critical processes in the health of mitochondria and cells (5, 26).

The results of this study found that the balance of mitochondrial fusion and fission has been broken in DCM rats compared with CON rats via detecting mitochondrial dynamics related proteins. Besides, ADR can upregulate the relative expression of Mitofusin-1 and Mitofusin-2 proteins and downregulate the expression of Drp1 protein in DCM rats. Thapa et al. (27, 28) had also conducted a series of studies concerning the effect of ADR in cardiac cells and pre-diabetic obese mice. *In vitro* experiments, the stimulation of cultured cardiac cells with ADR may lead to decreased expression of the pyruvate dehydrogenase negative regulator PDK4, which reduced inhibitory PDH phosphorylation. *In vivo* study, cardiac glucose oxidation reduced in high fat diet animals. Acute ADR treatment increased cardiac glucose oxidation. ADR can modulate the expression of the mitochondrial acetyltransferase enzyme GCN5L1, altering the activity of fuel metabolism enzymes to favor glucose utilization. These findings suggest that ADR may be a key regulator of fuel substrate utilization in the heart and may provide a future therapeutic avenue in the treatment of DCM.

There are some limitations in this study. Firstly, much of the current knowledge regarding DCM is the result of studies performed using adult streptozotocin-induced diabetic rodents. However, the mechanism of DCM in this model may be

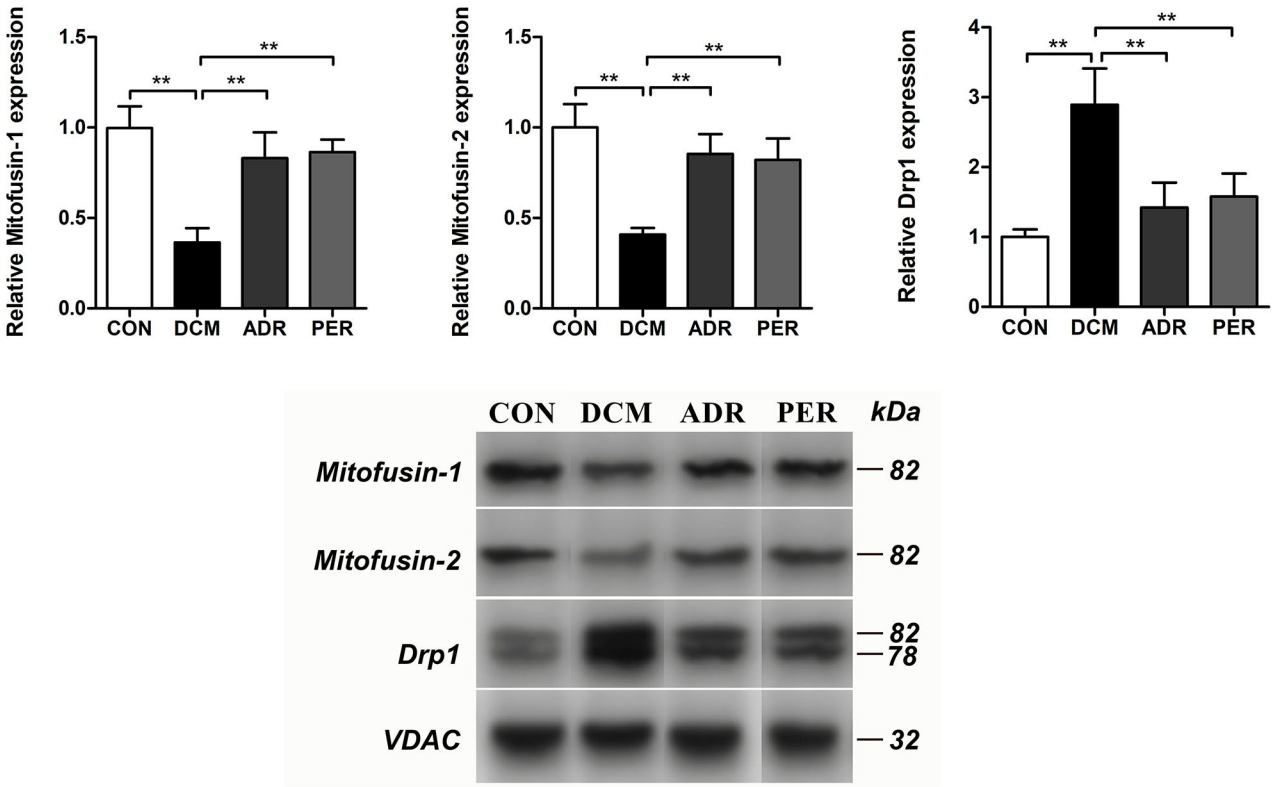

FIGURE 6 | Effects of ADR on the relative proteins of mitochondrial dynamics in DCM rats. Compared to DCM group, the relative expression of Mitofusin-1 and Mitofusin-2 proteins increased in ADR and PER group. The relative expression of Drp1 protein decreased in ADR and PER group in comparison with DCM group. CON, control; DCM, diabetic cardiomyopathy; ADR, adropin; PER, perindopril. Values are expressed as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

related the direct actions of streptozotocin on muscle fibers independent of the diabetic phenotype. Therefore, future studies should be repeated in alternative models of DCM, such as Ins2 (+/Akita) mouse and high fat diet-fed models. Secondly, this is a preliminary study on the effect of ADR in DCM rats. Even though several mitochondrial dynamics related proteins had been detected, other signaling pathways or parameters related to energy metabolism and mitochondrial respiration have not been tested. Besides, this study did not report the results of *in vitro* experiments. The result mainly implied the phenomenon. Further study should be conducted to observe the effects of ADR on different specific cells in myocardial tissue. Moreover, this study did not explore the involvement of mitochondrial apoptosis in DCM. Therefore, further studies are needed in the future to confirm the mechanism of ADR in DCM.

CONCLUSIONS

In summary, the present study demonstrated ADR could alleviate myocardial fibrosis and improve diastolic function in DCM rats. Our present study is the first to describe the effect of ADR on myocardial fibrosis in DCM rats. ADR may be a putative candidate for the treatment of DCM.

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 reviewed and approved by Ethics Committee of North Sichuan Medical College.

AUTHOR CONTRIBUTIONS

ML and JA: conception and design. ML: administrative support. ZS and JA: provision of study materials or patients. ZS, JA, and KT: collection and assembly of data. JA, KT, ZL, and YH: data analysis and interpretation. ML, JA, and YH: manuscript writing. 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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frontiers in Cardiovascular Medicine

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Impact of Metabolic Syndrome and It's Components on Prognosis in Patients With Cardiovascular Diseases: A Meta-Analysis

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Background: Patients with metabolic syndrome (MetS) have a higher risk of developing cardiovascular diseases (CVD). However, controversy exists about the impact of MetS on the prognosis of patients with CVD.

Methods: Pubmed, Cochrane library, and EMBASE databases were searched. Cohort Studies and randomized controlled trials *post hoc* analyses that evaluated the impact of MetS on prognosis in patients (≥18 years) with CVD were included. Relative risk (RR), hazard rate (HR) and 95% confidence intervals (CIs) were calculated for each individual study by random-effect model. Subgroup analysis and meta-regression analysis was performed to explore the heterogeneity.

Results: 55 studies with 16,2450 patients were included. Compared to patients without MetS, the MetS was associated with higher all-cause death [RR, 1.220, 95% CI (1.103 to 1.349), *P*, 0.000], CV death [RR, 1.360, 95% CI (1.152 to 1.606), *P*, 0.000], Myocardial Infarction [RR, 1.460, 95% CI (1.242 to 1.716), *P*, 0.000], stroke [RR, 1.435, 95% CI (1.131 to 1.820), *P*, 0.000]. Lower high-density lipoproteins (40/50) significantly increased the risk of all-cause death and CV death. Elevated fasting plasma glucose (FPG) (>100 mg/dl) was associated with an increased risk of all-cause death, while a higher body mass index (BMI>25 kg/m^2) was related to a reduced risk of all-cause death.

Conclusions: MetS increased the risk of cardiovascular-related adverse events among patients with CVD. For MetS components, there was an increased risk in people with low HDL-C and FPG>100 mg/dl. Positive measures should be implemented timely for patients with CVD after the diagnosis of MetS, strengthen the prevention and treatment of hyperglycemia and hyperlipidemia.

Keywords: cardiovascular disease, metabolic syndrome, all-cause death, prognosis, meta-analysis

INTRODUCTION

Cardiovascular disease (CVD) has attracted worldwide attention and accounts for 46.2% of deaths from non-communicable diseases (1). CVD is one of the main causes of premature death and disability. Metabolic syndrome (MetS), including dysglycemia, obesity (especially central obesity), high blood pressure, low high-density lipoprotein cholesterol (HDL-C), and elevated triglyceride levels, is a complex of risk factors for type 2 diabetes and CVD (2). Patients with MetS have a higher risk of developing CVD compared with those without MetS in the next 5–10 years, and the long-term risk is even higher (3). The National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III criteria also considered MetS as the second major target for CVD prevention (4).

The prevalence of MetS is higher in patients with CVD than in patients without MetS. The prevalence of MetS in hospitalized patients with acute myocardial infarction (AMI) is 46%, similar to that of the acute coronary syndrome (43.4%) (5); This finding indicates that MetS is associated with CVD. Boulon et al. (6) suggested that despite active management, patients with MetS have a higher long-term risk of cardiovascular events (6). However, Selcuk et al. (7) suggested that the main determinant of long-term prognosis of AMI is heart failure rather than metabolic disorder (7). But some researchers suggested that MetS does not increase the mortality among patients with CVD (8). Therefore, controversy exists about the impact of MetS on patients with CVD.

MetS is a disease associated with multiple factors, and the main diagnostic indicators (components) include blood pressure, overweight and obesity, HDL-C, and fasting blood glucose (9, 10). Most studies have focused on the overall effect of MetS on the prognosis of CVD. However, whether a correlation exists between each component and prognosis and which factor is more important have not been elucidated. Considering these inconsistencies, we performed a meta-analysis of cohort studies and RCT *post-hoc* analysis from CVD patients to evaluate associations between different definitions of MetS and the risk of all cause death, CV death and cardiovascular events.

METHODS

The study was registered with PROSPERO (CRD42021147609), and reported in accordance with the PRISMA statement (11).

Inclusion Criteria

Eligible Studies

(1) Influencing factors and study types: Studies that evaluated the influence of MetS and its components on patients with CVD were included. We included cohort and randomized controlled trials *post hoc* analyses and excluded single-group observational studies. (2) Types of patients: Patients with CVD were aged ≥18. (3) Outcomes: Primary outcomes were all-cause death, cardiovascular (CV) death, incidence of MI and stroke. Secondary outcomes were TVR, heart failure, cardiac arrest, angina pectoris, cardiogenic shock. All-cause death of high TG, low HDL-C, high BP, FPG>100 mg/dl, BMI>25kg/m^2, high WC. CV death of high TG, low HDL-C, high BP, FPG>100 mg/dl, BMI>25kg/m^2.

The definition of cardiovascular disease in this meta-analysis was history (comorbidity) of cardiovascular or cardiac disease. Hypertension/Cardiovascular Infections/Cardiovascular Abnormalities/Pregnancy Cardiovascular Complications/cardiomyopathy in specific terms was excluded because these diseases often overlap and potentially result in overestimation of cases.

Exclusion Criteria

(1) Studies that had incomplete or unavailable original data. (2) The diagnostic criteria for MetS were not specified. (3) Repeated published data. (4) Studies that evaluated the relationship between MetS and congenital heart disease.

Data Sources and Searches

We searched Pubmed, EMBASE, and Cochrane library from inception to October 18, 2020. The following subject and keywords were used in search: "cardiovascular disease," "cardiovascular event," "cardiocerebrovascular disease," "cerebrovascular disease," "cerebrovascular disorder," "cerebrovascular attack," "stroke," "cerebral infarction," "coronary artery disease," "coronary heart disease," "ischemic heart disease," "myocardial infarction;" "metabolic syndrome," "metabolic syndrome x," "Metabolic X Syndrome;" "Randomized controlled trial," "RCT," "Clinical Trials, Randomized," "Cohort Studies," "Follow-Up Studies," "Longitudinal Studies," "Prospective Studies," and "Retrospective Studies". **Supplementary Table 1** presents the search strategy. No date, language, or other restriction were incorporated into the searches. Two researchers (XL and YJZ) performed the data search.

Study Selection

Endnote X9 was used to manage and screen the literature. Title, abstract, and full texts were selected based on inclusion/exclusion criteria. We designed a standardized form to extract data including study characteristics, diagnostic criteria, characteristics of the study population, risk of bias, and outcome measures.

Risk of Bias Analysis

We used the Newcastle–Ottawa Scale (NOS) to assess the quality of the cohort studies (12). To be specific, studies with scores >7 were treated as high quality, 4–6 as medium quality, and below 4 as low quality (13). Cochrane Collaboration's tool for assessing the risk of bias was applied to determine the quality of the included RCT *post-hoc* studies (12, 14). Two researchers (X Li, YJ Zhai) independently screened and extracted the data, and a third researcher (J Lyu) resolved any disagreements. Quality evaluation results are reported in **Supplementary Table 2**.

The diagnostic criteria for MetS vary among different regions and institutions, but the majority of them included central obesity, hypertension, low HDL-C, and high TG and fasting blood glucose (FBG) levels. Other diagnostic criteria also included dyslipidemia, chronic mild inflammation, endothelial dysfunction, insulin resistance, increased oxidative stress. The

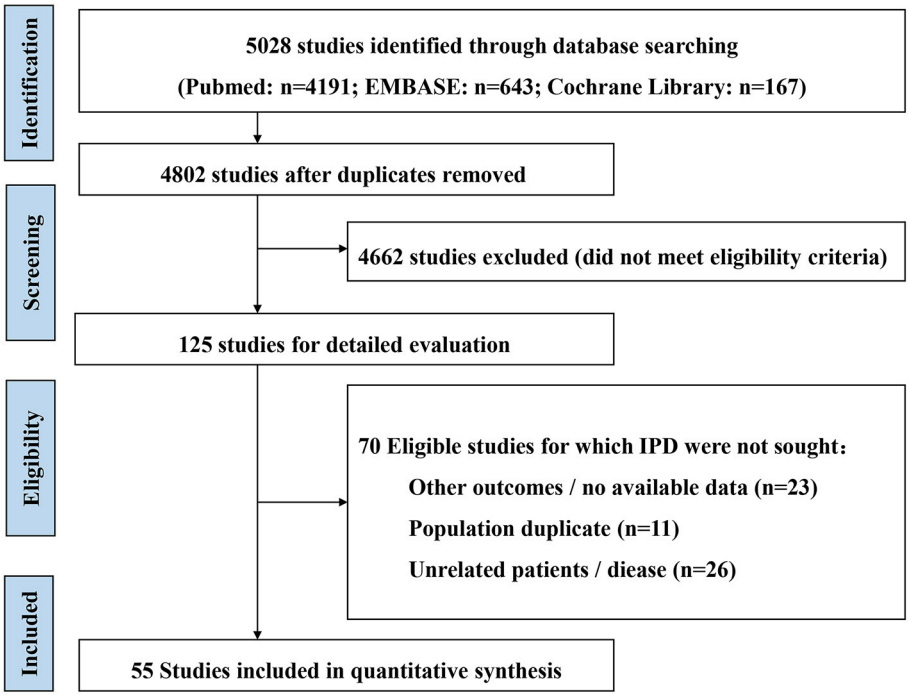

FIGURE 1 | Flow chat of study selection.

diagnostic criteria used in the included studies were NCEP2001 criteria (9), NCEP2005 criteria (4), and The International Diabetes Federation (IDF) criteria (10) (details reported in **Supplementary Table 3**). For specific diagnostic criteria, we compared the above criteria and divide into subgroups based on the comparison results.

Statistical Analysis

Statistical analysis was performed using STATA 13 and R software. For dichotomous outcomes (all-cause death, CV-death, the incidence of MI, stroke, TVR, heart failure, cardiac arrest, angina pectoris, and cardiogenic shock), relative risk (RR) and 95% confidence intervals (CIs) were calculated for each individual study. For the impact of MetS components on patients with CVD (all-cause death and CV death), hazard rate (HR) and 95% confidence intervals (CIs) were determined for each study. The heterogeneity across studies was examined using the Chi-square test and I-square statistics. The results were pooled by the D-L random-effect model due to the large statistical heterogeneity among the studies.

To explore the sources of clinical heterogeneity and methodological heterogeneity, we performed subgroup analysis based on the following: (1) diagnostic criteria, studies were divided into four subgroups (NCEP2001, NCEP2005, IDF and "others") and (2) study type, studies were divided into three subgroups (prospective cohort study, retrospective cohort study, and RCT *post-hoc* study). Meta-regression analysis of three covariates (follow-up time, male proportion, and patient age) was performed to explore the size and source of heterogeneity.

Effect measures [risk ratio (RR) vs. odds ratio (OR) vs. risk difference (RD)] and statistical models (D-L random-effects model vs. M-H fix-effects model) were used to examine the robustness of the results. We evaluated publication bias by Begg's tests and drew contour-enhanced funnel plots to assess whether the asymmetry of the funnel plots was caused by publication bias or other biases.

RESULTS

Overview of the Characteristics of the Studies

A total of 5,028 unique records were identified from the literature search. After excluding 226 duplicate articles, 125 studies were initially included by reading the title and abstract. Fifty-five studies were finally included after further reading the full text, including six RCT *post-hoc* studies (15–20) and 49 cohort studies (3, 5–8, 21–64) (**Figure 1**).

Study Characteristics

A total of 162,450 patients from 25 countries and regions were included, the sample size for each individual study varies from 57 to 44 548. Forty-one studies (145,390 patients) evaluated the risk of all-cause death among patients with CVD and MetS. Twenty-one studies with 95,049 patients reported CV death, 23 studies with 77,618 patients reported the incidence of MI, and 11 studies with 59,770 patients reported the incidence of stroke.

Twenty-six studies adopted NCEP-ATPIII (2005) criteria, 21 studies mainly adopted NCEP-ATPIII (2001) criteria, and 7

TABLE 1 | Characteristics of included studies.

No.	Author	Year	Country	Study design	Follow-up (Years)	Sample	Male (%)	Age (years)	Endpoints	Definition of MetS
1	Anderson	2004	America	Retrospective cohort study	2.80 ± 2.30	2,035	76.00	65 ± 11	②③	NCEP2001
2	Marroquin	2004	America	Prospective cohort study	3.50 (2.80–4.70)	284	0.00	58 ± 12	①②③④⑦	NCEP2001
3	Rana	2005	Netherlands	Prospective cohort study	at least 0.75	901	NA	62 ± 11	②⑤③	NCEP2005
4	Saely	2005	Australia	Prospective cohort study	2.30 ± 0.40	750	67.90	62.6 ± 10.4	②③④⑤	NCEP2001
5	Schwartz	2005	America	RCT *post-hoc* analysis	0.33	3,038	65.00	65 ± 12	①③⑥⑧⑩	NCEP2001
6	Zeller	2005	France	Prospective cohort study	6.90	633	75.00	66.2	①③④⑨	NCEP2001
7	Aguilar	2006	America, New England, Canada	RCT *post-hoc* analysis	3.10	3,319	81.70	62 ± 11	①③⑤⑧	NCEP2001
8	Boulon	2006	France	Prospective cohort study	1.60	480	82.20	61.6 ± 13	①④⑤⑦	NCEP2001,IDF
9	Briand	2006	Canada	Retrospective cohort study	2.30 ± 1.10	105	62.00	69 ± 12	①	NCEP2001
10	Hu	2006	China	Retrospective cohort study	2.30 ± 1.00	2,596	77.70	60.3 ± 10.3	②③④⑤⑦	IDF
11	Kasai	2006	Janpan	Retrospective cohort study	12.00 ± 3.60	748	87.00	59 ± 10	①②⑩	NCEP2001
12	Nigam	2006	Canada	Retrospective cohort study	12.60 ± 5.10	24,958	75.60	52.9 ± 9.3	①③④⑦	NCEP2001
13	Ovbiagele	2006	America	RCT *post-hoc* analysis	1.80	476	61.60	63 ± 11.4	②③④⑩	NCEP2001
14	Espinola-Klein	2007	Germany	Retrospective cohort study	6.70	811	75.10	62.7 ± 9.3	②③④	NCEP2005
15	Hajer	2007	Netherlands	Prospective cohort study	2.80 (0.10–7.50)	2,060	78.00	59.6 ± 10.3	②④	NCEP2001
16	Nakatani	2007	Janpan	Prospective cohort study	2.00	3,858	76.00	64.7 ± 11.4	②③	NCEP2001
17	Canibus	2008	Italy	Prospective cohort study	1.00	148	79.70	61 ± 11	②⑤	NCEP2001
18	Espinola-Klein	2008	Germany	Prospective cohort study	6.10 (0.70–7.70)	1,263	74.40	61.6 ± 10.1	②	NCEP2005
19	Iturry-Yamamoto	2009	Brazil	Prospective cohort study	1.00	159	71.70	60.7 ± 10.6	②③⑤	NCEP2005
20	Kasai	2009	Janpan	Retrospective cohort study	11.40 ± 2.90	1,836	85.10	59.2 ± 9.0	①②④⑩	NCEP2005
21	Protack	2009	America	Retrospective cohort study	4.50	921	64.00	71 ± 10	②③④	Custom
22	Selcuk	2009	Turkey	Prospective cohort study	2.30 (1.20–3.50)	188	82.40	56.9 ± 11.6	②③⑤	NCEP2005
23	Solymoss	2009	Canada	Retrospective cohort study	12.60 ± 3.40	1,080	73.40	58.1 ± 9.8	①②③④⑧	NCEP2005
24	Suwaidi	2010	Bahrain, Kuwait, Qatar, Oman, United Arab Emirates, and Yemen	Prospective cohort study	0.50	6,701	75.70	56.4 ± 12.2	①③④⑦	NCEP2005
25	Lee	2010	Korea	Prospective cohort study	1.00	1,990	73.00	63.4 ± 12.6	①②③⑤	NCEP2005
26	Miller	2010	Mexico	Prospective cohort study	/	971	70.00	62.3 ± 11.5	①⑤⑦	NCEP2005
27	Petersen	2010	America	Prospective cohort study	5.00	5,744	64.60	62(53–71)	①③④⑩	NCEP2005
28	Van Kuijk	2010	Netherlands	Retrospective cohort study	6.00 (2.00–9.00)	2,069	81.40	/	②③⑤⑩	NCEP2001
29	Hoshida	2011	Janpan	Prospective cohort study	1.00	1,173	72.50	67	①②③④⑤	NCEP2005
30	Hu	2011	China	Prospective cohort study	2.95	1,224	71.70	60 ± 10	②③	IDF
31	Kalahasti	2011	America	Retrospective cohort study	1.00	2,362	73.00	64	①③⑤⑩	Custom
32	Maron	2011	America	RCT *post-hoc* analysis	4.60 (2.50–7.00)	2,248	85.10	62.1 ± 9.9	①③⑤⑦⑩	NCEP2005
33	Capoulade	2012	Canada	Prospective cohort study	3.40 ± 1.30	243	62.00	57 ± 13	②	NCEP2001
34	Marso	2012	Netherlands	Prospective cohort study	3.00	673	75.80	58.2(50.1–70.8)	②③⑧	NCEP2001

(Continued)

TABLE 1 | Continued

No.	Author	Year	Country	Study design	Follow-up (Years)	Sample	Male (%)	Age (years)	Endpoints	Definition of MetS
35	Mi	2012	China	Prospective cohort study	1.00	701	64.80	61.4 ± 11.7	①④⑩	IDF
36	Arnold	2013	America	Prospective cohort study	1.00	1,129	66.00	59.7 ± 11.6	①	NCEP2001
37	Balti	2013	France	Prospective cohort study	5.00	57	56.00	61.9 ± 12.9	①	NCEP2005
38	Hossain	2014	Bangladesh	Prospective cohort study	1.00	210	70.00	53.2 ± 12	①④⑥⑨	NCEP2005
39	Mehta	2014	New England, Canada, America, Australian	RCT *post-hoc* analysis	1.00	9,406	68.40	68(60–75)	①	NCEP2005
40	Mornar	2014	Croatia	Prospective cohort study	1.00	250	/	/	②③	NCEP2005
41	Udell	2014	America	Prospective cohort study	4.00	44,548	64.60	68.7 ± 10.4	①②③④	NCEP2005
42	Won	2014	Korea	Prospective cohort study	3.00	963	75.60	62 ± 12	①②③⑩	NCEP2005
43	Ao	2015	China	Retrospective cohort study	5.00	1,238	84.40	59.5 ± 9	①③④	NCEP2005
44	Arbel	2015	Russia	Prospective cohort study	4.40 ± 1.90	3,525	72.00	66 ± 22	①	NCEP2005
45	Fan	2015	China	Retrospective cohort study	2.30	997	69.90	64.29 ± 13.13	⑦①	Custom
46	Perrone-Filardi	2015	Italy	Substudy of RCT	3.00	6,648	78.20	67.2 ± 10.6	①②⑩	IDF
47	Simao	2015	Brazil	Retrospective cohort study	1.00	148	56.80	69.5(55–81.5)	①	NCEP2005
48	Chen	2016	China	Prospective cohort study	4.90	3,351	63.00	64 ± 2.4	①②⑩	NCEP2005
49	Fang	2016	China	Prospective cohort study	3.40	1,087	51.20	65.1 ± 8.9	②③④	Custom
50	La Carrubba	2016	Italy	Prospective cohort study	1.80	1,920	56.30	60(50–69)	②③④⑤	IDF
51	Tadaki	2016	Janpan	Retrospective cohort study	3.20 ± 1.10	4,566	68.00	68.8 ± 1.4	①③⑤⑧	NCEP2001
52	Bhagat 2017	2017	Indian	Prospective cohort study	2.00	358	74.90	56.19 ± 11.56	①⑧⑨	NCEP2005
53	Lovic	2018	Serbia	Prospective cohort study	4.00	507	77.71	58.57 ± 11.30	①②③④⑤	AHA-NHLBI(NCEP2005), NCEP2001 and IDF
54	Vest	2018	USA	Prospective cohort study	5.10 (2.20–8.20)	1,953	74.00	55 (48–63)	①	NCEP2001
55	Polovina	2018	Serbia	Prospective cohort study	5.00	843	61.40	62.5 ± 12.2	②③⑩	NCEP2005

① All-cause death, ② CV death, ③ MI, ④ Stroke, ⑤ TVR, ⑥ Cardiac arrest, ⑦ HF, ⑧ Angina pectoris, ⑨ Cardiogenic shock, ⑩ MetS components.

TABLE 2 | The main results of meta-analysis.

Outcome	Number of studies	Number of volunteers	Random effect model RR (95%CI)	*P*	I^2(%)	Begg's Test *P*
Primary outcomes						
All-cause death	41	145,897	1.22 (1.10–1.35)	<0.01	89	0.01
CV death	21	94,542	1.36 (1.15–1.61)	<0.01	87	0.02
MI	23	77,125	1.46 (1.24–1.72)	<0.01	72	0.13
Stroke	11	60,297	1.44 (1.13–1.82)	<0.01	75	0.01
Secondary outcomes						
TVR	13	17,072	1.241 (1.06–1.45)	<0.01	81	0.16
Angina pectoris	3	5,147	1.28 (0.97–1.69)	0.03	71.5	–
Heart failure	8	12,369	1.50 (1.12–2.01)	<0.01	88.5	–
Cardiac arrest	4	4,171	1.46 (0.88–2.43)	0.52	0.0	–
Cardiogenic shock	3	7,309	1.28 (0.97–1.69)	0.03	71.5	–

studies adopted IDF (2005). Baseline characteristics are listed in **Table 1**. Risk of bias was assessed in all of the 55 studies (**Supplementary Table 2**). The cohort studies comprised 16 medium-quality studies, and 33 high-quality studies. For RCT *post-hoc* studies, the risk of bias was deemed low in 2 studies and moderate in 4 studies.

Meta-Analysis Results

All-Cause Death and CV Death

Forty-one studies (145,897 patients) reported all-cause death. MetS was associated with higher all-cause death [RR = 1.220, 95% CI (1.103, 1.349), $P = 0.000$] according to the heterogeneity test I2 = 89% (**Table 2**, **Figure 2**). Subgroup analysis showed that among different diagnostic criteria of MetS, the results from NCEP-ATPIII (2001) and NCEP-ATPIII (2005) subgroups were consistent with the overall result (**Table 3**). Among different study types, the cohort study subgroup was in the same direction with the overall results. No statistically significant difference was found in the RCT *post-hoc* studies. Diagnostic criteria and study type were the factors that affected heterogeneity. Meta-regression showed that the follow-up time and male proportion were not the sources of heterogeneity ($P > 0.05$), and age only explained 1.6% of the heterogeneity ($P = 0.022$). The Begg's test result showed bias ($P = 0.012$), and the contour-enhanced funnel plots showed that the bias may be due to other reasons rather than publication bias.

Twenty-one studies with 94,542 patients reported CV-related death. The MetS group had higher CV death than the non-MetS group [RR = 1.360, 95% CI (1.152, 1.606), $P = 0.000$] according to the heterogeneity test I^2 =87.0% (**Table 2**, **Figure 3**). Subgroup analysis showed that among different diagnostic criteria of MetS, NCEP-ATPIII (2001) and NCEP-ATPIII (2005) subgroups were consistent with the overall result (**Table 3**). Among different study types, the subgroups were consistent with the overall results. Diagnostic criteria affected the heterogeneity. Meta-regression showed that follow-up time, age, and male proportion were not the source of heterogeneity ($P > 0.05$). The Begg's test and the contour-enhanced funnel plots showed that bias may be caused by publication bias and other reasons.

Risk of MI and Stroke

Twenty-three studies with 77,125 patients reported the risk of MI. Patients with CVD and MetS had a higher risk of MI [RR = 1.460, 95% CI (1.242, 1.716), $P = 0.000$] according to the heterogeneity test $I^2 = 72\%$ (**Table 2**, **Figure 4**). Subgroup analysis showed that among the diagnostic criteria of MetS, the results of NCEP-ATPIII (2001) and NCEP-ATPIII (2005) were consistent with the overall results (**Table 3**). Other subgroups had no statistically significant difference. Among the study types, the subgroup results were in the same direction as the overall results. Meta-regression showed that follow-up time, age, and male proportion were not the source of heterogeneity ($P > 0.05$). The Begg's test and the contour-enhanced funnel plots reported no publication bias ($P = 0.125$).

Eleven studies with 60,297 patients reported the risk of stroke. Patients with CVD and MetS had a higher risk of stroke [RR = 1.435, 95% CI (1.131, 1.820), $P = 0.000$] according to the heterogeneity test $I^2 = 75\%$ (**Table 3**, **Figure 5**). The Begg's test and the contour-enhanced funnel plots showed that the bias may be caused by other reasons rather than publication bias.

Other Adverse Cardiovascular Indicators

The results of the TVR (13 studies) reported that patients with CVD and MetS had a higher risk to develop TVR [RR = 1.241, 95% CI (1.063, 1.448), $P = 0.000$]. Subgroup analysis showed that diagnostic criteria and study type explained the partial heterogeneity. The risk of heart failure was evaluated in eight studies. Patients with CVD and MetS were more likely to have heart failure [RR = 1.497, 95% CI (1.116, 2.007), $P = 0.000$]. Subgroup analysis showed that diagnostic criteria partly explained the heterogeneity (**Table 2**).

Other indicators include risk of cardiac arrest (4 studies), angina pectoris (3 studies), and cardiogenic shock (3 studies). We found no statistically significant difference in the risk of cardiac arrest [RR = 1.457, 95% CI (0.875, 2.429), $P = 0.518$], angina pectoris [RR = 1.280, 95% CI (0.967, 1.694), $P = 0.030$], and cardiogenic shock [RR = 0.923, 95% CI (0.752 1.132), $P = 0.764$].

Impact of MetS Component

Among MetS components, low HDL (40/50) was significantly associated with increased risks of all-cause death and CV death.

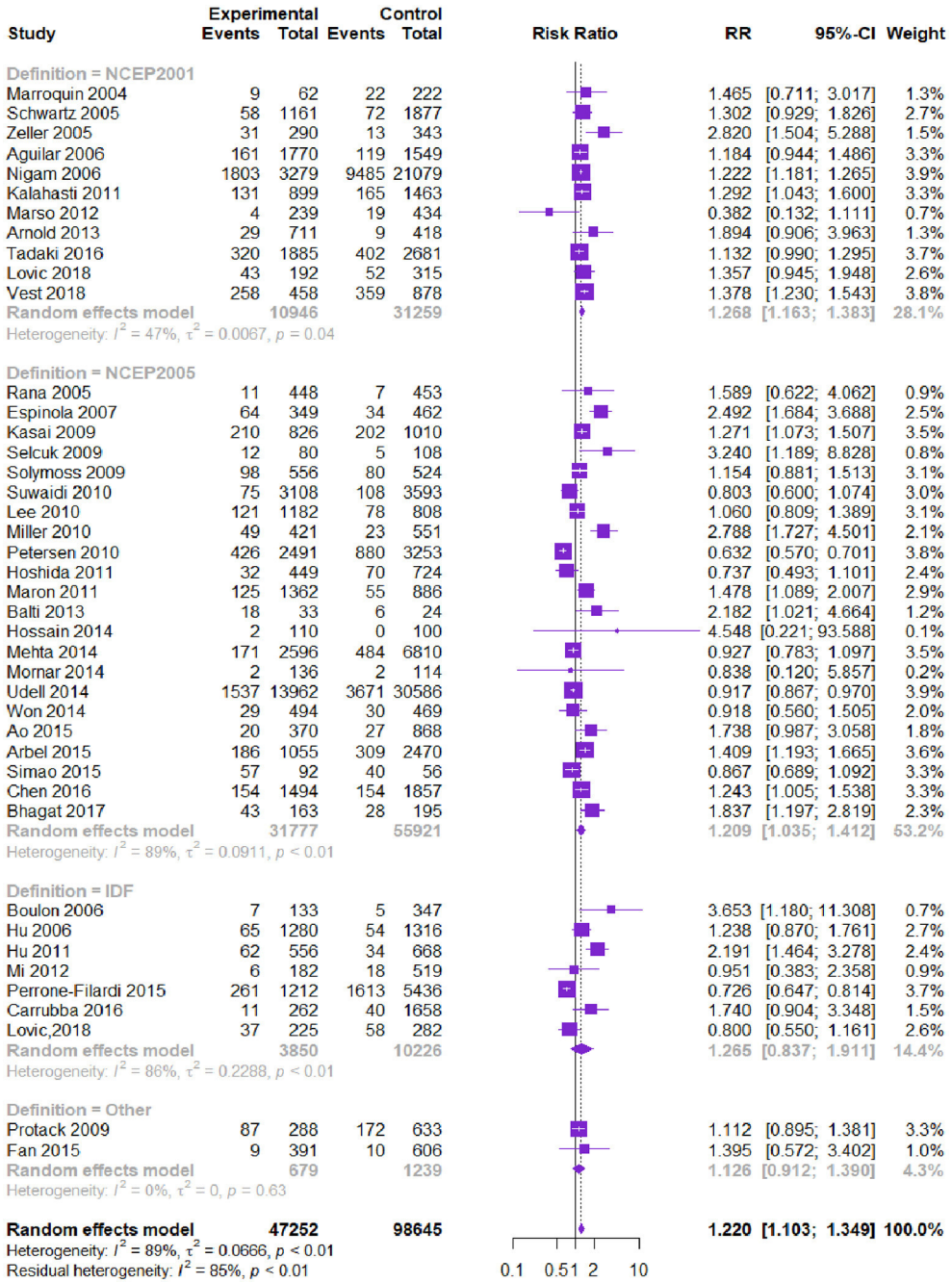

FIGURE 2 | Meta-analysis of the risk of all-cause death in patients with CVD and MetS compared with that of patient without MetS.

Elevated FPG (>100 mg/dl) was significantly associated with an increased risk of all-cause death, whereas body mass index (BMI) > 25 kg/m^2 was related to a reduced risk of all-cause death (**Table 4**).

Sensitivity Analysis

We examined the robustness of our results. The sensitivity analysis of the effect measures showed that the OR increased the effect size and did not change the direction of the results, except for angina pectoris. The RD did not change the direction of the results. The sensitivity analysis of the statistical models did not change the direction of the results. Hence, the results of this meta-analysis were robust.

DISCUSSION

Summary of Main Results

Fifty-five studies with 162,450 patients from 25 countries or regions were included. Most studies defined MetS using NCEP2001, NCEP2005, and IDF criteria, and other works

TABLE 3 | The results of subgroup analysis based on diagnostic criteria.

Outcome	Subgroup	Number of studies	RR (95%CI)	*P*	I^2(%)
All-cause Death	NCEP2001	11	1.27 (1.16–1.38)	<0.01	47
	NCEP2005	22	1.21 (1.04–1.41)	0.02	89
	IDF	7	1.27 (0.84–1.91)	0.19	86
	Other	2	1.13 (0.91–1.39)	0.27	0
CV Death	NCEP2001	5	1.67 (1.15–2.43)	0.01	68
	NCEP2005	11	1.45 (1.13–1.86)	<0.01	83
	IDF	4	1.02 (0.58–1,81)	0.93	80
	Other	/	/	/	/
MI	NCEP2001	7	1.57 (1.04–2,36)	0.03	81
	NCEP2005	12	1.18 (1.08–1.28)	<0.01	7
	IDF	3	1.58 (0.96–2.59)	0.07	16
	Other	2	2.24 (0.91–5.51)	0.08	91
Stroke	NCEP2001	3	1.77 (1.25–2.51)	<0.01	0
	NCEP2005	4	1.21 (0.89–1.64)	0.22	81
	IDF	3	1.79 (1.04–3.11)	0.04	0
	Other	2	1.45 (1.05–2.02)	0.03	25
TVR	NCEP2001	4	1.34 (0.91–1.96)	0.14	74
	NCEP2005	6	1.22 (1.08–1.37)	<0.01	0
	IDF	3	1.33 (0.84–2.09)	0.22	86

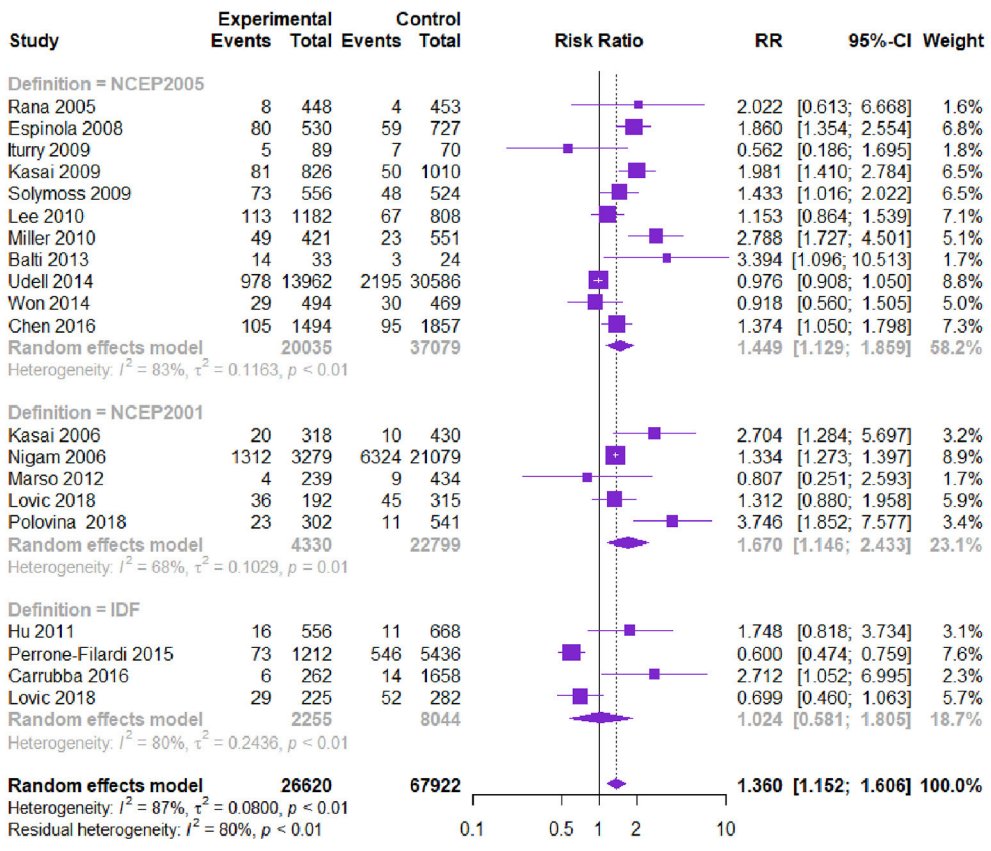

FIGURE 3 | Meta-analysis of the risk of CV death in patients with CVD and MetS compared with that of patient without MetS.

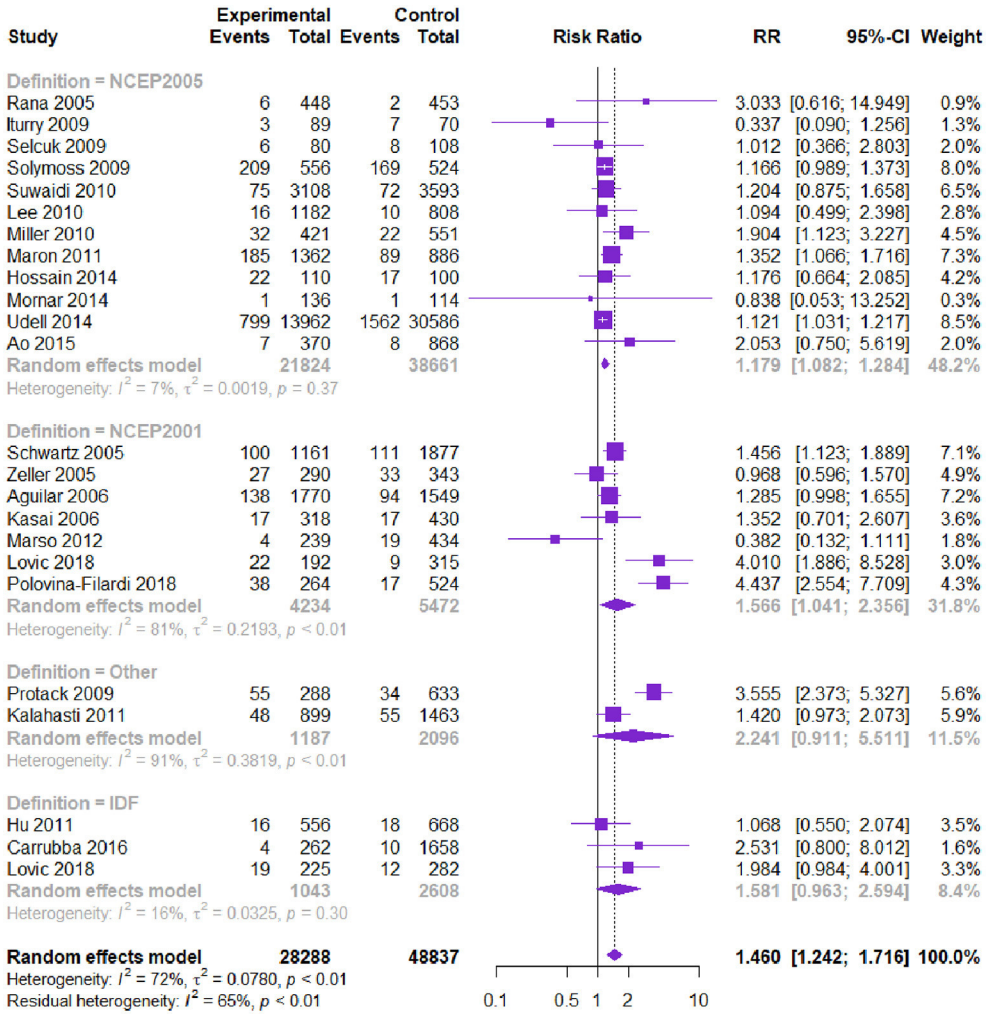

FIGURE 4 | Meta-analysis of the risk of MI in patients with CVD and MetS compared with that of patients without MetS.

adopted specific diagnostic criteria. Our results suggested that patients with CVD and MetS had an increased risk of all-cause death, CV-related death, MI, stroke, TVR, and heart failure. In the analysis of MS components, BMI>25 kg/m^2 was negatively correlated with the prognosis of patients with CVD. Dyslipidemia and abnormal glucose metabolism were the main risk factors for the prognosis of CVD. Different spectrum within patients with cardiovascular diseases may be the sources of heterogeneity.

Potential Biases in the Review Process

MetS and its components are a complex of risk factors for CVD and diabetes (21). Ford (65) reported that the population attributable fractions for CVD, diabetes, and all-cause death among patients with MS were 12–17%, 30–52%, and 6–7%, respectively (65). However, for patients with CVD, whether MetS and its components is associated with the risk of CV events remains controversial.

Obesity is an independent risk factor for hypertension, CVD, and diabetes (66). Given the known association between obesity and CVD, the adverse consequences of obesity may persist after the onset of CVD. However, previous studies suggest a contradictory U-shaped relationship between obesity and CVD-related death; hence, overweight and mild obesity are related to lower short-term and long-term mortality (67–69) based on the concept of "Obesity paradox" or "reverse epidemiology" (66). Although the setting of obesity indicators was involved in different MetS diagnostic criteria, the core of the diagnosis was consistent. In NCEP-ATP III (2001) and NCEP-ATP III (2005) criteria, obesity is one of the five elements and is not a necessary condition; however, in IDF (2005), obesity is the first prerequisite. Interestingly, our result discovered that the diagnosis of MetS under different standards has a distinct prognosis of CVD. The result of the subgroup analysis of all-cause mortality and cardiovascular mortality as two core factors demonstrated that IDF (2005) standards were consistently different from the final result. However, under the standards of NCEP-ATP III (2001) and NCEP-ATP III (2005) who didn't consider obesity as a necessary condition, MetS is a significant risk factor of prognosis.

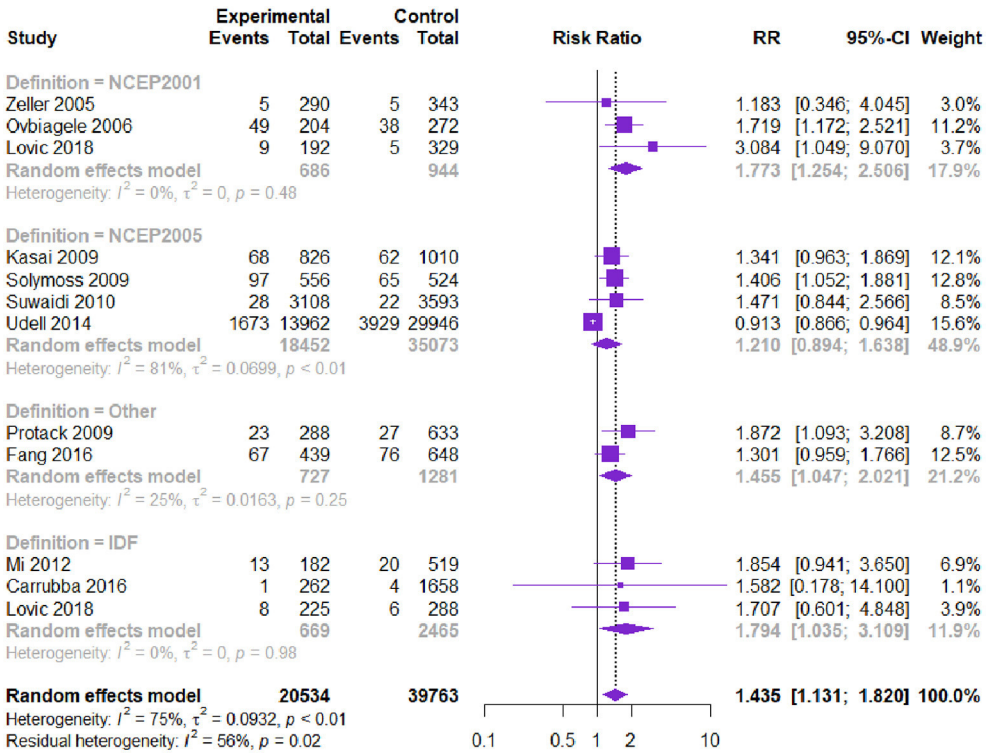

FIGURE 5 | Meta-analysis of the risk of stroke in patients with CVD and MetS compared with that of patients without MetS.

TABLE 4 | The results of metabolic syndrome's components.

Components	Outcomes [HR (95%CI)]					
	Number of studies	All-cause death	I^2 (%)	Number of studies	CV death	I^2 (%)
High TG	9	0.97 (0.93–1.01)	68	2	0.89 (0.77–1.03)	0
Low HDL-C	8	1.17(1.09–1.26)	56	2	1.39 (1.00–1.94)	74
High BP	9	0.98(0.94–1.01)	71	2	0.82 (0.58–1.18)	69
FPG>100 mg/dl	11	1.29 (1.23,1.35)	61	2	1.24 (0.96–1.60)	53
BMI>25 kg/m^2	5	0.88(0.79, 0,97)	89		/	
High WC	2	0.91(0.49–1.69)	36		/	

Hence, we need to reconsider which diagnostic criteria can predict the prognosis of MetS among patients with CVD more accurately. The heterogeneity in this study may be associated with the proportion of obese patients included.

AHA/NHLBI 2009 diagnostic criteria were not adopted in all of the included studies, which may be related to the fact that the indicators and numerical intervals of abdominal obesity were not clearly given in the criteria. BMI was used in most of the studies as a proxy for waist circumference, but the cutoffs for the inclusion criteria in each study were different. This phenomenon may be related to two factors: (1) BMI is easier to obtain than waist circumference, and (2) BMI can be effectively docked with the WHO's definition of obesity. However, existing evidence suggests that MetS might be caused by excessive central obesity (70). Therefore, in future research on MetS, we suggest that BMI and waist circumference data should be collected at the same time for strict implementation of MetS diagnostic criteria.

Impact of Follow-Up Time on Results

The span of follow-up time included was very large, ranging from 0.33 years to 12.6 years. A 32-year prospective cohort study of male residents without MI or stroke in the community showed that the CV-related mortality curves among patients with MetS varied at 10–15 years of follow-up (70). The findings of Kasai et al. (26) and Nigam et al. (46) show that MetS and its components had a significantly positive association with all-cause death of patients with CVD during 4–5 years of follow-up (20, 26). However, the impact of MetS on patients with CVD might be underestimated in these studies.

MS Components of Study

In this study, the potential influences of the five components of MetS [TG, HDL, BP, FPG, BMI/Waist circumference (WC)] on CVD prognosis was analyzed. We found that abnormal blood glucose and lipid metabolism are important factors that could lead to poor prognosis of CVD. As such, these factors should be considered as intervention targets for predicting the prognosis of patients with CVD. BMI was negatively correlated, which was manifested as the obesity paradox. Waist circumference was included in only two studies with relatively small sample sizes and conducted among Chinese patients only. Further studies are needed to explore the rationality, applicability, and the risk prognosis of BMI and waist circumference.

Prediabetes is an intermediate metabolic state between normoglycemia and diabetes, includes impaired glucose tolerance and impaired fasting glucose (71). Compared with NCEP-ATP III (2001) criteria, the NCEP-ATP III (2005) reduced the fasting plasma glucose from 6.1 mmol/L to 5.6 mmol/L. Our results showed that the two diagnostic criteria had the same contribution in predicting the prognosis of patients with CVD. The results of Huang 2016 also found that prediabetes with impaired fasting glucose or impaired glucose tolerance is associated with an increased risk of composite cardiovascular events, coronary heart disease, stroke, and all-cause mortality (71). Our findings indirectly supported the modification of the American ADA guidelines to reduce the standard of pre-diabetes from 6.1 mmol/L (72) to 5.6 mmol/L (73). In response to this result, lifestyle intervention is the fundamental management approach for prediabetes (73, 74).

Limitations

The span of follow-up time included was very large, ranging from 0.33 to 12.6 years, most studies were followed up for <5 years, the impact of MetS on patients with CVD in this study might be underestimated. As one of the diagnostic indicators of MetS, WC was only included in two studies, reflected the problems in the implementation of MetS diagnostic criteria and possibly underestimated the impact of central obesity on patients with CVD.

CONCLUSIONS

This meta-analysis was conducted using cohort studies and RCT *post-hoc* studies. MetS was found to be associated with an increased risk of CV-related adverse events among patients with CVD. For MetS components, there was an increased risk in people with low HDL-C and FPG>100 mg/dl. Positive measures should be implemented timely for patients with CVD after the diagnosis of MetS to reduce risk factors and strengthen the prevention and treatment of hyperglycemia and hyperlipidemia. Further studies need to clarify the selection of MetS diagnostic indicators (particularly the BMI or waist circumference).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JL, AX, XL, and YZ conceived the study. YZ and HH designed the search strategy and XL performed the literature search. JZ and YZ screened studies for eligibility. HH, YLi, YLiu, and AF performed data extraction. XL, YZ, LL, and TH assessed the risk of bias. XL, YZ, HH, and JZ performed data analysis. JL interpreted the data analysis and assessed the certainty of evidence. XL and YZ wrote the first draft of the manuscript and all other authors revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.704145/full#supplementary-material

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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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Triglyceride–Glucose Index Independently Predicts New-Onset Atrial Fibrillation After Septal Myectomy for Hypertrophic Obstructive Cardiomyopathy Beyond the Traditional Risk Factors

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The triglyceride–glucose index is a valuable marker of insulin resistance. However, the predictive value of this index for postoperative atrial fibrillation in patients undergoing septal myectomy remains unclear. A total of 409 patients with hypertrophic obstructive cardiomyopathy who underwent septal myectomy were recruited in this study. The triglyceride–glucose index was calculated for all patients preoperatively. All patients underwent clinical data collection, blood sampling, and standard echocardiographic examinations during hospitalization. The prevalence of postoperative atrial fibrillation was approximately 15% in the present study. Multivariate logistic regression revealed that age (odds ratio [OR]: 1.053, 95% CI: 1.016–1.090, $P = 0.004$), hypertension (OR: 2.399, 95% CI: 1.228–4.686, $P = 0.010$), left atrial diameter (OR: 1.101, 95% CI: 1.050–1.155, $P < 0.001$), and triglyceride–glucose index (OR: 4.218, 95% CI: 2.381–7.473, $P < 0.001$) were independent risk factors for postoperative atrial fibrillation in patients undergoing septal myectomy. In receiver operating characteristic curve analysis, the triglyceride–glucose index could provide a moderate predictive value for postoperative atrial fibrillation after septal myectomy 0.723 (95% CI: 0.650–0.796, $P < 0.001$). Moreover, adding the triglyceride–glucose index to conventional risk factor model could numerically but not significantly increase our ability to predict postoperative atrial fibrillation (area under the receiver: 0.742 (0.671–0.814) vs. 0.793 (0.726–0.860), $p = 0.065$) after septal myectomy. In our retrospective cohort study, the triglyceride–glucose index was identified as an independent predictor of postoperative atrial fibrillation in patients undergoing septal myectomy.

Keywords: insulin resistance, TyG index, hypertrophic cardiomyopathy, postoperative atrial fibrillation, septal myectomy

INTRODUCTION

Postoperative atrial fibrillation (POAF) has a prevalence of up to 24.6% in patients with hypertrophic obstructive cardiomyopathy (HOCM) who undergo septal myectomy (1). It is associated with increased morbidity and mortality as well as prolonged intensive care unit and hospital stay (2, 3). Several risk factors, including age, male sex, valvular surgery, and left atrial enlargement, have been reported to be associated with POAF in patients who underwent cardiac surgery (4, 5); however, no effective tool based on clinical parameters has been developed to predict POAF to date. It is crucial to identify patients at high risk of developing POAF before surgery so that modifiable risk factors can be addressed. Additionally, further determination of such factors is essential.

Insulin resistance (IR) is a known risk factor for cardiovascular disease (6) that is observed in patients with hypertrophic cardiomyopathy (7). It is associated with atrial remodeling (8) as well as atrial fibrillation (AF) (9). IR may predispose to AF by increasing left atrial size or impairing left ventricular diastolic function in hypertrophic cardiomyopathy (10); however, its role in predicting new-onset POAF after septal myectomy in these patients has not been well investigated.

The hyperinsulinemic–euglycemic clamp, which is considered the "gold standard" test for IR assessment, can accurately assess the severity of IR by measuring the glucose metabolism rate and the sensitivity of an individual's peripheral tissues to insulin (11). However, it is not commonly used in the clinical setting because of the complexity of the test and the associated costs. The more commonly used homeostasis model assessment of IR method requires measurement of fasting insulin levels, which is not routinely done. Therefore, the triglyceride–glucose (TyG) index, which combines triglyceride and fasting plasma glucose levels, has been proposed alternatively. According to previous studies, the TyG index can assess IR, more accurately than homeostasis model assessment of IR (12, 13). This study aimed to assess the impact of preoperative TyG levels on new-onset POAF in patients with HOCM undergoing septal myectomy.

METHODS

A total of 409 patients without a history of preoperative atrial arrhythmia, who underwent septal myectomy in Beijing Anzhen Hospital between September 2009 and June 2020 were enrolled in this study. The diagnostic criteria for HOCM include unexplained septal hypertrophy with a septal thickness of >15 mm and left ventricular outflow tract gradient (LVOTG) of ≥30 mmHg, according to previous guidelines (14). Septal myectomy was performed if patients had severe symptoms despite optimal medical therapy and LVOTG of ≥50 mmHg at rest or with exertion. The surgical method is described as follows: septal myectomy was performed through transverse aortotomy extending into the non-coronary sinus. Continuous resection was commenced at the nadir of the right aortic sinus leftward toward the mitral annulus and apically to the base of the papillary muscles. All areas of papillary muscle fusion to the septum or ventricular free wall were divided, and anomalous chordal structures and fibrous attachments of the mitral leaflets to the ventricular septum or free wall were divided or excised (15). Patients with conditions that could affect IR or the TyG index, such as severe liver, kidney, and thyroid disease were excluded. The study was approved by the ethics committee of Beijing Anzhen Hospital, Capital Medical University and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

The demographics and clinical characteristics of each patient were recorded. All patients underwent routine laboratory tests (after a fasting period of 12 h) on admission. Fasting serum triglyceride and glucose levels were measured using an automated biochemistry analyzer (Roche Cobas 801, Germany). The TyG index was calculated using this formula: ln [fasting TG (mg/dL) × fasting plasma glucose (mg/dL)/2]. Data were collected by two independent researchers.

Patients underwent continuous electrocardiographic monitoring or a standard 12-lead routine electrocardiograph daily during the postoperative period after septal myectomy and until discharge. POAF was defined as the presence of AF that lasted ≥5 min or required cardioversion with antiarrhythmic drugs.

Echocardiographic studies were performed using a commercially available E9 ultrasound system. Left atrial diameter (LAD), left ventricular end-diastolic dimension, left ventricular ejection fraction, maximal wall thickness, and LVOTG were measured. Values were analyzed according to the American Society of Echocardiography guidelines (16).

Continuous data are presented as median and interquartile range or mean ± standard deviation. Categorical variables are presented as number and percentage. Intergroup comparisons of normally distributed numerical variables were performed using an independent sample *t*-test. The Mann–Whitney U test was performed for intergroup comparisons of non-normally distributed numerical variables. Categorical variables were compared using the chi-square test. The cut-off value for the TyG index was determined by the area under the receiver operating characteristic (ROC) curve. Multivariate logistic regression models were used to determine the risk factors for POAF. Variables with a *P*-value of <0.1, on univariate analysis, were entered into the multivariate analysis. Co-linearity and intercorrelations among variables were also taken into consideration when constructing the multivariate logistic regression analysis model to control for confounding variables. Finally, the c-statistic of the conventional risk factor model vs. the conventional risk factor plus TyG index model were compared using the Delong test to examine whether adding the TyG index to conventional risk factors could improve discrimination power for POAF. All reported probability values were two-tailed, and a *P*-value of <0.05 was considered statistically significant. SPSS version 26.0 (IBM) and MedCalc were used for data analysis.

Abbreviations: AF, Atrial fibrillation; IR, Insulin resistance; LAD, Left atrial diameter; LVOTG, Left ventricular outflow tract gradient; POAF, Postoperative atrial fibrillation; ROC, Receiver operating characteristic.

TABLE 1 | Perioperative clinical variables of patients.

Variables	Whole cohort (n = 409)	No POAF (n = 348)	POAF (n = 61)	*P-value*
Preoperative data				
Age (years)	50.94 ± 12.37	49.92 ± 12.35	56.75 ± 10.85	<0.001
Male (%)	213 (52.1)	181 (52.0)	32 (52.5)	0.949
Hypertension (%)	104 (25.4)	74 (21.3)	30 (49.2)	<0.001
Diabetes mellitus (%)	39 (9.5)	25 (7.2)	14 (23.0)	<0.001
Hyperlipemia (%)	61 (14.9)	42 (12.1)	19 (31.1)	<0.001
CAD (%)	59 (14.4)	47 (13.5)	12 (19.7)	0.206
Body mass index (kg/m^2)	25.28 ± 3.34	25.08 ± 3.33	26.41 ± 3.22	0.004
Medication				
Calcium channel blockers (%)	270 (66.0)	231 (66.4)	39 (63.9)	0.710
Beta-blockers (%)	291 (71.1)	245 (70.4)	46 (75.4)	0.426
Laboratory factors				
Glucose (mmol/L)	5.32 ± 1.48	5.17 ± 1.19	6.21 ± 2.42	0.002
ALT (IU/L)	19.00 (14.00–29.00)	19.00 (14.00–29)	19.50 (15.00–32.75)	0.482
AST (IU/L)	21.00 (18.00–27.00)	21.00 (18.00–26.50)	21.50 (18.00–28.00)	0.630
Creatinine (μmol)	68.95 (59.30–79.68)	68.45 (58.93–79.00)	72.95 (62.53–89.00)	0.068
TC (mmol/L)	4.49 ± 0.89	4.41 ± 0.86	4.92 ± 0.95	<0.001
HDL-C (mmol/L)	1.12 ± 0.25	1.12 ± 0.24	1.09 ± 0.28	0.400
LDL-C (mmol/L)	2.81 ± 0.78	2.76 ± 0.76	3.08 ± 0.84	0.004
TG (mmol/L)	1.47 ± 0.88	1.38 ± 0.79	1.99 ±1.16	<0.001
TyG index	6.97 ± 0.60	6.90 ± 0.55	7.41 ± 0.67	<0.001
Concomitant operative procedures				
Coronary artery bypass grafting (%)	40 (9.8)	31 (8.9)	12 (14.8)	0.156
Myocardial unroofing (%)	16 (3.9)	13 (3.7)	3 (4.9)	0.935
Mitral valve replacement (%)	87 (21.3)	71 (20.4)	16 (26.2)	0.305
Mitral valve repair (%)	67 (16.4)	59 (17.0)	8 (13.1)	0.455
Aortic valve replacement or repair (%)	25 (6.1)	20 (5.7)	5 (8.2)	0.655
Tricuspid valve replacement or repair (%)	28 (6.8)	23 (6.6)	5 (8.2)	0.859
Postoperative data				
Mechanical ventilation time (hours)	17.25 (13.46–22.00)	16.92 (13.02–21.56)	18.92 (16.04–38.42)	<0.001
Aortic clamp time (minutes)	74.00 (55.50–102.00)	73.50 (54.00–101.00)	75.00 (58.50–117.50)	0.133
operation time (h)	4.25 (3.58–5.17)	4.25 (3.58–5.17)	4.25 (3.88–5.30)	0.396
cardiopulmonary bypass time (minutes)	114.00 (88.00–149.00)	113.00 (86.00–148.75)	118.00 (98.50–153.50)	0.126
Post-operative hospital stays (days)	8.00 (6.00–11.00)	8.00 (6.00–11.00)	9.00 (7.00–12.50)	0.028

POAF, postoperative atrial fibrillation; CAD, coronary artery disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglyceride; TyG index, triglyceride glucose index.

RESULTS

As presented in **Table 1**, 409 patients who underwent septal myectomy were included. The enrolled patients were followed up for a median of 8 days (6–11). POAF was diagnosed in 61 (15%) patients during their postoperative course. Compared with patients without POAF, those with POAF were older (49.92 ± 12.35 vs. 56.75 ± 10.85 years, $P < 0.001$), had higher body mass index (25.08 ± 3.33 vs. 26.41 ± 3.22, $P < 0.004$), and had longer mechanical ventilation time (16.92 [13.02–21.56] vs. 18.92 [16.04–38.42] h, $P < 0.001$) and postoperative hospital stay (8 [6–11] vs. 9 [7–12.50] days, $P = 0.028$). Patients in the POAF group were more likely to have hypertension (21.3% vs. 49.2%, $P < 0.001$), diabetes (7.2% vs. 23.0%, $P < 0.001$), hyperlipidemia (12.1% vs. 31.1%, $P < 0.001$), and elevated serum glucose ($P = 0.002$), total cholesterol ($P < 0.001$), triglycerides ($P < 0.001$), and low-density lipoprotein cholesterol ($P = 0.004$) levels. The TyG index was significantly higher (6.90 ± 0.55 vs. 7.41 ± 0.67, $P < 0.001$) in the POAF group. The echocardiographic data are summarized in **Table 2**. Patients with POAF had larger LAD both preoperatively (42.31 ± 6.27 vs. 5.10 ± 7.70 mm, $P = 0.003$) and postoperatively (37.00 ± 6.17 vs. 39.66 ± 6.57 mm, $P = 0.004$). There were no significant associations between POAF and other echocardiographic variables.

Patients were divided into the "low TyG index group" (<7.60) and "high TyG index group" (≥7.60) according to the cut-off point which is identified by ROC curve analysis. The high TyG index group ($n = 60$) had higher rates of hypertension (41.7% vs. 22.6%, $P = 0.002$), diabetes (31.7% vs. 5.7%, $P < 0.001$), hyperlipidemia (46.7% vs. 9.5%, $P < 0.001$), and coronary artery

TABLE 2 | Perioperative echocardiographic variables of patients.

Variables	Whole cohort ($n = 409$)	No POAF ($n = 348$)	POAF ($n = 61$)	*P-value*
Preoperative data				
Maximum wall thickness (mm)	21.14 ± 5.28	21.31 ± 5.35	20.21 ± 4.83	0.143
Left atrial diameter (mm)	42.75 ± 6.58	42.31 ± 6.27	45.10 ± 7.70	0.003
Left ventricular end-diastolic diameter (mm)	43.65 ± 5.29	43.63 ± 5.21	43.74 ± 5.75	0.886
Left ventricular ejection fraction (%)	67.36 ± 6.39	67.41 ± 6.34	67.07 ± 6.73	0.710
Peak LVOT gradients (mmHg)	84.00 (60.00–106.00)	83.00 (57.75–106.00)	85.00 (73.00–109.00)	0.120
Moderate or severe MR (%)	252 (61.6)	210 (60.3)	42 (68.9)	0.208
Postoperative data				
Left atrial diameter (mm)	37.40 ± 6.30	37.00 ± 6.17	39.66 ± 6.57	0.004
Left ventricular end-diastolic diameter (mm)	42.25 ± 5.31	42.14 ± 5.32	42.86 ± 5.23	0.342
Left ventricular ejection fraction (%)	60.87 ± 7.67	61.20 ± 7.61	59.09 ± 7.85	0.053
Peak LVOT gradients (mmHg)	18.00 (12.00–25.50)	18.00 (12.00–25.00)	20.50 (12.00–37.50)	0.281
Moderate or severe MR (%)	15 (3.7)	12 (3.4)	3 (4.9)	0.846

POAF, postoperative atrial fibrillation; LVOT, left ventricular outflow tract; MR, mitral regurgitation. Peak LVOT gradients was defined as the maximal value at resting or after provocation.

disease (25.0% vs. 12.6%, $P = 0.012$), as well as higher serum glucose ($P < 0.001$), total cholesterol ($P < 0.001$), and triglyceride ($P < 0.001$) levels. In addition, POAF occurred more frequently in the high TyG index group (45.0% vs. 9.7%, $P < 0.001$) (Details shown in **Supplementary Table 1**).

Univariate analysis showed that preoperative variables including age (odds ratio [OR]: 1.054, 95% CI: 1.027–1.082, $P < 0.001$), body mass index (OR: 1.126, 95% CI: 1.037–1.223, $P = 0.005$), hypertension (OR: 3.583, 95% CI: 2.039–6.297, $P < 0.001$), diabetes mellitus (OR: 3.849, 95% CI: 1.869–7.924, $P < 0.001$), hyperlipemia (OR: 3.296, 95% CI: 1.754–6.192, $P < 0.001$), LAD (OR: 1.063, 95% CI: 1.020–1.108, $P = 0.004$), and TyG index (OR: 4.163, 95% CI: 2.551–6.794, $P < 0.001$) were risk factors for the occurrence of POAF, whereas male sex, left ventricular ejection fraction, maximum wall thickness, LVOTG, and moderate or severe mitral regurgitation were not. In the multivariate analysis, age (OR: 1.053, 95% CI: 1.016–1.090, $P = 0.004$), hypertension (OR: 2.399, 95% CI: 1.228–4.686, $P = 0.010$), LAD (OR: 1.101, 95% CI: 1.050–1.155, $P < 0.001$), and TyG index (OR: 4.218, 95% CI: 2.381–7.473, $P < 0.001$) were independent risk factors for POAF in patients undergoing septal myectomy (**Table 3**).

A ROC curve was constructed to assess the ability of the TyG index to identify patients at risk of POAF (**Figure 1**). The optimal value for predicting POAF after septal myectomy was 7.60, with a sensitivity of 44.3% and specificity of 90.5%. The area under the receiver curve for TyG index was 0.723 (95% CI: 0.650–0.796, $P < 0.001$). Moreover, as shown in **Figure 2**, the TyG index could numerically but not significantly increase the ability of the conventional risk factor model to predict the incidence of POAF in patients after septal myectomy (area under the receiver curve: 0.742 (0.671–0.814) vs. 0.793(0.726–0.860), $P = 0.065$).

TABLE 3 | Logistic analysis for predictors of postoperative atrial fibrillation.

Characteristics	OR	95% CI	*P-value*
Univariate Logistic regression analysis			
Age	1.054	1.027–1.082	<0.001
Male	0.982	0.570–1.694	0.949
Body mass index	1.126	1.037–1.223	0.005
Hypertension	3.583	2.039–6.297	<0.001
Diabetes mellitus	3.849	1.869–7.924	<0.001
Hyperlipemia	3.296	1.754–6.192	<0.001
Left atrial diameter	1.063	1.020–1.108	0.004
Left ventricular ejection fraction	0.992	0.949–1.036	0.709
Maximum wall thickness	0.958	0.905–1.015	0.144
LVOT gradients	1.004	0.997–1.010	0.319
Moderate or severe mitral regurgitation	1.453	0.811–2.602	0.209
TyG index	4.163	2.551–6.794	<0.001
Multivariate Logistic regression analysis[a]			
Age	1.053	1.016–1.090	0.004
Hypertension	2.399	1.228–4.686	0.010
Left atrial diameter	1.101	1.050–1.155	<0.001
TyG index	4.218	2.381–7.473	<0.001

OR, odds ratio; CI, confidence interval; LVOT, left ventricular outflow tract; TyG index, triglyceride glucose index. [a] Age, Male, Body mass index, Hypertension, Diabetes mellitus, Hyperlipemia, Left atrial diameter, and TyG index were included in the multivariate logistic regression analysis.

DISCUSSION

The predictive value of IR for new-onset POAF in patients who undergo septal myectomy for HOCM has not been adequately studied. In the present study, we used the TyG index as a biomarker of IR to answer this question. The main finding of our study is that the TyG index is an independent predictor of POAF in patients who underwent septal myectomy beyond the traditional risk factors. Additionally, our present study suggested that the TyG index could provide moderate predictive value for POAF in patients who underwent septal myectomy. And adding the TyG index to conventional risk factor model could numerically but not significantly increase our ability to predict

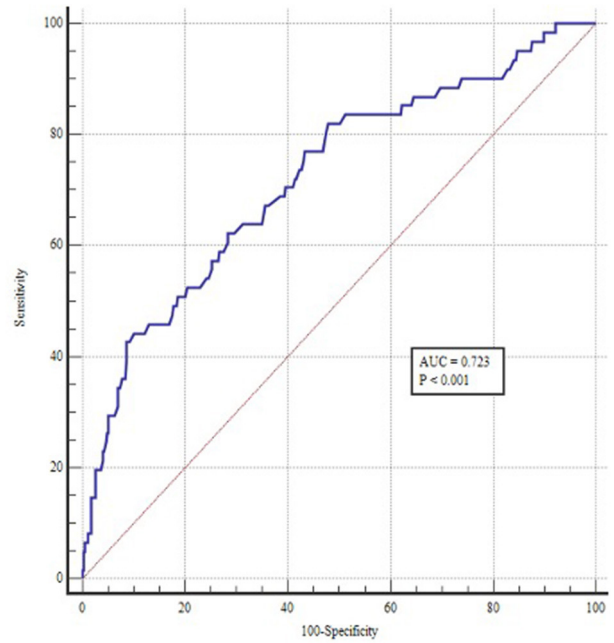

FIGURE 1 | ROC curve of TyG index for predicting POAF in patients undergoing septal myectomy. The optimal cut-off point was 7.60 with sensitivity and specificity of 44.3 and 90.5% (area under the curve 0.723, 95% CI: 0.650 to 0.796, $P < 0.001$).

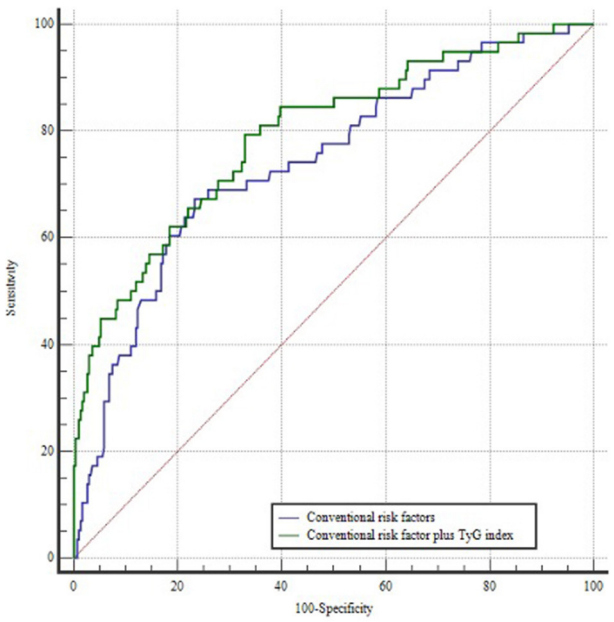

FIGURE 2 | ROC curves of the conventional risk factor model and the conventional risk factor plus TyG index model. The conventional risk factor model includes age, hypertension, and left atrial diameter.

POAF after septal myectomy. To the best of our knowledge, this is the first study to capture the value of the TyG index for predicting new-onset POAF in patients who underwent septal myectomy.

POAF is a common complication of cardiac surgery (5, 17). In this study, the prevalence of POAF was approximately 15%, which is lower than the previously reported 20% in patients undergoing septal myectomy (18). The lower incidence of POAF in our study is partly because we excluded patients with preoperative AF. It has been reported that patients with POAF after coronary revascularization or valvular surgery have increased postoperative morbidity and mortality as well as prolonged intensive care unit and hospital stay (2, 19). Consistent with these studies, we also found that patients with POAF have longer mechanical ventilation time and postoperative hospital stay than those without. Long-term outcomes in inpatients with POAF are worse than in those with sinus rhythm, but similar to those with preoperative AF (3, 20, 21). Considering the important effects of POAF on short- and long-term clinical outcomes, it is necessary to identify its risk factors in patients undergoing septal myectomy. Some of these risk factors, including advanced age, hypertension, and LAD, have been previously identified (3, 22).

The TyG index has been demonstrated as a useful predictor of cardiovascular events (23, 24). It has been proposed to be a reproducible, reliable, and valid surrogate marker of IR because it is simpler and more cost-effective than the gold standard hyperinsulinemic–euglycemic clamp (25–27). In this study, we used the TyG index to further investigate the relationship between IR and POAF in patients undergoing septal myectomy. The results showed that patients with POAF had a higher TyG index than those without POAF.

The TyG index had a moderate predictive value for POAF in patients who underwent septal myectomy. The relationship between TyG index and POAF was still significant even after adjusting for sex, body mass index, and other variables. When we divided patients according to the optimal TyG value for predicting POAF, we found that the TyG index was associated with common cardiovascular comorbidities. Adding the TyG index to the conventional risk factors model could numerically but not significantly increase our ability to predict POAF in patients who underwent septal myectomy due to HOCM.

However, patients after septal myectomy are continuously monitored using telemetry, which is much faster and more accurate in detecting AF. Our present study is significant for clinical practice to optimize the management of patients who underwent septal myectomy for the following reasons. First, we may identify patients at high risk of POAF preoperatively using the TyG index. Then, we may give those patients at high risk of POAF the intensive pre-treatment such as statin, which may reduce the risk of POAF according to previous study (1). Additionally, our present study indicated that the TyG index could serve as an independent risk factor for the incidence of POAF after septal myectomy beyond the traditional risk factors. From the translational outlook, this finding may improve our understanding of the pathogenesis of POAF and open new avenues for the primary prevention of POAF. However, before using it in clinical practice, we should further check the discrimination power of TyG in the validation cohort.

The exact mechanism by which TyG index is closely related to POAF is still unclear. However, we speculate that TyG is a reliable marker of IR, which may be mainly based on the

intrinsic association. IR refers to a clinical condition in which the biological effect of insulin is lower than expected. It can lead to metabolic syndrome and associated organ dysfunction (28). IR is a possible mechanism for the occurrence of AF and commonly occurs in patients with hypertrophic cardiomyopathy (7, 10). IR can induce atrial electrical and structural remodeling through activation of the MAPK pathway (8) and disrupt the functionality of insulin-sensitive glucose transporters, thus predisposing to AF (29). In addition, IR may lead to AF by increasing the left atrial size and left ventricular diastolic dysfunction (10).

The TyG index is a readily available and convenient IR biomarker, which, as our study shows, can be used for the identification of individuals at high risk of POAF among patients undergoing septal myectomy and can be used in conjunction with other established risk factors for this purpose.

Our study has some limitations. First, due to the inherent limitation of retrospective study, not all potential confounders affecting IR and /or POAF, such as lifestyle, dietary habits, and area of septal myectomy, were determined. Second, we only measured fasting plasma triglyceride and glucose levels once on admission; thus, we could not determine the effect of dynamic changes of the TyG index on POAF. Third, we do not have long-term follow-up information on the impact of the TyG index on POAF. Fourth, we failed to determine the discriminative power of TyG in the validation cohort due to the relatively small sample size; therefore, future studies are required to confirm our findings. Finally, we only enrolled patients with HOCM who underwent septal myectomy, which may limit the generalizability of our findings to other cardiac surgery populations.

In conclusion, we identified TyG index as an independent risk factor for POAF in patients undergoing septal myectomy in our retrospective cohort study. However, before putting it into clinical practice, validation of these results by large prospective, multicenter trials is needed.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical ethics committee, Beijing Anzhen Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZW: conceptualization, methodology, and writing—original draft preparation. EZ: data curation and software. CR: visualization and investigation. JD: supervision. JL: software and validation. YL: writing—reviewing and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.692511/full#supplementary-material

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Metformin Suppresses the Progress of Diabetes-Accelerated Atherosclerosis by Inhibition of Vascular Smooth Muscle Cell Migration Through AMPK–Pdlim5 Pathway

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Backgrounds: Our previous work revealed that AMP-activated protein kinase (AMPK) activation inhibits vascular smooth muscle cell migration *in vitro* by phosphorylating PDZ and LIM domain 5 (Pdlim5). As metformin is an AMPK activator, we used a mouse vascular smooth muscle cell (VSMC) line and a *Myh11-cre-EGFP* mice to investigate whether metformin could inhibit the migration of VSMCs *in vitro* and in a wire-injury model *in vivo*. It is recognized that VSMCs contribute to the major composition of atherosclerotic plaques. In order to investigate whether the AMPK–Pdlim5 pathway is involved in the protective function of metformin against atherosclerosis, we utilized ApoE$^{-/-}$ male mice to investigate whether metformin could suppress diabetes-accelerated atherosclerosis by inhibition of VSMC migration *via* the AMPK–Pdlim5 pathway.

Methods: The mouse VSMC cell line was exogenously transfected wild type, phosphomimetic, or unphosphorylatable Pdlim5 mutant before metformin exposure. *Myh11-cre-EGFP* mice were treated with saline solution or metformin after these were subjected to wire injury in the carotid artery to study whether metformin could inhibit the migration of medial VSMCs into the neo-intima. In order to investigate whether the AMPK–Pdlim5 pathway is involved in the protective function of metformin against atherosclerosis, ApoE$^{-/-}$ male mice were divided randomly into control, streptozocin (STZ), and high-fat diet (HFD)-induced diabetes mellitus; STZ+HFD together with metformin or Pdlim5 mutant carried the adenovirus treatment groups.

Results: It was found that metformin could induce the phosphorylation of Pdlim5 and inhibit cell migration as a result. The exogenous expression of phosphomimetic S177D-Pdlim5 inhibits lamellipodia formation and migration in VSMCs. It was also demonstrated that VSMCs contribute to the major composition of injury-induced

neointimal lesions, while metformin could alleviate the occlusion of the carotid artery. The data of ApoE$^{-/-}$ mice showed that increased plasma lipids and aggravated vascular smooth muscle cell infiltration into the atherosclerotic lesion in diabetic mice were observed Metformin alleviated diabetes-induced metabolic disorders and atherosclerosis and also reduced VSMC infiltration in atherosclerotic plaques, while the Pdlim5 phospho-abolished mutant that carried adenovirus S177A-Pdlim5 undermines the protective function of metformin.

Conclusions: The activation of the AMPK–Pdlim5 pathway by metformin could interrupt the migratory machine of VSMCs and inhibit cell migration *in vitro* and *in vivo*. The maintenance of AMPK activity by metformin is beneficial for suppressing diabetes-accelerated atherosclerosis.

Keywords: metformin, Pdlim5, AMPK, diabetes, vascular smooth cells

INTRODUCTION

Atherosclerosis is a chronic artery disease and responsible for one in four deaths induced by cardiovascular diseases (1, 2). Atherosclerosis is initiated with a regional endothelial injury and followed by monocyte adhesion, infiltration, and differentiation into macrophages, with the latter one taking up oxidized LDL and becoming foam cell (3–6). However, the major cell type in atherosclerotic plaques is vascular smooth muscle cell (VSMC), which can also become a foam cell (7). The VSMCs accumulated in the intima are thought to be the major source of extracellular matrix (ECM) and foam cells in fatty streaks (pre-atherosclerotic plaques) (3, 8). With time, these early fatty streak lesions develop into advanced lesions, some of which will eventually become unstable and rupture, resulting in the adverse clinical events of cardiovascular disease (CVD) (7, 9, 10). As described above, atherosclerotic lesions are formed through the complex interactions of various factors, and insulin resistance and hyperglycemia in diabetes mellitus (DM) accelerate all these interactions, with greater vascular inflammation, larger necrotic core, and more diffuse atherosclerosis in the coronary arteries (3). The underlying mechanism is still not very clear, probably due to the excessive and prolonged production of reactive oxidative species (10). Many clinical trials showed that intensive glucose therapy in patients with type 2 diabetes mellitus (T2DM) reduces the risk of a cardiovascular disease (11, 12). Even in individuals with prediabetes, the risk of CVD was increased (13). It was found that metformin, a hypoglycemic agent, exhibits abilities to suppress the progression of common carotid intima-media thickness in T2DM patients and also reduces the incidence of myocardial infarction (11, 12, 14, 15). However, it is paradoxical that several recent clinical trials showed that the anti-atherogenic effect of metformin seems independent of its hypoglycemic function because other regular therapies, such as insulin and sulfonylurea, possess less beneficial cardiovascular effects (16–19). A possible target of metformin is AMP-activated protein kinase (AMPK), a cellular energy sensor activated under metabolic stress (20). It has been reported that the activation of AMPK by metformin reduces endothelial mitochondrial fragmentation and suppresses atherosclerotic plaques in diabetic mice (15). Our previous findings found that AMPK phosphorylates PDZ and LIM domain 5 (Pdlim5), a protein involved in cytoskeleton organization, on Ser177 to inhibit vascular smooth muscle cell migration by suppressing the Rac1-Arp2/3 signaling pathway (21). Recent genetic lineage tracing studies showed that VSMCs get involved in every stage of atherosclerosis development and are a major cell type in an atherosclerotic plaque (8). The single-cell omics also reveals the heterogeneity and plasticity of VSMCs in the vessel wall during atherogenesis (22). The VSMCs not only take part in ECM synthesis and fibrous cap formation but also switch into macrophage-like cells/foam cells and even contribute to calcification and necrotic core, which are crucial to plaque instability (8, 23, 24). Considering the complexity of the roles that VSMCs played in the development of atherosclerosis and the importance of VSMCs in atherosclerotic plaques, we assume that activation of the AMPK–Pdlim5 pathway by metformin may be beneficial for suppressing diabetes-accelerated atherosclerosis *via* the inhibition of VSMC migration. In this study, we identified that metformin could induce the phosphorylation of Pdlim5 at Ser177 site through AMPK and inhibit cell migration *in vitro*. With vascular smooth muscle lineage tracking mice, we found that VSMCs from media contribute to neointima formation after artery injury, and metformin reduces VSMC migration and the area of the neointima. Using streptozotocin (STZ)-induced diabetic ApoE$^{-/-}$ mice, we found that metformin reduces atherosclerotic plaques, while S177A-Pdlim5, an unphosphorylatable mutant that carried adenovirus, undermines metformin's anti-atherosclerosis function. Taken together, metformin reduces the motility of vascular smooth muscle cells through the activation of the AMPK–Pdlim5 pathway, which contributes to the protective effects of metformin against diabetes-accelerated atherosclerosis and is beneficial for the therapy of metabolic syndrome.

Abbreviations: AMPK, AMP-activated protein kinase; CTL, control; CVD, cardiovascular disease; DM, diabetic mellitus; ECM, extracellular matrix; KDR, knockdown-rescue; LDL, low-density lipoprotein; STZ, streptozotocin; Met, metformin; Pdlim5, PDZ and LIM domain 5; T2DM, type 2 diabetic mellitus; VSMCs, vascular smooth muscle cells.

MATERIALS AND METHODS

Animal Experiments

Eight-week-old male ApoE$^{-/-}$ mice (body weight, 20–25 g) on C57/BL6 background were purchased from Beijing Biocytogen Co., Ltd. (Beijing, China) and kept with free access to water and food in a specific pathogen-free room under 24°C and 12-h light/dark cycle at the laboratory animal center of Southern Medical University. ApoE$^{-/-}$ mice were injected intraperitoneally with 50 mg/kg STZ for 5 days to induce DM. At 2 weeks later, the diabetic mice were randomly divided into eight groups ($n = 10$ per group): control group, metformin hydrochloride (*via* gastric gavage, 300 mg/kg/day, Sigma-Aldrich) group, wild-type Pdlim5 (Pdlim5 WT) that carried adenovirus (Ad) group, Ad Pdlim5 WT and Met group, Ad Pdlim5 S177A group, Ad Pdlim5 S177A and Met group, Ad Pdlim5 S177D group, and Ad Pdlim5 S177D and Met group. At 3 days after virus infection, the mice were fed with a high-fat diet subsequently. At the end of the experiments, the mice were euthanized with terminal anesthetic (isoflurane >4% in 95% O_2 and 5% CO_2). All the animal experiments were approved and performed according to the Institutional Animal Care and Use Committee (IACUC) of Southern Medical University, which conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. The high-fat and high-cholesterol diets were purchased from Guangdong Experimental Animal Center. The 2-kg pack of high-fat diet (HFD) contains 4.4 kcal/g of energy, and the components per pack as listed by the manufacturer were as follows: 17% lard, 1.2% cholesterol, 0.2% sodium cholate, 10% casein, 0.6% calcium hydrogen carbonate, 0.4% stone powder, 0.4% premix, and 52.2% basic feed.

Blood Glucose and Plasma Lipid Measurements

Blood glucose level was determined 2 weeks later after STZ induction with OneTouch Ultra2 Glucose Monitors (LifeScan, Milpitas, CA, USA). Mice whose blood glucose level was above 16.6 mmol/L were diagnosed as having DM. Plasma total cholesterol, triglyceride, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were determined with biochemical kits (Jiancheng Biotechnology, Nanjing, China).

Carotid Artery Injury

Twelve- to 15-week-old *Myh11-cre-EGFP* male mice, with background in C57BL/6 WT, were purchased from Shanghai Model Organisms Center, Inc. The carotid arterial intima of the mice were mechanically damaged with a beaded guidewire as described in the reference (25). The mice were anesthetized by inhalation with a mixture of isoflurane (2%) and oxygen (98%). The animal work was also approved and performed according to the IACUC of Southern Medical University, which conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Tissue Collection, *En face* Analysis of the Aortic Arch, and Immunohistochemical Staining

The mice were perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde, after euthanasia. The hearts, together with a short segment of the aorta, were collected and embedded or quickly frozen. Immunohistochemical staining was performed as described previously (26). All immunofluorescence micrographs and images of Oil Red O-stained areas of the atherosclerotic lesion were acquired with an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan), and morphometric analysis was performed using ImageJ software (NIH).

Western Blotting

Western blotting was performed according to the description in the reference (21).

Cell Culture

The mouse aortic smooth muscle cells and 293T cells were purchased from the American Type Culture Collection. The cells were cultured in DMEM supplemented with 10% serum (Gibco) under a humidified environment at 37°C in 5% CO_2 and 95% air. The cells were sub-cultured when grown to 80–90% confluence. Cells within 10 generations were used for the experiments. The AMPK-α1 knock-out knockdown-rescue (KDR)/EGFP-Pdlim5 VSMC was a gift generously provided by Professor Takashima (Osaka University).

Adenoviral Infection

All adenoviruses, a replication-defective adenoviral vector expressing wild-type Pdlim5 or Pdlim5 mutant fused with flag or EGFP tag, were gifts from the Takashima group (Osaka University). The Pdlim5 S177A or S177D adenoviral vector expressed a mutant of Pdlim5 in which serine 177 was substituted with alanine (S77A) or aspartate (S77D), respectively. For the animal experiments, the diabetic mice were infected with adenovirus in an open-chest myocardium injection after having been anesthetized with a mixture of isoflurane (2%) and oxygen (98%). For the cellular model work, VSMCs were infected with Ad-Pdlim5 WT, Ad-Pdlim5-S177A, or Ad-Pdlim5-S177D overnight in a medium supplemented with 2% fetal calf serum. The cells were then washed and incubated in fresh VSMC growth medium without fetal calf serum for an additional 12 h prior to experimentation. These conditions typically produced an infection efficiency of at least 80% as determined by EGFP or flag expression.

Scratch Assay

The mouse aortic smooth muscle cells were seeded on 35-mm glass dishes at an initial density of 5×105 cm^2. A scratch was made with a P-200 pipette tip 8 h after seeding. Then, 1 mM of metformin hydrochloride (Sigma-Aldrich) was added after changing the medium, with PBS as the control. The lesions were observed with a Zeiss inverted microscope, measured with the ImageJ software (NIH) once per hour, and totally observed for 8 h.

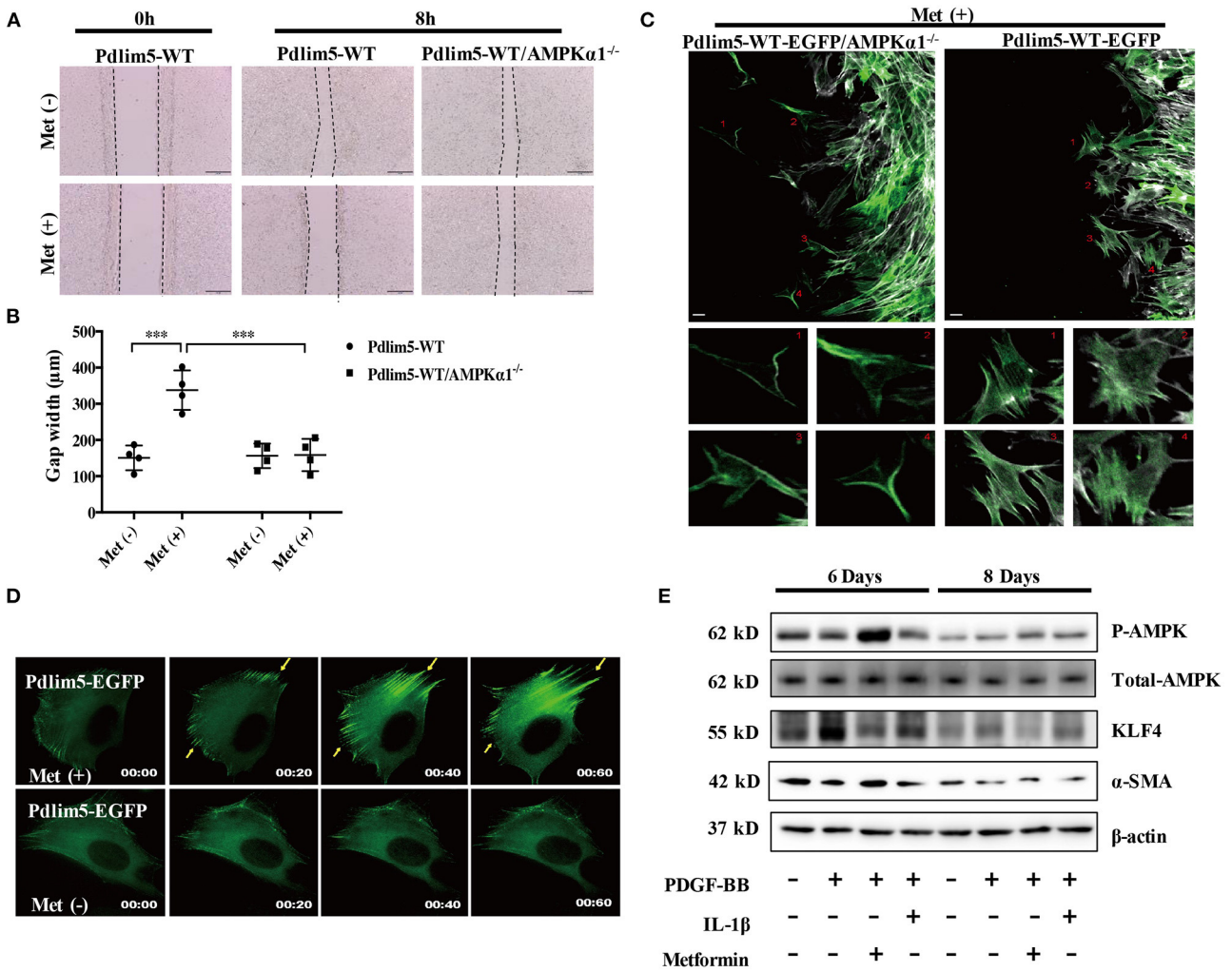

FIGURE 1 | Metformin inhibits vascular smooth muscle cell (VSMC) migration through the AMPK–Pdlim5 pathway. **(A)** EGFP-Pdlim5 VSMCs and AMPK α1-null/EGFP -Pdlim5 VSMCs were stimulated with metformin (1 mM) for 8 h in a scratch assay. **(B)** Bar graph showing the gap width 8 h after scratching [from **(A)**]. Data are representative of means ± SEM from four independent experiments. The significance of differences between series of results was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. $^{***}P < 0.001$, $n = 4$. **(C)** GFP images of AMPK α1-null/KDR/EGFP-WT-Pdlim5 cells before and after metformin stimulation (1 mM). The magnified images below show the cells labeled by the number. Scale bar, 10 mm. **(D)** Time-lapse images of GFP signal in EGFP-WT-Pdlim5 cells with or without metformin stimulation (1 mM). Yellow arrowheads indicate the growth of dorsal stress fibers from the opposite side in EGFP-WT-Pdlim5 cells after metformin stimulation. Scale bar, 10 mm. **(E)** Western blot of vascular smooth muscle cell (VSMC) contractile markers SMA, transcriptional regulator KLF4, and phosphorylated AMPK in control, PDGF-BB-treated, metformin combined with PDGF-BB and IL-1β combined with PDGF-BB-treated VSMCs.

Time-Lapse Imaging of VSMC Cells

EGFP-Pdlim5-WT cells or EGFP-Pdlim5/AMPK-α1 KO cells were plated on 35-mm glass dishes coated with collagen at an initial density of 4×104 cm^2. At 5 h after plating, the cells were treated with metformin (1 mM). The fluorescence images were recorded as described before (21).

Statistical Analyses

The data in the graphs are presented as means ± SEM. Two-tailed Student's *t*-test was utilized to compare the two groups. Differences among multiple experimental groups were analyzed by one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method utilizing SPSS 16 (IBM). $P < 0.05$ was considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$).

RESULTS

Metformin Inhibits VSMC Migration Through the AMPK–Pdlim5 Pathway and Independent of VSMC Phenotype Transition

To investigate the role of the AMPK–Pdlim5 pathway in cell migration, we established the KDR system in wild-type

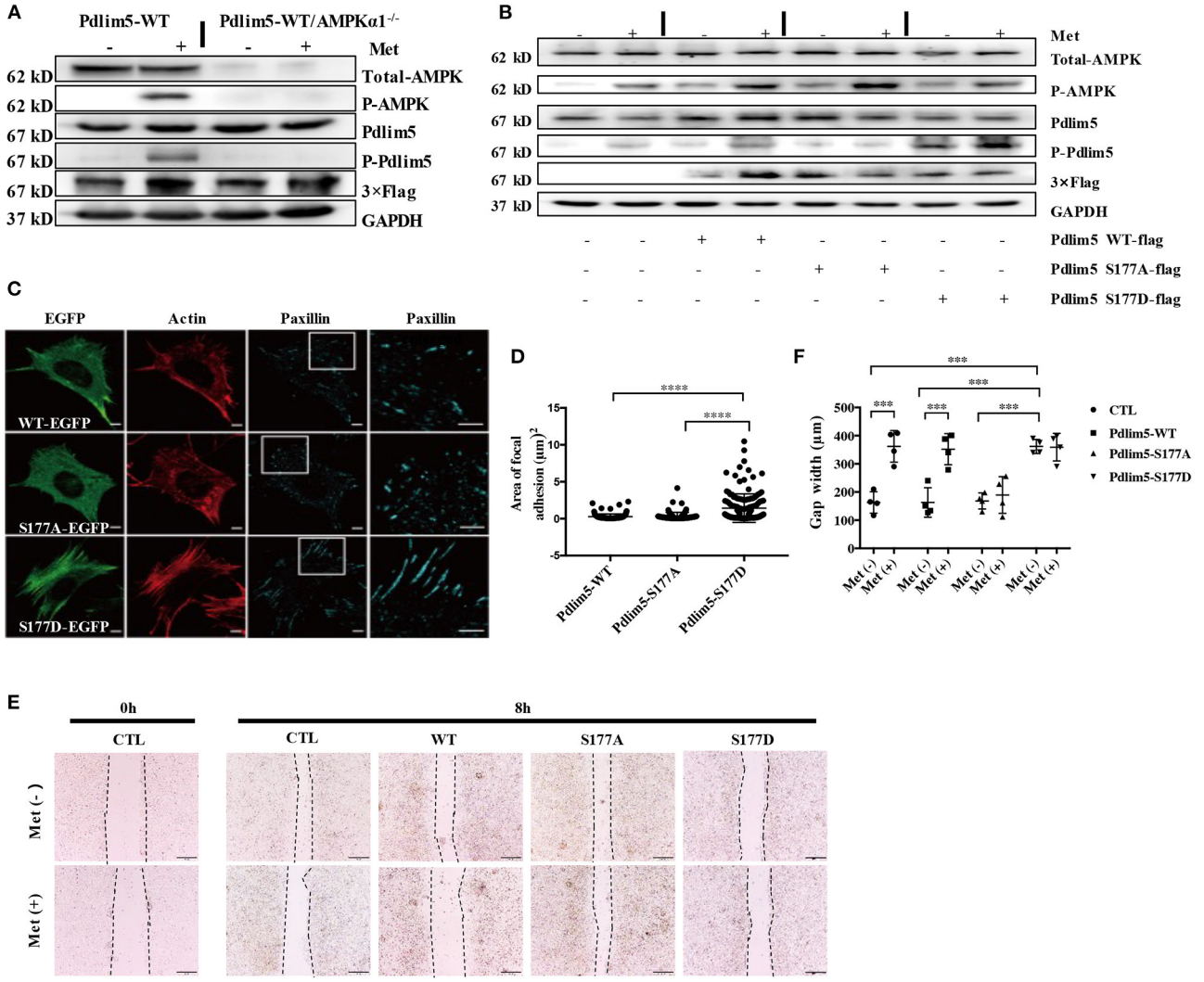

FIGURE 2 | Metformin inhibits lamellipodia formation and enforces focal adhesions in vascular smooth muscle cells (VSMCs) through the AMPK–Pdlim5 pathway. **(A)** Flag-WT-Pdlim5 VSMCs and AMPK α1-null/EGFP-WT-Pdlim5 VSMCs were stimulated with metformin before detecting the phosphorylation level of AMPK and Pdlim5 with immunoblotting. **(B)** Metformin activates the AMPK–Pdlim5 signaling pathway. VSMCs were transfected with CTL siRNA or Pdlim5 siRNA. Pdlim5 siRNA-resistant flag-Pdlim5 (WT, S177A, or S177D) was added back by adenoviral-mediated gene delivery. The phosphorylation levels of AMPK and Pdlim5 were detected with western blotting. **(C)** GFP signal and immunostaining of actin and paxillin in KDR/EGFP-Pdlim5 cells (WT, S177A, and S177D). Actin and paxillin were stained with phalloidin and anti-paxillin antibody to display actin microfilaments and focal adhesions, respectively. The right panels are the magnified images of the region outlined by white boxes. Scale bar, 10 mm. **(D)** The focal adhesion area of EGFP-Pdlim5 cells (WT, S177A, and S177D) were measured according to the paxillin-positive region in **(B)** and compared using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. $^{****}P < 0.0001$, $n = 100$. **(E)** Scratch assay of metformin-stimulated EGFP-Pdlim5 cells. Phase-contrast images of EGFP-Pdlim5 cells (WT, S177A, and S177D) 8 h after scratching with or without metformin. Scale bar, 0.5 mm. **(F)** Bar graph showing the gap width 8 h after scratching [from **(E)**]. Data are representative of means ± SEM from four independent experiments. The significance of the results was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. $^{***}P < 0.001$, $n = 4$.

VSMCs or AMPKα1 null VSMCs as described before (21), in which endogenous Pdlim5 was replaced with EGFP or flag-fused Pdlim5, Pdlim5 S177A (an unphosphorylatable mutant), or Pdlim5-S177D (a phosphomimetic mutant), respectively (**Figures 1**, **2**).

In the scratch assay, it was observed that metformin inhibits the wounding healing ability of WT-Pdlim5 VSMCs. However, metformin failed to inhibit wound healing in the AMPKα1 absent WT-Pdlim5 VSMCs (**Figures 1A,B**). Enhanced stress fiber and reduced VSMC migration were also observed with time-lapse imaging (**Figure 1C**). It was shown that metformin reduced lamellipodia formation and promoted the enhancement of the EGFP signals from the side opposite to the lamellae, a pattern similar to the growth of dorsal stress fibers (**Figure 1C**).

It has been reported that the migration of VSMCs is related to phenotype transition, which means the loss of contractile

proteins and the expression of specific transcriptional factors. To investigate whether metformin inhibit VSMC migration by influencing phenotype switching, wild-type VSMCs were treated with PDGF-BB, a phenotype transition inducer, or PDGF-BB combined with metformin or inflammatory cytokine IL-1 as a control for 8 days. It was shown that PDGF-BB increased the expression of phenotype switching regulator KLF4 from day 6, reducing the expression of contractile protein SMA from day 8. However, metformin activated AMPK effectively and reduced the KLF4 expression but had no significant influence upon SMA expression (**Figure 1E**). These results suggest that metformin inhibits VSMC migration through AMPK activation but not via the phenotype transition pathway.

Metformin Inhibits VSMC Migration Through the Enforcement of Focal Adhesions and Reducing Lamellipodia Formation

To investigate whether metformin could induce the phosphorylation of Pdlim5 through AMPK, EGFP or flag-fused WT-Pdlim5 Pdlim5, Pdlim5 S177A (an unphosphorylatable mutant), or Pdlim5-S177D (a phosphomimetic mutant) were overexpressed in wild-type VSMCs or AMPKα1 null VSMCs before metformin exposure. In **Figure 2A**, it was shown that metformin could induce the phosphorylation of AMPK and Pdlim5 in wild-type VSMCs but not in WT-Pdlim5/AMPKα1 null VSMCs. The phosphorylation of Pdlim5 was not observed in Pdlim5-S177A VSMCs, while Pdlim5-S177D was recognized by the Ab-pS177 antibody even without Met stimulation (**Figure 2B**). In **Figure 2C**, both WT- and Pdlim5-S177A cells possessed smooth lamellipodia-like edges, whereas Pdlim5-S177D cells displayed decreased lamellipodia formation and jagged edges with excessive filopodia-like protrusions and ventral stress fibers. In addition, it was found that both WT- and S177A-Pdlim5 cells had tiny and scattered spots of focal adhesions at the junction between the lamellipodia and lamella; by contrast, in S177D-Pdlim5 cells, focal adhesions were displaced to the edge of the cell and significantly enlarged in size (**Figure 2C**).

Next, a scratch assay was performed to observe whether decreased lamellipodia and enhanced stress fiber would inhibit cell migration (**Figure 2E**). It was shown that the wound healing ability of S177D cells is lower than that of WT- and Pdlim5-S177A cells, while Met inhibited the migration of both WT- and Pdlim5-S177D cells only, except S177A cells (**Figure 2E**).

Metformin Attenuates Intimal Hyperplasia After Artery Injury in Myh11-Cre/Rosa26-EGFP Mice

As metformin could inhibit VSMC migration *in vitro*, a wire injury-induced vascular remodeling model was utilized to verify this function of Met *in vivo* (**Figure 3**). To accomplish this experiment, 6- to 8-week-old Myh11-Cre/Rosa26-EGFP mice, which express EGFP in mature VSMCs, were subjected to ligation of the left carotid artery. An accumulation of VSMC-derived cells in the intimal hyperplasia of the left carotid artery was observed by confocal microscopy with arterial cross-sections. It was found that many Myh11-expressing VSMC-derived EGFP-positive cells contributed to the neo-intima lesions and constituted a significant proportion of the total cell number within lesions. Interestingly, metformin treatment reduced the intima hyperplasia (**Figure 3A**) and patch size (**Figure 3B**) than the saline treatment significantly.

Metformin Alleviates Atherosclerotic Lesions in Diabetic ApoE$^{-/-}$ Mice

To study whether metformin could be used to prevent diabetes-accelerated atherosclerosis, DM and atherosclerosis were induced in ApoE$^{-/-}$ mice with streptozocin and HFD. The diabetic mice were treated with metformin as described in "Materials and Methods." It was found that the diabetic ApoE$^{-/-}$ mice possessed obvious *en face* lesions in the aortic arch and thoracic and abdominal aorta and greater atherosclerotic lesions in the aortic root (**Figures 3C–E**). Metformin intervention significantly reduced the lesion areas in the aortic root and the aortic arch in diabetic ApoE$^{-/-}$ mice. The SMA-positive deposition in the aortic root was also smaller in metformin-treated mice than in diabetic mice, which suggests that metformin inhibited VSMC accumulation in atherosclerotic plaques (**Figures 3F,G**).

The Activation of the AMPK–Pdlim5 Pathway Is Involved in the Protective Function of Metformin Against Diabetes-Accelerated Atherosclerosis in ApoE$^{-/-}$ Mice

To study the role of AMPK–Pdlim5 pathway in the development of atherosclerosis, ApoE$^{-/-}$ animals were divided randomly into streptozocin-induced diabetes mellitus together with or without metformin, Pdlim5 phosphomimetic mutant that carried adenovirus (Pdlim5 S177D) or Pdlim5 unphosphorylatable mutant that carried adenovirus (Pdlim5 S177A). It is shown in **Figure 4** that metformin reduced hyperglycemia significantly (**Figure 4B**), while it did not exhibit an obvious influence upon dyslipidemia induced by STZ and HFD (**Figures 4C–F**). However, the manipulation of Pdlim5 phosphorylation with adenovirus has no significant influence on metabolic disorders in DM mice (**Figures 4B–F**). The diabetic ApoE$^{-/-}$ mice developed significantly larger *en face* lesions in the aortic arches (**Figures 4G–J**) and larger SMA- and phosphorylated Pdlim5-positive lesions in the aortic roots compared with those in the metformin treatment group (**Figures 5A–C**). The Pdlim5-negative mutant adenovirus (S177A) alleviated the protective function of metformin as mentioned above and increased the plaque area significantly (**Figures 4G–J**, **5A–C**). However, Pdlim5 constitutively active adenovirus (S177D) suppressed atherosclerosis obviously even under the absence of metformin (**Figures 4G–J**, **5A–C**).

DISCUSSION

The blood vessels mainly contain two cell types: endothelial cells (EC) and vascular smooth muscle cells. Injury of the endothelium

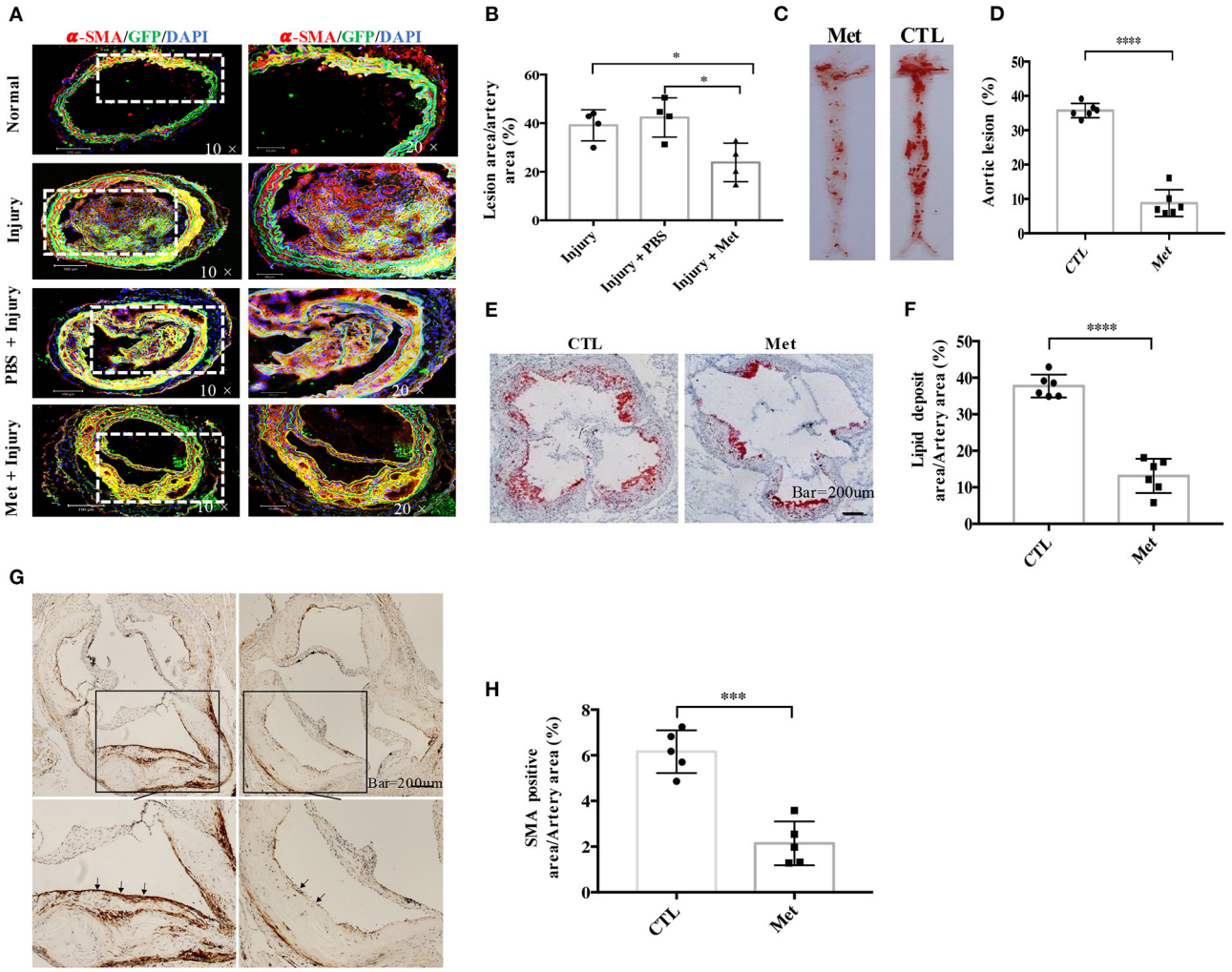

FIGURE 3 | Metformin inhibits medial vascular smooth muscle cell (VSMC) migration *in vivo* and reduces VSMC infiltration in atherosclerotic plaques in ApoE$^{-/-}$ mice. **(A)** Myh11cre-eGFP mice were treated with saline or metformin for a week after left carotid wire injury ($n = 5$ per group). Representative images of the immunostaining of SMA- and EGFP-positive cells in an artery cross-section. **(B)** Quantitative analysis of the percentage of lesion area compared to the cross-sectional area of the artery. Data are representative of means ± SEM from five independent experiments. The significance of differences between the series of results was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. *$P < 0.05$, $n = 5$. **(C)** ApoE$^{-/-}$ mice were injected with streptozotocin for 5 days to induce diabetes and then fed with high-fat diet and metformin intervention (Met) or control (CTL) for 8 weeks. The atherosclerotic lesions in the aorta were stained with Oil Red O. **(D)** Quantification of the *en face* lesion area in the aorta. The significance of differences was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. ****$P < 0.0001$, $n = 6$. **(E)** Oil Red O staining of atherosclerotic lesions at the aortic root. **(F)** Quantification of the lesion size in the aortic root. The significance of differences was analyzed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. ****$P < 0.0001$, $n = 6$. **(G)** Immunochemistry staining of α-SMA in the aortic root. **(H)** Quantitative analysis of α-SMA-positive area in the aortic root. The significance of differences between series of results was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. ***$P < 0.001$, $n = 5$.

leads to the initiation of atherosclerosis, while the abnormal proliferation and migration of vascular smooth muscle cells result to the development of atherosclerotic plaques (27). An aberrant EC–VSMC interaction could promote atherogenesis (27). It is widely accepted that the effects of endothelial dysfunction on VSMCs are reduction of NO bioavailability and/or augmentation of vasoactive constrictors released from the endothelium (28). The injured EC can also recruit inflammatory cells and release cytokines that induce a phenotype change of VSMCs from the "contractile" phenotype to the "synthetic" state that can migrate and proliferate from the media to the intima (29). An accumulation of VSMC in the vascular intima is a hallmark of atherosclerosis, but their exact origins are still in controversy (8, 22). In humans, both pre-existing intimal and medial VSMCs can contribute to plaque VSMCs (30). In mice, the VSMCs in the fibrous cap are unambiguously derived from media, which suggests the importance of cell migration in the pathogenesis of atherosclerosis (31). This is

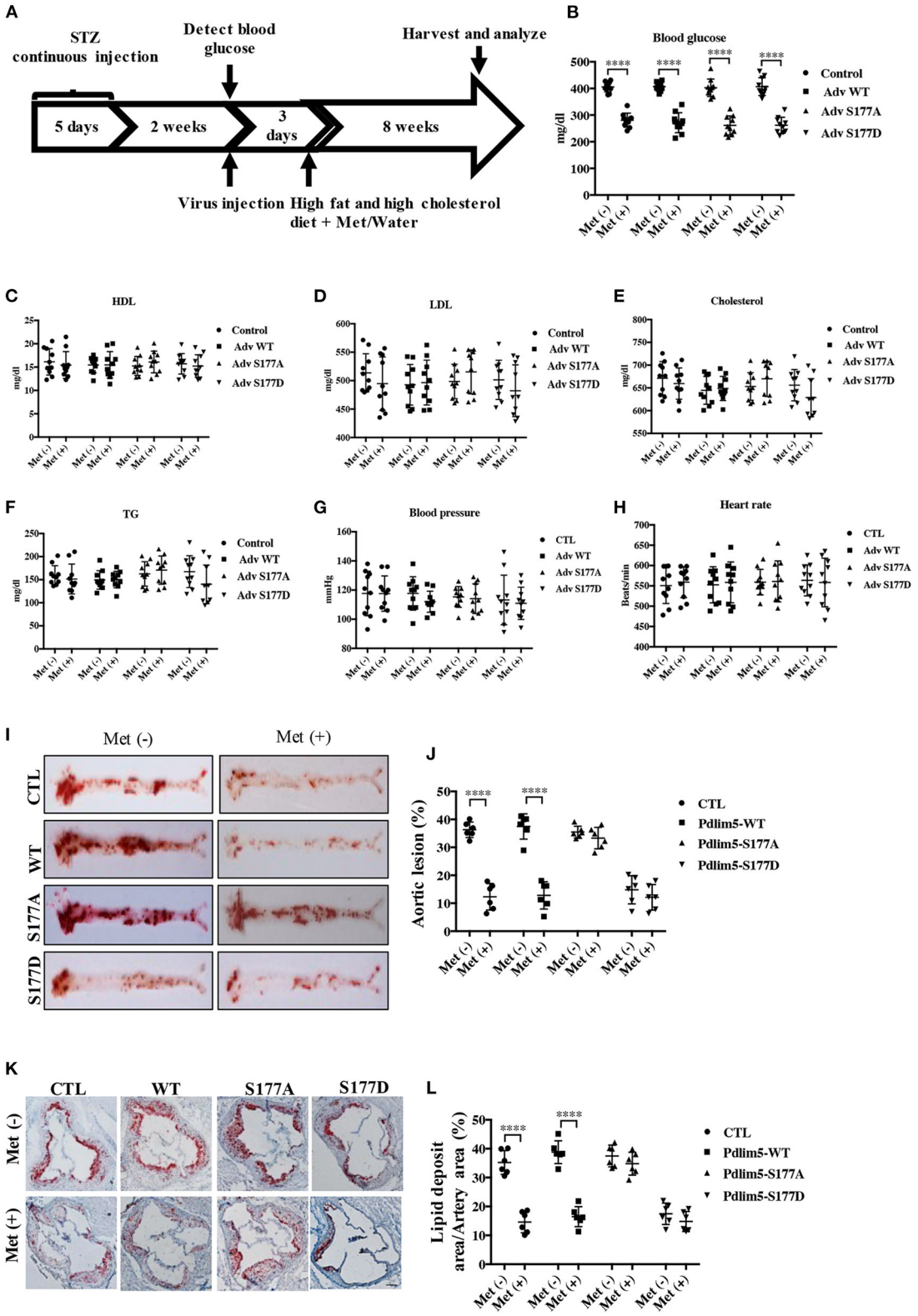

FIGURE 4 | Phosphorylation of Pdlim5 is involved in the anti-atherosclerosis function of metformin in diabetic ApoE$^{-/-}$ mice. The ApoE$^{-/-}$ mice had induced diabetic atherosclerosis and intervened with metformin as described in "Materials and Methods" for 8 weeks ($n = 10$ per group). At the same time, the mice were randomly

(Continued)

FIGURE 4 | separated into four groups: vehicle group, adenovirus WT Pdlim5 group, Ad S177A Pdlim5 group, and Ad S177D Pdlim5 group. **(A–H)** Blood glucose, plasma HDL, LDL, cholesterol, triglyceride, blood pressure, and heart rate were determined with a commercial kit. The data are representative of means ± SEM from 10 independent experiments. The significance of differences between the series of results was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. $^{****}P < 0.0001$, $n = 10$. **(I)** Oil Red O staining of atherosclerotic lesions at the aorta. **(J)** Quantification of the *en face* atherosclerotic lesion area in the aorta. Data are representative of means ± SEM from six independent experiments. The significance of differences was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. $^{****}P < 0.0001$, $n = 6$. **(K)** Oil Red O staining of atherosclerotic lesions at the aortic root. **(L)** Quantification of the atherosclerotic lesion size in the aortic root. Data are representative of means ± SEM from six independent experiments. The significance of differences of the results was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. $^{****}P < 0.0001$, $n = 6$.

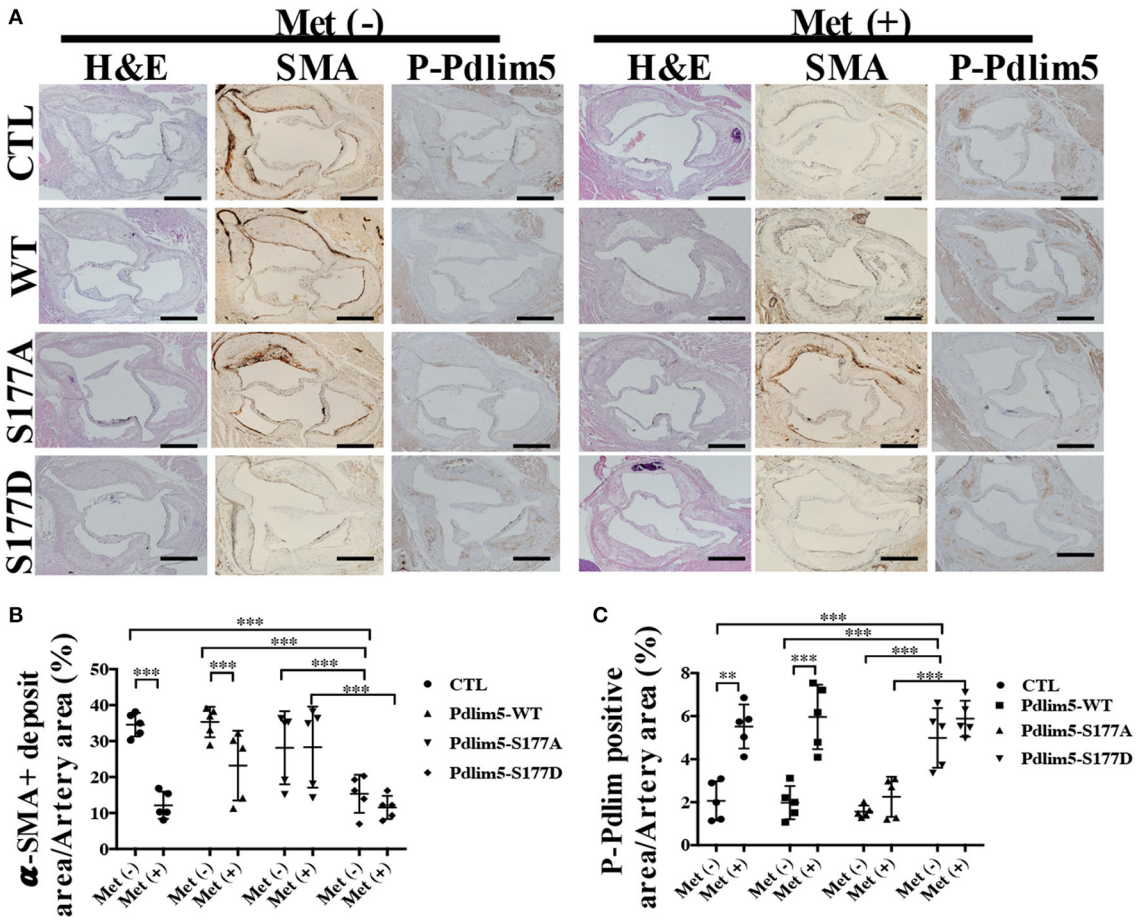

FIGURE 5 | The phosphorylation of Pdlim5 induced by AMP-activated protein kinase was related to metformin's anti-atherosclerosis function. **(A)** Representative images of H&E staining and immunostaining of α-SMA and phosphorylated Pdlim5 at the aortic root of diabetic ApoE$^{-/-}$ mice. Scale bar = 200 μm. **(B)** Quantification of α-SMA-positive area in the aortic root. Data are representative of means ± SEM from five independent experiments. The significance of differences between the series of results was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. $^{***}P < 0.001$, $n = 5$. **(C)** Quantification of phosphorylated Pdlim5-positive area in the aortic root. Data are representative of means ± SEM from five independent experiments. The significance of differences was assessed using one-way analysis of variance, followed by a *post-hoc* comparison with Dunnett's method for multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$, $n = 5$.

consistent with our finding that inhibiting VSMC migration through the activation of AMPK reduces neointima formation induced by artery injury and STZ/HFD-induced atherosclerosis. The mechanics of VSMC migration in atherosclerotic lesions involves the formation of plasma membrane-leading lamellae (leading edge) and the disengagement of focal adhesions that are in contact with the ECM (32). We found that metformin could inhibit cell migration through the enforcement of focal adhesions and reducing lamellipodia formation *in vitro*. The underlying mechanism has been elucidated by our publication and those of others, such that phosphorylation of Pdlim5 by AMPK disrupts the binding between Pdlim5 with Arhgef6 at the cell periphery (21). The dissociation suppresses Rac1 activity and dislocates the Arp2/3 complex from the leading edge of cells which impairs lamellipodia formation and cell migration (21, 32, 33).

VSMC migration in atherosclerosis has also been related to phenotype switching—a synthetic, de-differentiated state (34). The phenotype-switched VSMCs exhibit a reduced production of contractile proteins but with a higher expression of ECM-related products and increased levels of secretory organelles and pro-inflammatory cytokines (35). At the molecular level, the VSMC phenotype transition is governed by transcription factors myocardin serum response factor 58 and Krüppel-like factor 4 (KLF4) (23, 36). Diabetes mellitus-associated pathological factors exacerbate the synthetic phenotype of VSMCs through the up-regulation of KLF4 (37). Although our work has shown that metformin-induced AMPK activation has an insignificant influence on the expression of KLF4 and "contractile proteins" (**Figure 2E**), the relationship between VSMC migration and phenotype switching in atherosclerotic plaques still needs further investigation.

AMPK is a vital enzyme for regulating cellular energy homeostasis (38). The activation of AMPK depends on phosphorylation at its T172 site and binding with AMP and/or ADP (39). Compelling evidence has indicated an inverse correlation between diabetes and AMPK activity (38). Therefore, AMPK-activating agents have the potential to be utilized as precaution or therapies against diabetes and DM-related complications. Indeed metformin, an indirect AMPK activator and well-known T2DM drug, could reduce atherosclerosis in patients with diabetes (19, 39). However, the underlying mechanism is not clear yet. Recent work found that AMPK also plays an important role in the regulation of cell polarity and motility (21), which throws a light on the research of metformin's anti-atherosclerosis function. There are two different α isoforms (α1 and α2) that are differentially expressed in different tissues. Several references found that AMPKα2 plays an important role in the aberrant migration of vascular smooth muscle cells in atherosclerosis. However, our previous work found that AMPKα1 is more important for the migration of vascular smooth muscle cells *in vitro* (21), and the phosphorylation of Pdlim5 by AMPKα1, but not AMPKα2, plays an important role in the anchoring of vascular smooth muscle cells (21). In this study, we found that metformin activates AMPK, which phosphorylates Pdlim5 at Ser177, resulting in the attenuation of lamellipodia formation and the inhibition of vascular smooth muscle cell migration from the medial to intima. We also demonstrated that metformin reduces VSMC accumulation in atherosclerotic plaques *via* an AMPK–Pdlim5-dependent manner in STZ- and HFD-induced diabetic ApoE$^{-/-}$ mice. It is consistent with the existing concept that metformin has multiple beneficial effects on vascular cells (endothelial cells, vascular smooth muscle cells, and macrophages), many of which are AMPK-mediated (15, 40–43).

Our previous work found that AMPK has many substrates—CLIP170, VASP, and Pdlim5, for instance (21, 44, 45), but their inductions need a different activated level of AMPK. Basically, AMPK has three activated levels which are low activity, physiological activity, and augmented activity (21). Different activated AMPK situations induce different substrates, but only physiologically activated AMPK stimulates cell migration, while others both inhibit migration (21). Less activated AMPK inhibits migration through CLIP170, which is a component of a microtube (21), while augmented activated AMPK inhibits cell migration through the phosphorylation of Pdlim5, which is a component of actin (21). Metformin, as a well-accepted oral drug for type 2 diabetes patients, could induce the augmented activation of AMPK *in vitro* and *in vivo* efficiently (46), so our work first found that Pdlim5, a component of muscle cytoskeleton that is related to many cardiovascular diseases (47), as a substrate of AMPK could be phosphorylated only by augmented activated AMPK, which induces the abolishment of the migratory machine in VSMCs *in vitro* and *in vivo*. This work partially explains the beneficial effects of metformin toward diabetes-accelerated atherosclerosis. This work suggests that Pdlim5 has the potential to be a drug target to suppress the development of atherosclerosis. Moreover, we also found that Pdlim5 plays an important role in the pathology of some cardiovascular diseases through other post-translational modifications instead of phosphorylation. Hence, there are still lots of work to be accomplished to translate the knowledge of atherogenesis in the clinic.

CONCLUSION

Nevertheless, our research revealed that augmentation of AMPK activity could inhibit VSMC migration from the media to the atherosclerotic plaques through the phosphorylation of Pdlim5. It may be useful to develop novel therapies toward atherosclerosis and other complications in diabetes.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Southern Medical University.

AUTHOR CONTRIBUTIONS

YY, WW, and ZZ conceived and designed the studies, wrote the manuscript, and completed all analyses. YY and TL performed the experiments and data analysis. ZL, MH, DW, YX, XY, YB, and YL contributed to the acquisition of data. All authors contributed to the article and approved the submitted version.

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Diabetes Promotes Retinal Vascular Endothelial Cell Injury by Inducing CCN1 Expression

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Purpose: Diabetic retinopathy (DR) is one of the most common diabetic microvascular complications. However, the pathogenesis of DR has not yet been fully elucidated. This study aimed to discover novel and key molecules involved in the pathogenesis of DR, which could potentially be targets for therapeutic DR intervention.

Methods: To identify potential genes involved in the pathogenesis of DR, we analyzed the public database of neovascular membranes (NVMs) from patients with proliferative diabetic retinopathy (PDR) and healthy controls (HCs) (GSE102485, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102485). Further, we compared these findings by performing RNA-sequencing analysis of peripheral blood mononuclear cells (PBMC) from patients with DR, control patients with non-complicated diabetes mellitus (DMC), and HCs. To determine the critical role of candidate genes in DR, knockdown or knockout was performed in human retinal vascular endothelial cells (HRVECs). The oxidative stress pathway, as well as tight junction integrity, was analyzed.

Results: Transcriptional profiles showed distinct patterns between the NVMs of patients with DR and those of the HCs. Those genes enriched in either extracellular matrix (ECM)-receptor interaction or focal adhesion pathways were considerably upregulated. Both pathways were important for maintaining the integrity of retinal vascular structure and function. Importantly, the gene encoding the matricellular protein CCN1, a key gene in cell physiology, was differentially expressed in both pathways. Knockdown of CCN1 by small interfering RNA (siRNA) or knockout of CCN1 by the CRISPR-Cas9 technique in HRVECs significantly increased the levels of VE-cadherin, reduced the level of NADPH oxidase 4 (NOX4), and inhibited the generation of reactive oxygen species (ROS).

Conclusion: The present study identifies CCN1 as an important regulator in the pathogenesis of DR. Increased expression of CCN1 stimulates oxidative stress and disrupts tight junction integrity in endothelial cells by inducing NOX4. Thus, targeting the CCN1/NOX4 axis provides a therapeutic strategy for treating DR by alleviating endothelial cell injury.

Keywords: diabetic retinopathy, CCN1, oxidative stress, NOX4 NADPH oxidase, tight junction

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INTRODUCTION

Both diabetes (1) and pre-diabetes (2, 3) could lead to microvascular and macrovascular complication. Diabetic retinopathy (DR) is the most common complication of diabetes mellitus (DM); it has long been recognized as a microvascular disease (4) that affects nearly one-third of all patients with diabetes (5) and is the leading cause of vision loss globally (6). The condition results in a poor quality of life and increases mortality in patients. Etiological therapy is paramount for the management of DR and improvement of the prognosis. However, the pathogenesis of DR is not yet thoroughly understood. Thus, to date, no specific therapy is available to effectively treat DR.

The retina is the most metabolically active human tissue, and it is highly sensitive to hypoxia (7) DR has been shown to be closely related to hypoxia (8). Previous studies (9–11) have shown that CCN1, the first cloned member of the CCN family (12), is upregulated by hypoxia in several different cell types, including retinal vascular endothelial cells, along with the concurrent regulation of the transcriptional factors HIF-1a and c-Jun/AP-1(13). The CCN family consists of six distinct members, named according to the acronyms of the first three members: cysteine-rich (CYR61), connective tissue growth factor (CTGF), and nephroblastoma overexpressed (NOV) (14). They regulate cellular processes such as adhesion, migration, mitogenesis, differentiation, and survival (12). In addition to hypoxia, CCN1 expression in rat retinas is increased by more than 3-fold after 6 weeks of diabetes, and increased by 4-fold in the advanced glycation end product (AGE)-treated mouse retina (15). This indicates that the expression of CCN1 is positively correlated with the occurrence of DR.

Angiogenesis and fibrosis are the main characteristics of DR. The CCN1 family has been associated with both the fibrotic processes (16) and neovascularization of DR (17). According to one report, CCN1 induces fibroblast senescence and restricts cutaneous fibrosis; thus, tissue healing possibly occurs through the reactive oxygen species (ROS)-dependent activation of the p16INK4a/pRb pathway (18). The loss of retinal pericytes is another distinctive feature of DR, which is characterized by retinal capillary obliteration. Furthermore, CCN1 has been proven to induce pericyte detachment and anoikis (19). Previous studies suggest that CCN1 is involved in the pathogenesis of DR; however, the precise mechanism is still unknown.

The ROS are associated with one of the leading pathogenic mechanisms during DR progression. The NADPH oxidase (NOX) family produces ROS, which causes oxidative injuries and stimulates neovascularization in the retinal vasculature (20). In addition, NOX4 is the most abundant isoform in vascular endothelial cells (21). One study has shown that NOX4-generated ROS induces neuronal and blood-brain barrier injury after intracerebral hemorrhage (22). Our previous study (23) also demonstrated that NOX4 can stimulate inflammation in human retinal vascular endothelial cells (HRVECs) by activating the NLR family pyrin domain containing 3 (NLRP3). Both CCN1 and NOX4 are upregulated in DR (20, 24) and play important roles in the regulation of retinal inflammation and angiogenesis. However, whether CCN1 interacts with NOX4 in the pathogenesis of DR remains unknown.

In the current study, we reanalyzed the published transcriptome data of human retinal tissue (GSE102485) (25) to compare the mRNA levels of CCN1 and oxidative stress-related genes from DR patients and HCs. We also modulated CCN1 levels by overexpression or knocking-down to examine ROS generation, the expression levels of NOX4, and the junction molecule VE-cadherin in HRVECs. The current study demonstrated that CCN1 affects oxidative stress by modulating NOX4 and provides new insights into the mechanism of DR and a novel target for predicting or treating DR.

MATERIALS AND METHODS

Ethics Statement

All experiments related to human samples were approved by the Clinical Research Ethics Committee of The Third Affiliated Hospital of Sun Yat-Sen University, according to the guidelines of the Declaration of Helsinki. All patients were recruited from the Department of Endocrinology at the same hospital. The C57BL/6J mice were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, Guangdong, China). All experiments were conducted according to the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals, and approved by the Animal Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-Sen University. All mice were housed under a standard 12-h light–dark cycle and specific pathogen-free conditions, and were allowed free access to food and water.

Construction of Diabetic Mouse Model and Hyperlipidemic Mouse Model

The C57BL/6J mice, aged 6 weeks, were randomly divided into the diabetes mellitus (DM) group and normal control (NC) group. Mice were fed a high-fat diet and treated with streptozocin (STZ) to produce DM. The DM group was subjected to intraperitoneal injection of STZ at 40 mg/kg/day for 5 days, and the NC group was injected with a comparable amount of vehicle buffer. Blood glucose levels were measured daily before and after injection. Mice with random blood glucose levels above 16.7 mmol/L were considered to have DM. The $ApoE^{-/-}$ mice, aged 6 weeks, were used for the hyperlipidemia model, and age-matched C57BL/6J mice were used as the control group.

Sample Collection, RNA Extraction, and RNA Sequencing

We recruited three groups as follows: DR, DM control (DM without DR), and healthy controls (HCs). The main exclusion criteria for each group are shown in **Supplementary Table 1**. A volume of 6 ml of peripheral blood was collected, mixed with three times the volume of TRIzol, and stored in a refrigerator at −80°C for subsequent total RNA extraction. Furthermore, 3 μg RNA per sample was used as input for the RNA sample preparations. The RNA sequencing was performed by Novogene Inc. (Beijing, China).

Database Mining

We searched the Gene Expression Omnibus (GEO) database that stores curated gene expression and original series and platform records in a repository associated with research studies about DR. We downloaded, from the GEO database, the accession number (GSE102485, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102485) (25) containing 19 neovascular membrane (NVM) samples from patients with proliferative diabetic retinopathy (PDR). Two PDR samples were not included, as the total number of sequencing reads were 10 times lower than the average. The FastQ files of our in-house RNA-seq data of peripheral blood from DR, DMC, and HC samples, as well as the downloaded RNA-seq data of NVM samples from patients with PDR (GSE102485) (25), were first mapped to the human genome (hg19) using the STAR tool. Gene expression was quantified using feature counts after normalization, based on the total number of mappable counts. Gene expression counts were further normalized among samples based on the total numbers of all mapped reads. Differentially expressed genes between any two groups (DMC vs. DR, HC vs. DR, etc.) were identified by using the cutoff values for fold enrichment at 2 and *p*-values of 0.05. Heatmaps and volcano plots were generated for the visualization of the deferentially expressed genes (DEGs). Principal component analysis (PCA) was also performed for the expression levels of all samples to illustrate the consistency of the replicates and possible similarities among the three groups. The PCA plots of the first principal components (PC1 and PC2) were used to interpret the potential sample similarity, only when the loss of information was below 25% in the two components. We further explored the functional annotation of theDGEs. The significantly enriched (FDR-q < 0.05) gene ontology (GO) terms were identified using the DAVID tools (https://david.ncifcrf.gov/). The Gene Set Enrichment Analysis (GSEA) tool (26) was further utilized to identify enriched gene sets (MSigDB 7.1) between any two groups of samples.

Cell Culture

The HRVECs were obtained from Otwo Biotech (Shanghai, China) and cultured at 37°C with 5% CO_2. Cells were maintained in Dulbecco's modified Eagle's medium (D6046; Merck, Darmstadt, Germany) containing 10% fetal bovine serum (SH30406.05: Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin (SV30010-5; Hyclone). The HRVECs from passages 3–7 were used for further experiments.

siRNA Treatment

Double-stranded small interfering RNA (siRNA) oligonucleotides against human NOX4 were synthesized by Obio Technology (Guangzhou, China) and a non-silencing siRNA was used as the negative control. Transfection was carried out using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 6 h. Cells were recovered after being left in normal culture medium overnight and were then treated with high concentrations of glucose or palmitate.

CRISPR/Cas9-based CCN1 Knockout in HRVECs

The generation of CCN1-deficient HRVECs was performed as follows: Single guide RNA (sgRNA) oligos for human CCN1, 5′ GTTGTCATTGGTAACTCGTG, was used. The sgRNA oligo was inserted into lentiCRISPR v2 (Addgene plasmid 52961). We used second-generation lentiviral packaging plasmids (VSV-G and PAX2) to deliver CRISPR constructs into the cells. Twenty-four hours after infection, cells were cultured in a medium containing 2 μg/ml puromycin for 48 h, and then further cultured 24 h in normal growth medium.

Antibodies

The anti-CCN1 antibody (26689-1) and anti-NOX4 antibody (14347) were purchased from Proteintech (Chicago, IL, USA). The anti-CD31 antibody (3528S) and tubulin (2128s) were purchased from Cell Signaling Technology (Cambridge, MA, USA). The anti-actin antibody (A5441) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-HSP90 antibody (ab13492), anti-ZO-1 antibody (ab59720), and anti-VE-cadherin antibody (ab33168) were purchased from Abcam (Cambridge, MA, USA). Isolectin GS-IB 4 from *Griffonia simplicifolia* and Alexa Fluor 488 conjugate (I21411) were purchased from Invitrogen (Carlsbad, CA, USA).

Immunostaining Assays

The HRVECs were cultured on coverslips and fixed at the indicated time points with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. They were then subjected to 5 min of permeabilization with 0.3% Triton X-100. The coverslips were then blocked with 5% fetal calf serum, 1% bovine serum albumin, and 0.025% Tween 20 in PBS for 30 min at 37°C, followed by 4°C overnight incubation with primary antibodies. Subsequently, the Alexa Fluor conjugated secondary antibodies were applied for 1 h at room temperature and the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The coverslips were then mounted onto glass slides and analyzed with fluorescence microscopy.

Western Blot Analysis

The retinal tissue of mice and cell lysis was evaluated using western blot analysis. Both the homogenate of retinal tissues and HRVECs were extracted using radioimmunoprecipitation (RIPA) lysis buffer and placed on ice for 30 min. The lysates were centrifuged for 15 min at 12,000 $\times g$ at 4°C and the supernatants were collected. Protein concentration was determined using a bicinchoninic acid (BCA) assay kit. A 20-μg sample of each protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis at a specific voltage and then transferred onto a polyvinylidene difluoride (PVDF) membrane using a wet electro transferring system (Bio-Rad, Hercules, CA, USA). The PVDF membranes were incubated with a specific primary antibody overnight at 4°C, and then further incubated with appropriate secondary antibodies at room temperature for 1 h. The blots were visualized using an enhanced chemiluminescence (ECL) system (Bio-Rad, Hercules, CA, USA). The signal intensity of the target protein bands was quantitatively

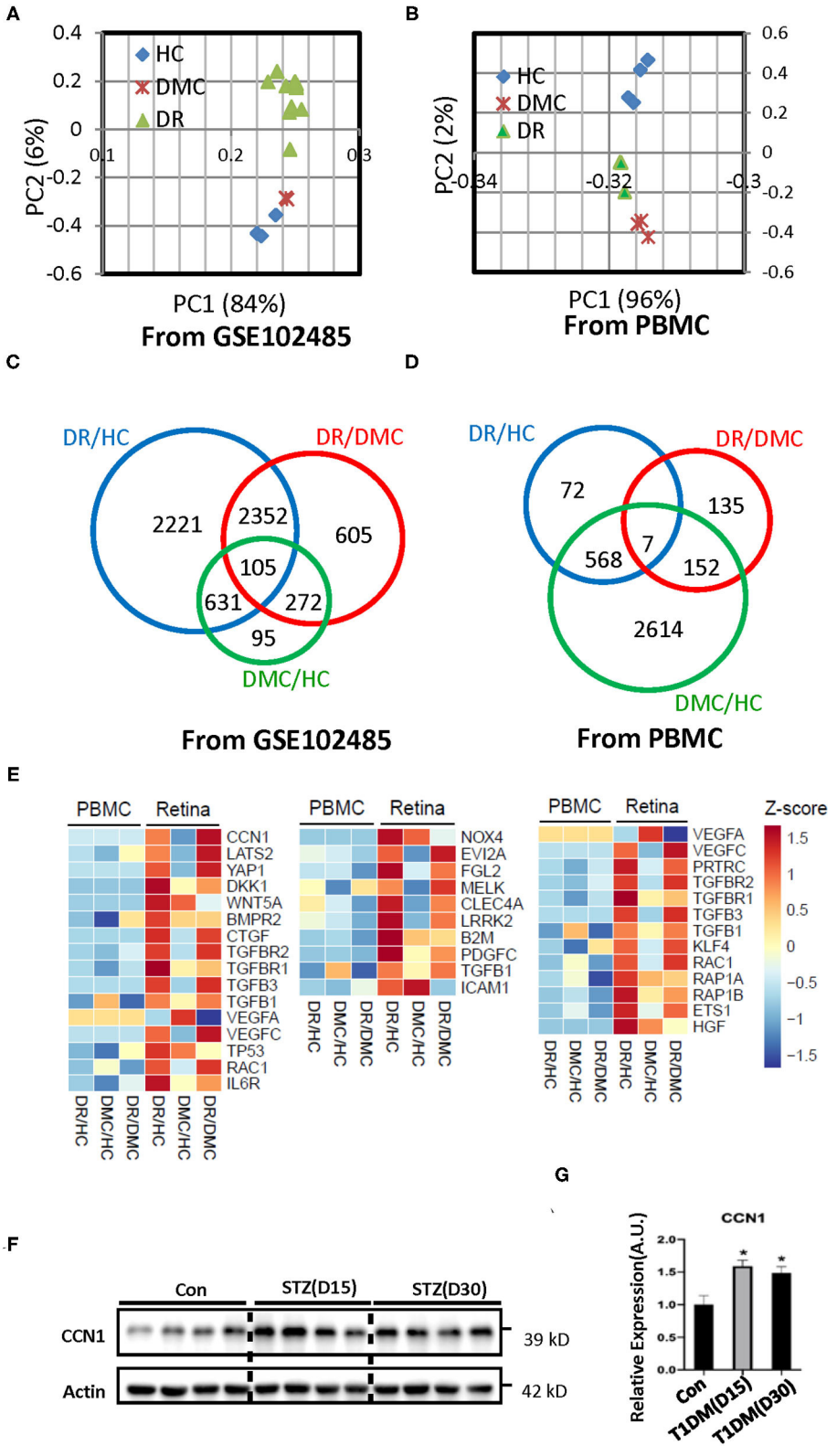

FIGURE 1 | Transcriptome profiles of retina tissue (the raw data of GSE102485) and peripheral blood samples from healthy controls (HCs), the diabetes mellitus control (DMC), and patients with diabetic retinopathy (DR). **(A,B)** Principal component analysis of the sequenced samples revealed the separation between DR

(Continued)

FIGURE 1 | samples and the other samples. **(C,D)** Venn diagrams summarize the different expression profiles among the DR, DMC, and HC groups. **(E)** The heat maps show different transcriptome profiles between retinal tissue and peripheral blood mononuclear cells (PBMCs); the image on the left represents the gene group associated with CCN1, the image in the middle represents the gene group associated with oxidative stress, and the image on the right represents the gene group associated with VE-cadherin. **(F)** Western blot analysis and **(G)** densitometry quantification of CCN1 in retinas from streptozotocin (STZ)-induced hyperglycemic mice. $^*p < 0.05$.

analyzed using the Image Lab software (Bio-Rad, Hercules, CA, USA) and normalized to the intensity of the internal control protein, such as actin or HSP90.

Fluorescent Quantitative PCR

Total RNA was extracted from HRVECs using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Quantitative real-time (RT)-PCR was conducted using the FastKing RT kit and SuperReal PreMix Plus kit (SYBR Green) (KR116-02 and FP205-02; Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions. The GAPDH mRNA level was used as the internal control. The PCR program included one cycle at 95°C for 15 min; followed by 40 cycles at 95°C for 10 s; 60°C for 20 s; and 72°C for 30 s. Analysis of each sample was repeated three times. The primer sequences used are listed as follows:

GAPDH,
Forward 5′-GATGCTGGTGCTGAGTATGRCG-3′
Reverse 5′-GTGGTGCAGGATGCATTGCTCTGA-3′.
CCN1,
Forward 5′-GGTCAAAGTTACCGGGCAGT-3′
Reverse 5′-GGAGGCATCGAATCCCAGC-3′.
NOX4,
Forward 5′- CAGATGTTGGGGCTAGGATTG -3′
Reverse 5′- GAGTGTTCGGCACATGGGTA -3′.
VE-cadherin,
Forward 5′-TTGGAACCAGATGCACATTGAT-3′
Reverse 5′-GTGGTGCAGGATGCATTGCTCTGA-3′.

Measurement of ROS Generation

The generation of ROS in HRVECs after palmitic acid (PA) treatment, or PA treatment together with CCN1 modulation, was determined using an ROS assay kit (Nanjing KeyGen Biotech Co. Ltd., Nanjing, Jiangsu, China) according to the manufacturer's instructions.

Retina Flat-Mount and Immunostaining

Retinas were fixed in 4% paraformaldehyde (E672002-0500; Sangon Biotech, Shanghai, China) for 15 min at room temperature immediately after being resected from the mice, after which they were washed three times in PBS. Retinas were then flat-mounted, blocked, and permeabilized in blocking buffer for 1 h at room temperature. Isolectin GS-IB4 (I21411; Invitrogen, Carlsbad, CA, USA) was applied and retinas were left to incubate at 4°C overnight, followed by further incubation with anti-NLRP3 (cryo-2, AdipoGen) at 4°C overnight. The anti-mouse secondary antibody (ab150115, Abcam) was applied at room temperature for 1 h. After three washes with PBS, samples were mounted using the Vectashield HardSet mounting medium (H-1700-10; Vector Laboratories, Burlingame, CA, USA). The retinal vasculature was imaged using a confocal fluorescence microscope (TSC SP8; Leica Microsystems, Wetzlar, Germany). The density and lesions of the microvasculature were analyzed using the Image J software.

Immunostaining of Frozen Section

The retinal frozen sections were blocked and permeabilized by a blocking buffer for 1 h at room temperature and further incubated with specific antibodies at 4°C overnight. After being washed three times with PBS, the sections were incubated with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (4413; Cell Signaling) or Alexa Fluor 647 anti-mouse secondary antibody (ab150115; Abcam) at room temperature for 1 h. An inverted fluorescence microscope (Eclipse Ti-S; Nikon, Tokyo, Japan) was used for closer observation. The results were quantified using the Image-Pro Plus software (Media Cybernetics Inc., Rockville, MD, USA).

Statistical Analyses

The results of continuous variables in the cohort study were presented as the mean ± SD. The baseline characteristics of each group were compared using the χ^2 test or a one-way analysis of variance test. Statistical analyses were performed using the SPSS V.19.0 statistical software (SPSS). $p < 0.05$ was considered significant. To describe the biologically functional significance of selected gene sets, we used the hypergeometric test to determine the enrichment of GO terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The significance of differences was evaluated with either the Student's *t*-test when only two groups were compared, or the hypergeometric test for a Venn diagram. Hierarchical clustering was performed using the Cluster 3.0 software.

RESULTS

Distinct Patterns Are Observed in the Transcriptome of Retinal Tissues and Peripheral Blood Mononuclear Cells From Patients With DR

Because of limited sample resources, only a few studies have demonstrated the RNA expression profiles of retinal samples from patients with DR. One such study, GSE102485 (25), analyzed three retinal samples from healthy controls (HCs), two retinal samples from patients with type 2 diabetes, and 25 retinal neovascular membranes (NVMs). Among the NVM samples, three were from patients with branch retinal vein occlusion, three from those with type 1 diabetes, two from those with retinal periphlebitis, and 17 from those with type 2 diabetes and PDR. We reanalyzed the raw data from that study and included 17 samples that fit our purpose (**Supplementary Figures 1A–C**).

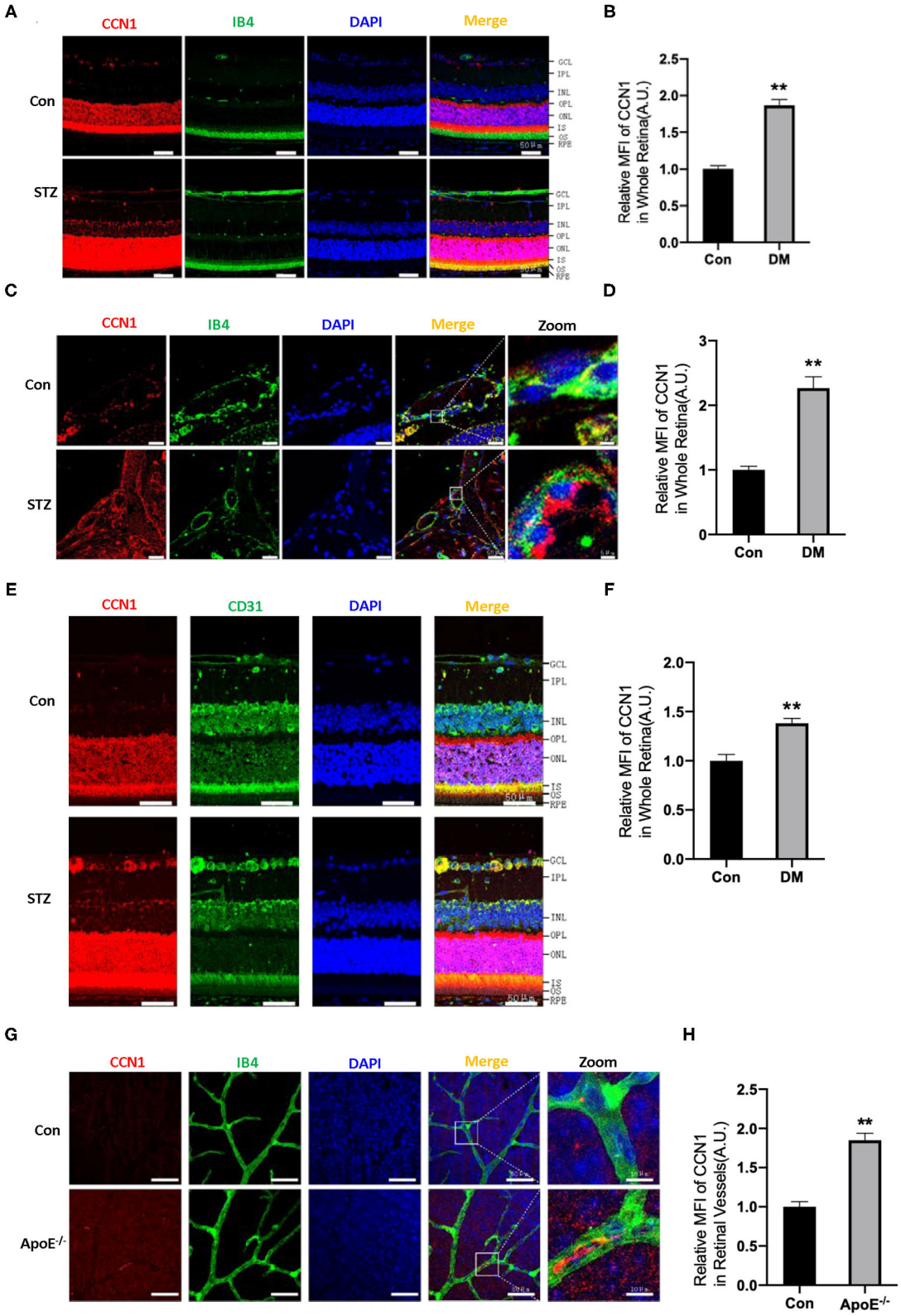

FIGURE 2 | CCN1 expression is increased in the retinal vessels of mice with diabetes mellitus (DM) and ApoE$^{-/-}$ mice. **(A)** Representative images of CCN1 expression patterns and **(B)** quantification of CCN1 mean fluorescence on frozen sections of the retina from control mice and streptozotocin (STZ)-induced hyperglycemic mice

(Continued)

FIGURE 2 | on day 15 after modeling; Isolectin B4 (IB4) was the vascular marker. **(C)** Representative images and **(D)** quantification of CCN1 mean fluorescence of central vessels on frozen sections of the retina from control mice and STZ-induced hyperglycemic mice on day 15 after modeling. **(E)** Representative images of CCN1 expression patterns and **(F)** quantification of CCN1 mean fluorescence on frozen sections of the retina from control mice and STZ-induced hyperglycemic mice on day 15 day after modeling; CD31 was the vascular marker. **(G)** Representative images of CCN1 expression pattern and **(H)** quantification of CCN1 mean fluorescence on flat-mount sections of the retina of ApoE$^{-/-}$ mice and control mice; IB4 was the vascular marker. **(A,C,E,F)** Scale bar = 50 μm. $^{**}p < 0.01$.

Therefore, we included 12 retinal NVM samples, three normal samples, and two type 2 diabetes samples.

The PCA of the transcriptomes showed congruent expression profiles among the three groups, whereas the similarity between the DM group and the HC group was greater (**Figure 1A**). In the peripheral blood samples, the PCA characteristics of the DR and DM groups showed greater similarity (**Figure 1B**). The Venn diagram constructed identified 2,221 differentially expressed genes between the NVMs of patients with DR and the retinal tissues of HCs. In addition, 95 differentially expressed genes were identified between retinal tissues of patients with DM and those of HCs (**Figure 1C**). Those genes were in the main categories related to extracellular matrix (ECM)–receptor interaction, tight junctions, apoptosis, and metabolism, among other categories (**Supplementary Figure 2A**). Although 72 differential gene expressions were identified in the peripheral blood of patients with DR and HCs, 2,614 gene differences were identified in the peripheral blood of patients with DM and HCs (**Figure 1D**). The genes involved in the GO category related to tight junctions were also more involved with inflammation or immune-related categories (**Supplementary Figure 2B**).

The transcriptome profiles of the retinal tissue are related to the pathophysiology of DR. The whole body is connected by the circulatory system and the blood is an accessible sample to show the distinct biomarker profile of heart failure with type 2 diabetes mellitus (27). Thus, we sought to determine whether the features of the DR transcriptome profile can be reflected in the peripheral blood sample. We enrolled three patients with DR, three patients with DM, and four HCs. The peripheral blood samples were collected and total RNA was extracted. The transcriptome profiles were obtained through the deep sequencing technique. The sequence quality was good enough for subsequent analysis (**Supplementary Figure 1B**). To discriminate between expression profiles between the retinal tissue and peripheral blood samples, we constructed a heat map of genes associated with DR-related pathways (**Figure 1E**; **Supplementary Figures 3A–D**). The transcriptome characteristics of retinal tissue cannot be reflected in peripheral blood.

As one of our aims was to illustrate the pathogenesis of DR, we chose to focus on the results of the retinal tissue. The cap junction molecular, oxidative stress, and the endoplasmic reticulum membrane protein complex (EMC) molecular pathways are considered to be related to the pathogenesis of DR. The heat map indicated the absolutely differential expression levels of genes associated with DR-related pathways. The expression of most of the VE-cadherin-, NOX4-, and CCN1-related genes was increased in the retina of patients with DR. VE-cadherin-related genes upregulated the body's ability to compensate for diseases. The EMC molecule, CCN1, is positively associated with DR, but its underlying mechanism is still unclear. Our results suggest that CCN1 influenced the tight junction through NOX4-induced oxidative stress. Interestingly, our results showed that the expression level of CCN1 in the retina of mice with diabetes was significantly higher than that of control mice (**Figures 1F,G**).

CCN1 Is Upregulated in Retinal Vessels From Mice With Hyperglycemia or Hyperlipidemia

The ECM is important for maintaining the normal function of blood vessels. Although CCN1 is an ECM component, closely related to DR, its role in the pathogenesis of DR is unclear. In order to determine whether CCN1 is involved in the pathogenesis of DR, we established a DM mouse model through STZ injection. Mice with blood glucose levels higher than 16.7 mmol/L were considered diabetic. Metabolic disorders, especially hyperlipidemia, are another characteristic of patients with DM. Therefore, ApoE$^{-/-}$ mice were used to study the effects of hyperlipidemia on the expression of CCN1 in retinal vascular tissue. *In situ* immunofluorescence experiments indicated that, compared with control mice, the expression of CCN1 in the whole retinal tissue of mice with DM was significantly increased (**Figures 2A,B,E,F**). To locate retinal blood vessels, we used IB4 and CD31 as vascular markers. We found the expression of CCN1 to be significantly increased in the retinal vascular tissue of diabetic mice using either marker. We also detected and analyzed the expression of CCN1 in the central artery and vein. As expected, CCN1 expression around the IB4-marked vessels was significantly increased in diabetic mice (**Figures 2C,D**). As both hyperglycemia and hyperlipidemia are the main metabolic features of DM, we investigated how hyperlipidemia affects CCN1 expression in ApoE$^{-/-}$ mice. We observed increased CCN1 expression in ApoE$^{-/-}$ mice (**Figures 2G,H**).

CCN1 Expression Is Induced by Palmitic Acid or High Glucose Treatment in HRVECs

Both lipotoxicity and glucotoxicity play important roles in promoting the progression of diabetic microvascular complications; however, which play a more important role is not clear. Hyperglycemia is considered the driving cause of DR pathology (28), while several studies found DR pathology in patients with relatively normal glucose tolerance and suggest that glucose may not be the primary driver of DR (29, 30). Recent studies indicate a strong association between dyslipidemia and DR (31, 32). In order to explore the influence of the DM environment on CCN1 expression in retinal microvascular cells, we chose HRVECs as the cell model. After 24 h of treatment with palmitic acid, the immune-stained HRVECs showed that PA can significantly increase the levels of CCN1, and most of the CCN1

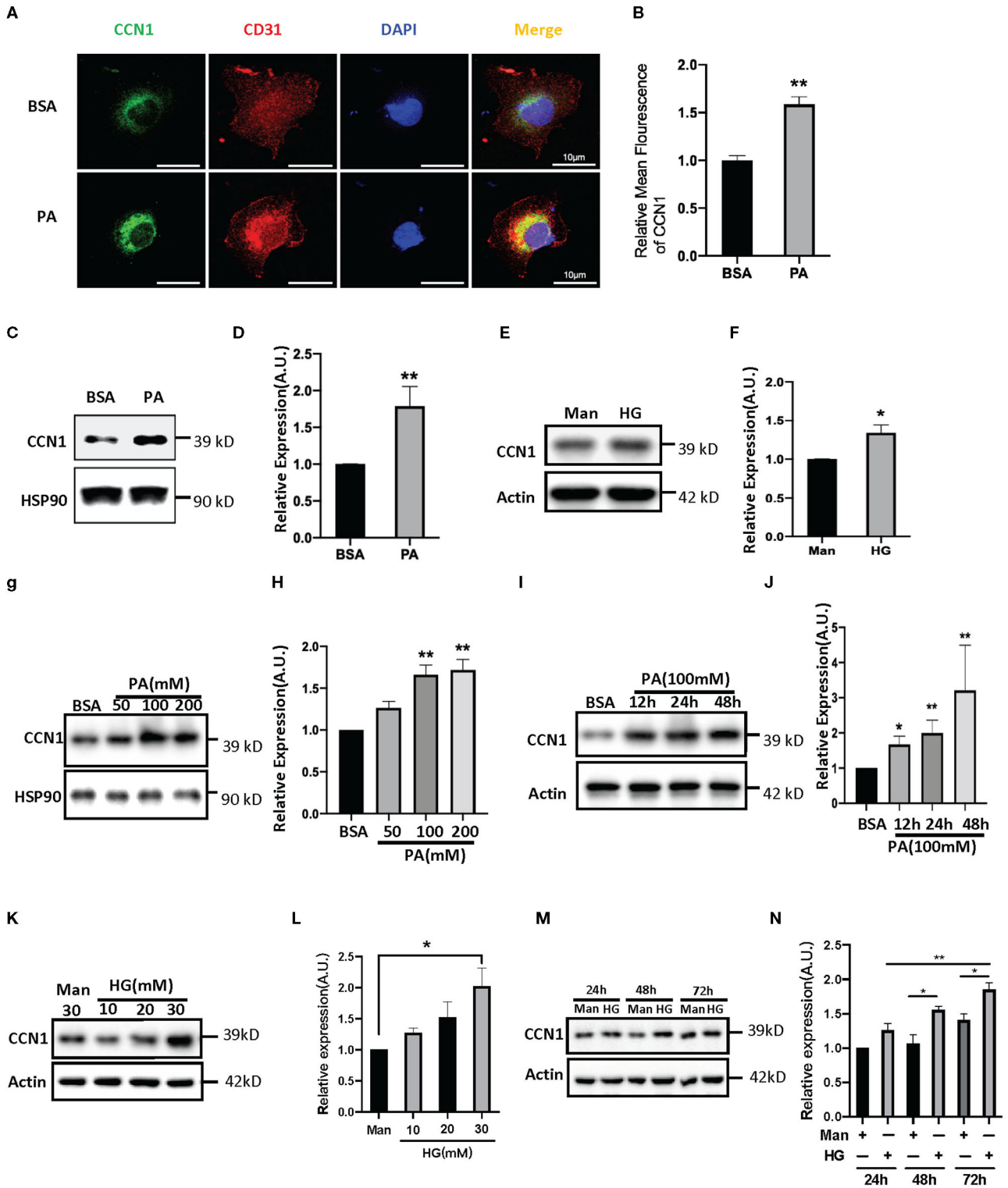

FIGURE 3 | Palmitic acid (PA) and high glucose (HG) levels induce CCN1expression in human retinal vascular endothelial cells (HRVECs). **(A)** Representative images and **(B)** quantification of CCN1 mean fluorescence in HRVECs treated with 100 mM PA for 24 h, scale bar = 10 μm. **(C,E)** Western blot analysis and **(D,F)** densitometry quantification of CCN1 in HRVECs under 100 mM PA or 30 mM HG treatment, respectively. **(G)** Western blot analysis and **(H)** densitometry quantification of CCN1 in HRVECs under 50, 100, or 200 mM PA for 24 h. **(I)** Western blot analysis and **(J)** densitometry quantification of CCN1 in HRVECs under 100 mM PA treatment for 12, 24, and 48 h. **(K)** Western blot analysis and **(L)** densitometry quantification of CCN1 in HRVECs under 10, 20, or 30 mM HG for 48 h. **(M)** Western blot analysis and **(N)** densitometry quantification of CCN1 in HRVECs under 30 mM HG treatment for 24, 48, and 72 h. $^{*}p < 0.05$, $^{**}p < 0.01$.

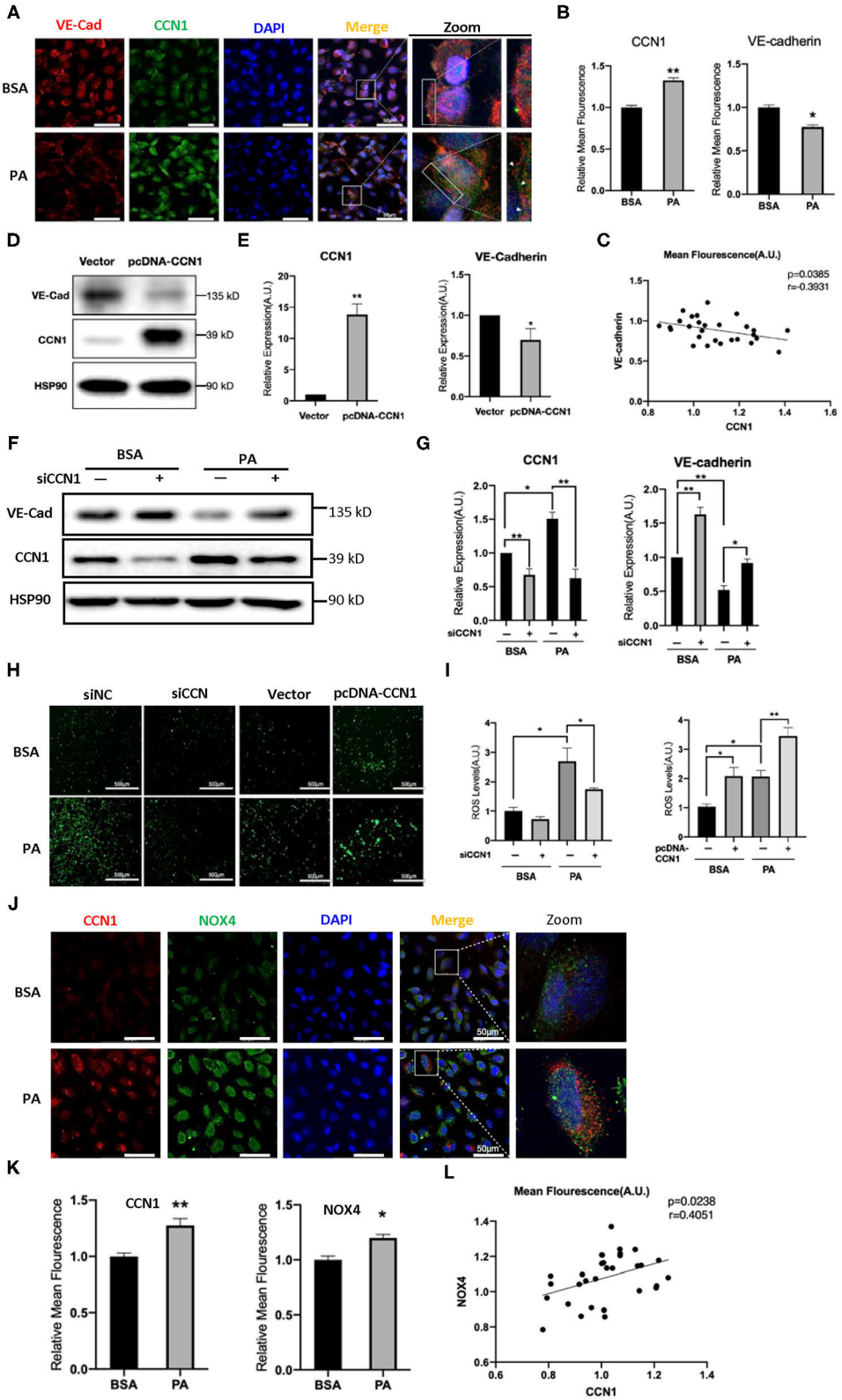

FIGURE 4 | CCN1 downregulates VE-cadherin by activating the NADPH oxidase (NOX4)/reactive oxygen species (ROS) axis. **(A)** Representative images, **(B)** mean density, and **(C)** relevance analysis of fluorescence of CCN1 and VE-cadherin in human retinal vascular endothelial cells (HRVECs). **(A)** White arrows indicate the

(Continued)

FIGURE 4 | defect of VE-cadherin on the cell membrane, scale bar = 50 μm. **(D)** Western blot analysis and **(E)** densitometry quantification of VE-cadherin and CCN1 in HRVECs after transfection with CCN1 plasmids or vector. **(F)** Western blot analysis and **(G)** densitometry quantification of VE-cadherin and CCN1 in HRVECs transfected with siCCN1 or the negative control, followed by 100 mM PA or bovine serum albumin (BSA) treatment for 24 h. **(H)** Representative images and **(I)** measurement of ROS generation in HRVECs transfected with siCCN1 or the negative control (siNC) followed by 100 mM PA or BSA treatment for 24 h, scale bar = 500 μm. **(J)** Representative images, **(K)** mean density, and **(L)** relevance analysis of fluorescence of CCN1 and NOX4 in HRVECs, scale bar = 50 μm. $^{*}p < 0.05$, $^{**}p < 0.01$.

can be found around the nucleus (**Figures 3A,B**). Western blot analysis also confirmed that CCN1 levels were upregulated after both PA (100 mM, **Figures 3C,D**) and high glucose (HG 30 mM, **Figures 3E,F**) treatment over 24 h; however, the difference was not as significant as that of the PA-treated cells. We then treated HRVECs with different concentrations of PA and HG for different durations. The Western blot analysis showed that PA treatment over 24 h dose-dependently induced both the protein levels of CCN1 in HRVECs (**Figures 3G,H**). We also treated HRVECs with PA (100 mM) for different durations (**Figures 3I,J**) and optimized the PA stimulation conditions. The results of Western blot analysis showed that HRVECs treated with PA at a concentration of 100 mM for 24 h were the best conditions. We also treated HRVECs with different concentrations of HG for different durations and the effect on the CCN1 expression is not significant than PA (**Figures 3K–N**).

CCN1 Inhibits VE-cadherin Expression in HRVECs

The results above indicated that lipotoxicity in DM can stimulate the expression of CCN1, but it is unclear whether increased CCN1 affects the function of retinal vascular endothelial cells. VE-cadherin is an endothelium-specific member of the tight junctions, and its reduction is related to the leakage of the retinal vasculature (33). We performed immunofluorescence staining to observe the expression patterns of VE-cadherin and CCN1 in HRVECs treated with 100 mM PA for 24 h. We found that PA can increase the expression of CCN1 and reduce the expression of VE-cadherin, which indicates that the levels of CCN1 and VE-cadherin are negatively correlated (**Figures 4A–C**).

To determine whether CCN1 actually regulates the expression of VE-cadherin, we induced the overexpression of CCN1 in HRVECs using plasmids. The vacant adenovirus vector was used as a control (vector group). Compared with the control group, the expression of CCN1 in the pcDNA-CCN1 group was significantly increased, whereas the expression of VE-cadherin was reduced (**Figures 4D,E**). Furthermore, we downregulated the expression of CCN1 using small interfering RNA (siCCN1). Both siCCN1-1 and siCCN1-2 can effectively inhibit the expression of CCN1 (**Supplementary Figure 5**). Consistent with the previous results (**Figure 3C**), PA treatment upregulated CCN1 expression in HRVECs, but downregulated VE-cadherin expression. In addition, knockdown of CCN1 significantly upregulated the expression of VE-cadherin, compared with the scramble control group (**Figures 4F,G**). Therefore, CCN1 can directly regulate VE-cadherin expression in HRVECs.

Moreover, CCN1 reportedly stimulates ROS generation (34) and ROS can potently downregulate the expression of VE-cadherin (35). As there is a clear relationship between ROS overproduction and DR (36), we proposed that ROS may mediate the inhibitory effect of CCN1 on VE-cadherin in HRVECs. Therefore, we measured ROS levels in the siCCN1 and pcDNA-CCN1 groups. Our results showed that PA treatment significantly increased the ROS levels, whereas siCCN1 attenuated ROS induction, and pcDNA-CCN1 potentiated ROS induction (**Figures 4H,I**). These findings indicate that the inhibitory effect of CCN1 on the expression of VE-cadherin is mediated through ROS production. As NOX4, which produces ROS, is abundantly expressed in vascular endothelial cells (37) and reportedly causes the destruction of the blood-brain barrier (22), we examined the levels of CCN1 and NOX4 in HRVECs after PA treatment. Immunostaining findings showed that levels of both CCN1 and NOX4 were increased in PA-treated HRVECs (**Figures 4J–L**), suggesting a positive correlation between the expression levels of CCN1 and NOX4. These results indicate that CCN1 may inhibit the expression of VE-cadherin by activating the NOX4/ROS axis.

CCN1 Inhibits VE-cadherin Expression by Upregulating NOX4 in HRVECs

Oxidative stress is one of the pathogenic factors leading to DR (38). Our findings above indicate a positive correlation between the levels of CCN1 and NOX4. To further determine whether NOX4 mediates the inhibition of VE-cadherin expression by CCN1, we tested the levels of CCN1, NOX4, and VE-cadherin in the retinas of mice with STZ-induced DM. Western blot analysis showed that, compared with the control group, CCN1 and NOX4 were significantly increased in the retinas of mice after 30 days of initial STZ treatment, whereas VE-cadherin was significantly reduced (**Figures 5A,B**). A similar expression pattern was also observed in PA-treated HRVECs (**Figures 5C,D**).

Moreover, we tested the expression levels of NOX4 and VE-cadherin after CCN1 overexpression. Our results showed that the expression of NOX4 was significantly increased in the pcDNA-CCN1 group compared with the vector group (**Figures 5E,F**). Furthermore, we measured the expression levels of NOX4 and VE-cadherin after CCN1 downregulation using siCCN1. In contrast to the effects of overexpression, NOX4 was significantly downregulated, whereas VE-cadherin was upregulated (**Figures 5G,H**). In addition, we constructed the CCN1 knockout (CCN1-KO) HRVECs using CRISPR-Cas9 plasmids. The target sequence and efficiency map of CCN KO is shown in **Supplementary Figure 4**. Consistently, similar effects to those of siCCN1 were detected in CCN1-KO HRVECs (**Figures 5I,J**). These results confirm that CCN1 negatively regulates the expression of VE-cadherin and positively regulates the expression of NOX4.

In order to determine the NOX4 regulation of VE-cadherin expression, we modulated the levels of NOX4 in HRVECs using

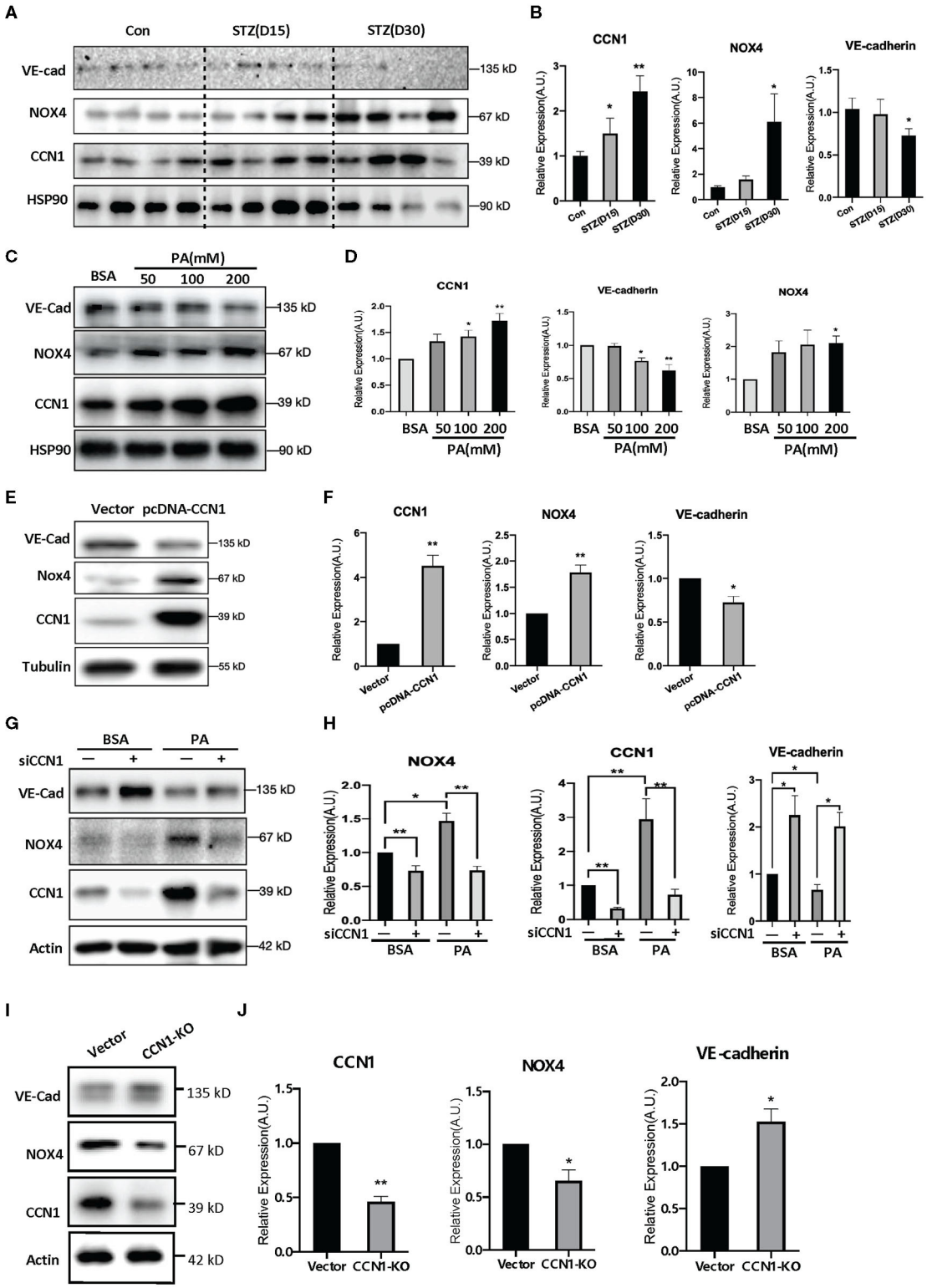

FIGURE 5 | NADPH oxidase (NOX4) mediates the negative regulatory effects of CCN1 on VE-cadherin expression. **(A,C,E,G,I)** Western blot analysis and **(B,D,F,H,J)** densitometry quantification of protein expression levels of VE-cadherin, NOX4, and CCN1 in **(A,B)** retina tissue of control mice and streptozotocin (STZ)-induced hyperglycemic mice on days 15 and 30 after modeling; **(C,D)** human retinal vascular endothelial cells (HRVECs) treated with serial dosages (50, 100, or 200 mM) of palmitic acid (PA) for 24 h; **(E,F)** HRVECs transfected with CCN1 plasmids or vector; **(G,H)** HRVECs transfected with siCCN1 or the negative control (siNC), followed by treatment with 100 mM PA or BSA for 24 h; **(I,J)** CCN1-KO HRVECs via CRISPR. $^{*}p < 0.05$, $^{**}p < 0.01$.

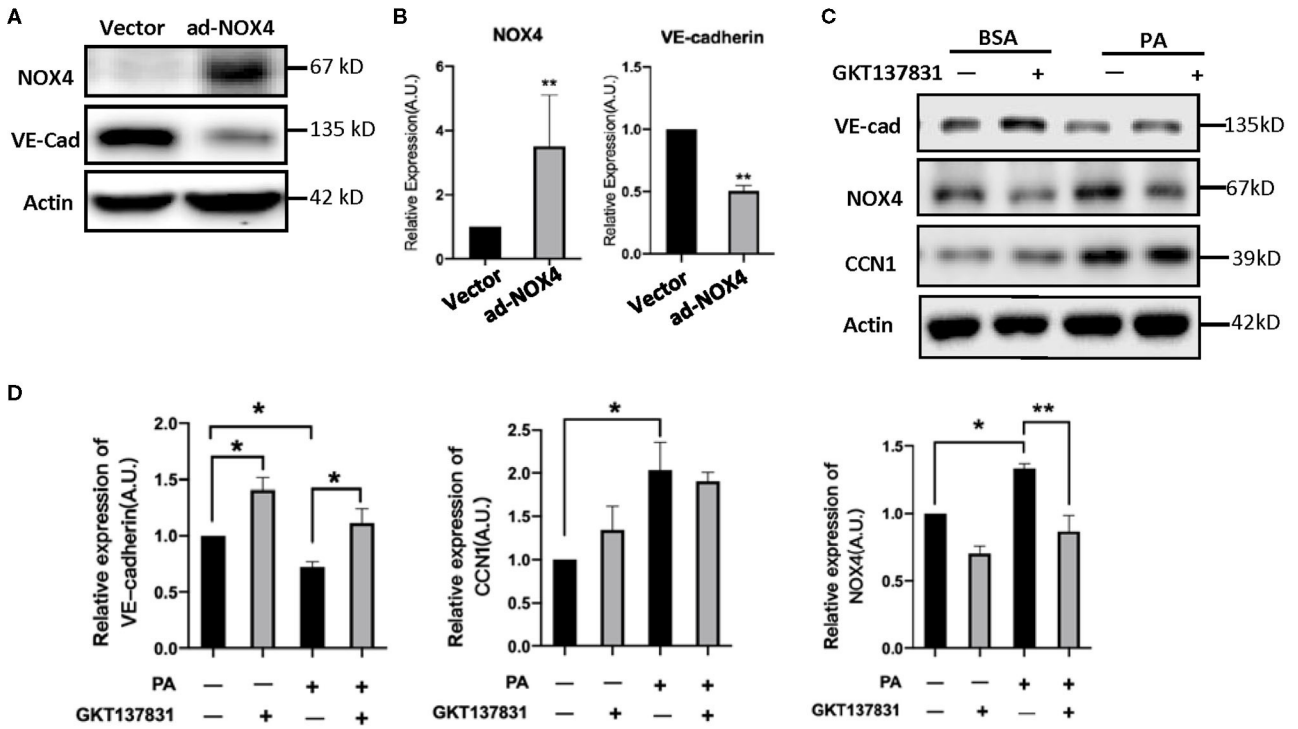

FIGURE 6 | CCN1 positive regulate the expression of NOX4 in HRVECs. **(A,B)** HRVECs infected with NOX4-overexpressed adenovirus or vector; **(C,D)** The active of NOX4 was inhibited by GKT137831 in HRVECs. *$p < 0.05$, **$p < 0.01$.

ad-NOX4. In order to clarify the regulation relationship between NOX4 and VE-Cadherin, we treat the HRVECs with the NOX4 inhibitor GKT137831. Western blot analysis showed that the overexpression of NOX4 can significantly reduce the levels of VE-cadherin (**Figures 6A,B**), while GKT137831 increases the level of VE-cadherin (**Figures 6C,D**) indicating that NOX4 negatively regulates the expression of VE-cadherin.

DISCUSSION

Although DR is the leading cause of blindness in developed and developing countries (39), the underlying pathogenic mechanisms have not been fully elucidated. The CCN family proteins are composed of four domains (IGFBP, VWC, TSP1, and CT) and functions at the cell matrix boundary or within the nucleus to modulatory gene transcription (40). The first identified member of the CCN family was CCN1, which was described as an angiogenic factor (41). The functionality of retinal small vessels depends on the subendothelial matrix, which is rich in CCN1 (42, 43). Previous reports have shown that CCN1 expression is increased in the retinas from mice with DM and from patients with DR, as well as in the vitreous humor of patients with PDR (15, 16, 44). Furthermore, circulating CCN1 levels are significantly correlated with the severity of peripheral arterial disease in diabetic patients (45). Other studies have shown that CCN1 regulates VEGF signaling during DR progression (17, 45). Those studies suggest a possible role of CCN1 in the pathogenesis of DR. However, the detailed mechanism underlying the involvement of CCN1 in DR pathogenesis remained unclear.

In the current study, we demonstrated that the expression of CCN1 was significantly upregulated in the microvascular endothelial cells from DM patients, as well as in diabetic mouse models. We further confirmed that CCN1 expression could be induced by either high glucose or fatty acid, while CCN1 induction inhibited tight junction protein expression by activating the NOX4/ROS axis. Overall, as summarized in **Figure 7** our results revealed a previously unappreciated mechanism of CCN1 regulation and function in promoting oxidative stress and tight junction damage in microvascular endothelial cells, which also suggested a potential strategy for the treatment of DR by targeting CCN1.

Previous reports have shown that ROS overproduction induced by hyperglycemia or hyperlipidemia plays key roles in endothelial damage in patients with DM (46). NOX4 is the main enzyme generating ROS in endothelial cells, and CCN1 induction leads to an accumulation of ROS (21). We compared the RNA expression profiles between retinal tissues and PBMCs from DR patients and non-diabetic controls. We noticed that the mRNA expression profile of endothelial cells from the retinas of DR patients (GSE102485) (25) showed increased levels of CCN1and oxygen stress-related genes compared with non-diabetic controls. These findings were further confirmed in the retina tissues from mice with STZ-induced diabetes. Furthermore, we demonstrated that both high glucose levels and PA treatment induced the expression of CCN1 in HRVECs *in vitro*. These results are

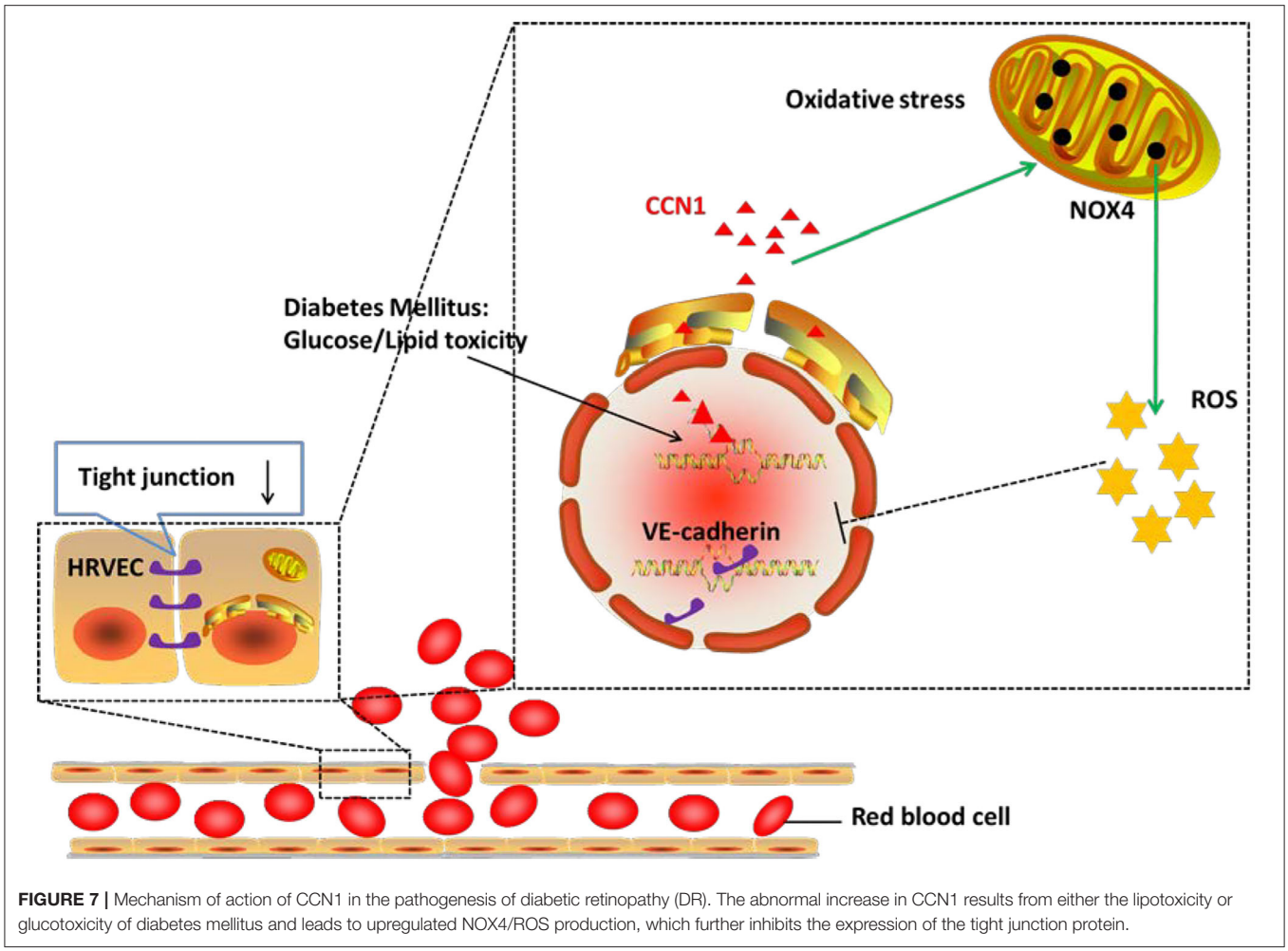

FIGURE 7 | Mechanism of action of CCN1 in the pathogenesis of diabetic retinopathy (DR). The abnormal increase in CCN1 results from either the lipotoxicity or glucotoxicity of diabetes mellitus and leads to upregulated NOX4/ROS production, which further inhibits the expression of the tight junction protein.

consistent with previous studies in that both the volume of NOX4/ROS and the expression level of CCN1 are upregulated under DM. Our results also confirm that DM status can promote NOX4/ROS production through CCN1 upregulation.

Damage of the blood-retinal barrier (BRB) is an early feature of DR that results in vascular leakage, as well as macular edema, which can cause distortion and loss of central vision (17). The proteolytic degradation of VE-cadherin plays an important role in the breakdown of the BRB and increases retinal vascular permeability (33). The interactions between the endothelium and ECM are important for maintaining the normal functions of blood vessels. A previous study has shown that NOX4-generated ROS induces neuronal and blood-brain barrier injury after intracerebral hemorrhage (22). Besides, AGE treatment promoted NOX4 membrane translocation, ROS production, and VE-cadherin phosphorylation and degradation in human umbilical vein endothelial cells (47). Acarbose treatment in diabetic rats blocked NOX4-dependent superoxide generation in rat aortic endothelial cells and ameliorated vascular leakage by upregulating VE-cadherin expression (48). Moreover, CCN1 is critical for retinal vascular development (49). Through the analysis of public database, clinical samples, mouse models, as well as HRVECs *in vitro*, the current study demonstrates that CCN1 is induced in vascular endothelial cells under diabetic conditions, and that CCN1 disrupts the intercellular tight junction of the retinal vascular endothelium via NOX4 induction and ROS overproduction to negatively regulate VE-cadherin protein level. However, the mechanism on how CCN1 is regulated by hyperglycemia or hyperlipidemia and how CCN1 regulates NOX4 expression remains unclear. Further, whether CCN1 regulates VE-cadherin by inducing NOX4 *in vivo* and whether inhibiting CCN1 expression will protect against DR progression in animal models remain to be explored.

In summary, we demonstrated that up-regulating CCN1 expression under DM activated the NOX4/ROS axis in microvascular endothelial cells and led to vascular leakage through inhibiting VE-cadherin expression. The current study has identified a novel and important role of CCN1 in the pathogenesis of DR and may thus provide a potential target for DR therapy through inhibiting CCN1 expression or signaling.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found below: SRA, PRJNA722765.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-Sen University.

AUTHOR CONTRIBUTIONS

HL wrote the original version of the manuscript. HL, TL, and HW performed most of the experiments. YLi and HY helped with the bioinformatic analysis of the GSE102485 and the PBMC RNA-seq data. YLu, SW, RP, and YN provided technical assistance. XH helped to organize and write the manuscript and performed some of the experiments. TL and HW helped to write the manuscript. YC, GS, and YY initiated and designed the study and experiments and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.689318/full#supplementary-material

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ORIGINAL RESEARCH
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β-Hydroxybutyric Inhibits Vascular Calcification via Autophagy Enhancement in Models Induced by High Phosphate

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Background: Vascular calcification (VC) is a landmark of aging, while β-hydroxybutyric acid (BHB) induced by calorie restriction has been identified as a promising factor to extend the lifespan. However, the effect of BHB on VC and the potential mechanism remain unknown.

Methods: A total of 160 subjects with or without metabolic abnormalities (MAs) were assigned to four groups according to different calcification severities. The association between BHB, MAs, and VC was investigated *via* mediation analysis. Then, with high phosphate-induced calcification models, the effect of BHB on arterial ring calcification and osteogenic phenotypic differentiation of vascular smooth muscle cells (VSMCs) was investigated. Hereafter the expressions of autophagy biomarkers, autophagy flux, and effects of autophagy inhibitors on VC were detected.

Results: Severe VC was observed in the elderly, accompanied with a higher proportion of hypertension, chronic kidney disease, and lower estimated glomerular filtration rate. The serum BHB level was an independent influencing factor of VC severities. With mediation analysis, BHB was determined as a significant mediator in the effects of MAs on VC, and the indirect effect of BHB accounted for 23% of the total effect. Furthermore, BHB directly inhibited arterial ring calcification and osteogenic phenotypic differentiation in VSMCs, accompanied with autophagy enhancement in VSMCs. In accordance, the inhibition of autophagy counteracted the protective effect of BHB on VC.

Conclusion: The present study demonstrated that BHB mediated the effects of MAs on VC; then, it further elucidated that BHB could inhibit arterial and VSMC calcification *via* autophagy enhancement.

Keywords: vascular calcification, β-hydroxybutyric acid, metabolic abnormalities, osteogenic phenotypic differentiation, vascular smooth muscle cells

INTRODUCTION

Vascular calcification (VC), characterized by the accumulation of calcium phosphate crystals and the phenotypical transition of vascular smooth muscle cells (VSMCs) inside the vessels, is related to a variety of diseases, such as diabetes, chronic kidney disease (CKD), atherosclerosis, and other cardiovascular diseases (1–3). Although the incidence of VC remains high in clinics, there are still no approved or substantially effective therapies for the treatment of VC. Therefore, exploring the novel therapy for VC has been an important tough problem waiting to be solved.

It has become increasingly clear that metabolic syndrome (METs) is a major risk factor of VC (4, 5). Emerging evidence has demonstrated that calorie restriction (CR), a nutritional intervention of reduced energy intake but with adequate nutrition, effectively regulates the metabolic balance, ameliorates metabolic abnormalities (MAs), and reduces VC (6–9). Meanwhile, β-hydroxybutyric acid (BHB), which could be generated by CR, has been proven to promote autophagy and prevent VSMC senescence (10). However, whether BHB can prevent arterial calcification in populations with MAs or VC and the potential mechanism remain unclear.

Among the numerous etiologies, autophagy, an important biological process that contributes to cellular homeostasis, has been recognized as an important influencing factor of survival and function in VSMCs in the process of VC (11, 12). Autophagy exists in three separate forms: microautophagy, chaperone-mediated autophagy, and macroautophagy (13). The term autophagy usually refers to macroautophagy, which is the most prevalent and the only form of autophagy mentioned here. Alterations in autophagy have been documented in VSMCs in response to various stimuli, resulting in the modulation of VSMC functions, including VC (14, 15). On the contrary, the activation of autophagy stimulates VSMC survival, maintains normal vascular cell function, and protects against VC (16, 17). Notably, the accumulated evidence has confirmed that BHB plays a broad role in regulating longevity and affects the development of aging (18). Hence, it may discreetly propose that BHB intervention may protect VC and the underlying mechanism is related to autophagy.

Based on the uncertainties mentioned above, we hypothesized that insufficient autophagy is associated with VC, and autophagy enhancement induced by BHB maintains the VSMC function and protects against VC. To address these assumptions, the role of BHB in the correlation of MAs and VC was investigated, and then the effect of BHB administration on high phosphate-induced VC models and the potential mechanism related to autophagy were further explored. This study may provide herein novel insights into the mechanism of autophagy in VC and shed novel light on the promising therapeutic target of the early prevention of VC.

METHODS

Subjects

This study was designed to investigate the relations between BHB and calcification under different metabolic status. The study recruited 160 patients from the Affiliated Hospitals of Sun Yat-sen University from January 2015 to July 2020. The participants, ages between 42 and 91, all underwent chest computerized tomographic (CT) scan and serum β-hydroxybutyric acid measurement and had no missing data on any components of the METs. Patients with severe diseases that might affect nutrient absorption and energy metabolism were excluded, including inflammatory bowel disease, cachexia, cirrhotic hepatic disease, malignant tumor, *etc*. Demographic data and medical history were acquired using standard questionnaires. The trial was conducted according to the International Conference on Harmonization—Good Clinical Practice guidelines and the Declaration of Helsinki. All patients were enrolled after informed consents were obtained.

Computerized Tomographic Analysis

All the patients underwent a non-enhanced chest CT scan to evaluate thoracic aorta calcification (TAC). As described by Agatston et al. (19), the calcium score of each lesion was calculated by multiplying the area of the calcified speck by the density factor derived from the maximal Hounsfield units within this area (20). The grouping criteria of TAC severity are as follows: TAC score = 0: no TAC; TAC score = 1–100: mild TAC; TAC score = 101–500: moderate TAC; and TAC score >500: severe TAC.

The chest CT scans were performed using the same equipment (SOMATOM Sensation 64 CT). The details of the scanning acquisition were described previously (21). Two experienced investigators, blinded to all clinical information, randomly analyzed all the CT images.

Definition of the Metabolic Abnormalities

The definition of METs was diverse from each other, and developing a standard one has been challenging. One of the best accepted definitions was provided by the US National Heart, Lung, and Blood Institute and the American Heart Association Consensus Statement (22). In this study, we referred to the METs definition and determined MAs with one and more of the following parameters: large waist circumference (women >80 cm and men >90 cm, according to the standard of abdominal obesity in the Chinese population), elevated triglycerides [≥1.7 mmol/L (150 mg/dl)], low high-density lipoprotein cholesterol (HDL-C) [men <1.03 mmol/L (40 mg/dl) and women <1.29 mmol/L (50 mg/dl)], impaired fasting glucose [≥ 6.1 mmol/L (110 mg/dl) or on antidiabetic medication), and elevated blood pressure (≥130/85 mmHg or self-reported use of medications for hypertension).

Collection of Laboratory Parameters

Blood samples were collected from the patients in the morning after more than 10 h of fasting. The following parameters were tested or determined in the Institutional Central Laboratory of the Affiliated Hospital of Sun Yat-sen University: fasting glucose, total cholesterol, triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDLC), serum calcium (Ca), serum phosphate (Pi), and estimated glomerular filtration rate (eGFR).

All the biochemical parameters were analyzed by using a standardized and certified TBA-120 autoanalyzer (Toshiba Medical Systems, Japan).

Establishment and Examination of Calcification Models in Arterial Rings and VSMCs

The arterial rings and VSMCs were isolated or extracted as previously described (23). Calcification was induced by adding inorganic phosphate (Na_2HPO_4, Sigma, USA) to normal medium at a concentration of 2.6 mM (calcification medium). To evaluate the impact of BHB on calcification, BHB (Sigma, USA) was administrated into a calcification medium at a concentration of 4.0 mM (BHB intervention medium) (24). The arterial rings were cultured for 14 days, while VSMCs were cultured for 7 days, with the medium for both refreshed every 2 to 3 days (23).

To evaluate whether autophagy inhibition could reverse the protective effects of BHB on VC, the autophagosome inhibitor 3-methyladenine (Selleck, USA), at a concentration of 20 μM, and the autophagic flux inhibitor chloroquine (Selleck, USA), at a concentration of 10 μM, were administrated to the calcification models with BHB intervention (25).

Von Kossa Staining and Alizarin Red S Staining

Von Kossa staining was used to detect the calcified depositions of arterial rings. The staining experiments were performed as previously described (25). The calcium deposits displayed black or dark brown colors under the microscope.

For Alizarin Red S staining, VSMCs in six-well plates were washed three times with phosphate-buffered saline (PBS) after the indicated treatments and then fixed with 4% paraformaldehyde for 30 min. After that, the fixed VSMCs were washed and then stained with 2% Alizarin Red S solution (Servicebio, China) for 10 min. The stained calcium deposits were observed and photographed under a microscope. The area positive for Alizarin Red S staining displayed a red color.

Calcium Assay

VSMCs were washed three times with PBS and then decalcified with 0.6 M hydrochloric acid for 12 h at 4°C. The supernatant was collected, of which the calcium concentration was determined as previously described (25).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted, reverse-transcribed, and amplified to detect some specific mRNA transcription as previously described (26). The primers were designed on Primerbank and verified with primer-BLAST on PubMed. The PCR primers were as follows:

GAPDH, forward 5′-AGGTCGGTGTGAACGGATTTG-3′ and reverse 5′-GGGGTCGTTGATGGCAACA-3′; Runx2, forward 5′-GACTGTGGTTACCGTCATGGC-3′ and reverse 5′-ACTTGGTTTTTCATAACAGCGGA-3′; BMP-2, forward 5′-GGGACCCGCTGTCTTCTAGT-3′ and reverse 5′-TCAAC TCAAATTCGCTGAGGAC-3′; SM22a, forward 5′-CCAAC AAGGGTCCATCCTACG-3′ and reverse 5′-ATCTGGGCGGC CTACATCA-3′; α-SMA, forward 5′-CCCAGACATCAGGGA GTAATGG-3′ and reverse 5′-TCTATCGGATACTTCA GCGTCA-3′.

The qPCR experiments were independently repeated three times, and the quantification was performed using the ΔΔCt method. The results were normalized to GAPDH transcription.

Western Blot Analysis

VSMCs were washed three times with PBS and collected after treatment with RIPA lysis buffer as described previously (26). The proteins were boiled, and the same amount of proteins was subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Merck-Millipore, the USA). The membranes were incubated successively with 5% bovine serum albumin for 1 h and then stained with primary antibodies for 12 h at 4°C, including anti-smoothelin (1:1,000 dilution, Abcam, USA), anti-Runx2 (1:500 dilution, CST, USA), anti-BMP2 (1:1,000 dilution, Novus, USA), anti-GAPDH (1:1,000 dilution, CST, USA), and anti-SM22α (1:1,000 dilution, Abcam, USA). The membranes were then incubated with secondary goat anti-rabbit IgG antibody (1:5,000 dilution, Santa Cruz, USA) for 1 h. Finally, the reaction was visualized by a chemiluminescence image system (Proteinsimple, USA), and the density of the bands was semi-quantified *via* the image software Image J (National Institutes of Health, *V* 1.53, USA).

Autophagy Detection Using mRFP-GFP-LC3 Adenoviral Vector

mRFP-GFP-LC3 double-labeled adenovirus was purchased from HanBio Technology Co., Ltd., and adenoviral infection was performed according to the instructions of the manufacturer (25). Isolated mouse VSMCs were plated in six orifice plates, and the cells were transduced with adenovirus after reaching 50% confluence by Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS) for 24 h at 37°C. After infection, the VSMCs were then grown in a medium with 2.5% FBS for 24 h before changing to a complete culture medium administrated with 2.6 mM Pi and 4.0 mM BHB. Autophagy was observed through a fluorescence microscope (Nikon Eclipse Tis). Autophagic flux was determined by evaluating the number of GFP and RFP puncta (puncta/cell was counted).

Statistical Analysis

Data of the continuous variables are presented as mean and standard deviation (SD), skewed data as median (25th and 75th percentiles), and categorical variables as absolute numbers and percentages. Comparisons among groups were attained by one-way ANOVA (for distributed variables) or chi-square test (for categorical variables). For skewed data, the cases were ranked as new variables, and then an analysis of variance was performed.

Regression analysis was performed to examine whether BHB concentration was an independent influencing variable for TAC severities. A univariate linear regression model (model 1) was initially analyzed. Then, model 1 was adjusted with significant and potential confounding variables, which formed multivariate linear regression models (models 2 and 3), to further investigate

TABLE 1 | Clinical characteristics of all participants according to different severities of TAC.

	No TAC ($n = 43$)	Mild TAC ($n = 35$)	Moderate TAC ($n = 32$)	Severe TAC ($n = 50$)
Age, year	52 (49, 60)	62 (56, 66)***	64 (57, 68)***	72 (62, 79)***###&
Male, *n* (%)	11 (25.6)	11 (31.4)	9 (28.1)	18 (36.0)
BMI, kg/m^2	23.27 (20.31, 25.94)	21.40 (18.97, 23.90)	23.33 (21.69, 24.67)	23.03 (20.12, 25.31)
WC, cm	78 (73.50, 81.00)	77.00 (72.75, 83.25)	79.00 (73.00, 84.00)	77.00 (70.25, 84.00)
HTN, *n* (%)	8 (21.6)	10 (30.3)	10 (32.3)	30 (63.8)***##&
T2DM, *n* (%)	6 (15.4)	7 (20.6)	3 (10.0)	16 (32.0)
CKD, *n* (%)	19 (47.5)	23 (65.7)	24 (75.0)	45 (90.0)***###
FBG, mmol/L	5.08 ± 1.06	5.30 ± 1.30	5.45 ± 2.55	5.81 ± 2.14
TC, mmol/L	4.95 ± 1.23	4.92 ± 1.40	5.05 ± 1.37	5.01 ± 1.54
HDL-C, mmol/L	1.24 (1.03, 1.46)	1.26 (1.00, 1.46)	1.15 (1.01,1.39)	1.27 (1.08, 1.47)
LDL-C, mmol/L	2.84 (2.45, 3.31)	2.59 (2.18, 3.59)	3.16 (2.63, 3.85)	2.99 (2.12, 3.93)
TG, mmol/L	1.22 (0.91, 1.66)	1.27 (0.96, 1.76)	1.34 (0.92, 1.81)	1.38 (0.87, 1.32)
eGFR, ml/min/1.73 m^2	90.00 (55.23, 100.20)	81.87 (73.42, 92.5)	75.64 (65.77, 89.80)	65.06 (44.56, 80.16)***##
Ca, mmol/L	2.27 (2.19, 2.35)	2.22 (2.13, 2.32)	2.28 (2.13, 2.35)	2.22 (2.13, 2.32)
Pi, mmol/L	1.15 (1.08, 1.28)	1.15 (1.02, 1.24)	1.17 (1.02, 1.24)	1.14 (1.04, 1.33)
TAC score	0	19.90 (5.6, 59.6)	255.10 (194.3, 353.1)	1,812.10 (800.1, 7164.4)

*Data are presented as mean ± SD or median (25th and 75th percentiles). HTN, T2DM, and CKD are presented as n (%). Compared with the normal group: ***$P < 0.001$; compared with the mild TAC group: ##$P < 0.01$, and ###$P < 0.001$; compared with the moderate TAC group: &$P < 0.05$.*
TAC, thoracic aorta calcification; BMI, body mass index; WC, waist circumference; HTN, hypertension; T2DM, type 2 diabetes mellitus; CKD, chronic kidney disease; FBG, fast blood glucose; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; eGFR, estimated glomerular filtration rate; Ca, calcium; Pi, phosphate.

TABLE 2 | Linear regression analyses between BHB and TAC severities.

Model	β	95%CI	*P*-value	Adjusted R^2
Model 1	−0.260	(−0.491, −0.129)	0.001	0.062
Model 2	−0.256	(−0.562, −0.108)	0.004	0.326
Model 3	−0.234	(−0.545, −0.066)	0.013	0.307

Model 1: unadjusted; model 2: adjusted with age, sex, BMI, WC, HTN, and T2DM; model 3: adjusted with age, sex, BMI, WC, HTN, T2DM, TG, HDL-C, eGFR, Ca, and Pi.
BHB, β-hydroxybutyric; BMI, body mass index; WC, waist circumference; HTN, hypertension; T2DM, type 2 diabetes mellitus; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; Ca, calcium; Pi, phosphorus.

the association between BHB and TAC severities. Specifically, model 2 was adjusted with age, sex, BMI, waist circumference, hypertension (HTN), and type 2 diabetes mellitus (T2DM), while model 3 was adjusted with age, sex, BMI, waist circumference, HTN, T2DM, TG, HDL, eGFR, Ca, and Pi.

Mediation analysis was conducted on SPSS 22.0 with model 4 in PROCESS plugin, which was introduced by (27). Before the analysis, we hypothesized that MAs (the independent variable) influenced TAC severities (dependent variable) directly as well as indirectly *via* BHB (mediator variable). Mediation analysis was used to validate the hypothesis, which was composed of three steps. Firstly, the direct effect of MAs on TAC severities ($c^{'}$) was evaluated. Secondly, the effect of MAs on serum BHB level (a) was analyzed. Thirdly, the indirect effect of MAs on TAC severities *via* serum BHB level ($a * b$) was evaluated, while b referred to the effect of BHB on TAC severities controlling for MAs. The sum of the direct effect and the indirect effect was considered as the total effect of MAs on TAC severities (c), meaning that $c = c^{'} + a * b$. In this research, age was included as a control variable. The effects were significant if their 95% confidence interval (CI) did not cross zero (28). BHB would be determined as a mediating factor if the results showed that both the total effect and the indirect effect were evident.

We used IBM SPSS Statistics 22.0 for all data analyses, and a two-tail $P < 0.05$ has been considered significant after Bonferroni correction.

RESULTS

Baseline Characteristics

A total of 160 participants (69.4% are female; ages between 42 and 91) were finally enrolled within the specified time, and the baseline characteristics of the subjects by different degrees of TAC are described in **Table 1**. The severity of TAC increased with aging; in addition, a higher occurrence of hypertension and CKD and a lower eGFR were observed in the severe TAC group. Meanwhile, no significant differences were found in gender, glucolipid, and Ca/Pi metabolism between groups.

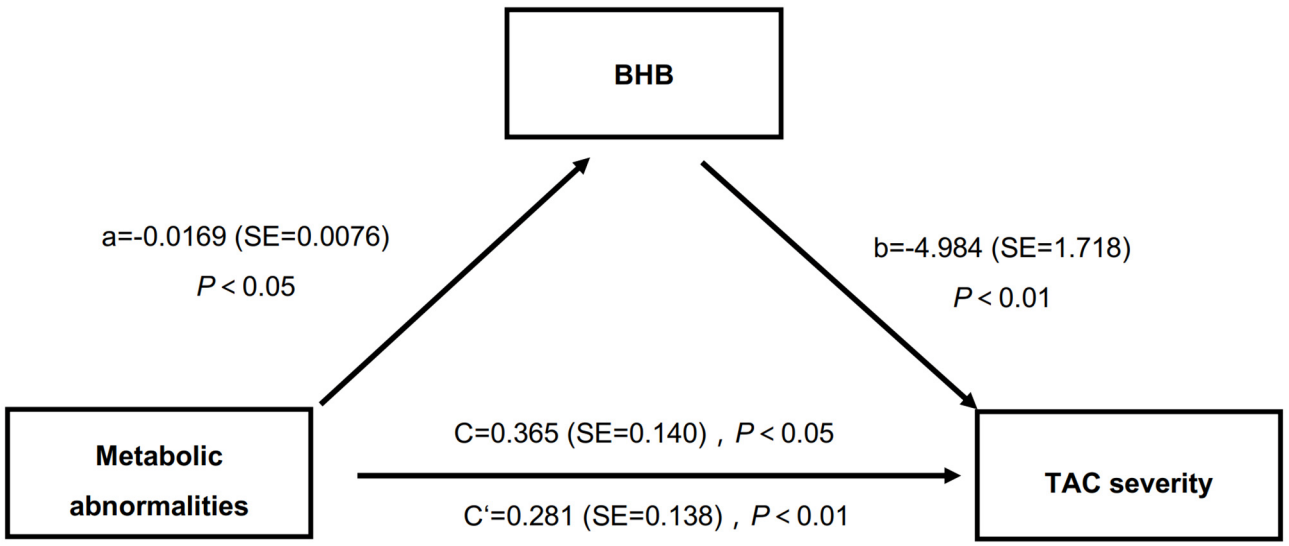

FIGURE 1 | BHB partly mediated the relations between MAs and TAC severities. BHB, β-hydroxybutyric acid; MAs, metabolic abnormalities; TAC, thoracic aorta calcification.

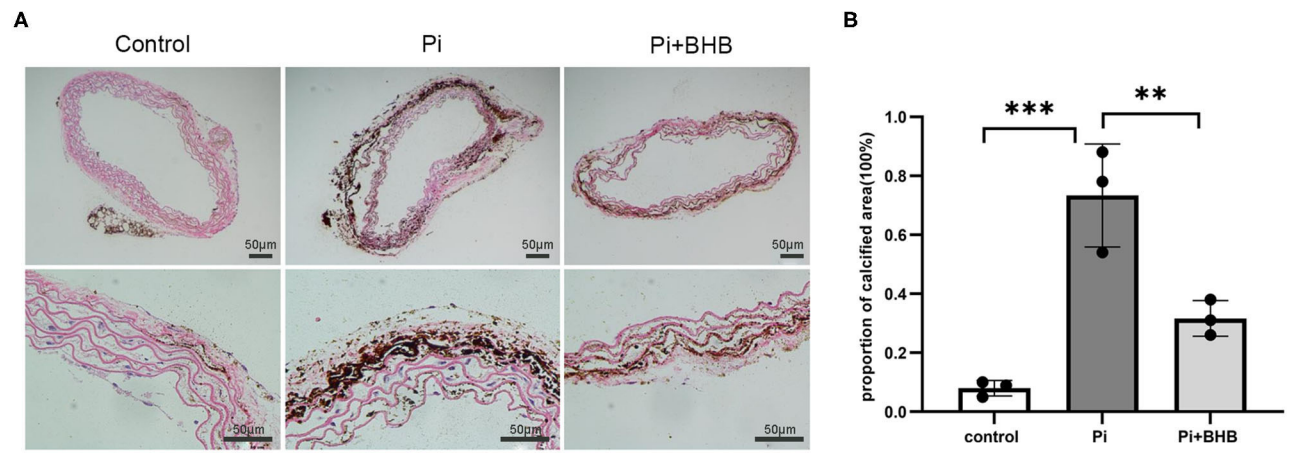

FIGURE 2 | β-Hydroxybutyric acid ameliorated the Pi-induced arterial calcification. **(A)** Von Kossa staining of arterial rings. Pieces of mice aorta were cultured in calcification medium (Pi: 2.6 mM) for 14 days. Scale bar: 50 μm. **(B)** Proportion of calcium areas in the total cross-sectional aorta area. The analysis was performed by Image J. Pi, phosphate; $^{**}P < 0.01$; $^{***}P < 0.001$.

BHB Was Related With MAs and VC and Partly Mediated the Effects of MAs on VC

In this study, the patients with MAs had higher TAC scores than those without MAs [205.3 (4.7, 1,031.5) vs. 26.1 (0.0, 310.3), $P < 0.01$]. Furthermore, we investigated the relationship between serum BHB levels and TAC severities with linear regression analysis (**Table 2**). The results showed that, in the univariate linear regression (model 1), BHB was a protective factor for TAC. After adjusting for some potential influencing factors and metabolic parameters, including age, sex, WC, HTN, T2DM, TG, HDL-C, *etc.*, the results still showed that BHB was an independent protective factor for TAC ($P < 0.05$).

It has been well-accepted that MAs contribute to the occurrence of VC, while BHB affected the metabolic status (4, 29). Here we performed a mediation analysis to further analyze whether BHB mediated the effects of MAs on TAC severities. The results showed that, in this study (**Figure 1**), the presence of MAs had a negative impact on serum BHB level ($\beta = -0.0169$, $P < 0.05$), and the BHB level also had a negative impact on TAC ($\beta = -4.984$, $P < 0.01$). Moreover, the presence of MAs had a significant positive effect on TAC severities ($\beta = 0.365$, $P < 0.001$), and after the mediator variable BHB was placed, the effect of MAs on TAC was still significant ($\beta = 0.281$, $P < 0.05$). In other words, MAs negatively affect BHB, while BHB

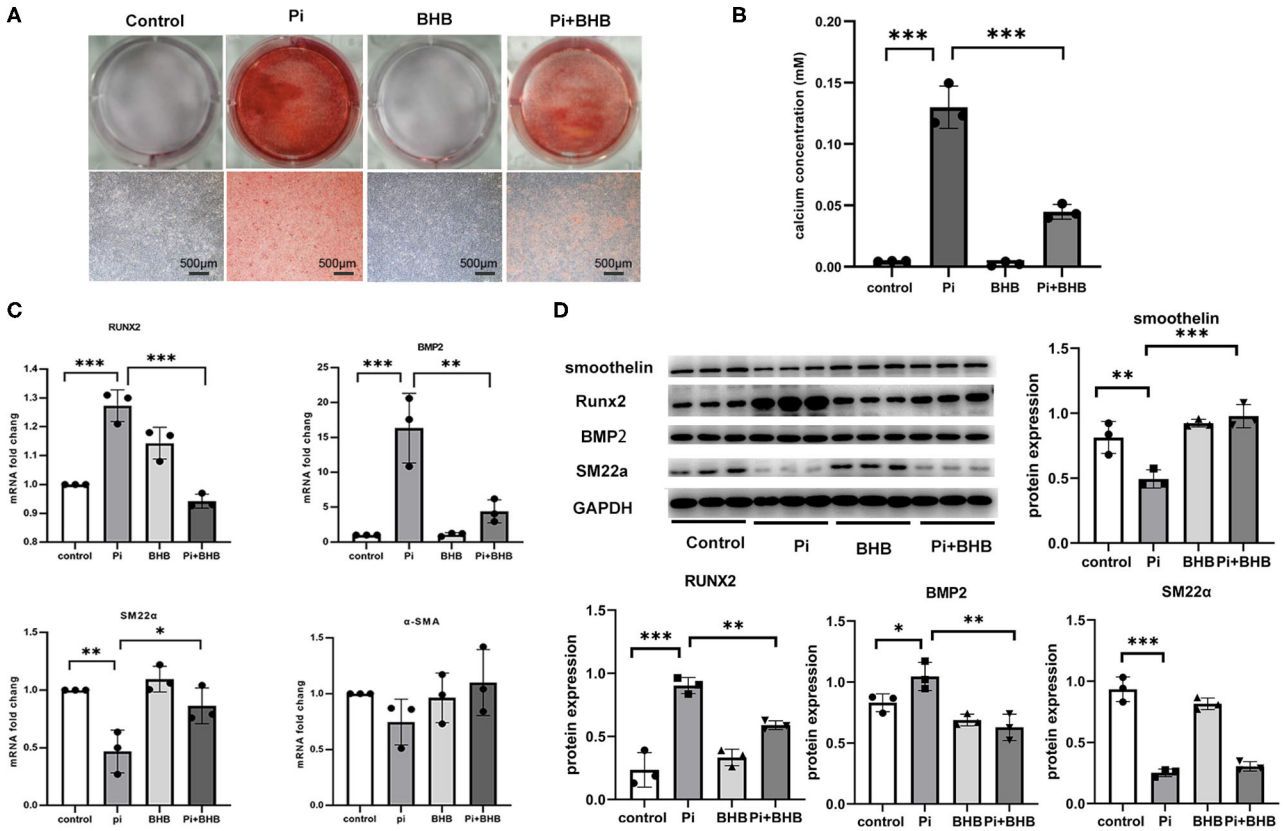

FIGURE 3 | β-Hydroxybutyric acid (BHB) inhibited the osteogenic phenotypical transition of vascular smooth muscle cells (VSMCs). **(A)** Alizarin Red S staining of VSMCs. Primary VSMCs were treated with Pi (2.6 mM), BHB (4.0 mM), or Pi (2.6 mM) + BHB (4.0 mM) for 7 days. Scale bar: 500 μm. **(B)** Calcium density analysis examined the calcium concentration in supernatant collected from VSMCs. **(C)** qPCR detected the differences in the transcription levels of Runx2, BMP2, SM22α, and α-SMA. **(D)** Representative Western blot bands and semi-quantitative analysis of the protein expression of smoothelin, Runx2, BMP2, and SM22a. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

partly mediated the effects of MAs on TAC. Specifically, BHB accounted for 23% of the association between MAs and TAC (direct effect: $\beta = 0.281$, 95%CI: 0.023–0.548; indirect effect: $\beta = 0.084$, 95%CI: 0.015–0.170).

BHB Inhibited High Phosphate-Induced VC

To further evaluate the effects of BHB on VC, we established calcification models with arterial rings and VSMCs and found that BHB co-incubation delayed the progression of the arterial calcification (**Figure 2**), and BHB intervention (2.6 mM Pi + 4.0 mM BHB) ameliorated VSMC calcification and decreased the calcium content in VSMCs (**Figures 3A,B**). Furthermore, with the analysis of RT-PCR and WB, the expressions of osteogenic markers, Runx2 and BMP2, were decreased in VSMCs treated with BHB intervention medium compared to cells treated with phosphate (2.6 mM Pi) alone, whereas BHB maintains the expressions of contractile proteins, smoothelin, and SM22α (**Figures 3C,D**). These results demonstrated that BHB may serve as an effective intervention for VC *via* regulating VSMC phenotypical transition.

Autophagy Enhancement Induced by BHB Was Found to Inhibit VC

To further investigate whether autophagy is the potential mechanism in BHB-mediated VC inhibition, this study performed several *ex vivo* and *in vitro* experiments. The results showed that BHB introduction postponed the progression of arterial calcification, enhanced the expression of autophagy-related protein LC3B, and upregulated the ratio of LC3II/LC3I (**Figures 4A,B**). To investigate the effects of BHB on autophagy flux, VSMCs were infected with mRFP-GFP-LC3 adenovirus (multiplicity of infection = 100). As a result, BHB administration induced a considerable increase in RFP-positive autolysosomes (red dots) in cells compared with those treated with high Pi alone (**Figures 4C,D**), while no differences were shown in GFP/RFP double-positive autophagosomes (yellow dots) between groups. Taken together, these results indicated that phosphate inhibits autophagy mainly by suppressing autophagic flux at late stage, while BHB offset this effect and promoted the fusion of autophagosomes with lysosomes to form autolysosomes. The inhibition of autophagy in VSMCs counteracted the protective

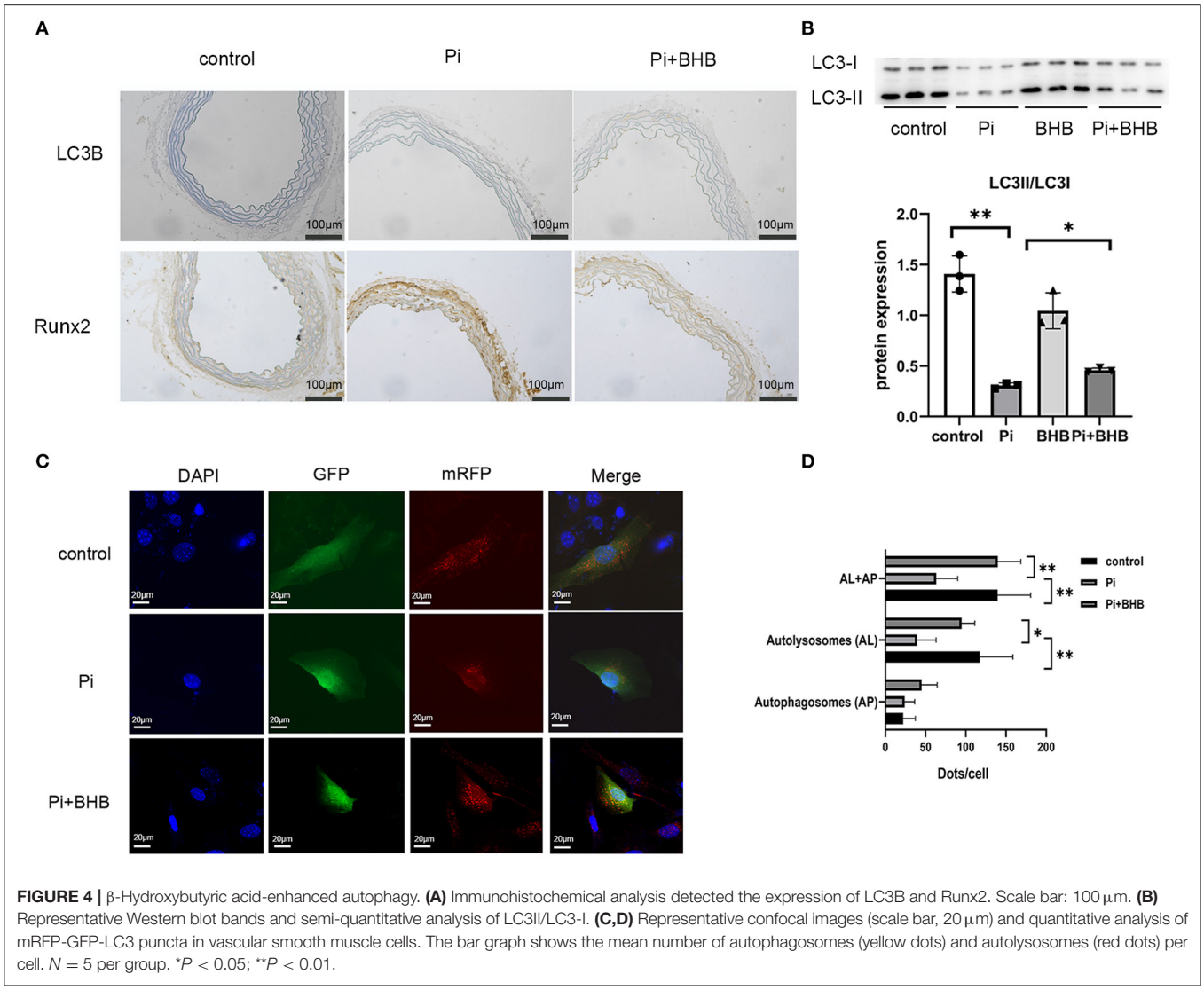

FIGURE 4 | β-Hydroxybutyric acid-enhanced autophagy. **(A)** Immunohistochemical analysis detected the expression of LC3B and Runx2. Scale bar: 100 μm. **(B)** Representative Western blot bands and semi-quantitative analysis of LC3II/LC3-I. **(C,D)** Representative confocal images (scale bar, 20 μm) and quantitative analysis of mRFP-GFP-LC3 puncta in vascular smooth muscle cells. The bar graph shows the mean number of autophagosomes (yellow dots) and autolysosomes (red dots) per cell. $N = 5$ per group. $^{*}P < 0.05$; $^{**}P < 0.01$.

effect of BHB on arterial calcification and increased the calcium content in VSMCs (**Figure 5**). In conclusion, the findings demonstrated that the BHB-induced autophagy enhancement may be a potential mechanism that inhibited the development of VC.

DISCUSSION

The aim of this study was to determine whether BHB ameliorated arterial calcification and to investigate the potential mechanism related to autophagy. The major findings of our present study are as follows: (1) BHB was negatively correlated with VC and mediated the effects of MAs on VC, (2) BHB postponed arterial calcification and inhibited the osteogenic phenotype transdifferentiation of VSMCs induced by high phosphate, and (3) autophagy enhancement promoted by BHB in VSMCs was found to contribute to the inhibition of arterial calcification. For the first time, this study demonstrated the role and effect of BHB in VC and MAs. Furthermore, the results may provide a potential interventional target for the prevention of VC.

MAs were common in the aging population. Data from the National Health and Nutrition Examination Survey showed that the prevalence of MAs was about 46.7% among subjects of age >60 years (30). It has been well-accepted that aging is a powerful risk factor for VC and METs (31). In accordance, interventions toward aging or MAs may possess potency in preventing VC (32, 33). We confirmed, with a univariate linear regression analysis, that serum BHB levels were negatively related with TAC severities. Moreover, after including various potential variables into the regression model, we showed that BHB was still an independent protective factor for severe TAC. To figure out whether BHB transmitted the effects of MAs to VC, we performed a mediation analysis, and the result confirmed that BHB was the mediator (intervening variable), indicating that BHB is a potential target to regulate metabolic conditions and therefore affects the pathogenesis of VC. However, the underlying effects and mechanisms of BHB on VC remain unclear.

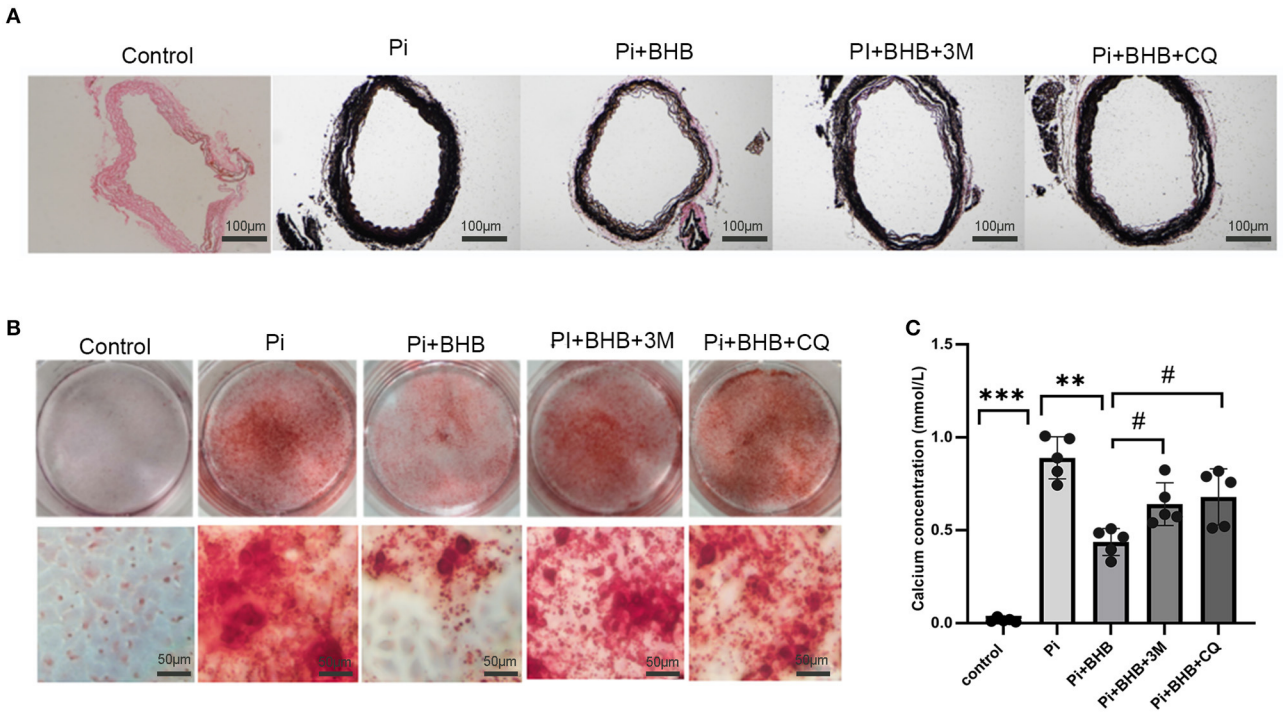

FIGURE 5 | Autophagy inhibitors counteracted the protective effect of β-hydroxybutyric acid (BHB) on vascular calcification. **(A)** Von Kossa staining of arterial rings. **(B,C)** Alizarin Red S staining and calcium deposition analysis of vascular smooth muscle cells (VSMCs). Scale bar: 50 μm. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. VSMCs treated with Pi alone; $^{\#}P < 0.05$ vs. VSMCs treated with Pi+BHB.

Accumulating data from observational and randomized clinical trials indicated that CR might improve cardiovascular health and metabolic status (34, 35). CR upregulates the production of ketone bodies, in which BHB accounted for more than 70%, a multifunctional metabolic substance which could be physiologically elevated under glucose deprivation. Recent studies suggested that BHB played an important role in delaying aging and improving metabolic conditions (18, 24, 29, 36).

Exercise and calorie restriction diets are believed to improve metabolic status. The metabolic status amelioration may be linked to the upregulation of BHB. Interestingly, sodium–glucose cotransporter 2 inhibitors, such as dapagliflozin, an effective drug for T2DM, could induce mild ketonemia and downregulate serum glucose, reduce body weight, and blood pressure (37). BHB is the most promising effector during the process, for researchers have confirmed that the administration of BHB precursor could attenuate hypertension and protect against heart diseases (29).

Moreover, BHB takes a part in maintaining the functions of the cardiovascular system—for example, BHB could delay vascular aging, improve cardiac ischemia/reperfusion injury, and inhibit inflammation (38, 39). However, it remains unclear whether BHB affects VC, and the underlying mechanism needs to be elucidated. In this study, we established vascular calcification models and evaluate the effects of BHB on VC. The Von Kossa staining of the aorta rings showed that BHB significantly alleviated arterial calcification, while the Alizarin Red S staining demonstrated that BHB reduced the calcium content of VSMCs. Moreover, we further demonstrated that BHB inhibited the osteogenic phenotype transdifferentiation of VSMCs, which was characterized by the upregulation of osteogenic phenotype markers (RUNX2 and BMP2) and downregulation of contractile phenotype markers (smoothelin and SM22a).

However, the underlying mechanisms in the regulation of BHB on VSMC calcification need further studies. It has been proven that autophagy, an evolutionarily conserved mechanism linking to several cellular pathways, impacts VSMC survival and functions. Dai et al. demonstrated that the inhibition of autophagy enhanced the phosphate-induced matrix vesicle release, thereby exacerbating VSMC calcification (40). Otherwise, BHB was found to enhance autophagy regulated by the histone acetylation of specific genes and improve proteostasis through increased chaperone expression (41). Thus, it is meaningful to further explore whether BHB can promote autophagy in VSMCs to protect against osteogenic phenotype transdifferentiation, resulting in inhibiting VC. In the present study, BHB enhanced the expression of autophagy-related protein LC3B and promoted the formation of autolysosomes. The results may raise the perspective attention for the intervention of BHB on inhibiting VC.

Our study has some limitations which must be appreciated. Firstly, the clinical study was retrospective; confounding factors or selection bias may have affected our findings. A prospective study for the effect of BHB on VC should be considered in the

future. Secondly, the current project should be further validated in BHB-knock-out animal models; however, the model is not available because BHB is important for animal survival.

In conclusion, we identified for the first time that BHB mediated the correlation between MAs and VC; then, we further demonstrated BHB could promote autophagy to inhibit VC. Our present study provides a novel insight into the potential of BHB as an effective strategy for the treatment of arterial calcification.

PERSPECTIVES

This is the first study to demonstrate that BHB mediated the correlation between MAs and arterial calcification. In addition, the enhancement of autophagy in VSMCs induced by BHB treatment may be an effective intervention target to ameliorate arterial calcification and delay aging.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Eighth Affiliated Hospital, Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Ethics Committee of the Eighth Affiliated Hospital, Sun Yat-sen University.

AUTHOR CONTRIBUTIONS

JL: funding acquisition, writing—original draft, and writing—review and editing. JH: conceptualization, data curation, formal analysis, and investigation. WH: data curation, formal analysis, and investigation. GS: computerized tomographic analysis. JC: supervision. HH: funding acquisition and supervision. All authors contributed to the article and approved the submitted version.

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REVIEW
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Oral, Tongue-Coating Microbiota, and Metabolic Disorders: A Novel Area of Interactive Research

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Interactions between colonizing microbiota and the host have been fully confirmed, among which the tongue-coating microbiota have a moderate rate of renewal and disease sensitivity and are easily obtained, making them an ideal research subject. Oral microbiota disorders are related to diabetes, obesity, cardiovascular disease, cancer, and other systemic diseases. As an important part of the oral cavity, tongue-coating microbiota can promote gastritis and digestive system tumors, affecting the occurrence and development of multiple chronic diseases. Common risk factors include diet, age, and immune status, among others. Metabolic regulatory mechanisms may be similar between the tongue and gut microbiota. Tongue-coating microbiota can be transferred to the respiratory or digestive tract and create a new balance with local microorganisms, together with the host epithelial cells forming a biological barrier. This barrier is involved in the production and circulation of nitric oxide (NO) and the function of taste receptors, forming the oral-gut-brain axis (similar to the gut-brain axis). At present, the disease model and mechanism of tongue-coating microbiota affecting metabolism have not been widely studied, but they have tremendous potential.

Keywords: tongue coating microbiota, metabolic disorders, gut microbiota, mechanisms, oral microbiota

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INTRODUCTION

Microbial–host interactions closely influence human health status (1, 2). Microorganisms colonizing the human body can participate in the synthesis and metabolism of vitamins, proteins, and lipids, promote immunity, maintain the local ecological balance in organs, degrade nutrients, provide energy to the host (1, 3, 4) and have an important impact on host metabolic processes. In contrast, oral microbiota have gradually gained importance as easily detectable colonizing microorganisms, and research has shifted from oral diseases to a broader perspective, of which tongue-coating microbiota are an important part. Tongue-coating diagnosis is a pivotal aspect of traditional Chinese medicine (TCM). The TCM theory suggests that the tongue is fumigated by "stomach qi," which indicates differences in disease etiology and disease status (5) and is widely used in clinical practice (6, 7).

Oral microbiota are not identical to tongue-coating microbiota. The oral cavity contains several habitats, and microbiota are distributed in the tongue coating, saliva, teeth, buccal mucosa (cheek), soft and hard palate, gingival sulcus, tonsils, throat, and lips (8, 9). Factors influencing the formation of oral microbiota include temperature, humidity, saliva volume, pH, oxygen, and the rate of local mucosal shedding (10, 11). Therefore, there is specificity in the microbiota of different

loci. For example, there are significant differences in species and abundance between the tongue coating-derived conjugates of *Veillonella* and *Streptococcus* and dental plaque-derived complexes of *Veillonella* and *Streptococcus* (12). The tongue coating microbiota has stability with a moderate rate of shedding of biofilms formed by tongue epithelial cells and microbiota, making it a good site for study. In contrast, dental plaque-predictive sensitivity to disease may be inferior to that of tongue coating (13, 14). A higher rate of dental plaque shedding makes it less stable (14). Oral epithelial cells are renewed every 2.7 h (15), and rapid biofilm shedding affects the stability of the test results. The tongue dorsum is rich in filiform papilla, fungiform papillae, one row of annular papillae, and foliate papillae. It has a high diversity of bacterial communities, whereas, the non-keratinized epithelium at the base of the tongue can rapidly absorb small molecules and interact with the host. The proximity of the tongue to the tonsils allows compounds shed from epithelial cells and tongue-coating microbiota to be transported into the respiratory and digestive tracts. These characteristics make tongue-coating microbiota more likely to achieve oral-gut microbiota translocation and have broader metabolic effects.

Tongue-coating microbiota have also been associated with chronic systemic diseases, in which nutrients and metabolic disorders occur, such as gastritis and diabetes (16, 17) and different types of cancer (18–20). Due to its association with chronic non-oral diseases, tongue-coating microbiota are expected to serve as a potential markers for metabolic homeostasis and may be used as a future diagnostic tool. A metabolic disorder is an imbalance between uncoordinated digestion and absorption of substances in all diseases. Research directions in tongue-coating microbiota, its relationship with metabolic diseases, and its role in metabolism, are worth exploring.

OVERVIEW OF TONGUE COATING MICROBIOTA

The Oral Microbiology Database

The National Institutes of Health Common Fund Human Microbiome Project (https://commonfund.nih.gov/hmp) (21) was established in 2007 and has previously examined the microbiota from nine oral cavity sites, including the tongue (22). Oral microbiota are relatively stable at the phylum level, but inter-host microbiota variation is high at both the species and strain levels (23). While published articles are available online (24), no tests on tongue-coating microbiota have been reported. Additional sequence analysis websites or databases (CORE et al.) have been developed based on the database (25, 26). Comparing high-throughput epidemiologic investigations,16S rRNA gene sequencing and the Human Oral Microbe Identification Microarray (HOMIM) provide similar sequencing detection sensitivity (27).

The Human Oral Microbiome Database (HOMD, http://www.homd.org/) was established in 2007 and is the first website that provides tools to describe human oral microorganisms systematically. Currently, 775 microorganisms are included online, of which 445 are from the oral cavity. Furthermore, 57% of the microorganisms were formally named, 13% were unnamed but cultured, and 30% were uncultured. A basic local alignment search tool (BLAST) (28) is available to search for genes and their annotations and is linked to JBrowse (29) to obtain information on the sequence of genes and other relevant information. The site allows searching for microbial strains by species taxonomic ID, genus, species, habitats, and nomenclatural status and provides a complete biotaxonomy list. However, currently, the colonization site is only localized to the oral cavity, and no detailed information is provided for tongue-coating-associated microbiota.

Common Microbiota in Tongue Coating

The tongue-coating microbiota are structurally complex and contain not only monolayers of sparsely colonized bacteria but also equally free bacteria, bacteria on squamous epithelial cells, and structurally complex bacterial entities (consortia) (30). This shows that the epithelial cells of the tongue dorsum are a mixture of rapidly shed, sparsely colonized cells, and long-lived structures where more substantial biofilms can form. The microbiota and tongue papillae form a wide range of interspecies interactions that can be specifically classified as synergistic, signaling, or antagonistic (31, 32). Different species and genera of bacteria may have the same metabolic function, and the functional redundancy is widespread (30). Diversity and appropriate redundancy allow for greater stability (33) and metabolic efficiency (34). Therefore, the normal tongue coating microbiota diversity is higher than that of diseased individuals (20); however, microbiota abundance may be greater (35) in diseased than in healthy populations.

Tongue-coating microbiota are reported more consistently at the phylum level, but with contradicting literature on the species level, which may be related to sampling methods, inclusion criteria, ethnicity, and region (14, 20, 36–39). The results of 16S rRNA analysis showed that tongue-coating colonizing microbiota in healthy humans at the phylum level included the following: *Firmicutes*, *Bacteroidetes*, *Proteobacteria, Actinobacteria, Spirochaetes, Fusobacteria*, and *Synergistetes* (27, 38, 40, 41), and the abundance of the top three microbiota was consistent with the overall distribution of the oral cavity. However, the proportions of *Actinobacteria* and *Spirochaetes* were higher than those of the oral cavity (42). The dominant tongue microbiota at each taxonomic level is shown in **Figure 1** (14, 20, 43–50).

Common harmful oral bacteria can damage immunity and cause chronic inflammation (51, 52). Changes in the abundance of *Enterococcus sp.* and *Lactobacillus sp.* lead to the expansion of Th17 cells and the accumulation of IL-6 and IL-23 (53). Periodontitis not only originates from the gingival microbiota but is also affected by tongue-coating microbiota (14, 54). *Porphyromonas spp.* have been shown to induce atherosclerosis (55, 56) and diabetes (55). In obese individuals, tongue-coating *Bacteroidales* (order), *Enterococcus* (genus), and *Staphylococcus* (genus) were elevated, and *Prevotella* (genus) and *Butyricoccus* (genus) were decreased (50). In rheumatoid arthritis (RA) patients and at-risk individuals, tongue-coating *Prevotella* and *Veillonella* were higher in relative abundance than populations

FIGURE 1 | The dominant tongue coating microbiota at each taxonomic level. Different phyla are color-coded; layers from the inside to outside are phylum-class-order-genus. Black text indicates microbiota whose abundance is increased in metabolic diseases, and yellow text shows microbiota whose abundance is decreased in metabolic diseases (44–50).

in healthy controls (13). RA patients present with an increased relative abundance of pro-inflammatory species.

Studies on oral probiotics have found that probiotic products containing *Lactobacillus* improve oral health status and may improve disease symptoms (57), such as fatty liver and cancer (53). Therefore, tongue epithelial cells have a barrier function (58) and are involved in taste production (44, 59), the oral-gut axis (60, 61), and nitric oxide (NO) cycling (62, 63). Thus, the role of tongue-coating probiotics in the host can be explored in several ways. Among nitrate-reducing bacteria, *Veillonella*, *Actinomyces*, *Haemophilus*, and *Neisseria* are highly abundant in the tongue coating and are potential probiotics (64).

Tongue Coating Microbiota and Intestinal Microbiota

Intestinal microbiota are more extensively and intensively studied than tongue-coating microbiota (65, 66), and their relationships and whether the two have similar metabolic mechanisms deserve further exploration. Both tongue-coating microbiota and gut microbiota are associated with metabolic status (67), immune status, age (41), sex, genetic factors, environmental factors (68), antibiotic use, infant feeding status (69), and probiotic and prebiotic administration closely related to diet (70, 71). Colonization by intestinal and tongue-coating microbiota are dominated by anaerobic bacteria (69). The unweighted intestinal microbiota are separated from the oral microbiota compartment, indicating a large difference in the oral gut microbiota (72). Some can undergo microbiota displacement from the oral to the gut (37) or otherwise interact with each other. There are dozens of genera shared by the tongue coating and intestinal microbiota (72), such as *Lactococcus*, *Bilophila*, and *Akkermansia*. Oral and intestinal microbiota share nearly 45% homology but differ in their abundance.

Numerous studies have confirmed the strong relationship between gut microbiota and oral microbiota dysbiosis and systemic disease. However, the association between tongue coating microbiota and gut microbiota remains elusive. First, the tongue coating microbiota can translocate to the gut and lead to fluctuations in the gut microbiota. Recently, it was found that the abundance of 14 taxa has increased in tongue coating samples and stool samples from older adults (72), i.e., microbiota from other parts of the host may migrate to the oral

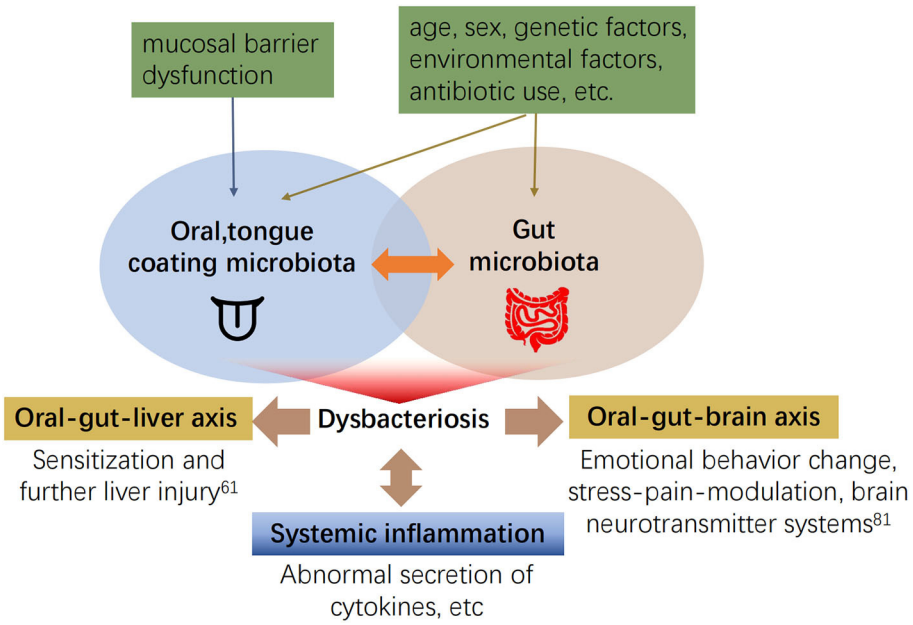

FIGURE 2 | The interaction between tongue coating and gut microbiota and its effects on other systems (60, 61, 81).

cavity. The mechanism could be a decline in gastrointestinal function, or decreased gastric and bile acid secretion in the elderly. Oral microbiota are not inactivated and reach the intestine, invasion of gingival or tongue tissue by tongue coating microbiota, the impaired mucosal barrier function of the tongue epithelium, decreased levels of ligand proteins in the tongue (73), or Fap2-mediation (74) blood diffusion to reach the intestine (75). Second, changes in abundance in tongue coating microbiota are consistent with intestinal microbiota in disease, and both abundances are altered during the activation of immune receptors or abnormal hormone levels (76–78). In patients with autoimmune liver disease, *Veillonella spp.* are increased, and positively correlated in the oral and intestinal tracts (46). The tongue and intestine are involved in digestion through reflex stimulation of the gastric system, pancreas, liver, and gallbladder (79). Microbiota can also influence metabolism by interacting with taste receptors on the tongue and intestine (80), which may be related to the specific effector mechanism of the oral-gut axis (see **Figure 2**).

There are differences in the metabolic effects of gut and tongue coating microbiota. The production and oxidation of intestinal microbiotic metabolites, such as short-chain fatty acids (SCAFs) and branched-chain fatty acids, regulate energy expenditure (82). Gut microbiota also participate in the regulation of bile acid metabolism and the TMAO pathway. At present, the effects of tongue coating on related mechanisms have not been identified. The main metabolic process used by oral microorganisms is anaerobic respiration, whereas, microbial fermentation is the main process in the intestine (83). Therefore, the mechanisms involved in developing chronic systemic diseases by tongue coating microbiota differ from those of the intestinal microbiota.

Tongue Coating Microbiota and Metabolic Status

The metabolites of tongue coating microbiota and chronic inflammation mutually promote each other. Tongue coating microbiota dysbiosis is directly associated with the development of periodontitis (14) and oral mucosal disease (84). Dental caries and periodontitis cause chronic infection, increase arterial inflammation and are high-risk factors for diabetes and cardiovascular disease (85–87). *Porphyromonas gingivalis* and *Fusobacterium nucleatum* have been shown to exacerbate chronic inflammation. C-reactive protein levels are elevated, and inflammatory responses are more severe in those with oral microbiota dysbiosis and are directly proportional to low-density lipoprotein (LDL) levels and the carotid intima-media area (60). The bacterial metabolites lipopolysaccharide (LPS) and IgG correspond to microbiota, and their activation of neutrophils can be detected in the blood of atherosclerosis patients (88), suggesting that the oral microbiota accelerates disease progression through endotoxin-activated immunity. Matrix-degrading metalloproteinases (MMPs) are important inflammatory mediators in cardiovascular disease and play a key role in the rupturing of atherosclerotic plaques (89), destruction of periodontal connective tissue, and exacerbating oral microbiota dysbiosis. Patients with myocardial infarction and periodontitis have elevated salivary MMPs and reduced tissue metalloproteinase inhibitor-1 levels, which may be related to oral microbiota metabolism (90). Periodontal microbiota causes widespread chronic inflammation and insulin resistance, exacerbating both the incidence and progression of Type 2 diabetes (91). Patients with periodontitis caused by chronic microbiota dysbiosis present with elevated serum

LDL levels, decreased HDL levels, and elevated triglycerides, while periodontal treatment improves dyslipidemia and reduces total cholesterol and serum LDL levels. The pathogenesis of atherosclerosis is associated with oral microbiota, and poor oral hygiene can cause increased levels of fibrinogen and cell adhesion proteins (90). Endothelial dysfunction caused by oral microbiota leads to increased blood pressure (64).

Chronic diseases can also remodel the microbiota. In a mouse model, diabetes increased IL-17 expression, and chronic inflammation led to changes in the abundance of oral microbiota. Transplantation of altered oral microbiota to germ-free mice resulted in increased susceptibility to diabetes (92). Diabetes can cause changes in the oral microbiota (93). Comparison of oral microbiota between Type 2 diabetics and non-diabetics revealed *Neisseria spp*., *Fusobacterium, Veillonella*, and *Streptococcus spp*. increased.

The above studies on oral microbiota suggest a close relationship between tongue coating microbiota and chronic inflammatory and systemic diseases, especially metabolic diseases. Tongue coating microbiota has been confirmed as a potential biomarker for gastritis (20) and have predictive value for gastric cancer (20) and pancreatic head cancer (94). The correlation of tongue coating microbiota with greater metabolism is worth exploring.

Mechanisms by Which Tongue Coating Microbiota Affects Metabolism

The mechanism by which the tongue coating microbiota affects metabolism may be similar to intestinal microbiota, namely the biological barrier effect, involvement in the nitric oxide (NO) cycle, and taste production (45, 59). Metabolism in the oral cavity produces various antimicrobial compounds and enzymes, such as lysozyme, amylase, immunoglobulins, and epithelial cell shedding, which influence the establishment and renewal of the tongue coating microbiota. In turn, the tongue coating microbiota produces different metabolites and endotoxins, forming a complex barrier between the microbiota and the local environment of the tongue. Oral microbiota dysbiosis and cell wall production of endotoxins (e.g., LPS) promote inflammation and tissue destruction, and disruption of epithelial integrity further aggravates the penetration of oral microbiota into the oral epithelium and connective tissue (95). Studies have shown that microbiota, such as *Porphyromonas gingivalis,* can downregulate ligand protein expression and disrupt the oral mucosal barrier (96). On the other hand, the main sources of nutrients for microorganisms are saliva, host-consumed food, and various by-products produced between species, which support the growth and reproduction of microbiota, forming a biological barrier that competitively inhibits opportunistic pathogenic bacteria (see **Figure 3**).

Tongue coating microbiota play an important role in exogenous NO production and uptake, regulating host NO homeostasis. As a gaseous signaling molecule, NO is bound to human proteins *via* S-nitrosylation and plays an important role in a variety of physiological processes, including the regulation of vascular tone, nerve transmission, mitochondrial respiration, and skeletal muscle systolic function, thereby alleviating the development of diseases such as diabetes, hypertension, and coronary heart disease (97, 98). There are endogenous and exogenous pathways for NO production, and the microbiota-nitrate-nitrite-NO pathway serves as the main source of exogenous NO (99). NO production is non-strain-specific, and relevant microbiota–host interactions exist in the tongue and gut. Some microbiota can convert dietary nitrate to nitrite and produce NO *via* nitrate reductase or react with endothelial and plasma proteins to form S-nitrosothiol (SNO). Nitrite, NO, and SNO have activated soluble guanylate cyclase (sGC) and increased cGMP levels in tissues. The nitric oxide synthase (NOS)-NO pathway improves vascular tone through cGMP/PKG and cellular signals that stimulate smooth muscle relaxation (64, 94, 97). *Veillonella*, *Actinomyces*, *Prevotella*, *Neisseria,* and *Haemophilus* are among the most abundant NO-producing tongue coating microorganisms (99). NO in the tongue crypt can diffuse directly into the circulation through the highly vascularized tissues of the tongue, and regular tongue coating removal increases nitrate reduction to produce more NO (100) while reducing the production of sulfur-containing compounds to reduce bad breath. Microbiota also regulate the host gene expression profile by binding to various host proteins. For example, binding to argonaute protein inhibits miRNA activity to regulate host development (101). *In vitro*, excessive NO production can lead to developmental deformities in the host (101). In humans, dietary nitrate lowers blood pressure in healthy populations (102, 103). In the absence of any dietary changes, the use of mouthwash with sterilizing effects can disrupt oral microbiota, thereby reducing oral and plasma nitrite levels in healthy populations, and is associated with a sustained increase in systolic and diastolic blood pressure (102, 103). Thus, NO production by the oral microbiota in healthy humans has positive implications for vascular endothelial homeostasis (see **Figure 3**).

In contrast, the chemosensory system in mammals is regulated by bacterial metabolites, and tongue coating microbiota may also be involved in taste formation, affecting eating and metabolism (104). In traditional Chinese medicine, the relationship between the five tastes and the internal organs was proposed as early as the period of the "Inner Canon of the Yellow Emperor: sour taste enters the liver, astringent taste enters the lung, and bitter taste enters the heart." These observations have been clinically corroborated in long-term clinical practice (105). The taste system mainly includes carbohydrate, amino acid, fat receptors (58), and bitter receptors. Through the above feeding sensations, the function of the tongue establishes a close relationship with the metabolic system. Obese children have significantly lower taste discrimination and fewer fungiform papillae, accompanied by reduced α-diversity of tongue coating microbiota. In healthy subjects, tongue coating microbiota was associated with taste function, thereby affecting dietary habits, such as preference for salty baked products, saturated fat-rich products. Beverages are consumed more frequently by those who are insensitive to salty flavors than by those who are sensitive.

Likewise, sweet foods are consumed more frequently by those who are insensitive to sweet flavors (106). *Prevotella* abundance

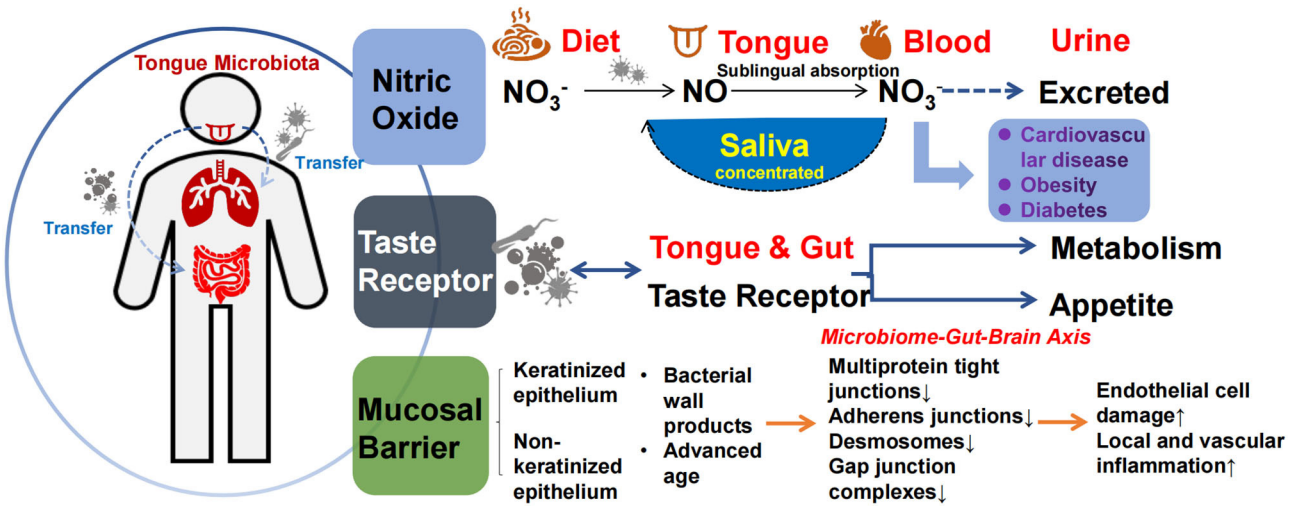

FIGURE 3 | Mechanisms of tongue coating microbiota involved in metabolism. The tongue coating microbiota transfers or co-presents with other parts, including the respiratory tract and the gut (left). These three pathways may be involved in metabolism: (1) The production of nitric oxide (2) Receptor function (3) The formation of consortia with oral epithelial cells and entering the blood when the epithelial barrier is impaired (right).

is positively associated with vegetable intake, whereas, *Clostridia* abundance is associated with protein/fat-rich diets (106). A recent study of the human microbiome found that commensal bacteria have developed strategies to stimulate chemosensory receptors and trigger host cell function (104). Thus, tongue coating microbiota may impact metabolic systems through interactions with chemosensors on the tongue. Similar to the gut microbiota, several previous studies have elucidated feedback mechanisms in gut microbiota-microbiota products-intestinal epithelial cells-endocrine metabolic homeostasis (107, 108) and have inhibitory effects on gastrointestinal motility and appetite through GLP-1, CCK-, ghrelin-, and peptide tyrosine tyrosine (PYY)-labeled EECs in the human small intestine and colon (109). It can be suggested that tongue coating microbiota and their hosts share similar interaction mechanisms.

CONCLUSIONS

Tongue coating microbiota, one of the important components of the oral microbiota, have high sampling stability. Disturbances in tongue coating microbiota have been shown to elevate various chronic inflammatory markers, as well as being closely linked to mechanisms such as local mucosal barriers, nitric oxide metabolism, and taste chemoreceptors. Although, there is a lack of studies of drug action on tongue-coating microbiota to treat disease, studies have shown that probiotics can modulate oral microbiota and improve health (110, 111). In contrast, frequent use of antibiotics or mouthwash adversely affects blood pressure (102, 103). Therefore, tongue coating microbiota are expected to become a new, easy, and non-invasive biological marker that can contribute to diagnostic and prognostic studies of chronic non-infectious diseases.

Research on tongue coating microbiota has not been extensively conducted, and the number of studies related to metabolic diseases and metabolic mechanisms is limited in the field of oral microbiota. Tongue coating microbiota and intestinal microbiota show some similarity in composition, as both are involved in food digestion. Based on studies on intestinal microbiota, tongue coating microbiota may also be involved in various metabolic mechanisms in the human body. The standard sampling procedure and research paradigm of tongue coating microbiota should be standardized, and multi-omics studies or tongue-gut clustering analysis should be performed in the future.

AUTHOR CONTRIBUTIONS

YueL contributed to the topic design, manuscript revision, and the decision to submit for publication. YiwL, JC, and YanL performed the literature retrieval, collation, analysis, and wrote the manuscript together. YiwL and JC were co-first authors. LH and KC revised the manuscript. All authors approved the final version of the manuscript.

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Using Restricted Cubic Splines to Study the Trajectory of Systolic Blood Pressure in the Prognosis of Acute Myocardial Infarction

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Background: Acute myocardial infarction (AMI) is still the most serious manifestation of coronary artery disease. Systolic blood pressure (SBP) is the best predictor of blood pressure in AMI. Thus, its influence on AMI is necessary to be explored.

Methods: A total of 4,277 patients with AMI were extracted from the Medical Information Mart for Intensive Care database. Chi-square test or Student's *t*-test was used to judge differences between groups, and Cox regression was used to identify factors that affect AMI prognosis. SBP was classified as low (<90 mmHg), normal (90–140 mmHg), or high (>140 mmHg), and a non-linear test was performed. Meaningful variables were incorporated into models for sensitivity analysis. Patient age was classified as low and high for subgroup analysis, and the cutoff value of the trajectory was identified. $P < 0.05$ indicates statistical significance.

Results: The effect of SBP on the prognosis of patients with AMI is non-linear. The risks in models 1–3 with low SBP are 6.717, 4.910, and 3.080 times those of the models with normal SBP, respectively. The risks in models 1–3 with high SBP are 1.483, 1.637, and 2.937 times those of the models with normal SBP, respectively. The cutoff point (95% confidence interval) of the trajectory is 114.489 mmHg (111.275–117.702 mmHg, all $P < 0.001$).

Conclusions: SBP has a non-linear effect on AMI prognosis. Low and high SBP show risks, and the risk of low SBP is obviously greater than that of high SBP.

Keywords: SBP, AMI, prognosis, non-linear, MIMIC

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INTRODUCTION

The mortality and morbidity of acute myocardial infarction (AMI) are declining in most countries, especially in countries with higher per capita income (1–3). However, the prevalence of long-term AMI is also increasing with the aging of the world population and rapid population growth; thus, the disease burden of AMI is increasing (1). Every year, 2.4 million people die in the United States, and four million people die in Europe and North Asia; coronary artery disease (CAD) causes up to

one-third of these deaths (4–7). AMI is the most serious manifestation of CAD and greatly increases the mortality rate of CAD (8).

The pathogenesis of acute coronary syndrome is a complex pathophysiological process accompanied by complex neuroendocrine changes (9). Neuroendocrine response after AMI results in the activation of the sympathetic nervous system and renin–angiotensin system and the release of vasopressin and atrial natriuretic peptide. The net effect of this response is vasoconstriction, cardiac stimulation, and regional flow redistribution, which may have a favorable effect in some situations and a deleterious effect in others. Therefore, blood pressure (BP) measurement can be used to reflect the overall potential performance of the cardiovascular and neuroendocrine systems after AMI (10).

As early as the 1990s, studies have shown that systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse pressure are risk factors for cardiovascular disease (11). A study in 2000 showed that the average values of SBP, DBP, and mean blood pressure (MBP) are important predictors of cardiovascular disease in young men (<60 years old), and mean SBP and pulse pressure are important predictors in elderly men (>60 years old) (12). In 2015, Sundström and Arima found that SBP and DBP are very important independent risk factors for cardiovascular and renal diseases (13). The various indicators of BP have played an irreplaceable warning role in the treatment and prognosis of cardiovascular diseases.

Admission SBP can be used as a predictor of rapid clinical evaluation and poor cardiovascular prognosis studies. If this indicator can be effectively used to assess the risk of adverse consequences, then a treatment plan for patients with new AMI can be quickly developed (14). Therefore, a more comprehensive and detailed understanding of the performance of SBP in the prognosis of AMI is important. The purpose of this study was to use restricted cubic splines to study the performance of SBP in patients with AMI from the Medical Information Mart for Intensive Care (MIMIC) database.

MATERIALS AND METHODS

Patients and Variables

The MIMIC [database jointly issued by the Massachusetts Institute of Technology (MIT) Computational Physiology Laboratory, Beth Israel Dikang Medical Center, and Philips Medical] is supported by the National Institutes of Health to promote the work of intensive medical research (15). MIMIC is a publicly available dataset developed by the MIT Computational Physiology Laboratory that includes unidentified patient health data related to ∼60,000 intensive care unit visits. The dataset includes demographic information, vital signs, laboratory tests, drugs, and other information (16). The database has a large number of samples, comprehensive information, and long-term patient tracking; can be used for free; and provides a wealth of resources for intensive care research (17). Access to the database (Certificate Number: 38489997) was granted after the completion of the National Institutes of Health's web-based training course, "Protecting Human Research Participants." In the present study, we extracted 4,277 AMI data from the MIMIC database. All patients were diagnosed with AMI for the first time upon admission. The following variables were extracted from the information of the patients with AMI in the MIMIC database: gender, atrial fibrillation, atrial flutter (AFL), ventricular fibrillation (VF), ventricular tachycardia (VT), SBP, drug, total calcium level, chloride level, creatinine level, phosphate level, potassium level, sodium level, nitrogen level, hemoglobin count, platelet count, red blood cell width (RDW), white blood cell (WBC) count, age, respiration rate, mean heart rate, mean BP, DBP, mean glucose level, Sequential Organ Failure

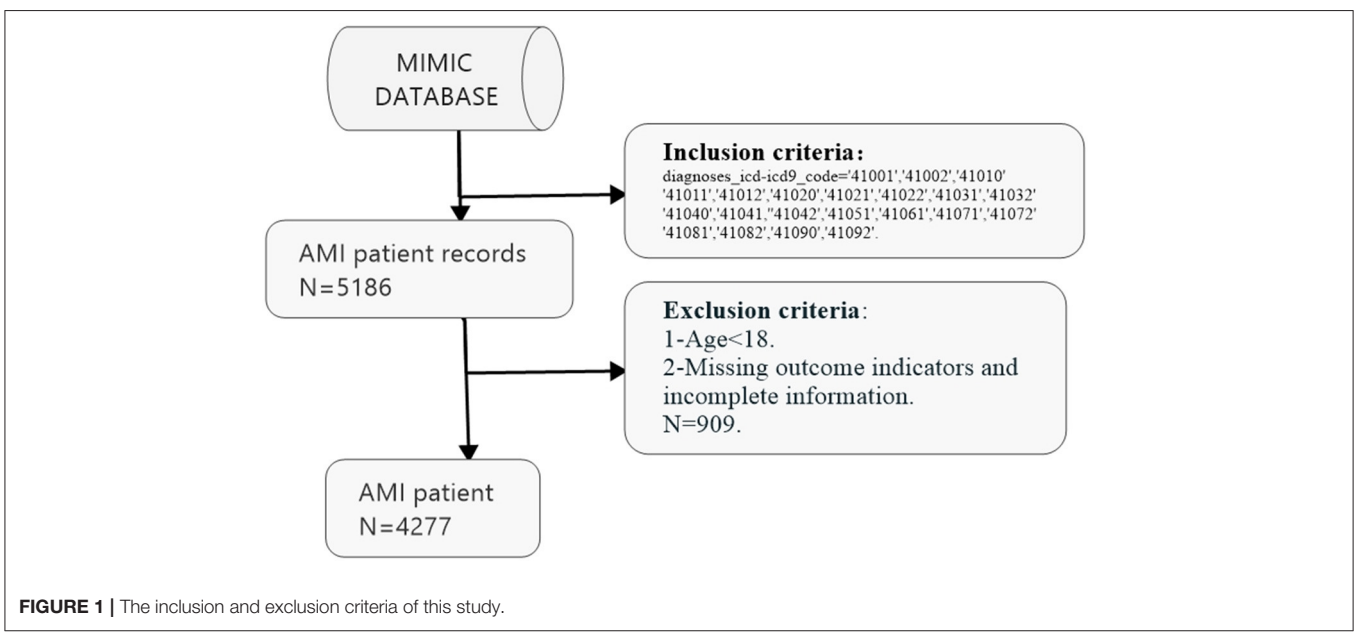

FIGURE 1 | The inclusion and exclusion criteria of this study.

Assessment (SOFA) score, and Acute Physiology Score (APS)-III when the patient was admitted to the hospital. Survival time (in months) and status were also extracted from the database. The inclusion and exclusion criteria are shown in **Figure 1**.

Statistical Analysis

The patients were divided into two groups according to life and death outcomes. In addition to status and survival time, the chi-square test was performed on categorical variables, and Student's *t*-test was performed on continuous variables. Then, Cox regression was performed on all variables to explore the variables that have an impact on AMI outcome. Finally, SBP was categorized as low (<90 mmHg), normal (90–140 mmHg), and high (>140 mmHg). A non-linear test was performed to judge whether the effect of SBP on AMI prognosis is non-linear. The patients were also divided into the "advanced age" and "low age" groups for subgroup analysis. Meaningful variables were incorporated in the Cox regression results to construct three models, and then sensitivity analysis was performed on the models. SBP alone was incorporated in model 1; some variables were collectively included in model 2, and all meaningful variables were included in the Cox regression result as model 3. The best cutoff point for the entire prognostic trajectory was determined. $P < 0.05$ was considered statistically significant. Statistical analyses were conducted using Excel, SPSS, and R (ggplot2, rms, survival, and segmented packages).

TABLE 1 | Baseline characteristics of patients in the study.

Variable	Live	Dead	*P*-value
N	3,434	843	
Sex			0.324
Male	1,234 (35.9)	287 (34.0)	
Female	2,200 (64.1)	556 (66.0)	
AF			<0.001
Yes	931 (27.1)	323 (38.3)	
No	2,503 (72.9)	520 (61.7)	
AFL			1
Yes	79 (2.3)	20 (2.4)	
No	3,355 (97.7)	813 (97.6)	
VF			0.01
Yes	139 (4.0)	52 (6.2)	
No	3,295 (96.0)	791 (93.8)	
VT			<0.001
Yes	281 (8.2)	105 (12.5)	
No	3,153 (91.8)	738 (87.5)	
Drug			0.001
Yes	1,829 (53.3)	395 (46.9)	
No	1,605 (47.7)	448 (53.1)	
Total_Ca	8.7 (8.2–9.1)	8.5 (7.9–9.0)	<0.001
Chloride	103.0 (100.0–106.0)	102.0 (98.0–106.0)	<0.001
Creatinine	1.0 (0.8–1.5)	1.5 (1.0–2.4)	<0.001
Phosphate	3.5 (2.9–4.1)	3.9 (3.2–5.0)	<0.001
Potassium	4.2 (3.8–4.6)	4.4 (3.9–4.9)	<0.001
Sodium	139.0 (136.0–141.0)	138.0 (135.0–141.0)	0.009
Nitrogen	21.0 (15.0–31.0)	33.0 (21.0–51.0)	<0.001
Hemoglobin	12.2 (10.6–13.7)	11.2 (9.9–12.5)	<0.001
Platelet	236.0 (188.0–294.0)	233.0 (173.0–308.5)	0.347
RDW	13.8 (13.1–14.9)	14.9 (13.7–16.7)	<0.001
WBC	10.4 (8.0–13.7)	12.3 (8.9–16.7)	<0.001
Age	69.0 (58.3–78.0)	77.0 (68.0–83.0)	<0.001
Respiration rate	25.0 (18.5–29.0)	28.0 (24.0–33.0)	<0.001
Mean HR	80.7 (71.2–90.1)	86.2 (75.1–97.7)	<0.001
MBP	58.0 (51.8–83.0)	53.0 (46.0–61.0)	<0.001
DBP	58.8 (53.0–65.7)	55.2 (49.5–62.0)	<0.001
SBP	113.5 (105.4–124.2)	108.5 (99.5–122.0)	<0.001
Mean glucose	134.8 (117.2–163.0)	149.0 (121.3–187.1)	<0.001
SOFA	3.0 (1.0–5.0)	6.0 (4.0–9.0)	<0.001
APS-III	37.0 (28.0–49.0)	55.0 (45.0–72.0)	<0.001

AF, atrial fibrillation; AFL, atrial flutter; VF, ventricular fibrillation; VT, ventricular tachycardia; RDW, red blood cell width; WBC, white blood cell count; Mean HR, mean heart rate; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; SOFA, Sequential Organ Failure Assessment; APS-III, Acute Physiological Score.

RESULTS

Among the 4,277 patients with AMI, 843 died during follow-up. According to our results, gender ($P = 0.324$) may not be a factor affecting AMI outcome. The proportion of male/female (%) in the survival group was 2,200/1,234 (64.1%/35.9%), and that in the death group was 556/287 (66.0%/34.0%). The mean value [95% confidence interval (95% CI)] of the

TABLE 2 | The results of cox regression analysis.

Variable	HR	95% CI	*P*-value
Age	1.0477	1.0382–1.0572	<0.001
VFI			<0.001
No	Reference		
Have	1.9324	1.3678–2.7302	
VT			<0.001
No	Reference		
Have	1.589	1.2232–2.0642	
Drug			0.027
No	Reference		
Have	0.8275	0.6999–0.9784	
Total_Ca	0.8470	0.7668–0.9357	0.001
Chloride	0.9721	0.9523–0.9922	0.007
Creatinine	0.9318	0.8817–0.9847	0.012
Phosphate	1.1209	1.0522–1.1940	<0.001
Platelet	0.9991	0.9984–0.9998	0.011
RDW	1.1483	1.1014–1.1972	<0.001
WBC	1.0115	1.0043–1.0188	0.002
Respiration rate	1.0095	1.0006–1.0185	0.036
Mean HR	1.0106	1.0044–1.0169	<0.001
Mean glucose	1.0035	1.0021–1.0049	<0.001
SOFA	1.0488	1.0106–1.0883	0.012
APS-III	1.0219	1.0162–1.0276	<0.001

TABLE 3 | Cox regression analyses of the relationship between SBP and AMI prognosis.

Variable	Model 1		Model 2		Model 3	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Normal	1		1		1	
Low	6.717 (5.044–8.946)	<0.001	4.910 (3.679–6.552)	<0.001	3.080 (2.162–4.385)	<0.001
High	1.483 (1.120–1.965)	0.01	1.637 (1.231–2.18)	<0.001	2.937 (1.918–4.498)	<0.001

Model 1: univariate. Model 2: adjust for aged, VF, VT, drug, SOFA, and APS-III. Model 3: adjusted for age, VF, VT, Total_Ca, chloride, creatinine, phosphate, platelet, RDW, WBC, respiration rate, mean HR, mean glucose, drug, SOFA, and APS-III.

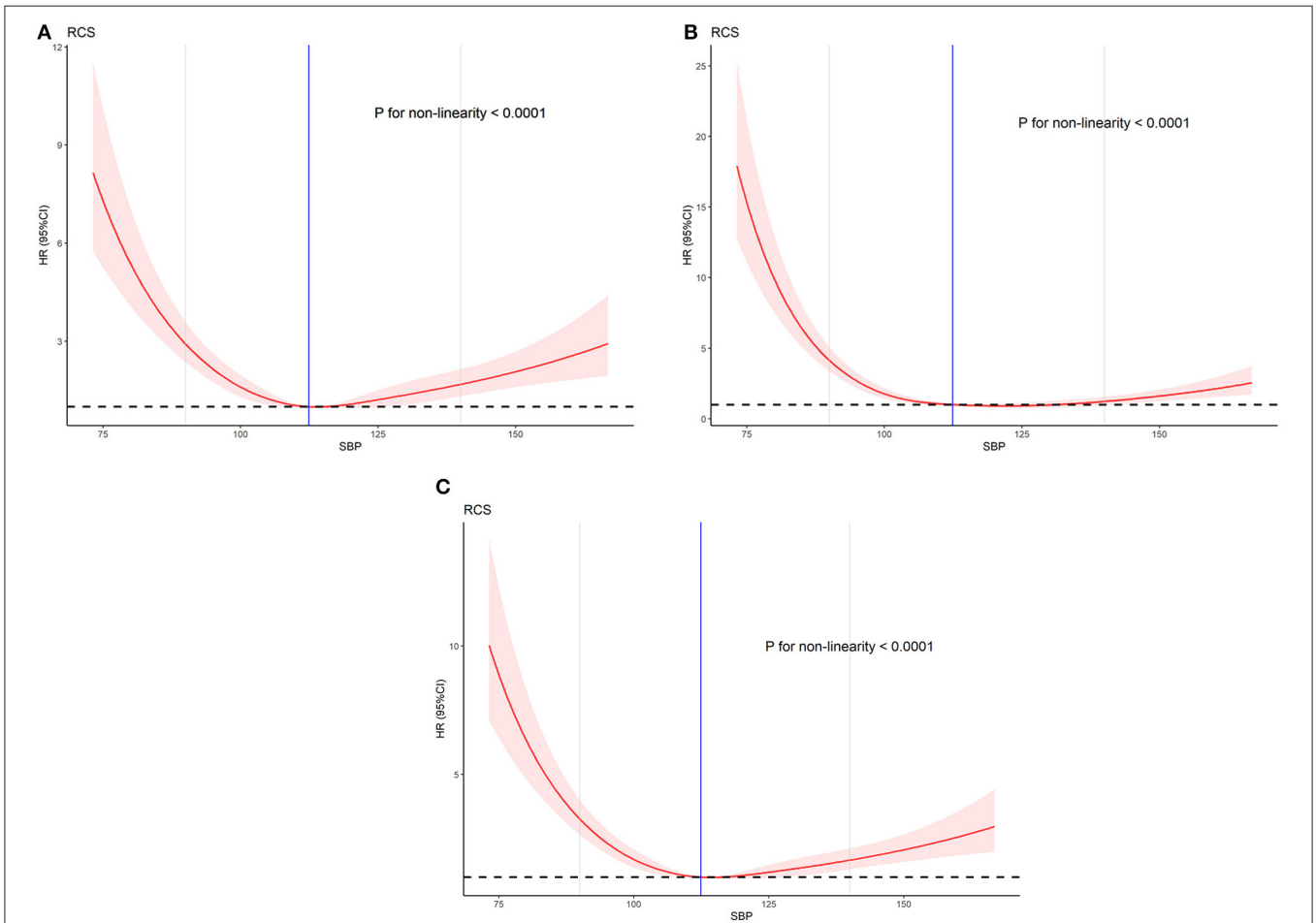

FIGURE 2 | The effect of different doses of SBP on the prognosis of AMI. **(A)** Univariate. **(B)** Adjusted for age, VF, VT, drug, SOFA, and APS-III. **(C)** Adjusted for age, VF, VT, Total_Ca, chloride, creatinine, phosphate, platelet, RDW, WBC, respiration rate, mean HR, mean glucose, drug, SOFA, and APS-III.

platelet count ($P = 0.347$) of the same patients in the survival group was 236.00 (188.00–294.00), whereas the mean value (95% CI) in the death group was 233.00 (173.00–308.50). The incidence of AFL in the survival and death groups was 2.3% ($N = 79$) and 2.4% ($N = 20$, $P = 1$), respectively. All variables except gender ($P = 0.324$), AFL ($P = 1$), and platelet ($P = 0.347$) showed differences in AMI outcome. The results of the chi-square test and Student's *t*-test are shown in **Table 1**.

All variables were incorporated into the Cox regression of model 3. The results showed that age, VF, VT, drug, total calcium level, chloride level, creatinine level, phosphate level, platelet count, RDW, WBC count, respiration rate, mean heart rate, mean glucose, SOFA score, and APS-III score are the prognostic factors of AMI (all $P < 0.001$, **Table 2**).

The statistical results of the RCS test are shown in **Table 3**. Model 1 incorporates a single variable (SBP) into the analysis as shown in **Figure 2A**. The hazard ratio (HR) with (95% CI)

of model 1 with low SBP is 6.717 (5.044–8.946, $P < 0.001$), and that of model 1 with high SBP is 1.483 (1.120–1.965, $P < 0.01$). Model 2 incorporates age into the analysis as shown in **Figure 2B**. The HR of model 2 with low SBP is 4.910 (3.679–6.552, $P < 0.001$), and that of model 2 with high SBP is 1.637 (1.231–2.18, $P < 0.001$). Model 3 incorporates all meaningful factors in all Cox regression results into the analysis as shown in **Figure 2C**. The HR (95% CI) of model 3 with low SBP is 3.080 (2.162–4.385, $P < 0.001$), and that of model 2 with high SBP is 2.937 (1.918–4.498). SBP is non-linear in the prognosis curves of AMI in all models. The trend of the curve changed at about SBP = 110 mmHg (**Figure 2**, all $P < 0.001$). Notably, the HRs of all models with low SBP are greater than those with high SBP ($P < 0.05$). This finding indicates that the risk of patients with AMI who have SBP <90 mmHg is higher than that of patients with SBP >140 mmHg. This performance becomes more obvious and statistically significant with the addition of more adjustment variables.

Age is a prognostic factor of AMI in the Cox regression analysis results. Therefore, we divided the patients into the low-age (≤65 years) and high-age groups (>65 years) and performed an age subgroup analysis. The baseline characteristics and COX regression analysis results of the subgroups are shown in **Table 4** and **Table 5**. The results of the two groups are all non-linear ($P < 0.001$). The non-linear curve performance of the two groups is shown in **Figure 3**. The slope of the low SBP part is steeper than the slope of the high SBP part. This result reflects that the risk of low SBP is higher than that of high SBP in patients with AMI. The results are similar to the performance of all models.

After the prognostic trajectory of SBP was verified as non-linear, a two-line piecewise linear model with a single change point was estimated by trying all possible values for the change point and choosing the value with the highest likelihood. The result showed that the cutoff point is 114.489 mmHg (111.275–117.702 mmHg). The HR (95% CI) of line 1 (SBP < 114.489 mmHg) is 0.952 (0.943–0.962, $P < 0.001$). This result means that for every 1 mmHg reduction in SBP, the risk of death in patients with AMI is reduced by 4.8% (1–0.952). The HR (95% CI) of line 2 (SBP > 114.489 mmHg) is 1.026 (1.018–1.035, $P < 0.001$), which also means that the risk of death in AMI patients increases by 2.6% (1.018–1) for every 1 mmHg increase in SBP (**Figure 4**).

TABLE 4 | Baseline characteristics between different age groups.

Variable	Low-age group (age < 65)	High-age group (age > 65)	*P*-value
n	1,616	2,661	
Status			<0.001
Dead	159 (9.8)	684 (25.7)	
Live	1,457 (90.2)	1,977 (74.3)	
Sex			0.01
Male	614 (38.0)	907 (34.1)	
Female	1,002 (62.0)	1,754 (65.9)	
AFI			<0.001
Yes	253 (15.7)	1,001 (37.6)	
No	1,363 (84.3)	1,660 (62.4)	
AFL			0.01
Yes	24 (1.5)	75 (2.8)	
No	1,592 (98.5)	2,586 (97.2)	
VFI			0.001
Yes	95 (5.9)	96 (3.6)	
No	1,521 (94.1)	2,565 (96.4)	
VT			0.01
Yes	1,445 (89.4)	215 (8.1)	
No	171 (10.6)	2,446 (91.9)	
SBP			0.64
Normal	1,472 (91.1)	2,403 (90.3)	
Lower	38 (2.4)	73 (2.7)	
Higher	106 (6.6)	185 (7.0)	
Drug			0.28
Yes	858 (53.1)	1,366 (51.3)	
No	758 (46.9)	1,295 (48.7)	
Total_Ca	8.7 (8.1–9.1)	8.6 (8.1–9.1)	0.83
Chloride	103.0 (100.0–106.0)	103.0 (100.0–106.0)	0.11
Creatinine	1.0 (0.8–1.4)	1.2 (0.9–1.7)	<0.001
Phosphate	3.5 (2.9–4.2)	3.6 (3.0–4.3)	<0.001
Potassium	4.2 (3.8–4.6)	4.2 (3.8–4.7)	<0.001
Sodium	138.0 (136.0–140.0)	138.0 (136.0–141.0)	0.06
Nitrogen	18.0 (14.0–27.0)	25.0 (18.0–39.0)	<0.001
Hemoglobin	12.8 (11.0–14.2)	11.6 (10.2–13.0)	<0.001
Platelet	245.0 (196.0–299.0)	230.0 (180.0–295.0)	<0.001
RDW	13.7 (13.0–14.7)	14.2 (13.4–15.5)	<0.001
WBC	11.0 (8.3–14.4)	10.5 (8.0–14.2)	0.66
Respiration rate	25.0 (17.5–29.0)	26.0 (23.0–30.0)	<0.001
Mean HR	82.5 (72.6–92.5)	81.2 (71.8–91.2)	0.02
Mean BP	61.0 (54.0–87.0)	55.0 (49.0–76.2)	<0.001
Mean glucose	134.1 (117.0–164.3)	139.1 (118.5–168.8)	0.01
SOFA	3.0 (1.0–5.3)	4.0 (2.0–7.0)	<0.001
APS-III	34.0 (25.0–48.0)	44.0 (34.0–57.0)	<0.001

DISCUSSION

In the past few decades, mortality from cardiovascular disease has been greatly reduced (18) partly because of the improved management of AMI (19). However, AMI is the most serious manifestation of CAD and the main cause of global cardiovascular disease morbidity and mortality (6, 20). A number of studies have pointed out that SBP is related to the prognostic risk of AMI (11–13, 21, 22). MBP may also be an important predictor. Pulse pressure has a little effect on the risk of cardiovascular disease; thus, SBP should be used in long-term monitoring as an indicator of AMI prognosis (23). Psaty and others pointed out that although DBP, SBP, and pulse pressure are directly related to the risk of coronary and cerebrovascular events, SBP is the best single predictor of cardiovascular events (22).

The results of our Cox multivariate analysis study showed that SBP ($P < 0.001$) is an influencing factor for AMI prognosis. All the prognostic curves of SBP in the RCS shows very similar performance. Our study sets a normal value range (90–140

TABLE 5 | Cox regression results for different age groups.

Variable	High-age group (age > 65)			Low-age group (age < 65)		
	HR	**95% CI**	***P***	**HR**	**95% CI**	***P***
Age	1.0496	1.0335–1.0659	<0.001	1.0307	1.00045–1.0618	0.047
VFI						
No	Reference					
Have	1.8323	1.1856–2.8319	0.006	2.2974	1.21526–4.3433	0.01
VT						
No	Reference					
Have	1.4291	1.0698–1.9090	0.016	2.7406	1.63084–4.6056	<0.001
Drug						
No	Reference					
Have	0.8316	0.6931–0.9979	0.047	0.955	0.65098–1.4009	0.814
SBP						
Normal	Reference					
Lower	3.2289	2.2317–4.6718	<0.001	4.6389	2.46920–8.7150	<0.001
Higher	1.8212	1.2879–2.5754	<0.001	1.199	0.51073–2.8146	0.677
Total_Ca	0.8928	0.7994–0.9971	0.044	0.9793	0.81501–1.1766	0.823
Chloride	0.9814	0.9581–1.0053	0.127	0.9361	0.89230–0.9821	0.007
Creatinine	0.9093	0.8432–0.9806	0.014	0.8397	0.73948–0.9534	0.007
Phosphate	1.2223	1.1329–1.3188	<0.001	1.0415	0.90282–1.2016	0.577
Platelet	0.9987	0.9979–0.9995	0.001	1.0001	0.99855–1.0017	0.864
RDW	1.1476	1.0962–1.2014	<0.001	1.2934	1.17824–1.4197	<0.001
WBC	1.0258	1.0156–1.0362	<0.001	1.0017	0.98456–1.0192	0.845
Respiration rate	1.0171	1.0068–1.0275	0.001	1.022	1.00210–1.0422	0.03
Mean HR	1.007	1.0003–1.0139	0.041	1.0229	1.01017–1.0357	<0.001
Mean glucose	1.0025	1.0008–1.0042	0.003	1.0001	0.99600–1.0042	0.972
SOFA	1.0458	1.0037–1.0896	0.033	1.171	1.07203–1.2791	<0.001
APS-III	1.0198	1.0134–1.0262	<0.001	1.0185	1.00570–1.0314	0.004

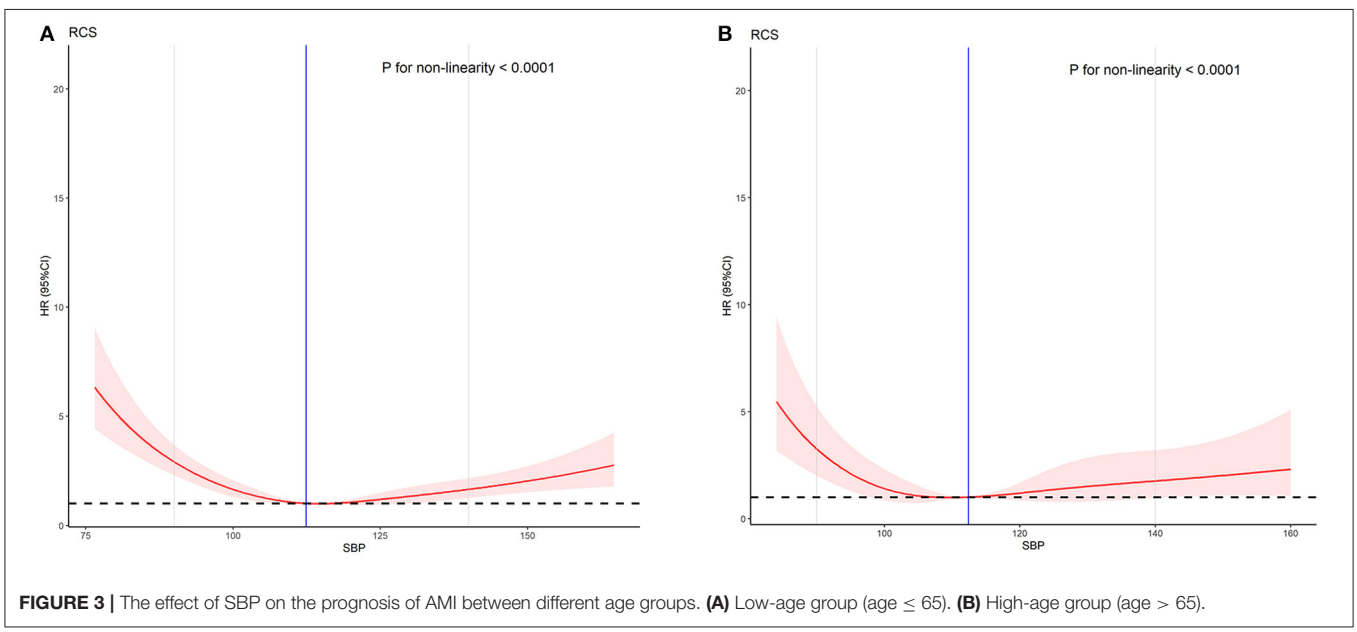

FIGURE 3 | The effect of SBP on the prognosis of AMI between different age groups. **(A)** Low-age group (age ≤ 65). **(B)** High-age group (age > 65).

mmHg) based on the normal physiological values in the human body, and the risk of AMI is the lowest within the normal value range. Notably, the performance of SBP in the prognosis of AMI in the RCS analysis is non-linear, and the non-linear test is statistically significant ($P < 0.001$). Moreover, in the sensitivity analysis, all models with low SBP had higher risks than those with

high SBP. The risks in models 1–3 with low SBP are 6.717, 4.910, and 3.080 times those with normal SBP, and the risks in models 1–3 with high SBP are 1.483, 1.637, and 2.937 times those with normal SBP, respectively. In the subgroup analysis, the low-age and high-age groups also showed a higher risk among patients with low SBP compared with those with high SBP.

When the SBP of a patient with AMI is low to a certain level, the blood supply to the coronary artery becomes obstructed, which leads to myocardial ischemia, myocardial hypoxia, degeneration and necrosis, and eventually myocardial infarction. Several observational studies have shown that lowering the BP below a certain threshold may be harmful as reflected by the "J curve phenomenon" (24–26). An observational study of 22,672 patients with stable CAD showed that hypertension, low SBP (<120 mmHg), and low DBP (<70 mmHg) are all related to the heart. The increased risk of vascular events supported the "J curve phenomenon"; thus, low BP may be harmful in patients with coronary heart disease (27). Shiraishi et al. found that patients with AMI who have SBP <106 mmHg often reach Killip ≥3 upon hospital admission. The right coronary artery and left main trunk or multivessels are the culprits, the number of diseased blood vessels increases, and the hospital mortality rate increased considerably (28). The results of the Israeli Acute Coronary Syndrome Survey showed that SBP is related to cardiovascular events and total mortality. Patients with SBP <110 mmHg have a remarkably higher 1-year mortality risk ratio within 7 days (HR = 2.37) compared with those with normal SBP (110–140 mmHg, HR = 1.92) upon admission (29). Our study determined that the cutoff value of the SBP prognostic curve is 114.489 mmHg, and we quantified the risk of the entire curve. The result means that for every 1 mmHg reduction in SBP before 114.489 mmHg, the risk of death in patients with AMI is reduced by 4.8% (1–0.952). Moreover, the risk of death among patients with AMI increases by 2.6% (1.018–1) for every 1 mmHg increase in SBP after 114.489 mmHg. Lewington et al. also pointed out that SBP = 115 mmHg is an important observation threshold (30, 31). This value is very close to our findings.

SBP can reflect cardiac output and systemic peripheral resistance. A higher SBP at admission may indicate increased systemic resistance, maintenance of cardiac function, and less myocardial damage in patients with AMI. Metabolic syndrome refers to the co-occurrence of several known cardiovascular risk factors, including insulin resistance, obesity, atherogenic dyslipidemia, and hypertension. These conditions are interrelated and share underlying mediators, mechanisms, and pathways (32). Abnormal metabolism and blood pressure characterized by metabolic syndrome are risk factors for cardiovascular disease (33), particularly in young patients with AMI. These patients present extensive atherosclerotic disease in angiographic studies (34). Average arterial BP levels and short-term BP variability are related to hypertension-mediated organ damage, increased carotid intima-media thickness (35), and hypoperfusion, which further increase the risk of adverse events in AMI. The short-term outcomes of patients with normal SBP and with high admission SBP are similar, but with the passage of time, the outcome of patients with excessively elevated SBP upon admission is death, and the probability of major adverse cardiac events has an upward trend (9). Although different studies set different standards for normal BP, the results obtained are consistent, that is, patients with low SBP have a higher risk compared with patients with high SBP. Future studies would be interesting to explore the optimal blood pressure cutpoint by other methods, such as ambulatory blood pressure monitoring or home blood pressure measurement (36, 37).

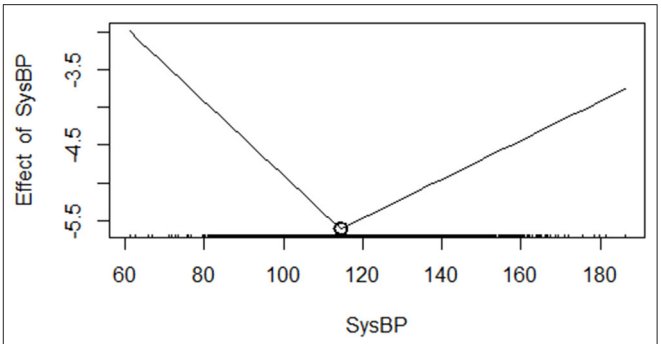

FIGURE 4 | Identification of the trajectory cutoff value.

CONCLUSION

The risk of patients with AMI who have low SBP is very high; therefore, maintaining a high degree of vigilance for patients with AMI and low SBP is necessary. Continuous BP monitoring is particularly important; a normal SBP can guarantee a good prognosis for AMI, and high SBP has a certain low risk. The SBP value of 114.489 mmHg is the inflection point of the prognostic trajectory of AMI. Patients with AMI have the lowest probability of death when the SBP value is 114.489 mmHg. Continuous in-depth research on SBP is very necessary. SBP has a non-linear performance in AMI prognosis. Thus, the establishment of an accurate and reliable prognostic model that uses SBP to predict AMI has still a long way to go. We will further study the impact of SBP on AMI and strive to establish a prediction model with accurate threshold, high sensitivity, and reliability to guide clinicians and assist in decision-making.

LIMITATIONS

First, our data were obtained from the MIMIC database. Most of the patients included in this database are from the United States; therefore, the generalization of the conclusion is limited. Second, parts of the index information of patients were not fully recorded, which led to the loss of information in the study. Finally, this study is a retrospective study, which may inevitably have some biases.

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

SZ: resources, investigation, validation, writing—original draft, and writing—review and editing. FZ: resources, investigation, validation, and writing—review and editing. RY: resources, investigation, and writing—review and editing. WW: resources, investigation, and formal analysis. HL, WM, FX, and DH: data curation and formal analysis. JL: project administration, supervision, and visualization. All authors contributed to the article and approved the submitted version.

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Association of Digoxin Application Approaches With Long-Term Clinical Outcomes in Rheumatic Heart Disease Patients With Heart Failure: A Retrospective Study

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Objective: This retrospective, case–control study was executed to assess the effects of digoxin (DGX) use approaches [continuous use of DGX (cDGX) vs. intermittent use of DGX (iDGX)] on the long-term prognosis in rheumatic heart disease (RHD) patients with heart failure (HF).

Methods: A total of 642 RHD patients were enrolled to this study after propensity matching. The associations of DGX application approaches with the risks of all-cause mortality, cardiovascular death (CVD), HF re-hospitalization (1-, 3-, and 5-year), and new-onset atrial fibrillation (AF) were analyzed by multivariate Cox proportional hazards or binary logistic regression models, respectively.

Results: cDGX was associated with increased risks of all-cause mortality (adjusted HR = 1.84, 95% CI: 1.27–2.65, $P = 0.001$) and CVD (adjusted HR = 2.23, 95% CI: 1.29–3.83, $P = 0.004$) in RHD patients with HF compared to iDGX. With exception of 1-year HF re-hospitalization risk, cDGX was associated with increased HF re-hospitalization risk of 3-year (adjusted OR = 1.53, 95% CI: 1.03–2.29, $P = 0.037$) and 5-year (adjusted OR = 1.61, 95% CI: 1.05–2.50, $P = 0.031$) as well as new-onset AF (adjusted OR = 2.06, 95% CI: 1.09–3.90, $P = 0.027$).

Conclusion: cDGX was significantly associated with increased risks of all-cause mortality, CVD, medium-/long-term HF re-hospitalization, and new-onset AF in RHD patients with HF.

Keywords: heart failure re-hospitalization, mortality, rheumatic heart disease, application approaches, digoxin, new-onset atrial fibrillation

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INTRODUCTION

Rheumatic heart disease (RHD) is a rheumatic fever–induced acquired heart disease. It remains one of the most major causes of mortality in low- and middle-income countries. Heart failure (HF) is an independent predictor of death in RHD patients (1). Congestion is one of the classic clinical features of decompensated HF, manifesting the characteristics of extracellular fluid accumulation (2). Digoxin (DGX), as an ancient drug for HF treatment, improves cardiac output and relieves congestion owing to its positive inotropic effect. However, the evidence of DGX on the HF prognosis mainly comes from non-valvular HF rather than valvular HF and has been controversial since its use for congestive HF treatment. Early randomized controlled trials (RCTs) with small sample sizes showed that DGX improved the symptoms of HF patients (3). The Digitalis Investigation Group (DIG) study, as the only large-scale RCT with DGX for HF therapy, reported that DGX did not reduce all-cause mortality in patients with HF during 37-month follow-up (4). Based on the database of the DIG trial, a cluster analysis found that DGX did not reduce HF hospitalization risk in specific subparticipants who were female and had hypertension or left ventricular ejection fraction (LVEF) >45% and even increased mortality (5). The Valsartan Heart Failure Trial (Val-HeFT) study showed that continuous DGX treatment was related to elevated risk of all-cause death, first morbid event, and HF hospitalization (6). A 2-year retrospective analysis by Freeman et al. (7) also found that continuous DGX treatment in HF patients was independently related to a higher mortality. Recent *post-hoc* randomization analysis of the DIG trial further confirmed that the mortality was significantly increased in subparticipants pre-treated with DGX (8). Meanwhile, another meta-analysis also showed that DGX was related to elevated risk of all-cause death in HF patients (9). These findings suggested that HF patients cannot benefit from DGX use (especially continuous DGX use) and DGX use can even be harmful.

One overlooked reason of this phenomenon is possibly related to the administration strategies of DGX, including continuous use of DGX (cDGX) and intermittent use of DGX (iDGX). According to the congestion status of HF patients, cDGX is defined as continuous use of DGX even after congestion is eliminated. iDGX is defined as adopting de-escalating therapy after the congestion is eliminated, which the DGX dose is gradually reduced and eventually discontinued. Observational and RCT studies showed an opposite association between cDGX and mortality as mentioned above. On the other hand, the Prospective Randomized Study of Ventricular Failure and the Efficacy of Digoxin (PROVED) (10) and Randomized Assessment of Digoxin on Inhibitors of Angiotensin-Converting Enzyme (RADIANCE) (11) studies were designed to assess the effect of DGX withdrawal on patients with stable systolic HF [New York Heart Association (NYHA) II–III and LVEF $\leq$ 35%]; they found that DGX withdrawal did not improve the life quality of patients with HF. Because the two studies were not designed to evaluate the "hard endpoints" related to DGX use and also based on the short-term follow-up, small sample size, and insufficient dose of renin–angiotensin system inhibitors (RSIs) at the baseline level, it still cannot be confirmed whether iDGX might be helpful for improving survival in HF patients, but at least it did not increase mortality. Simultaneously, some studies tried to explore the effect of reducing the dosage of DGX on prognosis of patients with HF. Ahmed et al. (12) reported that a low dose (125 μg po qod) of DGX was related to lower risk of mortality and hospitalization under condition of lower serum DGX concentration (SDC). In dialysis patients with HF receiving lower-dose DGX treatment (62.5 μg po qod), patients with iDGX had fewer DGX-related poisoning symptoms and were safer compared to those with cDGX (13). These findings suggested that the DGX use in HF management may be dynamic and change with congestion status. Based on DGX de-escalation therapy, iDGX may be a potential preferred approach for HF management.

However, the influence of this strategy on the clinical prognosis in HF patients is still unclear, especially in valvular HF. Current guidelines still recommend DGX for the treatment of RHD patients with congestive HF, but there is no evidence that it helps to improve long-term prognostic progress in RHD patients with HF. In fact, prescription of DGX is still subjective and unproven to a large extent in RHD patients with congestive HF. The Rheumatic Heart Disease Global Registry (REMEDY) study (14), as the only clinical trial to evaluate the effect of DGX use on clinical endpoints among RHD patients, showed that DGX use significantly increased the mortality of the overall population as well as recurrent HF *via* 2-year follow-up. However, this retrospective study did not distinguish the effect the administration approaches of DGX on prognosis in RHD patients with HF according to the congestive status. Whether the long-term prognosis of RHD patients with HF can be improved by the de-escalation therapy of DGX, it still lacks conclusive evidence from a large well-designed RCT with "hard" clinical endpoints. Hence, the aim of this study is to access the associations between DGX use approaches (cDGX vs. iDGX) and long-term clinical prognosis in RHD patients with HF.

METHODS

Study Subjects

A retrospective propensity score–matched case–control study from January 1, 1999, to December 31, 2020, was conducted at South China University of Technology. A total of 734 RHD patients were recruited into the study; 219 patients died during follow-up. Clinical information was collected from the four information sources, including interviews of patients and their families, review of medical records, contact with treating physicians, and the Center for Disease Control and Prevention. RHD was identified referring to the recommendations of the World Heart Federation. HF was diagnosed according to the Framingham Heart Study's criteria and American College of Cardiology (ACC)/American Heart Association (AHA) guidelines. Congestion in HF was evaluated referring to European Society of Cardiology (ESC) guidelines (2). According to the application approaches of DGX in RHD patients with

HF, RHD patients were divided into two groups: cDGX (N = 386) and iDGX (N = 348). The cDGX group of participants receives continued DGX therapy regardless of whether the congestion is eliminated. The iDGX group of participants only receives DGX therapy when congestion occurs. In particular, surgical intervention was defined as valve replacement or repair surgery *via* surgical or percutaneous intervention for any affected valve(s) with tissue or mechanical prosthesis referring to recommendations. Coexisting medical conditions, including AF, hypertension (HT), coronary heart disease (CHD), stroke, and type 2 diabetes (T2D), were also diagnosed referring to relevant recommendations. Blood sample analyses were executed by standard lab protocols. Echocardiography was carried out according to our previous protocol (15). The date of entry into the study was the RHD first diagnosis date. Survival duration was calculated from the date of initial diagnosis of RHD to the date of mortality or to the date of last follow-up (December 31, 2020).

Primary and Secondary Outcomes

All-cause death and cardiovascular death (CVD) were the primary outcomes. The all-cause death was defined as death due to any causes. The CVD was defined as death due to any cardio-/cerebrovascular diseases, such as CHD, HF, AF, stroke, and/or other cardio-/cerebrovascular causes. HF re-hospitalization and new-onset AF were the secondary outcomes. After first discharge for HF, HF re-hospitalization was defined as hospital re-hospitalization with HF treatment. The new-onset AF was defined as AF that first occurred during follow-up period.

Propensity Score Matching and Statistical Analysis

The 1:1 propensity score matching (PSM) and statistical analyses were executed by SPSS version 24 (SPSS, Chicago, IL). The 1:1 PSM was executed referring to our previous methods (16) by baseline characteristics at enrollment such as age at diagnosis, age of surgery, disease duration, follow-up time, levels of systolic/diastolic blood pressure (SBP/DBP) and heart rate (Hr), surgical intervention, cardiac valve damage, NYHA, medical condition, combined medication, blood test index, and echocardiographic index. The caliper width was 0.1 for PSM. Categorical and continuous variables were shown as numbers (percentages) and mean ± standard deviation (SD), respectively. Significant differences for categorical variables and continuous variables were determined by χ^2 test and independent-sample t-test, respectively. The multivariate Cox proportional hazards regression model was carried out to access the association [hazard ratio (HR) and 95% confidence interval (CI)] of different DGX use methods with all-cause death and CVD, adjusting by baseline characteristics. Binary logistic regression modeling was carried out to evaluate the association [odds ratio (OR) and 95% CI] of different DGX use methods with 1-, 3-, and 5-year HF readmission as well as new-onset AF. A P-value of 0.05 or less was statistically significant (two-tailed).

RESULTS

Characteristics of Study Subjects

Before PSM, a total of 734 patients with RHD were enrolled to this study, the median follow-up time was 5.8-years among those participants, and there were obvious differences between cDGX and iDGX in RHD patients on levels of SBP ($P = 0.025$), DBP ($P = 0.004$), Hr ($P = 0.042$), the constituent ratio of mitral stenosis (MS, $P = 0.044$), surgical intervention for valve repair ($P = 0.014$), NYHA ($P < 0.001$), coexisting disorder [e.g., HT ($P = 0.019$) and AF ($P = 0.011$)], drug use [e.g., diuretics ($P < 0.001$), RSIs ($P = 0.010$), mineralocorticoid receptor antagonist (MRA, $P < 0.001$), and calcium channel blockers (CCBs, $P = 0.037$)], and echocardiographic index for right ventricular end-diastolic diameter (RVD, $P = 0.001$), left atrial end-systolic diameter (LAD, $P = 0.001$), and LVEF ($P = 0.009$), as shown in **Tables 1**, **2**. After 1:1 PSM, a total of 642 patients with RHD were finally entered to the study with median follow-up of 5.9-years, and there was still significant difference on NYHA ($P = 0.001$) between the two groups.

Association of DGX Application Methods With All-Cause Death and CVD in RHD Patients

After PSM, cDGX was associated with elevated risks of all-cause death (unadjusted HR = 2.49, 95% CI: 1.85–3.34, $P < 0.001$) and CVD (unadjusted HR = 3.51, 95% CI: 2.23–5.30, $P < 0.001$) in RHD patients with HF compared to those with iDGX. Adjusting for confounding covariates, cDGX was still correlated with increased risks of all-cause death (model 1: adjusted HR = 1.86, 95% CI: 1.31–2.65, $P = 0.001$; model 2: adjusted HR = 1.81, 95% CI: 1.26–2.60, $P = 0.001$; model 3: adjusted HR = 1.84, 95% CI: 1.27–2.65, $P = 0.001$) and CVD (model 1: adjusted HR = 2.46, 95% CI: 1.47–4.12, $P = 0.001$; model 2: adjusted HR = 2.33, 95% CI: 1.36–3.99, $P = 0.002$; model 3: adjusted HR = 2.23, 95% CI: 1.29–3.83, $P = 0.004$) in RHD patients with HF, as shown in **Table 3**.

Association of DGX Application Methods With HF Re-hospitalization in RHD Patients

After PSM, cDGX was associated with elevated HF re-hospitalization risks of 1-year (unadjusted OR = 1.54, 95% CI: 1.05–2.26, $P = 0.026$), 3-year (unadjusted OR = 1.69, 95% CI: 1.20–2.38, $P = 0.003$), and 5-year (unadjusted OR = 2.08, 95% CI: 1.44–3.02, $P < 0.001$) in RHD patients with HF compared to those with iDGX. Adjusting for confounding covariates, cDGX was still correlated with increased HF readmission risk of 3-year (model 1: adjusted OR = 1.55, 95% CI: 1.08–2.22, $P = 0.018$; model 2: adjusted OR = 1.62, 95% CI: 1.08–2.42, $P = 0.020$; model 3: adjusted OR = 1.53, 95% CI: 1.03–2.29, $P = 0.037$) and 5-year (model 1: adjusted OR = 1.98, 95% CI: 1.34–2.94, $P = 0.001$; model 2: adjusted OR = 1.75, 95% CI: 1.14–2.68, $P = 0.011$; model 3: adjusted OR = 1.61, 95% CI: 1.05–2.50, $P = 0.031$), as shown in **Table 4**.

TABLE 1 | Baseline clinical characteristics of study subjects.

		Before PSM			After PSM		
		iDGX (*N* = 348)	**cDGX (*N* = 386)**	***P*-value**	**iDGX (*N* = 321)**	**cDGX (*N* = 321)**	***P*-value**
Male: female		105:243	126:260	0.472	95:226	99:222	0.731
Age at diagnosis (years)		47.4 ± 16.2	45.6 ± 15.5	0.117	47.2 ± 16.3	45.7 ± 15.4	0.206
Age of surgery (years)		47.1 ± 11.1	45.6 ± 15.5	0.904	47.4 ± 11.0	47.7 ± 11.5	0.852
Disease duration (years, *N*/%)	Duration <1	68 (19.5)	60 (15.5)	0.176	55 (17.1)	53 (16.5)	0.402
	1 ≤ Duration < 5	86 (24.7)	84 (21.8)		78 (24.3)	68 (21.2)	
	5≤ Duration < 10	52 (14.9)	55 (14.2)		49 (15.3)	42 (13.1)	
	10 ≤ Duration < 15	62 (17.8)	69 (17.9)		60 (18.7)	57 (17.8)	
	Duration ≥ 15	80 (23.0)	118 (30.6)		79 (24.6)	101 (31.5)	
Follow-up (years)		5.9 (3.5, 9.3)	5.6 (3.2, 9.7)	0.701	6.1 (3.6, 9.5)	5.7 (3.3, 9.8)	0.598
Average daily dose of DGX (μg)		125 (125, 125)	125 (125, 125)	0.423	125 (125, 125)	125 (125, 125)	0.872
Cumulative use time of DGX (years)		4.0 (1.8, 6.7)	5.7 (2.7, 9.0)	<0.001	3.1 (1.4, 6.5)	5.7 (3.3, 9.7)	<0.001
Smoking (*N*/%)		35 (10.1)	47 (12.2)	0.363	29 (9.0)	38 (11.8)	0.245
Drinking (*N*/%)		39 (11.2)	48 (12.4)	0.607	33 (10.3)	39 (12.1)	0.453
SBP at initial diagnosis (mmHg)		**119.9 ± 10.0**	**121.6 ± 11.1**	**0.025**	119.9 ± 10.2	121.5 ± 11.4	0.070
DBP at initial diagnosis (mmHg)		**72.6 ± 10.7**	**74.9 ± 10.8**	**0.004**	72.5 ± 10.8	73.2 ± 8.8	0.364
HR at initial diagnosis (bpm)		**82 ± 18**	**85 ± 17**	**0.042**	82 ± 18	84 ± 16	0.175
Cardiac valve damage (*N*/%)							
(AI) Valve damage (stenosis or regurgitation) types (single: combined)		165:183	170:216	0.360	163:158	151:170	0.343
(A-II) Types of single valve damage	MV	153 (92.7)	158 (92.9)	0.090	151 (92.6)	140 (92.7)	0.109
	AV	8 (4.8)	12 (7.1)		8 (4.9)	11 (7.3)	
	TV	4 (2.4)	0 (0.0)		4 (2.5)	0 (0.0)	
(A-III) Types of combined valve damage	MV+AV	99 (54.1)	105 (48.6)	0.506	78 (49.4)	81 (47.6)	0.919
	MV+TV	49 (26.8)	68 (31.5)		46 (29.1)	53 (31.2)	
	MV+AV+TV	35 (19.1)	43 (19.9)		34 (21.5)	36 (21.2)	
(B-I) MS		245 (70.4)	297 (76.9)	0.044	228 (71.0)	245 (76.3)	0.128
(B-II) MS degree	Mild	135 (55.1)	160 (53.9)	0.813	125 (54.8)	135 (55.1)	0.669
	Moderate	69 (28.2)	81 (27.3)		67 (29.4)	65 (26.5)	
	Severe	41 (16.7)	56 (18.9)		36 (15.8)	45 (18.4)	
(C-I) MR		210 (60.3)	208 (53.9)	0.078	198 (61.7)	178 (55.5)	0.109
(C-II) MR degree	Mild	75 (35.7)	80 (38.5)	0.495	69 (34.8)	67 (37.6)	0.582
	Moderate	58 (27.6)	47 (22.6)		56 (28.3)	42 (23.6)	
	Severe	77 (36.7)	81 (38.9)		73 (36.9)	69 (38.8)	
(D-I) AS		84 (24.1)	94 (24.4)	0.946	74 (23.1)	78 (24.3)	0.710
(D-II) AS degree	Mild	50 (59.5)	50 (53.2)	0.445	43 (58.1)	41 (52.6)	0.591
	Moderate	25 (29.8)	28 (29.8)		22 (29.7)	23 (29.5)	
	Severe	9 (10.7)	16 (17.0)		9 (12.2)	14 (17.9)	
(E-I) AR		125 (35.9)	136 (35.2)	0.846	108 (33.6)	109 (34.0)	0.934
(E-II) AR degree	Mild	60 (48.0)	62 (45.6)	0.437	53 (49.1)	48 (44.0)	0.374
	Moderate	48 (38.4)	61 (44.9)		40 (37.0)	50 (45.9)	
	Severe	17 (13.6)	13 (9.6)		15 (13.9)	11 (10.1)	
(F-I) TR		88 (25.3)	111 (28.8)	0.291	84 (26.2)	89 (27.7)	0.656
(F-II) TR degree	Mild	29 (33.0)	39 (35.1)	0.089	27 (32.1)	31 (34.8)	0.137
	Moderate	35 (39.8)	29 (26.1)		34 (40.5)	24 (27.0)	
	Severe	24 (27.3)	43 (38.7)		23 (27.4)	34 (38.2)	
(G-I) PAH		241 (69.3)	285 (73.8)	0.169	227 (70.7)	239 (74.5)	0.228
(G-II) PAH degree	Mild	141 (58.5)	154 (54.0)	0.161	130 (57.3)	134 (56.3)	0.657
	Moderate	72 (29.9)	81 (28.4)		70 (30.8)	69 (29.0)	
	Severe	28 (11.6)	50 (17.5)		27 (11.9)	35 (14.7)	

(Continued)

TABLE 1 | Continued

		Before PSM			After PSM		
		iDGX (*N* = 348)	**cDGX (*N* = 386)**	***P*-value**	**iDGX (*N* = 321)**	**cDGX (*N* = 321)**	***P*-value**
Surgical intervention (*N*/%)							
(A) Valve replacement (tissue or mechanical prosthesis)	MV	109 (65.7)	145 (70.4)	0.265	107 (70.4)	120 (71.4)	0.450
	AV	5 (3.0)	12 (5.8)		4 (2.6)	10 (6.0)	
	MV+AV	49 (29.5)	47 (22.8)		39 (25.7)	36 (21.4)	
	MV+TV	3 (1.8)	2 (1.0)		2 (1.3)	2 (1.2)	
(B) Valve repair	MV	**18 (69.2)**	**12 (41.4)**	**0.014**	18 (69.2)	8 (47.1)	0.094
	TV	**5 (19.2)**	**17 (58.6)**		5 (19.2)	9 (52.9)	
	AV	**2 (7.7)**	**0 (0.0)**		2 (7.7)	0 (0.0)	
	MV+TV	**1 (3.8)**	**0 (0.0)**		1 (3.8)	0 (0.0)	
NYHA (*N*/%)	I	**45 (12.9)**	**49 (12.7)**	**<0.001**	**41 (12.8)**	**49 (15.3)**	**0.001**
	II	**133 (38.2)**	**102 (26.4)**		**126 (39.3)**	**87 (27.1)**	
	III	**138 (39.7)**	**165 (42.7)**		**124 (38.6)**	**128 (39.9)**	
	IV	**30 (9.2)**	**70 (18.1)**		**30 (9.3)**	**57 (17.8)**	
Medical condition (*N*/%)							
HT		**102 (29.3)**	**84 (21.8)**	**0.019**	93 (29.0)	78 (24.3)	0.181
CHD		20 (5.7)	32 (8.3)	0.180	17 (5.3)	26 (8.1)	0.155
T2D		42 (12.1)	35 (9.1)	0.185	38 (11.8)	30 (9.3)	0.305
AF		**122 (35.1)**	**171 (44.2)**	**0.011**	119 (37.1)	134 (41.7)	0.226
Stroke		40 (11.5)	41 (10.6)	0.706	39 (12.1)	38 (11.8)	0.903
Combined medication (*N*/%)							
Antiplatelet drugs		60 (17.2)	60 (15.5)	0.535	56 (17.4)	52 (16.2)	0.673
Warfarin		218 (62.6)	216 (56.0)	0.066	202 (62.9)	182 (56.7)	0.107
Diuretics		**182 (52.3)**	**280 (66.4)**	**<0.001**	181 (56.4)	204 (63.6)	0.064
Nitrates		42 (12.1)	56 (14.5)	0.332	42 (13.1)	48 (15.0)	0.495
RSIs		**171 (49.1)**	**153 (39.6)**	**0.010**	154 (48.0)	146 (45.5)	0.527
BBs		127 (36.5)	120 (31.1)	0.122	118 (36.8)	107 (33.3)	0.363
MRA		**176 (50.6)**	**249 (64.5)**	**<0.001**	172 (53.6)	196 (61.1)	0.055
CCBs		**42 (12.1)**	**29 (7.5)**	**0.037**	38 (11.8)	28 (8.7)	0.194
Statins		40 (11.5)	41 (10.6)	0.706	37 (11.5)	36 (11.2)	0.901

The bold values mean P value < 0.05.

Association of DGX Application Methods With New-Onset AF in RHD Patients

After PSM, cDGX was associated with elevated new-onset AF risks (unadjusted OR = 1.80, 95% CI: 1.20–2.69, $P = 0.004$) compared to those with iDGX. Adjusting for confounding covariates, cDGX was still correlated with elevated new-onset AF risk (model 1: adjusted OR = 1.96, 95% CI: 1.07–3.58, $P = 0.028$; model 2: adjusted OR = 1.94, 95% CI: 1.05–3.57, $P = 0.034$; model 3: adjusted OR = 2.06, 95% CI: 1.09–3.90, $P = 0.027$), as shown in **Table 5**.

DISCUSSION

Association of DGX Application Methods With All-Cause Death, CVD and HF Readmission

Studies or *post-hoc* analyses showed that DGX use was associated with increased adverse cardiovascular events (e.g., all-cause death, CVD, and HF readmission) in HF patients (6, 7, 14, 17). When talking about possible explanations for this harmful effect, it is often attributed to its narrow effective window (18) that can easily cause drug poisoning (19). However, the influence of DGX application approaches on the prognosis of RHD patients with HF remains unclear. After reviewing the data on DGX application approaches from the earliest DIG study to the latest REMEDY study, all studies were designed to use the strategy of cDGX during its 2- to 10-year follow-up regardless of whether the congestion status of HF patients was eliminated or not, which tries to continuously improve heart function and obtain a better survival prognosis. The results of these studies found that cDGX was correlated with increased risks of all-cause death [e.g., by (21–72)%] (6, 7, 14, 17, 20), CVD [e.g., by (25–32)%] (21), and HF readmission [e.g., by (21–41)%] (22) in HF patients. In this real-world, retrospective study, our findings showed that cDGX was indeed related to increased risk of all-cause death (by 84%), CVD (by 1.23-fold), and HF readmission (by 53–61%) compared to iDGX, partially consistent with the recent study by Maciej

TABLE 2 | Baseline laboratory and echocardiography profile of study subjects.

		Before PSM			After PSM		
		iDGX (*N* = 348)	**cDGX (*N* = 386)**	***P*-value**	**iDGX (*N* = 321)**	**cDGX (*N* = 321)**	***P*-value**
Blood biochemical index							
WBC ($\times 10^9$/L)		7.46 ± 2.93	7.88 ± 3.36	0.068	7.42 ± 2.98	7.88 ± 3.48	0.076
HGB (g/L)		121.6 ± 18.8	121.9 ± 18.9	0.812	121.9 ± 18.9	121.7 ± 18.8	0.861
PLT ($\times 10^9$/L)		190.7 ± 72.0	186.2 ± 71.4	0.398	190.6 ± 71.9	191.1 ± 72.6	0.941
FBG (mmol/L)		5.84 ± 2.35	5.61 ± 2.01	0.159	5.67 ± 1.99	5.52 ± 1.92	0.361
ALT (U/L)		22.0 ± 18.6	23.6 ± 29.6	0.406	21.5 ± 16.4	22.6 ± 20.7	0.419
AST (U/L)		31.3 ± 23.2	33.2 ± 24.5	0.309	31.2 ± 22.8	33.1 ± 24.2	0.343
Cr (μmol/L)		86.8 ± 33.0	87.2 ± 36.4	0.878	87.1 ± 33.5	87.4 ± 36.5	0.905
CRP (mg/L)		15.4 ± 26.3	14.6 ± 27.8	0.723	15.5 ± 26.5	15.9 ± 30.0	0.882
ASO (U/ml)		65.3 ± 85.7	47.7 ± 69.7	0.088	66.2 ± 85.4	45.9 ± 58.7	0.053
RF (U/ml)		14.6 ± 31.7	9.5 ± 16.3	0.127	14.9 ± 32.5.8	9.0 ± 17.2	0.112
ESR (mm/h)		25.2 ± 24.4	21.5 ± 17.8	0.147	25.6 ± 24.8	21.8 ± 18.4	0.169
BNP (pg/ml)		1,031.5 ± 2,615.4	1,210.1 ± 3,130.2	0.410	1,031.6 ± 2,655.1	1,148.6 ± 3,165.1	0.615
TRIG (mmol/L)		1.23 ± 0.83	1.23 ± 1.17	0.964	1.19 ± 0.713	1.27 ± 1.26	0.322
TC (mmol/L)		4.25 ± 1.18	4.36 ± 1.30	0.246	4.27 ± 1.18	4.41 ± 1.30	0.143
LDL-C (mmol/L)		2.43 ± 0.90	2.45 ± 0.95	0.879	2.45 ± 0.91	2.48 ± 0.92	0.669
HDL-C (mmol/L)		1.12 ± 0.35	1.16 ± 0.79	0.358	1.14 ± 0.33	1.12 ± 0.34	0.490
Na^+ (mmol/L)		139.5 ± 4.7	139.3 ± 4.1	0.508	139.6 ± 4.7	139.4 ± 4.2	0.657
K^+ (mmol/L)		3.95 ± 0.44	3.99 ± 0.47	0.243	3.95 ± 0.44	3.96 ± 0.47	0.845
INR ratio	INR < 2.0	35 (10.8)	59 (16.5)	0.055	34 (11.3)	50 (16.8)	0.096
	2.0 ≤ INR <3.0	285 (87.7)	289 (81.0)		262 (87.0)	240 (80.5)	
	INR ≥ 3.0	5 (1.5)	9 (2.5)		5 (1.7)	8 (2.7)	
Echocardiography	LVD (cm)	4.58 ± 0.68	4.66 ± 0.79	0.116	4.58 ± 0.68	4.63 ± 0.76	0.369
	LAD (cm)	**4.55 ± 0.80**	**4.75 ± 0.82**	**0.001**	4.57 ± 0.80	4.71 ± 0.64	0.502
	RVD (cm)	**2.24 ± 0.81**	**2.05 ± 0.74**	**0.001**	2.09 ± 0.56	2.02 ± 0.71	0.180
	RAD (cm)	3.59 ± 0.64	3.67 ± 0.76	0.133	3.59 ± 0.65	3.64 ± 0.72	0.436
	LVEF (%)	**57.4 ± 8.5**	**55.6 ± 9.1**	**0.009**	57.3 ± 8.7	56.2 ± 8.9	0.111

The bold values mean P value < 0.05.

Tysarowski et al. reporting that DGX use was associated with increased mortality in patients with AF and without concomitant HF (*https://doi.org/10.1161/circ.142.suppl_3.16085*). Conversely, this suggested that iDGX was associated with better prognosis of RHD patients with HF, which may be the optimal therapy strategy of DGX for those patients.

Interestingly, studies showed that the mortality of patients with DGX was elevated with the increase of SDC (especially ≥1.0 ng/ml), and lower SDC (0.5–0.9 ng/ml) may be good for improving the overall risk of death and hospitalization. Under conditions of the two DGX use approaches, however, the effect of different SDC on all-cause mortality and CVD is still unclear. In this study, four issues were worth paying attention to as follows: Firstly, RHD patients with DGX treatment at SDC ≥ 1.0 ng/ml had relatively high risk of all-cause death while they had relatively low risk of CVD, and this phenomenon was observed in both DGX use approaches (**Supplementary Table 1**). The possible explanation is that the cardiovascular benefits increased with the increases of SDC, but the non-cardiovascular-related deaths increased more obviously, which may be attributed to the poisoning effect at high SDC. Secondly, RHD patients receiving iDGX above the corresponding SDC cutoff value of 0.6 to 1.0 ng/ml had relatively high risk of CVD compared to their counterparts receiving cDGX, especially at SDC ≥ 0.6 ng/ml (**Supplementary Table 1**). The difference between the two DGX application approaches (iDGX vs. cDGX) was whether or not to receive DGX treatment among RHD patients with HF during their compensated HF period. In other words, those subjects in the iDGX group were re-treated with DGX when congestion occurred again during the follow-up, which meant that they were the "new" users of DGX in one sense. Indeed, a retrospective analyses based on the Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation (ARISTOTLE) study reported that new DGX users had significantly higher risk of total mortality (increased by 78%), which was also related to higher sudden cardiac death risk (increased by 3-fold) owing to malignant arrhythmia (23). Those results suggested that iDGX was associated with better prognosis compared to cDGX, but it is necessary to closely monitor the SDC when DGX needs to be reused in HF patients when their cardiac function turns point from compensatory to decompensated stage. Thirdly, the optimal SDC for monitoring of the benefit–risk balance of a treatment in both DGX use approaches was <0.8 ng/ml, because it implies a relatively high

TABLE 3 | Association of DGX application methods with all-cause death and CVD in RHD patients.

Outcomes		Group	Before PSM			After PSM		
			Events (*N*/%)	HR (95% CI)	*P*-value	Events (*N*/%)	HR (95% CI)	*P*-value
All-cause mortality	Unadjusted	iDGX	68 (19.5)	Ref.		66 (20.6)	Ref.	
		cDGX	151 (39.1)	2.48 (1.87–3.31)	<0.001	130 (40.5)	2.49 (1.85–3.34)	<0.001
	Model 1[a]	iDGX	68 (19.5)	Ref.		66 (20.6)	Ref.	
		cDGX	151 (39.1)	1.88 (1.34–2.65)	<0.001	130 (40.5)	1.86 (1.31–2.65)	0.001
	Model 2[b]	iDGX	68 (19.5)	Ref.		66 (20.6)	Ref.	
		cDGX	151 (39.1)	1.73 (1.22–2.59)	0.002	130 (40.5)	1.81 (1.26–2.60)	0.001
	Model 3[c]	iDGX	68 (19.5)	Ref.		66 (20.6)	**Ref.**	
		cDGX	151 (39.1)	1.82 (1.28–2.59)	0.001	130 (40.5)	**1.84 (1.27–2.65)**	**0.001**
CVD	Unadjusted	iDGX	32 (9.2)	Ref.		31 (9.7)	Ref.	
		cDGX	105 (27.2)	3.65 (2.46–5.42)	<0.001	87 (27.1)	3.51 (2.33–5.30)	<0.001
	Model 1[a]	iDGX	32 (9.2)	Ref.		31 (9.7)	Ref.	
		cDGX	105 (27.2)	2.50 (1.54–4.04)	<0.001	87 (27.1)	2.46 (1.47–4.12)	0.001
	Model 2[b]	iDGX	32 (9.2)	Ref.		31 (9.7)	Ref.	
		cDGX	105 (27.2)	2.35 (1.42–3.89)	0.001	87 (27.1)	2.33 (1.36–3.99)	0.002
	Model 3[c]	iDGX	32 (9.2)	Ref.		31 (9.7)	**Ref.**	
		cDGX	105 (27.2)	2.24 (1.35–3.73)	0.002	87 (27.1)	**2.23 (1.29–3.83)**	**0.004**

[a]*Model 1: adjusting for baseline adjustment covariates, including age, gender, smoking, drinking, disease duration, NYHA, cardiac valve damage, surgical intervention, medical condition (HT, CHD, T2D, AF at enrolment, and stroke), blood biochemical index (WBC, HGB, PLT, FBG, ALT, AST, Cr, ASO, RF, ESR, BNP, TRIG, TC, LDL-C, HDL-C, serum sodium, and serum potassium), and echocardiography (LAD, LVD, RVD, RAD, and LVEF).*

[b]*Model 2: It was the same as model 1 and also included new-onset AF and combined medication (antiplatelet drugs, warfarin, nitrates, RSIs, BBs, MRAs, CCBs, and statins).*

[c]*Model 3: It was the same as model 2 and also included SDC.*

The bold values mean P value < 0.05.

TABLE 4 | Association of DGX application methods with HF re-hospitalization in RHD patients.

HF re-hospitalization (*N*/%)		Before PSM				After PSM			
		iDGX (Ref.)	cDGX	OR (95% CI)	*P*-value	iDGX (Ref.)	cDGX	OR (5% CI)	*P*-value
Unadjusted	1-year	62 (18.1)	102 (28.1)	1.77 (1.23–2.52)	0.002	59 (42.8)	79 (57.2)	1.54 (1.05–2.26)	0.026
	3-year	108 (35.9)	165 (52.4)	1.97 (1.42–2.72)	<0.001	101 (44.1)	128 (55.9)	1.69 (1.20–2.38)	0.003
	5-year	138 (51.7)	206 (70.3)	2.21 (1.56–3.13)	<0.001	130 (43.9)	166 (56.1)	2.08 (1.44–3.02)	<0.001
Model 1[a]	1-year	62 (18.1)	102 (28.1)	1.50 (1.03–2.18)	0.036	59 (42.8)	79 (57.2)	1.39 (0.93–2.08)	0.106
	3-year	108 (35.9)	165 (52.4)	1.75 (1.24–2.45)	0.001	101 (44.1)	128 (55.9)	1.55 (1.08–2.22)	0.018
	5-year	138 (51.7)	206 (70.3)	2.01 (1.40–2.90)	<0.001	130 (43.9)	166 (56.1)	1.98 (1.34–2.94)	0.001
Model 2[b]	1-year	62 (18.1)	102 (28.1)	1.31 (0.87–1.97)	0.191	59 (42.8)	79 (57.2)	1.25 (0.81–1.93)	0.305
	3-year	108 (35.9)	165 (52.4)	1.52 (1.05–2.21)	0.027	101 (44.1)	128 (55.9)	1.62 (1.08–2.42)	0.020
	5-year	138 (51.7)	206 (70.3)	1.67 (1.12–2.54)	0.012	130 (43.9)	166 (56.1)	1.75 (1.14–2.68)	0.011
Model 3[c]	1-year	62 (18.1)	102 (28.1)	1.29 (0.85–1.94)	0.230	59 (42.8)	79 (57.2)	1.22 (0.79–1.88)	0.366
	3-year	108 (35.9)	165 (52.4)	1.51 (1.04–2.19)	0.030	101 (44.1)	128 (55.9)	**1.53 (1.03–2.29)**	**0.037**
	5-year	138 (51.7)	206 (70.3)	1.68 (1.11–2.52)	0.013	130 (43.9)	166 (56.1)	**1.61 (1.05–2.50)**	**0.031**

[a]*Model 1: adjusting for baseline adjustment covariates, including age, gender, smoking, drinking, disease duration, NYHA, cardiac valve damage, surgical intervention, medical condition (HT, CHD, T2D, AF at enrolment, and stroke), blood biochemical index (WBC, HGB, PLT, FBG, ALT, AST, Cr, ASO, RF, ESR, BNP, TRIG, TC, LDL-C, HDL-C, serum sodium, and serum potassium), and echocardiography (LAD, LVD, RVD, RAD, and LVEF).*

[b]*Model 2: It was the same as model 1 and also included new-onset AF and combined medication (antiplatelet drugs, warfarin, nitrates, RSIs, BBs, MRAs, CCBs, and statins).* [c]*Model 3: It was the same as model 2 and also included SDC.*

The bold values mean P value < 0.05.

cardiovascular benefit and a relatively low risk of all-cause death, which may reflect the interplay of neurohormonal and inotropic effects (18). The finding was consistent with a previous report (24). Finally, the all-cause death risk in male RHD patients with cDGX above the corresponding SDC cutoff value (0.6, 0.8, and 1.0 ng/ml) was approximately increased by 40% compared to that in their female counterparts while CVD risk was at least 90% (**Supplementary Table 2**). A similar phenomenon was also seen in a previous study (25), but was inconsistent with another study that reported that DGX therapy was related to elevated all-cause

TABLE 5 | Association of DGX application methods with new-onset AF in RHD patients.

New-onset AF		Before PSM			After PSM		
		Events (*N*/%)	OR (95% CI)	*P*-value	Events (*N*/%)	OR (95% CI)	*P*-value
Unadjusted model	iDGXUD	91 (40.3)	Ref.		84 (41.6)	Ref.	
	cDGX	123 (57.2)	1.98 (1.36–2.90)	<0.001	105 (56.1)	1.80 (1.20–2.69)	0.004
Model 1[a]	iDGXUD	91 (40.3)	Ref.		84 (41.6)	Ref.	
	cDGX	123 (57.2)	1.73 (1.12–2.68)	0.014	105 (56.1)	1.96 (1.07–3.58)	0.028
Model 2[b]	iDGXUD	91 (40.3)	Ref.		84 (41.6)	Ref.	
	cDGX	123 (57.2)	1.58 (1.03–2.44)	0.038	105 (56.1)	1.94 (1.05–3.57)	0.034
Model 3[c]	iDGXUD	91 (40.3)	Ref.		84 (41.6)	Ref.	
	cDGX	123 (57.2)	1.61 (1.03–2.52)	0.036	105 (56.1)	**2.06 (1.09–3.90)**	**0.027**

[a]*Model 1: adjusting for baseline adjustment covariates, including age, gender, smoking, drinking, disease duration, NYHA, cardiac valve damage, surgical intervention, medical condition (HT, CHD, T2D, AF at enrolment, and stroke), blood biochemical index (WBC, HGB, PLT, FBG, ALT, AST, Cr, ASO, RF, ESR, BNP, TRIG, TC, LDL-C, HDL-C, serum sodium, and serum potassium), and echocardiography (LAD, LVD, RVD, RAD, and LVEF).*

[b]*Model 2: It was the same as model 1 and also included combined medication (antiplatelet drugs, warfarin, digoxin, nitrates, RSIs, BBs, MRAs, CCBs, and statins).*

[c]*Model 3: It was the same as model 2 and also included SDC.*

The bold values mean P value < 0.05.

death in women (26). The sex-related differences in the effect of DGX among HF patients need further research.

Association of DGX Application Methods With the Risks of New-Onset AF and Stroke

Many studies have shown that DGX increases predisposition to AF. In AF patients after electrical cardioversion, recurrence risk of AF in those patients receiving DGX increased by 2- to 3-fold, and the duration of AF lasted longer (27). In this study, cDGX was related to elevated risk of new-onset AF (by 1.06-fold), consistent with the above-mentioned study. DGX promotes the increase of calcium load in atrial cells and then increases the "trigger" activity, which is the initiating factor for the electrical remodeling of AF. DGX also promotes fibrosis and inflammation of cardiomyocytes and worsens cardiac structural remodeling, generating a vicious spiral (28).

Evidence confirmed that chronic low-grade inflammation status plays a central role of rheumatism-induced atrial structural and electrical remodeling, leading to higher risk of cardiovascular events (29). After standard antibiotics and surgical treatment, there is no clinical characteristic of rheumatic activity and evidence of subclinical persistent myocarditis in histomorphology, but chronic subclinical active non-specific inflammation still exists in the myocardium, atrium, and valvular tissue (30). A pilot study by Saikia et al. also confirmed the inflammation present in rheumatic valvular diseased tissue was non-specific interstitial inflammation (31). In patients with "end-stage" RHD, the presence of inflammatory cells and the increased expression of multiple cytokines reflected that the damage of chronic low-grade inflammation may be subclinical and sustained damage (32). Chronic subclinical inflammation of RHD was related to the progression of RHD, showing clinical manifestations with left atrial enlargement (LAE), or even "mega LA" (33). On the other hand, recent studies confirmed that the chronic inflammation status associated with uncontrolled rheumatic diseases may be a pathophysiologic driver in the development of metabolic disorder (34), and insulin resistance (IR) is independently associated with rheumatic disease–related inflammation status and metabolic disorder (35). In this study, there were no significant differences in serum level C-reactive protein (CRP), fasting blood glucose (FBG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-C) between the two groups (**Table 1**), but the average levels of CRP (>10.0 mg/L), FBG (>5.6 mmol/L), TC (>4.2 mmol/L), and LDL-C (>1.8 mmol/L) were higher than normal (**Table 2**). This suggested that RHD patients suffered a status of chronic low-grade inflammation and metabolic disorder (36). At the mouse model level, high-dose DGX promoted inflammation status and then caused myocardial necrosis (37). At the clinical level, a *post-hoc* analysis of the DIG trial found that the substantial risk of DGX toxicity had nothing to do with metabolic status among HF patients (38). The Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) study showed that CRP was a vital risk factor for mortality and vascular accidents in patients with AF (39). These findings suggested that DGX-related AF may be mainly owing to DGX-induced persistent chronic low-grade inflammation status.

Recent studies showed that the mechanism of occurrence and development of AF has far exceeded the influence of atrial-related remodeling but is also related to the adverse effects of coronary microvascular dysfunction (MVD). One of the important mechanisms on coronary microvascular dysfunction (CMD) mainly involves the abnormal production and release of nitric oxide (NO), which is mainly synthesized by endothelial nitric oxide synthase (eNOS) in the cardiovascular system. Under the circumstances of RHD, there is a potential association among DGX use, CMD, and arrhythmia based on NO as follows: Firstly, RHD is an autoimmune disease, with inflammatory factors upregulated, resulting in the inhibition of eNOS activity and then contributing to the reduction of NO bioavailability, which affected the function of coronary microvascular tissue (40). Secondly, Medow et al. (41) found that NO-mediated microvasodilation dysfunction was related to arrhythmia (e.g., postural tachycardia syndrome). Thirdly,

DGX use induced AF in eNOS-deficient mice (42). Lastly, intracellular magnesium deficiency may not only be related to MVD (43) but also an important risk factor for AF (44). cDGX may continuously inhibit the Na^+-K^+-ATPase pump, deplete intracellular magnesium ions (45), further lead to CMD, and eventually induce AF.

Theoretically, valvular AF (especially caused by RHD) is associated with increased risk of stroke, even cerebrovascular death. In this study, the two DGX use approaches were not associated with the risks of new-onset stroke (**Supplementary Table 3**) and cerebrovascular death (**Supplementary Table 4**). Possible explanations are as follows: Firstly, the main cause of cerebrovascular accident in RHD is cardiogenic embolism caused by LAE. Secondly, LAE is an independent risk factor for thrombosis (46). Thirdly, due to the existence of chronic low-grade inflammation of RHD (47), left atrial remodeling is continuously progressing. However, both iDGX and cDGX had no effect reversing left atrial remodeling and even promoted remodeling. The indifferent serum CRP levels between the two DGX use approaches may explain this at least partially.

Limitations

This research has produced some relief, but it still has some shortcomings. The main limitation is that this present study was only a retrospective investigation, and participants did not randomly receive DGX application strategies, which may lead to bias (e.g., similar to "per-protocol" analyses present in clinical trials). The neurohumoral condition in RHD patients with iDGX may be better than those with cDGX, which may be linked to better outcomes. This study can only carry out correlation analysis and cannot confirm that DGX application was the reason for increasing poor prognosis. The PSM was performed to eliminate potential bias, but it may also be possible to amplify hidden confounding variables. In addition, because there is still no consensus about the optimal therapy strategy for RHD patients with HF, the evidence-based drugs for HF treatment [e.g., RSIs, beta-receptor blockers (BBs), and MRA] in this retrospective study were not adequate. Hence, the results must be interpreted carefully.

CONCLUSION

cDGX was correlated with elevated risk of all-cause death, CVD, medium-/long-term HF readmission, and new-onset AF in RHD patients with HF. In contrast, DGX de-escalation approaches (e.g., iDGX) were related to a better prognosis in RHD patients with HF, suggesting that DGX should be adjusted with a de-escalation therapy as soon as possible after the congestion state of RHD patients with HF is relieved and the clinical condition is stable. It is uncertain whether this relationship observed is due to unnecessary continuous treatment with DGX or simply reflects the inevitable link between more severe HF and more frequent DGX treatment. These substitute hypotheses could be studied in further large well-designed RCTs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The study received ethics approval from the Institutional Review Board of Guangzhou First People's Hospital, South China University of Technology (K-2018-136-1). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CL: literature search, study format, writing protocol, collecting data, processing data, data interpretation, analyzing data, and writing manuscript. TG and QZ: processing data, data interpretation, and analyzing data. JP, SZ, YL, and DeW: recruiting patients, following up patients, and collecting data. DaW: echocardiography testing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.711203/full#supplementary-material

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GLOSSARY

AF, atrial fibrillation; ALT, alanine aminotransferase; AR, aortic regurgitation; AS, aortic stenosis; ASO, antistreptolysin O; AST, aspartate aminotransferase; AV, aortic valve; AVR, aortic valve replacement; BBs, beta-receptor blockers; BNP, B-type natriuretic peptide; cDGX, intermittent use of DGX; CHD, coronary heart disease; CI, confidence interval; CCBs, calcium channel blockers; Cr, creatinine; CRP, C-reactive protein; CVD, cardiovascular death; DBP, diastolic blood pressure; DGX, digoxin; eNOS, endothelial nitric oxide synthase; ESR, erythrocyte sedimentation rate; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; HF, heart failure; HGB, hemoglobin; HR, hazard ratio; Hr, heart rate; HT, hypertension; LAD, left atrial end-systolic diameter; iDGX, intermittent use of DGX; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; LVD, left ventricular end-diastolic diameter; MRA, mineralocorticoid receptor antagonist; MR, mitral regurgitation; MS, mitral stenosis; MV, mitral valve; MVD, microvascular dysfunction; NYHA, New York Heart Association; NO, nitric oxide; OR, odds ratio; PAH, pulmonary arterial hypertension; PLT, platelet count; PSM, propensity score matching; RAD, right atrial end-systolic diameter; RHD, rheumatic heart disease; RF, rheumatoid factor; RSIs, renin–angiotensin system inhibitors; RVD, right ventricular end-diastolic diameter; SBP, systolic blood pressure; TC, total cholesterol; T2D, type 2 diabetes mellitus; TR, tricuspid regurgitation; TV, tricuspid valve; TRIG, triglyceridemia; VAF, valvular atrial fibrillation; WBC, white blood cell count.

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Development and Validation of Risk Prediction Model for New-Onset Diabetes After Percutaneous Coronary Intervention (NODAP): A Study Protocol for a Retrospective, Multicenter Analysis

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Background: Type 2 Diabetes mellitus (T2DM) is a major risk factor for cardiovascular diseases and increase mortality. Clinical outcomes of patients after percutaneous coronary intervention (PCI) were worse in T2DM patients than those without T2DM. New-onset diabetes after PCI (NODAP) is often observed during long-term follow-up and this further aggravates cardiovascular diseases. Several studies had focused on patients after PCI with known T2DM. Previous studies showed that impaired glucose tolerance and aging are risk factors that promote NODAP. Considering the unique characteristics of patients after PCI, we will further study relevant risk factors. We sought to investigate the potential predictors of acute coronary syndrome patients with NODAP by a multicenter retrospective cohort study.

Methods: This is a multicenter retrospective cohort study including patients after PCI. Clinical medical records of these patients were collected from four hospitals in different areas in China, from 2010 to 2021. Patients' demographic information, medical history, diagnostic testing, PCI-related information, medication situation will be summarized using descriptive statistics, and correlation analysis was performed on the development of new-onset diabetes. Variation will be described and evaluated using χ^2 test or Kreskas-Wallis test. The prediction model will be verified by a validation set.

Discussion: A novel diabetes prediction model for patients after PCI is established, and this study can achieve advanced intervention for the occurrence of NODAP. Owing to its retrospective nature, this study has some limitations, but it will be further studied through supplement data collection or prospective study. The study has been registered for clinical trials by the Chinese Clinical Trial Registry (**ChiCTR2100047241**).

Keywords: cardiovascular disease, percutaneous coronary intervention, new-onset diabetes, risk factors, prediction model

INTRODUCTION

Type 2 Diabetes mellitus (T2DM) is a major health concern worldwide, with a prevalence of about 463 million by 2019, and a 62% increase since 2009 (1). In Chinese adults, the estimated T2DM and pre-T2DM prevalence are 10.9% and 35.7% (2), respectively. It is a strong risk factor for cardiovascular artery disease (CAD) and major adverse cardiovascular events (MACE) (3–5). A pooled analysis involving more than 1 million participants in Asia found that patients with T2DM had a higher risk (hazard ratio, 1.89) of all-cause death than those without T2DM (6). People are conferred an eight fold increase in death risk by unrecognized myocardial infarction (MI) with diabetes (7). As patients with acute ST-segment elevation myocardial infarction (STEMI) significantly improved after percutaneous coronary intervention (PCI), the 10-year survival rate was nearly 76% (8). Prolonged life expectancy makes chronic care management after PCI a concern. Nearly half of the inpatients with MI complicated with T2DM (9, 10) and good blood glucose control is one of the keys. There have been multiple studies regarding clinical outcomes in patients after PCI with known T2DM (11, 12). Patients after PCI with chronic T2DM emerge with poor clinical outcomes and different treatment strategies (13, 14). Patients approximately 30% who underwent PCI suffer from diabetes (13). Considering the counter-balancing effects of hemorrhagic and ischemic complications, the recent ultra-short DAPT strategy may not be suitable for patients with T2DM (15). New-onset of diabetes after PCI (NODAP) has attracted much attention; a 5-year survival of patients after revascularization with diabetes were worse (16). Among 3,683 acute coronary syndrome (ACS) patients (without T2DM before PCI), 436 patients (11.8%) developed NODAP after a follow-up of 3.41 ± .9 years (10).

However, the risk factors and pathophysiological mechanisms for NODAP remain unknown. The risk factors for NODAP may be different from those for simple diabetes. Patients after PCI are generally elderly, and are associated with a variety of metabolic diseases (obesity, hyperlipidemia, etc.) and stress states related to surgery; all the above predict the risk of T2DM. Previous studies show that abnormal heart rate variability is a risk factor for diabetes (17), and heart rate variability (low SDNN) is common in patients undergoing PCI (18). High intensity statins (19, 20) also has adverse effects on diabetes.

We propose leveraging advancements in machine learning by systematically comparing different modeling approaches (21), to develop a prognostic tool for NODAP. Therefore, we put forward the following hypothesis:

(i) Refer to common risk factors such as obesity and smoking and explore the risk factors for NODAP; probing the risk of NODAP in different types of ACS patients (STEMI, NSTEMI, and UA) is equal or not.
(ii) Systematically apply several machine learning algorithms to establish a predictive model of NODAP and verify its effectiveness.
(iii) In this study, we sought to investigate the potential predictors of ACS patients with NODAP and comparatively evaluate performances of these predictive models with each other, to identify which is the most accurate.

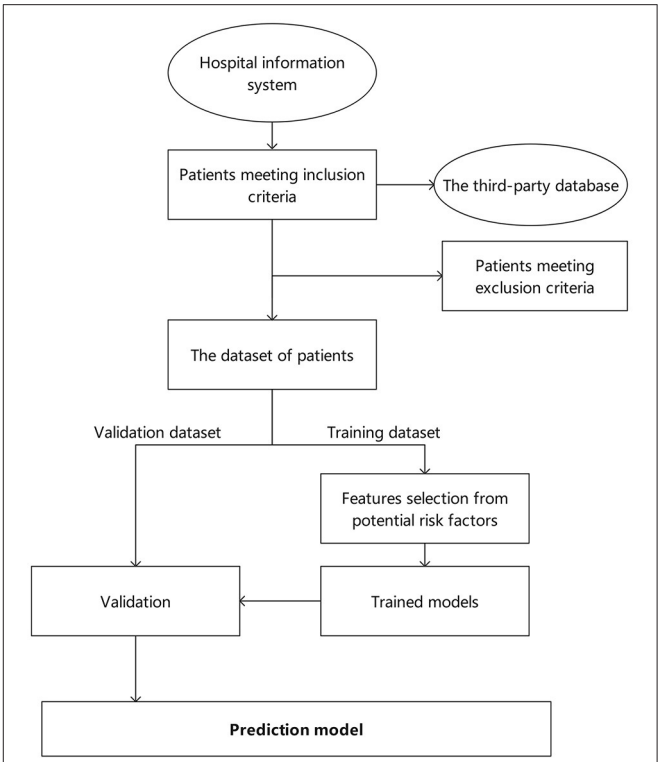

FIGURE 1 | Flowchart of specific steps in the methodology.

METHODS AND ANALYSIS

For Study Design

The study design and report are according to the methods section of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) (22) for cohort studies. The retrospective study used hospital health records and third party databases as the data source and management platform. It is based on the program "evidence-based study of integrated Chinese and Western medicine in metabolic diseases of glycolipids" hosted by the China Academy of Chinese Medical Sciences (project number: 2020YJSZX-4) to develop the risk factors of NODAP. This multicenter, retrospective, cohort study will be carried out between June 2021 and December 2023 in four top hospitals from different districts of China. The recruitment and research sites are 1. Xian Hospital, China Academy of Chinese Medical Sciences, Beijing. 2. Beijing Anshan Hospital, Capital Medical University, Beijing. 3. The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangdong. 4. Affiliated Hospital of Traditional Chinese Medicine of Southwest Medical University, Sichuan. Additional hospital sites may be added based on feasibility and resources available. The study has been registered for clinical trials by the Chinese Clinical Trial Registry (**ChiCTR2100047241**), which provided an overview of our methodology. Through the establishment of validation dataset and training dataset, machine learning is conducted and the prediction model is finally formed. The specific overview of our methodology is shown in **Figure 1.**

TABLE 1 | Inclusion criteria and exclusion criteria of the study.

Inclusion criteria	
i	Age > 18 years
ii	Adults with a primary diagnosis of an ACS (23)
iii	Did not meet the diagnostic criteria for diabetes (24, 25) before the PCI surgery*, no history of taking hypoglycemic drugs or insulin
iv	Only have received the initial PCI
v	outpatient follow-up ≥12 months
vi	Volunteer to participate in the study
Exclusion criteria	
i	Have received coronary artery bypass grafting (CABG)
ii	Patients with severe hepatic and renal insufficiency, severe respiratory, hematonosis, tumor, autoimmune diseases, or multiple organ failure
iii	Patients with Alzheimer's disease, mental illness, or depression
iv	Being pregnant or lactating
v	Did not take secondary prevention# for coronary heart disease after PCI (26)
vi	Participating in other clinical trials

**Diagnostic criteria for diabetes: fasting blood glucose greater than 7.0 mmol/ L, HbA1c ≥6.5% (23).*

 # Secondary prevention medications should include at least aspirin, P_2Y_{12} receptor inhibitors, statins.

Participants

We will identify eligible patients based on the diagnostic description and *China Classification and Code of Diseases National Clinical Edition 2.0* (CCCDNCE 2.0), which is based on the *International Classification of Diseases, Tenth Revision,* (ICD-10) and *International Classification of Diseases, Eleventh Revision,* (ICD-11). Diagnoses of ACS (23), including ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI) and unstable angina (UA), will be made by the attending physician at either admission to the hospital or during discharge. The inclusion criteria and exclusion criteria of the study are shown in **Table 1**.

Patient and Public Involvement

The patients included in this study were not involved in the development of the informed consent and will not be involved in the conduct of the study. Given its retrospective nature, it does not involve collecting identifiable information such as name, medical record number, and genetic tests.

Data

The data extracted from the hospital information system (HIS) will exclude the privacy of participants; detailed parameters will vary based on each institution, which may include outpatient and inpatient medical history and diagnostic imaging database. To ensure the unified management of mass data among multiple institutions, we have established a third-party data management platform (*http://121.30.232.162:10094/#/user/login?redirect=%2F).* Every participant will be assigned a systemic number separate from the medical record number. Based on the platform, the required information can be retrieved and excluded. After machine retrieval by a third-party platform, two researchers will review each participant for inclusion in the study.

Using data provided from decision support at four hospital sites, we expect at least eligible patients in the period 2010–2021, and data according to the fields included in the diagnosis (acute coronary syndrome, PCI, diabetes, etc.) and CCCDNCE 2.0 codes. A standardized case report form (CRF) that has been pilot tested and refined will be used for data extraction. Trained researchers will enter the identified data directly using the electronic CRF into the online platform managed in Beijing, China.

Data will be collected with traditional Chinese and western medicine characteristics that are important in the management of ACS patients with NODAP to confirm the baseline level and severity of blood stasis syndrome. Data will be included in two stages. First, ACS patients who were eligible for inclusion criteria were included. All these patients will be re-screened to ensure the duration of 12 months of follow-up and above. Structural data such as demographic information, diagnostic testing and medication situation can be directly collected in the database. Medical history and surgery records will be retained in the platform in unstructured text format. Researchers in charge of each institution will ensure consistency on extraction method and content, and train individuals for data identification and extraction, and entering DE identified data using the electronic CRF. Relevant medical history including history of ACS, history of T2DM, history of PCI surgery will be collected to confirm the diagnosis. New-onset diabetes was diagnosed by fasting blood glucose or HbA_1c levels in the outpatient or inpatient biochemical test during follow-up.

Potential Risk Factors

As the study is primarily retrospective, multiple initial information data will be collected and outcome data will be divided into one dimension: new-onset diabetes. Initial information data for five broad categories include (I) demographic information; (ii) medical history; (iii) diagnostic testing; (iv) PCI surgery-related information; and (v) medication situation. Detailed information refer to previous studies on risk factors for diabetes (27, 28) (**Table 2**). Variables with missing data other than demographic information (age, sex, ethnicity, marital status) will be dealt with using multiple imputation (29).

Outcomes Measures

The outcome for each model will be the occurrence of NODAP. For reference, new-onset diabetes after organ transplantation mainly occurs 3–6 months after the operation, and the incidence drops to 4–6% 1 year after the operation (30). The onset of NODAP mainly occur within 1 year after the operation, thus the follow-up time of outpatient was determined to be no less than 12 months.

Statistical Analysis

Baseline demographic characteristics and study outcomes conforming to normal distribution were expressed as mean ± standard deviation. The *t*-test was used for comparison between the two groups. The measurement data not conforming to normal distribution was represented as the median (quaternary interval), and the comparison between the two groups was

TABLE 2 | Patient information to be extracted.

Patient information	
Demographic information	age, sex, ethnicity (nationality/ethnic minorities), marital status, Occupation (physical and non-physical)
Medical history	the type of ACS (STEMI, NSTEMI, UA), smoking history, alcohol consumption history, history of hypertension, history of hyperlipidemia, history of chronic diseases, family history of T2DM, family history of CAD
Diagnostic testing	height, weight, body mass index (BMI), blood pressure, resting heart rate, fasting blood glucose, HbA1c, serum uric acid level, triglyceride level, total cholesterol level, low density lipoprotein level, left ventricular ejection fraction (LVEF)
PCI surgery-related information	number of diseased vessels, number of stent implantation, total length of the stent
Medication situation	statin medications (low intensity, high intensity), traditional Chinese medicine (Yes or No)

performed using the Mann–Whitney *U* test. Descriptive statistics will be used for the overall cohort and aggregated statistics will be calculated. Descriptive statistics are used for the entire queue and the aggregated statistics are calculated. Enumeration data were expressed as frequency or percentage, and $\chi 2$ test was used for comparison between the two groups. Univariate analysis was conducted for all factors first, and statistical learning methods such as tree integration algorithm and Lasso regression were adopted to compare and screen related risk factors from multi-dimensional perspectives. To avoid omission of important variables, covariates with $P < 0.01$ in univariate analysis and important factors screened by statistical learning method were included in multivariate logistic regression analysis. Independent risk factors for NODAP were analyzed by progressive forward regression analysis, and the OR value of each related factor and its 95%CI were estimated. $P < 0.05$ on both sides of all tests indicated statistically significant differences.

We will evaluate the potential predictors of NODAP informed by prior literature (10, 20). The data set will be randomly divided into a training dataset and an internal validation dataset in a 5:5 ratio. We will include all a priori selected variables in the final model; the Bootstrap method was used for model internal validation in this data set. If necessary, non-normally distributed variables will be transformed using the Box-Cox transformation. The validation will be established with the same inclusion and exclusion criteria and sensitivity analyses will be performed. All analyses were performed using R3.6.1 and SPSS® Statistics 21 software (IBM®, Armonk, NY, USA). Prism 5 (GraphPad Software Inc., San Diego, CA, USA) is used for drawing statistical graphs.

Sample Size

Based on prior studies and conditions of each hospital (31), the number of PCI surgical interventions is between 10,000 and 15,000 in the past 10 years and in which 6.1–11.6% patients (10, 32) may develop NODAP (at least 610 patients). For the exploratory analysis of multiple risk factors, ≥10 events per variable (EPV) are necessary to obtain sufficient degrees of freedom, with the assumption of at least 20 patients with the outcome (NODAP) per predictor variable (33). The ratio of the two groups is set to 5:5 (validation dataset: training dataset); the sample size could be calculated as 300 in both the training set and an internal validation set. The actual sample size may be larger than the calculated amount, thus it will be adjusted according to the situation.

Model Evaluation

Model evaluation will be carried out quantitatively via receiver operator characteristic (ROC) analysis (34). ROC curves will be constructed to evaluate and compare the predictive models. A ROC curve plots sensitivity and specificity at a range of threshold settings. The area under the curve provides an aggregate measure of model performance across all classification thresholds and will be used to compare predictive models.

Data Storage and Management

Protocols regarding data security have concluded between the research group and each hospital. Data will either be stored in structured query language form and entered into the third-party electronic platform. Data transfer and entry were carried out by the professional database engineer who also responsible for regular data quality checks. For data safety and security, the electronic data will be maintained under secured password-protected conditions, and access will only be given to licensed personnel (research group). Each patient will be reset a unique identification code fit for both outpatient and inpatient records. The code-breaking information will be kept separate from the data extraction files and inaccessible to individuals outside the research team and will only be available to the research group.

All electronic documents and data will be protected by passwords on the network in the platform. The required data will be exported in Excel format and further analyzed.

DISCUSSION

Strengths

The protocol for the study design and methods to evaluate a new model of patients with new-onset diabetes mellitus after PCI. This study represents the largest cohort study that addresses risk factors for NODAP and it will make available data as regards blood glucose management and T2DM risk prediction. Prior studies carried out in Taiwan (20) were based on a health insurance database and the use of statin was the only variable that was extracted; all subjects in the large academic tertiary care hospitals can be a source of bias. With increases in aging, more people will be diabetic after PCI over a long period. This proportion will continue to increase, emerging sensitive predictors (35). This is the first study that addresses the risk factors for diabetes after PCI. The related factors of PCI surgery were reviewed, and a more comprehensive evaluation of diabetes after PCI was performed.

A future direction for the study is to collect patient-reported outcomes through the questionnaires to supplement

the retrospectively available data, or to conduct a prospective cohort study where data will be collected at baseline and at 12-month follow-up, pending additional institutional review board (IRB) approval. Consent forms will be distributed to participants before data collection. Consent forms will highlight the voluntary nature and anonymity of the participation. Based on the above studies, our study went further to verify the risk factors, incidence and prognosis of T2DM after PCI.

Limitations

This study has some limitations. As the study is a non-randomized retrospective study, selection bias cannot be excluded. Based on diagnosis name or CCCDNCE 2.0 codes, eligible patients may miss some medical records that would have met eligibility criteria, including those that were given a broader diagnosis or incorrect ICD code. However, given a large number of sites and patients identified with decision support, we anticipated that only a small number of patients will be omitted. Given its retrospective design, included data will be limited by what is documented in the HIS, thus some of the data we are interested in cannot be obtained (e.g., waistline). Third, as an observational study, we were only able to address association rather than causation, if any predictors are identified with surgical outcome, education levels, or income levels. However, the analyses outlined are exploratory in nature. Fourth, given the 12-month follow-up period, it is not enough for all patients at risk to develop NODAP, which may act as unmeasured confounders.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Xiyuan Hospital, China Academy of Chinese Medical Sciences. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YuL conceived the study protocol and were responsible for the research design and statistical analysis plan. YiL and JC prepared the initial draft of the manuscript and then YaL and YiL did critical revision. KC, LH, and YuL drafted the final draft of the paper and helped to critically revise drafts of the manuscript. All authors contributed to the article and approved the submitted version.

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Inverse Associations Between Circulating Secreted Frizzled Related Protein 2 (sFRP2) and Cardiometabolic Risk Factors

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Background: Secreted frizzled-related protein 2 (sFRP2) plays an important role in metabolic syndrome and cardiovascular diseases (CVDs); However, its relevance with cardiometabolic diseases remains to be elucidated. We aimed to determine the serum levels of sFRP2 in patients at different stages of heart failure (HF) with or without type 2 diabetes mellitus (T2DM), and assess the correlation between circulating sFRP2 levels and cardiometabolic risk factors.

Methods: In this study, serum samples from 277 patients visiting Zhongshan Hospital affiliated to Fudan University were collected. These patients were clinically diagnosed and categorized as five groups, including the control group, pre-clinical HF group, pre-clinical HF+T2DM group, HF group and HF+T2DM group. Serum sFRP2 levels were measured with enzyme-linked immunosorbent assay (ELISA) tests and the clinical characteristics of each patient were recorded. Spearman rank correlation analysis and multiple stepwise linear regression analysis were conducted. Univariate and multivariate logistic regression analysis were performed to screen risk factors for HF in patients with CVDs.

Results: Serum sFRP2 levels were significantly lower in the HF+T2DM group compared with the other four groups. Spearman rank correlation analysis showed that sFRP2 was negatively correlated with parameters including patients' age, fasting plasma glucose (FPG), glycated hemoglobin A1c (HbA1c), cardiac troponin T (cTNT), N-terminal pro-B-type natriuretic peptide (NT-proBNP), high-sensitivity C-reactive protein (hs-CRP), left atrial dimension (LAD) and left ventricular posterior wall (LVPW), and positively correlated with hemoglobin, estimated glomerular filtration rate (eGFR), albumin, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and left ventricular ejection fraction (LVEF). However, in multiple regression analysis, significant associations with ln(sFRP2) were observed only in FPG, hs-CRP and LAD. Higher serum sFRP2 was significantly linked to lower odds of HF in patients with CVDs.

Conclusion: sFRP2 progressively decreased when glucose homeostasis and cardiac function deteriorated. sFRP2 acted as a risk factor for HF in patients with CVDs, especially in those with concomitant T2DM.

Keywords: secreted frizzled-related protein 2 (sFRP2), cardiometabolic risk factors, heart failure (HF), type 2 diabetes (T2DM), cross-section study

INTRODUCTION

Despite numerous advances in diagnosis and treatment, cardiovascular diseases (CVDs) remain the leading cause of global mortality. Heart failure (HF), characterized by dyspnea or limited activity due to fluid retention and/or reduced cardiac output, is the end stage of most CVDs and affects at least 23 million people worldwide (1). To emphasize the importance of early intervention and guide therapy, the American College of Cardiology Foundation/American Heart Association (ACCF/AHA) started to promote the concept of HF stages in 2001 (2) and have categorized HF into four stages. Stage A is defined as high risk for HF, including hypertension, metabolic syndrome, tobacco use, and known cardiotoxic agents. Stage B is defined as heart disease without signs or symptoms of HF. Stage C refers to HF with prior or current symptoms, and stage D refers to refractory HF requiring specialized interventions (3). The China Medical Association (CMA) has adapted this concept since 2007 and has classified HF into pre-HF, pre-clinical HF, clinical HF and refractory HF stages (4), which correspond to stages A to D in the American guideline, respectively. The progression of HF stages is irreversible and is associated with a decrease in 5-year survival (5). The rational for treating each stage of HF varies from controlling risk factors, treating structural heart disease to reducing morbidity and mortality.

In the past decade, the prevalence of type 2 diabetes (T2DM) has increased by 30% globally (6), with the number of influenced Chinese adults increasing from 128 million in 2007 to 155 million in 2017 (7). Previous studies have consistently demonstrated a 2- to 4-fold increased risk of HF in individuals with T2DM compared with their age- and sex-matched non-diabetic counterparts, even after adjustment for other cardiovascular risk factors (8, 9). The blood glucose control level also influences the development and progression of HF (10–12). Moreover, HF patients with concomitant T2DM exhibit significantly worse outcomes compared with those without T2DM (13). Therefore, investigating the T2DM-related characteristics in a HF patient cohort may give insight into our understanding of how the metabolic disorders incorporate with CVDs.

Secreted frizzled-related protein (sFRP) family consists of 5 members (sFRP1-5) that structurally resemble the Wnt frizzled receptors and play important roles in cardiac development and various cardiovascular pathophysiological conditions (14). Among these proteins, sFRP2 is considered to be the most potent modulator of the Wnt signaling (15). Previous studies have shown divergent roles of sFRP2 in myocardial fibrosis (16–18), hypertrophy (19), angiogenesis (20), cardiac cell death (21), and regeneration (22, 23) *via* bi-directional regulation of the canonical Wnt pathway, activation of non-canonical Wnt pathway or modulation of other signaling pathways (24). The expression of sFRP2 in cardiac tissues was increased in the early stage of HF and subsequently decreased with the progression of HF (19). In patients with severe decompensated HF, serum sFRP2 levels were higher in those experiencing primary outcome events (25). However, data on serum sFRP2 are still lacking in healthy subjects and in patients with cardiac abnormalities that have not developed into HF. As an adipokine, sFRP2 expression in the white adipose tissue (WAT) was upregulated in obesity (26) and downregulated with moderate weight loss (27). Serum sFRP2 was positively associated with circulating insulin, homeostasis model assessment of β cell secretory capacity (HOMA-β) and insulin resistance (HOMAIR), body mass index (BMI) and triglycerides (TG), and was increased in patients with abnormal glucose tolerance (AGT) (28). Circulating sFRP4 (29–32) and sFRP5 (33–36) levels were associated with the risk of prediabetes/T2DM, obesity and CVDs. Lower levels of serum sFRP5 was associated with worse prognosis in patients with HF, especially in those with concomitant T2DM (37). However, it is obscure whether sFRP2 is involved in the comorbidity of HF and T2DM.

In this study, we investigated the serum levels of sFRP2 among patients in different HF stages with or without T2DM and analyzed its association with fasting blood glucose (FBG), glycated hemoglobin (HbA1c), blood lipid levels, BMI and other cardiometabolic risk factors. We also explored whether sFRP2 could serve as an indicator of the comorbidity of HF and T2DM.

METHODS

Study Population

This study complied with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Zhongshan Hospital, Fudan University (B2020-078R). During the period of August 2020 to August 2021, a total of 425 serum samples from patients admitted to Zhongshan Hospital were initially collected. Those with T1DM, history of malignancy, autoimmune diseases, severe infection, severe renal failure (estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m^2 or under renal replacement therapy), severe hepatic disease (bilirubin $>$ 3$\times$ the upper limit of normal, or aspartate aminotransferase/alanine aminotransferase/alkaline phosphatase $>$ 5$\times$ the upper limit of normal, or cirrhosis), active bleeding or severe anemia (Hb $<$ 60 g/L) were excluded from the study.

Finally, 277 of these serum samples were included for the following analysis. Patients were classified and grouped according to the Guideline for the diagnosis and treatment of heart failure

in China (2018 edition) (38) and the guideline for T2DM (2020 edition) (39). The healthy subjects and pre-HF patients (stage A) were pooled as control group ($n = 53$) and none of them was diagnosed with T2DM. The pre-clinical HF patients (stage B) were subdivided into pre-clinical HF group without T2DM ($n = 57$) and pre-clinical HF+T2DM group ($n = 54$), depending on their medical history of T2DM. The HF patients, including both clinical HF (stage C) and refractory HF (stage D), were also stratified by the medical history of T2DM and thus subdivided into HF group without T2DM ($n = 63$) and HF+T2DM group ($n = 50$).

The cardiac conditions of these patients were further determined following the latest guidelines for hypertension (40), ischemic heart disease (41, 42), valvular heart disease (43), cardiomyopathy (44), and congenital heart disease (45). Based on echocardiography, patients with left ventricular ejection fraction (LVEF) $< 40\%$, 40–49%, and $\geq 50\%$ were subclassified as HF with reduced ejection fraction (HFrEF), midrange EF (HFmrEF), and preserved EF (HFpEF), respectively (46).

Clinical Characteristics and sFRP2 Detection

Medical records were carefully documented, including age, gender, BMI, smoking habit, history of hypertension and CVDs, medication, New York Heart Association (NYHA) functional class, biochemistry tests and echocardiographic parameters.

Fasting venous blood was collected with a serum separation tube, followed by stratification at room temperature for 15 min. Serum were separated and then stored at -80°C until analysis. An enzyme-linked immunosorbent assay (ELISA) kit purchased from Shanghai Yu Bo Biotech Co., Ltd was used to measure the levels of serum sFRP2 in accordance with the manufacturer's instruction manual. The intra-assay and inter-assay variations were 5.4% and 7.5%, respectively.

Statistical Analysis

Statistical analyses were performed in R software (Version 4.0.4) and GraphPad Prism Software (Version 8.3.0). Continuous variables were presented as mean ± standard error of mean or median and interquartile range. Categorical variables were exhibited as numbers or proportions. Differences among groups were compared using one-way ANOVA test for normally distributed continuous variables with homogeneous variance, otherwise Kruskal–Wallis test along with Dunn *post-hoc* test were used. Pearson's chi-squared test or Fisher's exact test was used for categorical variables. Spearman rank correlation analysis identified factors correlated with sFRP2. The levels of sFRP2 were $\log_e$-transformed (ln-transformed) for further analyses to optimize the fitted equation. Cross-sectional associations between ln(sFRP2) and cardiometabolic risk factors were estimated by multiple stepwise linear regression analysis. Multicollinearity was considered present for a variance inflation factor (VIF) > 2.5. Univariate logistic regression analysis was performed to screen risk factors for HF and the variables with significance were further investigated in the multivariate logistic stepwise regression analysis. All statistical tests were two-sided and a P-value < 0.05 was considered as statistically significant.

TABLE 1 | Demographic and laboratory information of the study population.

	Control ($n = 53$)	Pre-HF ($n = 57$)	Pre-HF +T2DM ($n = 54$)	HF ($n = 63$)	HF + T2DM ($n = 50$)	*P*-value
Demographic characteristics						
Age (years)	62 (56, 66)	65 (54, 67)	66 (56, 71)	64 (52, 72)	68 (62, 74)	0.15
Gender (male%)	32 (60.4)	40 (70.2)	41 (75.9)	46 (73.0)	31 (62.0)	0.33
BMI (kg/m^2)	23.8 ± 0.6	24.2 ± 0.5	24.8 ± 0.4	25.1 ± 0.6	24.7 ± 0.6	0.16
Smoking [n (%)]	15 (28.3)	19 (33.3)	19 (35.1)	17 (27.0)	8 (18.0)	0.22
Biochemistry indicators						
Hemoglobin (g/L)	142.8 ± 1.6	130.0 ± 2.5	128.7 ± 2.5	121.0 ± 1.9	111.0 ± 3.9	*<0.001*
Alb (g/L)	46.8 ± 0.5	40.8 ± 0.5	40.5 ± 0.7	38.4 ± 0.6	36.9 ± 0.7	*<0.001*
eGFR (mL/min/1.73 m^2)	87.3 ± 1.9	83.6 ± 2.2	77.6 ± 2.7	75.3 ± 3.0	62.8 ± 3.8	*<0.001*
FPG (mmol/L)	4.9 (4.6, 5.1)	5.5 (4.9, 6.1)	6.6 (5.6, 8.2)	5.4 (4.7, 6.4)	8.5 (6.1, 10.6)	*<0.001*
HbA1c (%)	5.7 (5.5, 5.9)	5.6 (5.4, 5.8)	6.6 (5.9, 7.8)	5.9 (5.4, 6.4)	7.6 (6.4, 8.1)	*<0.001*
cTNT (ng/mL)	0.005 (0.004, 0.006)	0.019 (0.007, 0.077)	0.015 (0.009, 0.056)	0.082 (0.025, 0.192)	0.055 (0.028, 0.142)	*<0.001*
NT-proBNP (pg/mL)	36.3 (27.5, 43.4)	310.0 (64.6, 698.0)	284.0 (77.1, 1410.0)	1263.0 (676.0, 2759.0)	2261.0 (647.0, 5270.0)	*<0.001*
TC (mmol/L)	4.97 (4.47, 5.48)	3.32 (2.88, 3.88)	3.63 (2.98, 4.88)	3.76 (3.14, 4.26)	3.32 (2.83, 3.93)	*<0.001*
TG (mmol/L)	1.47 (1.10, 2.21)	1.33 (0.94, 1.70)	1.58 (1.02, 2.03)	1.25 (0.93, 1.87)	1.51 (1.15, 2.12)	0.09
LDL-C (mmol/L)	2.82 (2.46, 3.32)	1.56 (1.26, 2.17)	1.64 (1.32, 2.56)	2.01 (1.57, 2.62)	1.56 (1.25, 2.02)	*<0.001*
HDL-C (mmol/L)	1.24 (1.08, 1.48)	1.08 (0.86, 1.31)	1.08 (0.83, 1.31)	0.99 (0.85, 1.13)	0.98 (0.80, 1.16)	*<0.001*
hs-CRP (mg/L)	1.0 (0.4, 1.3)	1.0 (0.1, 2.8)	1.4 (0.6, 5.4)	3.8 (1.0, 8.8)	3.5 (1.2, 26.2)	*<0.001*

Continuous variables are presented as mean ± standard error of mean or median (interquartile range). Categorical variables are expressed as number (percentages). Statistically significant values are indicated in italic. BMI, body mass index; Alb, albumin; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; cTNT, cardiac troponin T; NT-proBNP, N-terminal pro-B-type natriuretic peptide; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein.

RESULTS

Clinical Characteristics of the Study Population

The 277 participants were divided into 5 groups: control group ($n = 53$), pre-clinical HF group ($n = 57$), pre-clinical HF+T2DM group ($n = 54$), HF group ($n = 63$) and HF+T2DM group ($n = 50$). There was no significant difference in demographic characteristics such as age, gender, BMI and smoking habit among these groups. The pre-clinical HF+T2DM group and HF+T2DM group had higher FPG and HbA1c than their nondiabetic counterparts and the control group (**Table 1**). Compared with the control group, the levels of cardiac troponin T (cTNT), N-terminal pro-B-type natriuretic peptide (NT-proBNP) and left atrial dimension (LAD) increased in the pre-clinical HF group and further elevated in the HF group (**Tables 1**, **2**). Similar results were obtained in the pre-clinical HF+T2DM group and the HF+T2DM group. Patients with CVDs exhibited reduced LVEF and elevated pulmonary artery pressure (PAP) compared with the control group. Echocardiographic examinations showed enlarged left ventricular end-diastolic dimension (LVDd) and left ventricular end-systolic dimension (LVDs), as well as thickened interventricular septal thickness (IVS) and left ventricular posterior wall (LVPW) in the HF group and HF+T2DM group compared with the control group, pre-clinical HF group and pre-clinical+T2DM group (**Table 2**).

Serum sFRP2 Levels in Different Groups

Next, we compared the serum sFRP2 levels among the patient groups (**Figure 1**). There was no significant difference between the control group and the pre-clinical HF group ($P = 0.94$) or the HF group ($P = 0.43$). However, compared with the control group, serum sFRP2 level decreased significantly in the HF+T2DM group ($P < 0.0001$). Although no significant difference was found between the pre-clinical HF group and the pre-clinical HF+T2DM group ($P = 0.62$), the HF+T2DM group had lower levels of serum sFRP2 than the HF group ($P < 0.0001$). Our results showed that serum sFRP2 levels reduced

TABLE 2 | Medical history and echocardiography of the study population.

	Control ($n = 53$)	Pre-HF ($n = 57$)	Pre-HF+T2DM ($n = 54$)	HF ($n = 63$)	HF+T2DM ($n = 50$)	*P*-value
History of cardiovascular diseases						
IHD [n (%)]	/	47 (82.6)	51 (94.4)	30 (47.6)	27 (54.0)	*<0.001*
VHD [n (%)]	/	4 (7.0)	6 (11.1)	19 (30.2)	14 (28.0)	*<0.01*
Cardiomyopathy [n (%)]	/	2 (3.5)	2 (3.7)	8 (12.7)	5 (10.0)	0.16
CHD [n (%)]	/	3 (5.3)	3 (5.6)	6 (9.5)	3 (6.0)	0.83
Hypertension [n (%)]	19 (35.8)	33 (57.9)	45 (83.3)	27 (42.9)	40 (80.0)	*<0.001*
Chronic medication						
ACEI/ARBs [n (%)]	12 (22.6)	16 (28.1)	17 (31.5)	14 (22.2)	18 (36.0)	0.45
CCBs [n (%)]	11 (20.8)	15 (26.3)	17 (31.5)	13 (20.6)	22 (44.0)	*<0.01*
Beta-blockers [n (%)]	1 (1.9)	28 (49.1)	22 (40.7)	20 (31.7)	21 (42.0)	*<0.001*
Diuretics [n (%)]	3 (5.7)	6 (10.5)	7 (13.0)	29 (46.0)	19 (38.0)	*<0.001*
Digoxin [n (%)]	/	0 (0.0)	1 (1.9)	7 (11.1)	4 (8.0)	*<0.05*
Nitrate esters [n (%)]	/	8 (14.0)	11 (20.4)	11 (20.7)	16 (32.0)	0.12
Antiplatelet [n (%)]	/	40 (70.2)	28 (51.9)	31 (49.2)	28 (56)	0.10
Anticoagulant [n (%)]	/	9 (15.8)	3 (5.6)	15 (23.8)	4 (8.0)	*<0.05*
Insulin [n (%)]	/	/	9 (16.7)	/	17 (34.0)	*<0.05*
OAD [n (%)]	/	/	49 (90.7)	/	19 (38.0)	*<0.001*
Statins [n (%)]	7 (13.2)	33 (57.9)	19 (35.2)	13 (20.6)	24 (48.0)	*<0.001*
Echocardiographic parameters						
LVEF (%)	67 (63,69)	62 (56, 66)	63 (57, 66)	60 (35, 64)	58 (44, 65)	*<0.001*
ARD (mm)	33 (30, 34)	34 (30, 36)	33 (31, 36)	33 (31, 37)	34 (32, 38)	0.17
LAD (mm)	36 (33, 38)	40 (37, 43)	41 (38, 44)	47 (41, 51)	44 (42, 48)	*<0.001*
LVDd (mm)	46 (44, 47)	46 (44, 51)	47 (44, 53)	53 (46, 58)	51 (47, 59)	*<0.001*
LVDs (mm)	30 (28, 30)	30 (29, 34)	30 (28, 35)	35 (30, 47)	32 (30, 43)	*<0.001*
IVS (mm)	9 (8, 10)	10 (9,11)	10 (9, 13)	10 (9, 10)	10 (9, 10)	*<0.001*
LVPW (mm)	8 (8, 9)	9 (9, 10)	9 (9, 10)	10 (9, 11)	10 (9, 11)	*<0.01*
PAP (mmHg)	30 (29, 30)	31 (30, 35)	33 (31, 38)	36 (31, 44)	35 (31, 45)	*<0.001*

Continuous variables are presented as mean ± standard error of mean or median (interquartile range). Categorical variables are expressed as number (percentages). Statistically significant values are indicated in italic. IHD, ischemic heart disease; VHD, valvular heart disease; CHD, congenital heart disease; ACEI/ARB, angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers; CCB, calcium channel blocker; OAD, oral antidiabetic drugs; LVEF, left ventricular ejection fraction; ARD, aortic root dimension; LAD, left atrial dimension; LVDd, Left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; IVS, interventricular septal thickness; LVPW, left ventricular posterior wall; PAP, pulmonary artery pressure.

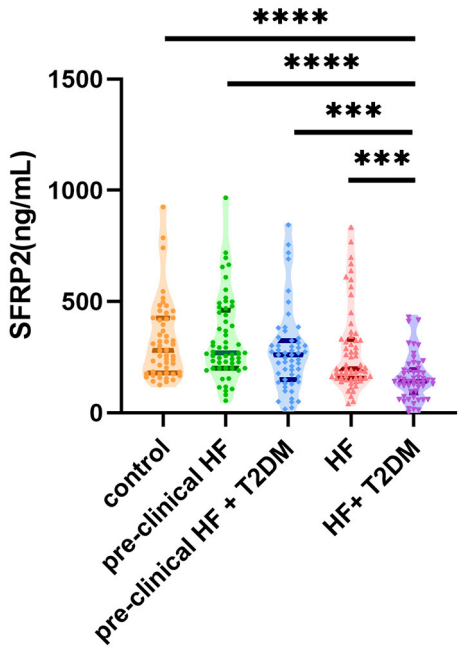

FIGURE 1 | Serum sFRP2 in different groups (***$P < 0.001$, ****$P < 0.0001$).

in the co-occurrence of HF and T2DM, rather than in either individual condition.

Association of sFRP2 With Clinical Variables

The correlation between sFRP2 and clinical variables were analyzed with Spearman rank correlation analysis using data from all the 277 participants. The presence and absence of diseases were assigned as 1 and 0, respectively. No, past and current tobacco use were assigned as 0,1,2, respectively. The detailed data are shown in **Table 3**. The Spearman correlation coefficient between sFRP2 and FPG was <-0.5, suggesting a moderate inverse correlation. SFRP2 had weak negative correlations with HbA1c, cTNT, NT-proBNP and weak positive correlations with hemoglobin and eGFR, as evidenced by the coefficients between −0.4 and −0.2 or 0.2 and 0.4. Although the *P*-values were <0.05, the correlation coefficients of sFRP2 with age, albumin, TC, LDL-C, high-sensitivity C-reactive protein (hs-CRP), LVEF, LAD and LVPW were between −0.2 and 0.2, indicating that the correlations were marginal.

With ln(sFRP2) serving as the dependent variable and the clinical variables correlated with sFRP2 in Spearman correlation analyses as independent variables, a multiple stepwise regression analysis was performed using data from all the 277 participants. During the stepwise regression, age, hemoglobin, Alb, eGFR, HbA1c, cTNT, NT-proBNP, LDL-C and LVPW were removed to reach the lowest Akaike information criterion (AIC) level. Potential confounders such as medications (with or without drugs were assigned as 1 and 0, respectively) were also added as independent variables, but no significant associations were found (data not shown). Finally, FPG, TC, hs-CRP, LVEF and LAD were selected as independent variables in the optimized model. The *P*-value of the model was <2.2e-16 and the adjusted *R*-squared was 0.5109, which indicated 51.09% of the changes in ln(sFRP2) could be attributed to these factors (**Table 4**). VIFs were <1.5 in all analyses, suggesting that no collinearity was observed. As shown in **Figure 2**, residuals were equally distributed and approximately accorded with a normal distribution.

TABLE 3 | Spearman's correlation of sFRP2 with clinical variables.

Indicator	ρ	*P*-value
Age	−0.1591595	*<0.01*
Gender	−0.0567796	0.35
BMI	−0.0980334	0.16
Smoking	−0.0538868	0.38
IHD	−0.0252443	0.68
VHD	−0.0699490	0.25
Hypertension	−0.1141791	0.06
Hemoglobin	0.2127307	*<0.001*
Alb	0.1946989	*<0.01*
eGFR	0.2082241	*<0.001*
FPG	−0.5345865	*<0.001*
HbA1c	−0.3071817	*<0.001*
cTNT	−0.2118334	*<0.001*
NT-proBNP	−0.3009578	*<0.001*
TC	0.1492633	*<0.05*
TG	−0.0672510	0.29
LDL-C	0.1639592	*<0.01*
HDL-C	0.1214094	0.06
hs-CRP	−0.1856972	*<0.01*
LVEF	0.1175641	*<0.05*
ARD	−0.0618573	0.32
LAD	−0.1438706	*<0.05*
LVDd	−0.1023572	0.10
LVDs	−0.116702	0.06
IVS	−0.093653	0.13
LVPW	−0.1213423	*<0.05*
PAP	−0.0072189	0.91

Statistically significant values are indicated in italic. BMI, body mass index; IHD, ischemic heart disease; VHD, valvular heart disease; Alb, albumin; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; cTNT, cardiac troponin T; NT-proBNP, N-terminal pro-B-type natriuretic peptide; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; LVEF, left ventricular ejection fraction; ARD, aortic root dimension; LAD, left atrial dimension; LVDd, Left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; IVS, interventricular septal thickness; LVPW, left ventricular posterior wall; PAP, pulmonary artery pressure.

Relationship Between sFRP2 and the Risk of HF

To investigate the risk factors of HF in patients with CVDs, a univariate logistic regression analysis was performed between pre-clinical HF patients (the pre-clinical HF group and pre-clinical HF+T2DM group) and HF patients (the HF group and HF+T2DM group). In total, 224 of the 277 participants were included in this analysis. As shown in **Table 5**, hemoglobin, albumin, eGFR and ln(sFRP2) were negatively associated with

the odds of HF, whereas FPG, HbA1c, and hs-CRP showed positive associations with the odds of HF. The 7 variables with significance were further investigated in the multivariate logistic stepwise regression analysis. Finally, hemoglobin, albumin, eGFR and ln(sFRP2) were included in the logistic regression equation **(Table 6)**.

DISCUSSION

In our study, sFRP2 decreased significantly in the HF+T2DM group compared with the control group and the pre-clinical HF+T2DM group. Serum sFRP2 was negatively correlated with cTNT, NT-proBNP, LAD, LVPW, and positively correlated with LVEF. In addition, higher sFRP2 was significantly associated to lower odds of HF in patients with CVDs. These results suggest that the decreased circulating sFRP2 may be a risk factor for HF. Several experimental studies supported our observations and revealed the potential mechanisms. sFRP2 attenuated cardiac fibrosis and HF induced by pressure overload (19) and autoimmune myocarditis (16) *via* the Wnt/β-catenin and TGF-β pathways, respectively. sFRP2 prevented cardiomyocyte apoptosis (21) and pathological hypertrophy (19). *SFRP2*

TABLE 4 | Multiple regression analysis of ln(sFRP2) and clinical variables.

	Estimate	Std. error	t value	*P*-value
(Intercept)	7.361267	0.414689	17.751	*<0.001*
FPG	−0.179817	0.015973	−11.258	*<0.001*
TC	0.058419	0.030584	1.910	0.06
hs-CRP	−0.006420	0.001744	−3.682	*<0.001*
LVEF	0.005843	0.003451	−1.693	0.07
LAD	−0.014792	0.006302	−2.347	*<0.05*

Statistically significant values are indicated in italic. FPG, fasting plasma glucose; TC, total cholesterol; hs-CRP, high-sensitivity C-reactive protein; LVEF, left ventricular ejection fraction; LAD, left atrial dimension.

TABLE 5 | Risk factors for HF in 244 cardiac patients: univariate logistic regression.

	OR (95% CI)	*P*-value
Age	1.0166780 (0.9958878, 1.0379020)	0.12
Gender	0.8284024 (0.4743534, 1.4467070)	0.51
BMI	1.0403330 (0.9532089, 1.1354200)	0.38
Smoking	0.7870194 (0.5334641, 1.1610890)	0.23
Hypertension	0.8865784 (0.5183912, 1.5162700)	0.66
Hemoglobin	0.9744476 (0.9620449, 0.9870103)	*<0.001*
Alb	0.8674282 (0.8141866, 0.9241514)	*<0.001*
eGFR	0.9779109 (0.9655423, 0.9904378)	*<0.01*
FPG	1.1219660 (1.0124180, 1.2433680)	*<0.05*
HbA1c	1.2742450 (1.0220770, 1.5886280)	*<0.05*
cTNT	1.0953920 (0.8179942, 1.4668600)	0.54
TC	1.0374960 (0.8384569, 1.2837850)	0.74
TG	0.9669338 (0.7421804, 1.2597490)	0.80
LDL-C	1.0332420 (0.7431301, 1.4366110)	0.85
HDL-C	0.5222179 (0.2337020, 1.1669200)	0.11
hs-CRP	1.0210930 (1.0052670, 1.0371690)	*<0.01*
ln(sFRP2)	0.5360921 (0.3668849, 0.7833375)	*<0.01*

Statistically significant values are indicated in italic. BMI, body mass index; Alb, albumin; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; cTNT, cardiac troponin T; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein.

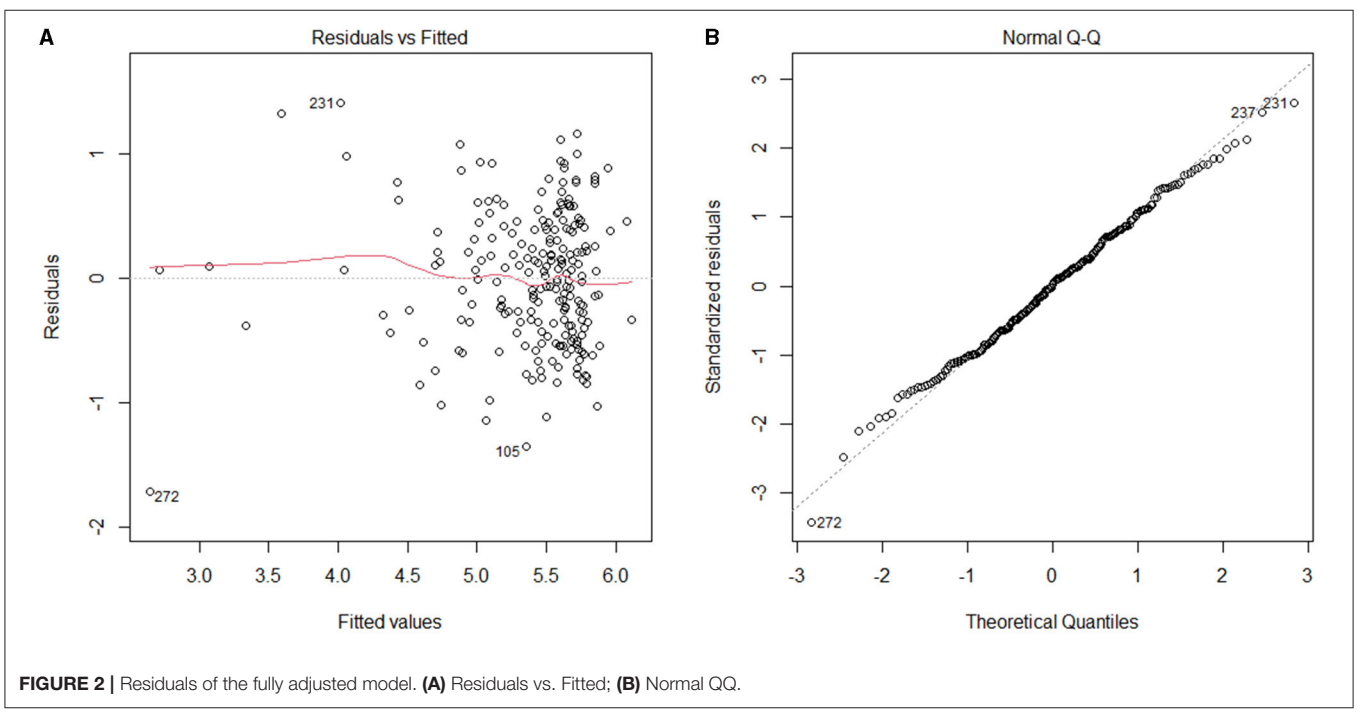

FIGURE 2 | Residuals of the fully adjusted model. **(A)** Residuals vs. Fitted; **(B)** Normal QQ.

TABLE 6 | Risk factors for HF in 244 cardiac patients: multivariate logistic regression.

	OR (95% CI)	*P*-value
Hemoglobin	0.9856071 (0.9714623, 0.9999579)	*<0.01*
Alb	0.9123722 (0.8501815, 0.9791121)	*<0.05*
eGFR	0.9835357 (0.9700838, 0.9971742)	*<0.01*
ln(sFRP2)	0.6633438 (0.4362906, 0.9985590)	*<0.05*

Statistically significant values are indicated in italic. Alb, albumin; eGFR, estimated glomerular filtration rate.

transgene mice exhibited higher LVEF and reduced infarct size due to the increased angiogenesis mediated by ATF6 signaling (20). In cardiac regeneration therapy, sFRP2 also improved bone marrow stromal cells (BMSCs) transplantation (22, 23) and enhanced the differentiation of cardiac progenitor cells (CPCs) (47). However, Yang *et al.* reported that in HF patients, sFRP2 was positively associated with extracellular volume (ECV) fraction, a parameter in cardiovascular magnetic resonance (CMR) imaging used to assess myocardial fibrosis. During follow-up periods, patients with primary cardiac events had higher levels of sFRP2 (25). Since they only enrolled patients with severe decompensated HF and NYHA functional class III–IV, the disparity may be attributed to different stages of HF and the elevated sFRP2 in their study may be a compensatory factor to counteract myocardial fibrosis. Indeed, an initial compensatory increase of cardiac sFRP2 level followed by a substantial decrease of sFRP2 during the progression of HF has already been reported in an animal model (19).

Our study also revealed that sFRP2 was lower in the HF+T2DM group than in the HF group and was negatively correlated with FPG and HbA1c. A previous study showed higher sFRP2 levels in patients with AGT (28). Since the diabetic patients in our study was subdivided from patients with CVDs, the effect of CVDs on sFRP2 may be the main reason for the difference. This discrepancy may also be attributed to their enrollment of pre-diabetes patients. Prediabetes is also associated with increased risk of CVDs (48) and HF (49). Further studies are needed to explore whether sFRP2 is associated with prediabetes and whether this association, if any, is involved in CVDs and HF.

It is intriguing that serum sFRP2 levels reduced in the co-occurrence of HF and T2DM, rather than in either individual disease. We performed a univariate logistic regression analysis specifically in patients with CVDs and T2DM (the pre-clinical HF+T2DM group and the HF+T2DM group, data not shown), and found a more obvious inverse association between ln(sFRP2) and HF (OR: 0.42, 95% CI: 0.24–0.74, $P < 0.01$) compared with that in all the patients with CVDs (OR: 0.54, 95% CI: 0.37–0.78, $P < 0.05$). Patients with the comorbidity of HF and T2DM have distinct biomarkers compared to those without T2DM (50) and sFRP2 is potentially one of them. A falling heart undergoes metabolic perturbations characterized by impaired lipid metabolism and a substrate preference switch toward glycolysis and ketone body oxidation (51). The hyperglycemia, insulin resistance, and hyperinsulinemia in T2DM trigger a cascade of deleterious effects that contribute to the development of HF (52). sFRP2 was reported to be associated with lipogenesis and insulin resistance (53), which indicated that sFRP2 might modulate heart function *via* regulating metabolism.

In our study, the level of hs-CRP in the HF+T2DM group was higher than that in other groups and sFRP2 was negatively correlated with hs-CRP. This is unsurprising since HF and T2DM are accompanied by chronic low-grade systemic inflammation (54). Previous studies have reported the association between sFRP2 and inflammation. sFRP2 was downregulated in inflammation-induced muscle atrophy and prevented inflammatory muscle atrophy (55). sFRP2 enhanced the osteo/odontogenic differentiation and paracrine potentials under inflammation conditions *via* inhibiting canonical Wnt/β-catenin and nuclear factor kappa B (NF-kB) signaling pathways (56). However, Zhou *et al.* demonstrated that serum sFRP2 was significantly upregulated in patients with COPD and knockdown of sFRP2 in peripheral blood mononuclear cells (PBMCs) attenuated airway inflammation (57). Thus, further studies are mandated to explore the detailed roles of sFRP2 in cardiovascular inflammation.

The main strengths of our study are the inclusion of people with different HF stages, which allow us to explore the association of sFRP2 and severity of HF. Furthermore, we collected and adjusted multiple cardiovascular risk factors. However, several limitations should be noted. First, the cross-sectional design precluded us from drawing casual conclusions and further studies are required to define the role of sFRP2 in cardiac function and glucose homeostasis. Second, there were significant differences in etiologies between each group, although there were no significant differences of sFRP2 between ischemic and non-ischemic heart diseases in each group (data not shown) and Spearman rank correlation analysis confirmed no significant association between sFRP2 and etiologies. Third, the measurement of serum sFRP2 by ELISA could not reflect its sources and targets. *SFRP2* gene is ubiquitously expressed in adipose tissue, small intestine, colon, heart, skeletal muscle and other organs, with the highest expression in adipose tissue (58, 59). As an adipokine, circulating sFRP2 is not linearly correlated with *SFRP2* gene expression in WAT (27), indicating that other sources of sFRP2 production should be investigated. Since the cardiac sFRP2 expression increased in the early stage of HF and subsequently decreased with the development of HF (19), less sFRP2 production from heart may explain the declined circulating sFRP2. Previous studies focused on the autocrine and paracrine function, while the changes in serum sFRP2 may indicate its endocrine function. SFRP2 exerts effects on cardiomyocytes, cardiac fibroblast cells, endothelial cells (24) and adipocytes (28) and its function exhibits a high degree of cell specificity. More researches are necessary to explore why serum sFRP2 reduced in the comorbidity of HD and T2DM and the relevant mechanism.

CONCLUSIONS

The level of sFRP2 was negatively correlated with age, FPG, HbA1c, cTNT, NT-proBNP, hs-CRP, LAD and LVPW, and positively correlated with hemoglobin, eGFR, albumin, TC, LDL-C and LVEF. Higher serum sFRP2 was significantly linked to lower odds of HF in patients with CVDs. sFRP2 is a promising

risk factor for the comorbidity of HF and T2DM, which might pave novel ways for the diagnosis and treatment of clinical HF from the perspective of metabolism.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Zhongshan Hospital affiliated to Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PG and MC designed the study. PG, MC, HW, and XW performed the experiments. WL and XJ contributed to the interpretation of the results. MC analyzed the data and wrote the first draft of the manuscript. WG, PG, and YZ supervised the project and revised the paper. All authors contributed to the article and approved the submitted version.

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Association Between Long-Term Visit-to-Visit Hemoglobin A1c and Cardiovascular Risk in Type 2 Diabetes: The ACCORD Trial

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Background: To explore the association between visit-to-visit variability of glycated hemoglobin (HbA1c) and cardiovascular outcomes in the patients with type 2 diabetes mellitus (T2DM) of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study.

Methods: We conducted a *post-hoc* analysis on the ACCORD population including 9,544 participants with T2DM. Visit-to-visit variability of HbA1c was defined as the individual SD, coefficient of variation (CV), and variability independent of the mean (VIM) across HbA1c measurements. The clinical measurements included primary outcome [the first occurrence of non-fatal myocardial infarction (MI), non-fatal stroke or cardiovascular death], total mortality, cardiovascular death, non-fatal MI event, non-fatal stroke, total stroke, heart failure, macrovascular events, and major coronary events (CHD).

Results: Over a median follow-up of 4.85 years, 594 and 268 participants experienced all-cause mortality and cardiovascular mortality, respectively. After adjusting for baseline HbA1c levels and confounding factors, the adjusted hazard ratio (HR) comparing patients in the highest vs. the lowest quartile CV of HbA1c variability was 1.61 (95% CI 1.29–2.00) for the primary outcome. Similar trends for secondary outcome were also observed. There was no association between HbA1c fluctuation and non-fatal stroke. Noticeably, there was 66% greater risk for the all-cause mortality among patients in the highest vs. the lowest quartile (HR 1.66, 95% CI 1.27–2.17).

Conclusions: Greater variability of HbA1c is associated with higher risk for cardiovascular complications and all-cause death in T2DM. Our study stresses the significance of well-controlled glycemic levels for improving cardiovascular outcomes. Further randomized clinical trials are required to confirm these findings.

Keywords: cardiovascular risk, mortality, ACCORD 07 trial, diabetes - quality of life, HbA1c - hemoglobin A1c

INTRODUCTION

Statistically speaking, diabetes was estimated by WHO as the 7th leading cause of mortality, which contributed to 1.6 million deaths in 2016. It has arisen the attention of the world not only because of its growing prevalence but also of increased higher risks for macrovascular and microvascular complications (1–3). Even in the patients with prediabetes, the risk of macrovascular and microvascular disease was increased (4–6). Glycated hemoglobin (HbA1c) presents the average plasma glucose concentration in the past 3 months (7). Recent randomized controlled trials have found that long-term glycemic fluctuation was closely associated with macrovascular and microvascular complications in both type 1 and type 2 diabetes mellitus (T2DM) (8–14). In Diabetes Control and Complications Trial (DCCT), HbA1c levels predicted risk for the renal disease and cardiovascular events in type 1 diabetes (15). A Chinese study contains 91,866 participants demonstrated that HbA1c variability contributed to the development of cardiovascular disease (CVD) and all-cause mortality, particularly in the elderly cohort (16). What is more, ADVANCE study replicated the similar results that visit-to-visit HbA1c fluctuation increased the risk of CVD in T2DM (10). Nevertheless, in the Renal Insufficiency and Cardiovascular Events (RIACE) study, HbA1c variability was associated with macrovascular complications, but not microvascular complication, particularly nephropathy (17). Furthermore, KIM et al. suggested that higher HbA1c fluctuation could not predict carotid artery intima-media thickness independently (18). Besides, participants with diabetes being treated with hypoglycemic agents usually fluctuate greatly in the blood glucose. Therefore, it is important to explore whether the HbA1c variability is an independent risk factor in participants with diabetes. As a result, we performed data analysis in ACCORD cohort to evaluate the association between visit-to-visit HbA1c variability and the risk of cardiovascular outcomes in the patients with T2DM.

METHODS

Study Design and Participants

The ACCORD trial is a randomized, multicenter, double-blind, 2 $\times$ 2 factorial trial, consisting of 10,251 patients with T2DM. The study design and results have been published previously (19, 20). Briefly, the trial recruited participants aged 40 to 79 years who had additional cardiovascular risk factors at 77 North American sites (21). Participants were randomly assigned to achieve intensive glycaemia therapy vs. standard glycaemia therapy (plus either antihypertensive or lipid-lowering intervention). Measurement of HbA1c was recorded every 4 months in the participants for up to 7 years (22). Participants who had <three documented HbA1c measurements and missing confounding data were excluded. Finally, we included 9,544 participants from this study.

Measurement of HbA1c Visit-to-Visit Variability

HbA1c variability was considered as intra-individual SD across visits (17). We calculated intra-personal CV, SD, and VIM of 7-year follow-up HbA1c in this study. In brief, CV for HbA1c was the ratio of SD and mean to correct for larger SD on account of the higher absolute value of HBA1C. VIM was calculated as 100 $\times$ SD/mean$^{\beta}$, and β means the regression coefficient independent of mean. Moreover, all the participants were divided into four quartiles of HbA1c variability for further analyses.

Outcomes

The primary outcome includes the first occurrence of non-fatal myocardial infarction (MI), non-fatal stroke or cardiovascular death. Secondary outcomes included MI, non-fatal MI, any stroke, non-fatal stroke, death from any cause, death from a cardiovascular cause, congestive heart failure, and revascularization (23).

Covariates

Baseline variables include sex, age, race, education, smoking and alcoholic status, BMI, waist, HbA1c, systolic blood pressure (SBP), cholesterol, CVD history, insulin usage, antihypertension, or lipid-lowering medications (24).

Statistical Analysis

The continuous variables were compared by Kruskal–Wallis or ANOVA test and described as mean $\pm$ SD. The categorical variables were compared by chi-squared test and described as a percentage. Kaplan–Meier estimates were used to compare the cumulative incident for the cardiovascular outcome and all-cause mortality within subgroups defined by the variability of HbA1c. Cox proportional hazards regression models were used to calculate adjusted hazard ratios (HR) and 95% CI for CVD outcomes. Subjects were divided into quartiles of CV of HbA1c. Model 1 was adjusted for sex, age, race, duration of DM at baseline. Model 2 was further adjusted for level of education, smoking, alcohol abuse, BMI, and waist at baseline. Model 3 included SBP, heart rate, cholesterol, CVD history, insulin usage, antihypertension, or lipid-lowering medications. Model 4 additionally accounted for the baseline HbA1c levels. All the analyses were performed using SPSS statistic (Version 20.0; IBM Corp., New York, USA) and R software (version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria). A two-sided $P < 0.05$ was considered as statistically significance.

RESULTS

Figure 1 shows the process of selection of the participants. Of the ACCORD cohort, 9,544 patients included in this study were followed up more than three times and had complete data on the covariates. A proportion of participants with ACCORD were excluded because of missing HbA1c measurements. Baseline

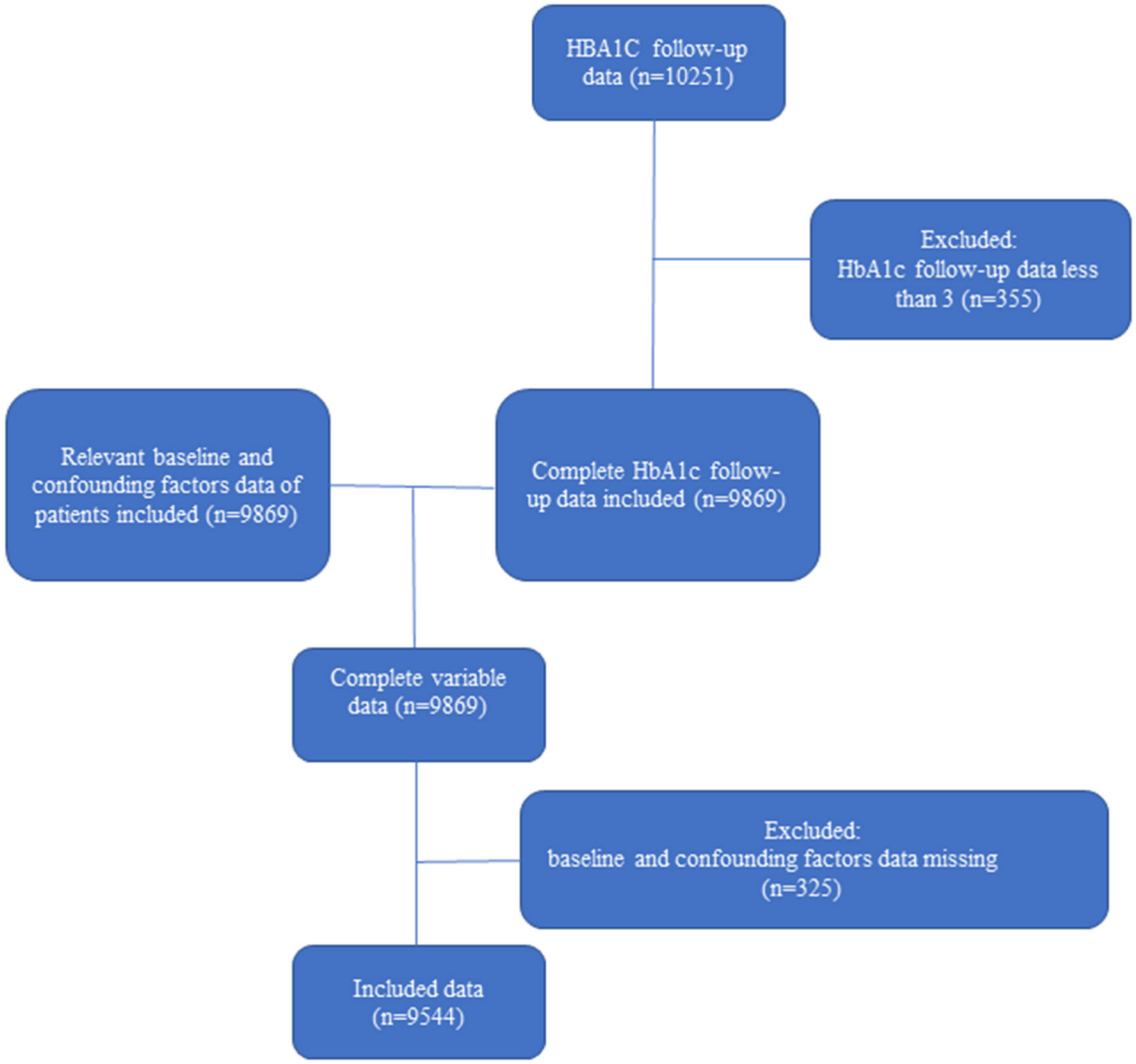

FIGURE 1 | Flow chart of this study.

characteristics grouped by quartiles of CV are presented in **Table 1**.

Individuals with higher variability of HbA1c than those with a lower variability of HbA1c tended to be younger, had a higher body mass index (BMI), waist, cholesterol, triglyceride (TRIG), and smoke more frequently, had a shorter duration of DM. Over a median follow-up period of 4.85 years, 943, 594, 268, 414 individuals, respectively, were adjudicated as having the primary outcome, all-cause mortality, CVD mortality, and heart failure, respectively.

The cumulative incidence curves for the cardiovascular outcome and all-cause mortality within subgroups defined by the variability of HbA1c are presented in **Figure 2**. In our fully adjusted Cox model, visit-to-visit variability of HbA1c including CV, SD, and VIM was associated with primary outcome, all-cause mortality, CVD death, nonfatal MI event, total stroke, heart failure, microvascular events, and major coronary disease but not with non-fatal stroke (**Table 2**). HR of primary and secondary outcomes are summarized in **Table 3** when HbA1c was calculated as a category. We found that the risk of CVD outcome increased with higher levels of HbA1c variability.

Compared with participants with the lowest quartile, after adjustment of potential confounding factors, the HR (95% CI) for primary outcome in the second, third, and the highest quartiles of variability of HbA1c were 1.26 (1.03–1.54), 1.24 (1.01–1.52), 1.61 (1.29–2.00), respectively. Similar trends were also noted in all secondary outcomes including non-fatal MI, any and all-cause mortality, major coronary events, and fatal and non-fatal heart failure except for non-fatal stroke (**Figure 3**). Noticeably, the adjusted HR comparing patients in the highest vs. the lowest quartile CV of HbA1c variability was 1.67 (95% CI 1.28–2.18) for all-cause mortality.

Participants in the highest quartile experienced the highest risk than other groups during 7 years of follow-up ($P < 0.05$).

TABLE 1 | Baseline characteristics grouped by quartiles of variability of HbA1c.

	Total	Q1 (0–0.071)	Q2 (0.071–0.094)	Q3 (0.094–0.122)	Q4 (>0.122)	*p*
Female	3658 (38.3%)	911 (9.5%)	913 (9.6%)	916 (9.6%)	918 (9.6%)	0.997
Age	62.7 ± 6.61	63.88 ± 6.66	63.12 ± 6.55	62.47 ± 6.54	61.42 ± 6.44	0.011
BMI	32.21 ± 5.38	31.24 ± 5.12	32.17 ± 5.40	32.61 ± 5.40	32.82 ± 5.45	0.01
Waist	106.75 ± 13.59	104.26 ± 12.98	106.81 ± 13.64	107.67 ± 13.52	108.27 ± 13.85	0.011
Race	9544 (100%)	2386 (25%)	2386 (25%)	2386 (25%)	2386 (25%)	<0.001
Black	1774 (18.6%)	361 (3.8%)	402 (4.2%)	449 (4.7%)	562 (5.9%)	
Hispanic	678 (7.1%)	130 (1.4%)	147 (1.5%)	180 (1.9%)	221 (2.3%)	
White	5985 (62.7%)	1544 (16.2%)	1574 (16.5%)	1502 (15.7%)	1365 (14.3%)	
Other	1107 (11.6%)	351 (3.7%)	263 (2.8%)	255 (2.7%)	238 (2.5%)	
Education	9544 (100%)	2386 (25%)	2386 (25%)	2386 (25%)	2386 (25%)	<0.001
<HS	1383 (14.5%)	299 (3.1%)	301 (3.2%)	348 (3.6%)	435 (4.6%)	
HS	2529 (26.5%)	626 (6.6%)	613 (6.4%)	646 (6.8%)	644 (6.7%)	
SC	3129 (32.8%)	783 (8.2%)	786 (8.2%)	789 (8.3%)	771 (8.1%)	
More	2503 (26.2%)	678 (7.1%)	686 (7.2%)	603 (6.3%)	536 (5.6%)	
HbA1c	8.29 ± 1.05	7.69 ± 0.65	7.99 ± 0.74	8.43 ± 0.89	9.06 ± 1.25	<0.001
Duration of DM	10.78 ± 7.57	11.18 ± 7.75	11.05 ± 7.65	10.84 ± 7.58	10.07 ± 7.24	0.001
SBP	136.28 ± 17.00	135.49 ± 16.54	136.1 ± 16.57	136.7 ± 17.27	136.8 ± 17.60	0.038
Heart rate	72.58 ± 11.70	71.08 ± 11.38	72.22 ± 11.75	72.91 ± 11.92	74.12 ± 11.52	0.115
cholesterol	183.27 ± 41.82	178.36 ± 38.74	181.5 ± 40.60	183.4 ± 40.45	189.7 ± 46.30	<0.001
TRIG	190.4 ± 149.7	168.8 ± 109.5	182.0 ± 125.5	194.8 ± 145.9	216.1 ± 198.5	<0.001
HDL	41.86 ± 11.56	43.43 ± 11.73	42.08 ± 11.62	41.24 ± 11.32	40.69 ± 11.37	0.127
CVD history	3335 (34.9%)	809 (8.5%)	817 (8.6%)	857 (9.0%)	852 (8.9%)	0.354
CHF history	439 (4.6%)	105 (1.1%)	96 (1.0%)	126 (1.3%)	112 (1.2%)	0.204
Smoke	9544 (100%)	2386 (25%)	2386 (25%)	2386 (25%)	2386 (25%)	<0.001
Current	1323 (13.92%)	289 (3%)	287 (3%)	331 (3.5%)	416 (4.4%)	
Ever	4246 (44.5%)	1084 (11.4%)	1079 (11.3%)	1053 (11%)	1030 (10.8%)	
No smoke	3975 (41.6%)	1013 (10.6%)	1020 (10.7%)	1002 (10.5%)	940 (9.8%)	
Alcohol	0.97 ± 2.70	1.11 ± 2.86	1.07 ± 2.91	0.80 ± 2.33	0.91 ± 2.62	<0.001
Thiazide	2651 (27.8%)	688 (7.2%)	689 (7.2%)	657 (6.9%)	617 (6.5%)	0.065
ARBs	1591 (16.7%)	445 (4.7%)	423 (4.4%)	394 (4.1%)	329 (3.4%)	<0.001
ACE-inhibitors	5217 (54.7%)	1274 (13.3%)	1292 (13.5%)	1333 (14.0%)	1318 (13.8%)	0.318
Beta-blocker	2882 (30.2%)	709 (7.4%)	711 (7.4%)	752 (7.9%)	710 (7.4%)	0.451
Any insulin	3348 (35.1%)	872 (9.1%)	851 (8.9%)	842 (8.8%)	783 (8.2%)	0.045
Statin	6086 (63.8%)	1635 (17.1%)	1544 (16.2%)	1533 (16.1%)	1374 (14.4%)	<0.001
IG	4760 (49.9%)	946 (9.9%)	1173 (12.3%)	1260 (13.2%)	1381 (14.5%)	<0.001

Values are mean ± SD for continuous variables and n (%) for categorical variables; BMI, body mass index; HS, high school; SC, some college; SBP, systolic blood pressure; DM, diabetes mellitus; TRIG, triglyceride; HDL, high-density lipoprotein; CVD, cardiovascular disease; ARBs, angiotensin receptor blocker; ACE, angiotensin-converting enzyme; IG, intensive glycaemia control; Q, quartile.

DISCUSSION

Previous studies had established HbA1c as an effective index of long-term glycemic control, lower HbA1c persisting at <7% was associated with less risks for diabetes related complications and per 1% higher HbA1c is related to 15–20% higher cardiovascular risk (25, 26). However, whether controlling HbA1c to a normal range by intensive glycemic therapy in patients with T2DM can reduce CVD remains controversial. ACCORD, VADT, and other large studies showed no beneficial effects of intensive glucose therapy targeting for low-level HbA1c (27, 28). Hence, whether the mean HbA1c level is the most appropriate factor to predict the risk for diabetes complication is still questionable. Many researchers highlighted the effects of HbA1c variability on the cardiovascular outcomes.

To our best knowledge, our study was the first one to explore the association between the long-term visit-to-visit fluctuation of HbA1c levels CVD outcomes using ACCORD data. We discovered patients with T2DM with higher variability of HbA1c tended to develop cardiovascular diseases and had a worse prognosis. This observation was consistent after adjusting for the baseline HbA1c and other confounding factors such as demographic characteristics, hypertension, dyslipidemia, smoking, and medications. In accordance with our results, a

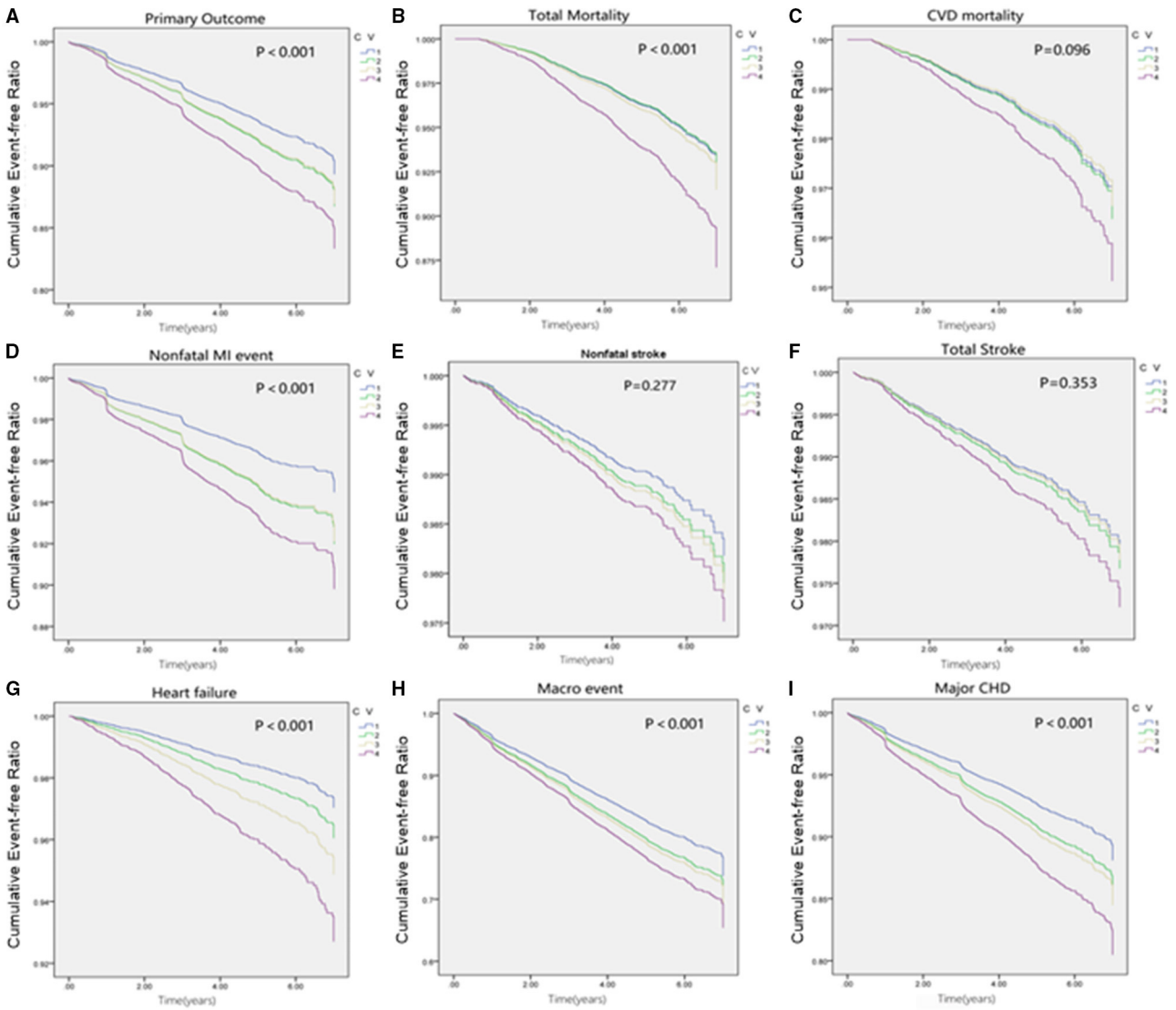

FIGURE 2 | Cumulative survival of outcomes grouped by quartiles of HbA1c fluctuation. **(A)** Cumulative survival of Primary outcome grouped by quartiles of HbA1c fluctuation. **(B)** Cumulative survival of Total mortality grouped by quartiles of HbA1c fluctuation. **(C)** Cumulative survival of CVD mortality grouped by quartiles of HbA1c fluctuation. **(D)** Cumulative survival of Non-fatal MI event grouped by quartiles of HbA1c fluctuation. **(E)** Cumulative survival of Nonfatal stroke grouped by quartiles of HbA1c fluctuation. **(F)** Cumulative survival of Total stroke grouped by quartiles of HbA1c fluctuation. **(G)** Cumulative survival of CHF grouped by quartiles of HbA1c fluctuation. **(H)** Cumulative survival of Macro event grouped by quartiles of HbA1c fluctuation. **(I)** Cumulative survival of Primary outcome grouped by quartiles of HbA1c fluctuation.

Chinese study had evaluated the association between HbA1c variability and vascular complication and mortality (11). Moreover, Prentice et al. conducted a retrospective study with T2DM and concluded that visit-to-visit HbA1c fluctuation might predict adverse outcomes (29). A recent review pooled 20 studies with 87,641 participants to investigate on HbA1c fluctuation and vascular complication in type 1 diabetes in T2DM. Particularly, in T2DM, higher HbA1c variability group had higher risk for cardiovascular disease, renal disease, mortality (14). In contrast, the RIACE study shown no association between HbA1c and macrovascular disease. The potential explanation for such disparity may be due to the study design. We collected the multiple HbA1c variability measurements of 7 years, with a median follow-up time of 4.85 years, while the RIACE obtained serial HbA1c values during 2-year period recruitment. Furthermore, our study held distinct baseline HbA1c and degree of HbA1c fluctuation and diabetes duration (17).

Until now, there has not been a standardized method to access HbA1c variability. We selected three measurements including SD, CV, VIM, all of which were independently correlated with the cardiovascular outcomes. Our results showed that the CV is possibly a more robust measure of visit-to-visit in HbA1c rather than average HbA1c levels. Unfortunately, researchers fail to carry out intervention study and conclude the cause-effect of

TABLE 2 | Visit-to-visit variability of HbA1Cas a continuous variable and cardiovascular outcomes.

CVD outcome	Model 1		Model 2		Model 3		Model 4	
	HR (95%CI)	P	HR (95%CI)	P	HR (95%CI)	P	HR (95%CI)	P
Primary outcome								
SD	1.31 (1.23–1.38)	<0.001	1.29 (1.22–1.36)	<0.001	1.25 (1.17–1.32)	<0.001	1.22 (1.15–1.31)	<0.001
CV	1.27 (1.20–1.35)	0.004	1.26 (1.18–1.33)	<0.001	1.23 (1.54–1.31)	0.071	1.20 (1.11–1.29)	<0.001
VIM	2.12 (1.81–2.49)	<0.001	2.06 (1.75–2.42)	<0.001	1.87 (1.59–2.20)	<0.001	1.79 (1.48–2.16)	<0.001
Total mortality								
SD	1.35 (1.26–1.45)	<0.001	1.32 (1.23–1.41)	<0.001	1.29 (1.20–1.39)	<0.001	1.31 (1.21–1.42)	<0.001
CV	1.32 (1.23–1.42)	<0.001	1.28 (1.19–1.38)	<0.001	1.27 (1.18–1.37)	<0.001	1.28 (1.17–1.40)	<0.001
VIM	2.34 (1.92–2.85)	<0.001	2.18 (1.78–2.66)	<0.001	2.05 (1.67–2.52)	<0.001	2.15 (1.70–2.71)	<0.001
CVD mortality								
SD	1.34 (1.20–1.49)	<0.001	1.30 (1.17–1.45)	<0.001	1.28 (1.15–1.43)	<0.001	1.26 (1.11–1.43)	0.001
CV	1.27 (1.14–1.43)	<0.001	1.24 (1.10–1.39)	<0.001	1.24 (1.10–1.40)	0.001	1.19 (1.03–1.37)	0.017
VIM	2.28 (1.69–3.07)	<0.001	2.14 (1.57–2.90)	<0.001	2.01 (1.47–2.75)	<0.001	1.92 (1.33–2.77)	<0.001
Non-fatal MI event								
SD	1.30 (1.21–1.39)	<0.001	1.29 (1.20–1.38)	<0.001	1.24 (1.16–1.33)	<0.001	1.25 (1.16–1.36)	<0.001
CV	1.27 (1.18–1.37)	<0.001	1.26 (1.17–1.36)	<0.001	1.23 (1.14–1.33)	<0.001	1.24 (1.13–1.35)	<0.001
VIM	2.09 (1.72–2.55)	<0.001	2.05 (1.68–2.51)	<0.001	1.85 (1.51–2.27)	<0.001	1.90 (1.51–2.39)	<0.001
Non-fatal stroke								
SD	1.40 (1.24–1.58)	<0.001	1.39 (1.23–1.58)	<0.001	1.33 (1.18–1.51)	<0.001	1.17 (0.99–1.38)	0.063
CV	1.40 (1.23–1.60)	<0.001	1.40 (1.22–1.60)	<0.001	1.36 (1.18–1.56)	<0.001	1.16 (0.97–1.39)	0.095
VIM	2.60 (1.84–3.66)	<0.001	2.55 (1.80–3.61)	<0.001	2.25 (1.57–3.21)	<0.001	1.55 (0.98–2.47)	0.064
Total stroke								
SD	1.42 (1.27–1.59)	<0.001	1.42 (1.26–1.59)	<0.001	1.37 (1.21–1.54)	<0.001	1.22 (1.04–1.42)	0.012
CV	1.43 (1.26–1.62)	<0.001	1.43 (1.26–1.62)	<0.001	1.39 (1.22–1.59)	<0.001	1.22 (1.03–1.43)	0.019
VIM	2.71 (1.96–3.74)	<0.001	2.69 (1.94–3.73)	<0.001	2.41 (1.72–3.37)	<0.001	1.74 (1.13–2.68)	0.011
CHF								
SD	1.47 (1.36–1.58)	<0.001	1.42 (1.31–1.54)	<0.001	1.39 (1.28–1.51)	<0.001	1.40 (1.27–1.53)	<0.001
CV	1.43 (1.31–1.55)	<0.001	1.37 (1.26–1.49)	<0.001	1.36 (1.25–1.49)	<0.001	1.35 (1.22–1.50)	<0.001
VIM	2.95 (2.38–3.67)	<0.001	2.72 (2.17–3.41)	<0.001	2.54 (2.01–3.21)	<0.001	2.59 (1.99–3.37)	<0.001
Macro event								
SD	1.20 (1.15–1.24)	<0.001	1.18 (1.13–1.26)	<0.001	1.15 (1.10–1.19)	<0.001	1.15 (1.09–1.20)	<0.001
CV	1.17 (1.24–1.22)	<0.001	1.16 (1.11–1.20)	<0.001	1.13 (1.09–1.18)	<0.001	1.13 (1.08–1.19)	<0.001
VIM	1.64 (1.47–1.84)	<0.001	1.59 (1.42–1.78)	<0.001	1.47 (1.30–1.65)	<0.001	1.48 (1.29–1.69)	<0.001
Major CHD								
SD	1.23 (1.17–1.30)	<0.001	1.22 (1.15–1.28)	<0.001	1.18 (1.12–1.25)	<0.001	1.20 (1.12–1.27)	<0.001
CV	1.21 (1.14–1.28)	0.034	1.19 (1.13–1.26)	<0.001	1.18 (1.11–1.28)	<0.001	1.18 (1.11–1.27)	<0.001
VIM	1.80 (1.54–2.10)	<0.001	1.75 (1.50–2.04)	<0.001	1.61 (1.38–1.88)	<0.001	1.67 (1.40–1.99)	<0.001

Model 1, adjusted for sex, age, race, duration of diabetes mellitus at baseline. Model 2, further adjusted for level of education, smoking, alcohol abuse, BMI, waist at baseline. Model 3 included systolic blood pressure, heart rate, cholesterol, CVD history, insulin usage, antihypertension or lipid-lowering medication. Model 4, accounted for baseline HbA1c.

HbA1c variability. The mechanisms linking the higher HbA1c fluctuation to the higher risk of CVD remain unclear and require further biological investigation. One of the hypotheses is that glycemic variability might cause endothelial dysfunction and atherosclerosis induced by inflammatory cytokines and oxidative stress (30–32). Another hypothesis is the "metabolic memory" in vascular cells, by which the cellular transduction system and extra oxygen and nitrogen lead to endothelial damage (33). Moreover, the glucose fluctuation may cause hypoglycemia which poses a threat to cardiovascular systems (34).

There are several limitations to our present study. First, the number and frequency of HbA1c value varied from different participants. To minimize the influences, we use CV and VIM which are independent of average of HbA1c levels. Second, the most of our participants had a DM duration of over 10 years, thus, it is uncertain whether our conclusion could be generalized to those with a shorter duration of DM. Third, we failed to conduct the trend of HbA1c variability. Whether decreasing the HbA1c variability could reduce cardiovascular outcomes and all-cause mortality remains obscure and needs further investigation.

TABLE 3 | Visit-to-visit variability of HbA1C as a categorical variable and cardiovascular outcomes.

	Model 1		Model 2		Model 3		Model 4	
	HR (95%CI)	***P***	**HR (95%CI)**	***P***	**HR (95%CI)**	***P***	**HR (95%CI)**	***P***
Primary outcome								
Q1	reference		reference		reference		reference	
Q2	1.30 (1.07–1.59)	0.008	1.30 (1.07–1.58)	0.009	1.29 (1.06–1.57)	0.012	1.26 (1.03–1.54)	0.022
Q3	1.41 (1.16–1.71)	0.001	1.37 (1.13–1.67)	0.002	1.30 (1.07–1.58)	0.009	1.24 (1.01–1.52)	0.041
Q4	1.95 (1.61–2.35)	<0.001	1.88 (1.55–2.28)	<0.001	1.76 (1.45–2.14)	<0.001	1.61 (1.29–2.00)	<0.001
Total mortality								
Q1	reference		reference		reference		reference	
Q2	0.99 (0.78–1.28)	0.977	0.99 (0.78–1.28)	0.977	0.99 (0.77–1.27)	0.941	0.99 (0.77–1.27)	0.933
Q3	1.18 (0.92–1.50)	0.187	1.18 (0.92–1.50)	0.187	1.08 (0.84–1.38)	0.563	1.07 (0.83–1.38)	0.592
Q4	1.87 (1.48–2.35)	<0.001	1.87 (1.48–2.35)	<0.001	1.67 (1.32–2.12)	<0.001	1.66 (1.27–2.17)	<0.001
CVD mortality								
Q1	reference		reference		reference		reference	
Q2	1.06 (0.74–1.52)	0.763	1.04 (0.72–1.50)	0.844	1.07 (0.74–1.55)	0.711	1.04 (0.72–1.51)	0.828
Q3	1.51 (0.80–1.66)	0.449	1.07 (0.74–1.55)	0.707	1.03 (0.71–1.48)	0.885	0.96 (0.66–1.40)	0.830
Q4	1.75 (1.23–2.47)	0.002	1.60 (1.13–2.27)	0.008	1.61 (1.13–2.29)	0.008	1.41 (0.94–2.12)	0.096
Non-fatal MI event								
Q1	reference		reference		reference		reference	
Q2	1.47 (1.15–1.88)	0.002	1.49 (1.16–1.89)	0.002	1.47 (1.15–1.88)	0.002	1.47 (1.15–1.88)	0.002
Q3	1.54 (1.20–1.97)	0.001	1.52 (1.19–1.96)	0.001	1.45 (1.13–1.87)	0.004	1.45 (1.12–1.88)	0.005
Q4	2.05 (1.60–2.61)	<0.001	2.01 (1.57–2.57)	<0.001	1.89 (1.48–2.43)	<0.001	1.89 (1.43–2.48)	<0.001
Non-fatal stroke								
Q1	reference		reference		reference		reference	
Q2	1.33 (0.79–2.23)	0,285	1,34 (0.80–2.26)	0.265	1.29 (0.77–2.16)	0.342	1.15 (0.68–1.95)	0.594
Q3	1.69 (1.03–2.79)	0.039	1.70 (1.03–2.81)	0.037	1.56 (0.94–2.58)	0.082	1.21 (.072–2.04)	0.478
Q4	2.53 (1.57–4.08)	<0.001	2.52 (1.55–4.08)	<0.001	2.25 (1.38–3.67)	0.001	1.38 (0.78–2.41)	0.277
Total stroke								
Q1	reference		reference		reference		reference	
Q2	1.24 (0.77–1.98)	0.383	1.25 (0.78–2.01)	0.351	1.20 (0.74–1.93)	0.458	1.08 (0.67–1.74)	0.765
Q3	1.42 (0.89–2.26)	0.140	1.43 (0.90–2.29)	0.129	1.31 (0.82–2.10)	0.254	1.02 (0.63–1.67)	0.928
Q4	2.32 (1.50–3.60)	<0.001	2.33 (1.50–3.62)	<0.001	2.09 (1.33–3.28)	0.001	1.28 (0.76–2.17)	0.353
CHF								
Q1	reference		reference		reference		reference	
Q2	1.37 (0.99–1.90)	0.061	1.37 (0.99–1.90)	0.061	1.35 (0.97–1.88)	0.072	1.35 (0.97–1.88)	0.077
Q3	2.07 (1.52–2.82)	<0.001	2.07 (1.52–2.82)	<0.001	1.77 (1.29–2.42)	<0.001	1.75 (1.27–2.41)	0.001
Q4	3.01 (2.22–4.08)	<0.001	3.01 (2.22–4.08)	<0.001	2.57 (1.88–3.49)	<0.001	2.52 (1.79–3.54)	<0.001
Macro event								
Q1	reference		reference		reference		reference	
Q2	1.21 (1.07–1.37)	0.003	1.20 (1.06–1.36)	0.004	1.19 (1.05–1.35)	0.006	1.19 (1.05–1.35)	0.007
Q3	1.34 (1.18–1.52)	<0.001	1.31 (1.16–1.48)	<0.001	1.24 (1.10–1.41)	0.001	1.23 (1.08–1.40)	0.002
Q4	1.55 (1.37–1.75)	<0.001	1.49 (1.32–1.69)	<0.001	1.42 (1.25–1.61)	<0.001	1.39 (1.21–1.60)	<0.001
Major CHD								
Q1	reference		reference		reference		reference	
Q2	1.25 (1.05–1.50)	0.014	1.24 (1.04–1.49)	0.018	1.25 (1.04–1.50)	0.016	1.26 (1.05–1.51)	0.013
Q3	1.40 (1.17–1.67)	<0.001	1.36 (1.14–1.63)	0.001	1.31 (1.09–1.57)	0.003	1.33 (1.10–1.60)	0.003
Q4	1.78 (1.49–2.12)	<0.001	1.71 (1.43–2.04)	<0.001	1.66 (1.39–1.99)	<0.001	1.71 (1.40–2.08)	<0.001

Participants were divided into four quartiles of CV of HbA1c. Model 1, adjusted for sex, age, race, duration of diabetes mellitus at baseline. Model 2, further adjusted for level of education, smoking, alcohol abuse, BMI, waist at baseline. Model 3 included systolic blood pressure, heart rate, cholesterol, CVD history, insulin usage, antihypertension or lipid-lowering medication. Model 4, accounted for baseline HbA1c.

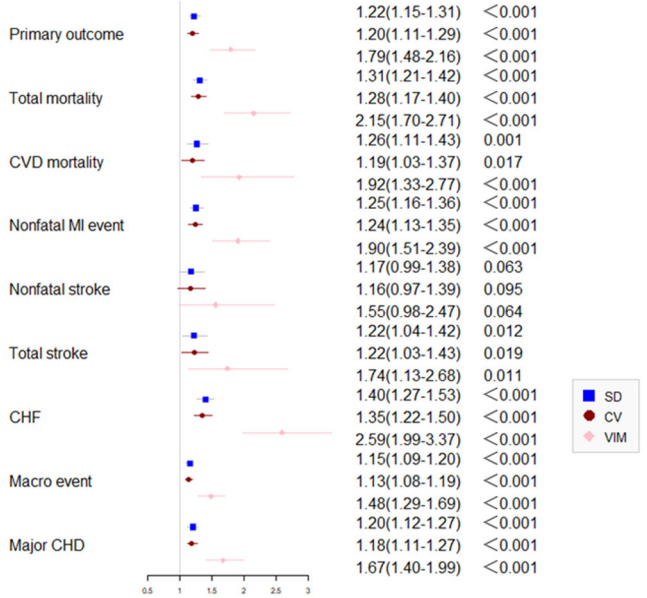

FIGURE 3 | Comparison of primary and secondary outcomes by level of HbA1c variability in the ACCORD cohort.

CONCLUSIONS

In conclusion, data from a 7-year follow-up cohort has revealed that high variability of HbA1c is an independent risk for cardiovascular outcomes of the ACCORD study. Moreover, it seems reasonable to include HbA1c variability as potential target in the routine management of T2DM.

DATA AVAILABILITY STATEMENT

The data of the Action to Control Cardiovascular Risk in Diabetes study is available free of charge from the NHLBI Biologic Specimen and Data Repository Information Coordinating Center, the datasets used during the current study are available from the corresponding authors on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Fifth Affiliated Hospital, Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YH and DH planned and interpreted the data. YH, DH, and Y-QH drafted the manuscript. Q-YZ and YC performed the statistical analyses. YH is the guarantor of the present study and took responsibility for the integrity and the accuracy of the data analysis. All authors read and revised the content and approved the final manuscript.

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Prognostic Significance of Triglyceride-Glucose Index for Adverse Cardiovascular Events in Patients With Coronary Artery Disease: A Systematic Review and Meta-Analysis

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Background: Insulin resistance (IR) represents a critical regulator in the development and progress of coronary artery disease (CAD). Triglyceride-glucose (TyG) index, a novel surrogate biomarker of IR, has been implicated in several cardiovascular diseases. Accordingly, we conduct a meta-analysis to elucidate the relationship between TyG index and adverse cardiovascular events in patients with CAD.

Methods: To identify the studies examining the predictive capacity of the TyG index for adverse cardiovascular events in the setting of CAD, we performed a comprehensive literature retrieval of Scopus, PubMed, EMBASE, and Web of Science, from the inception of databases to October 5, 2021. We pooled the adjusted hazard ratio (HR) along with 95% CI using a random-effects model. The primary outcome was a composite of major adverse cardiovascular events (MACEs), including all-cause death, cardiovascular death (CV death), myocardial infarction (MI), stroke, hospitalization for unstable angina or heart failure, and revascularization. The secondary outcomes were all-cause death, CV death, MI, stroke, and revascularization. Additionally, we conducted subgroup analyses stratified by diabetes status, age, body mass index (BMI), low-density lipoprotein cholesterol (LDL-C), category of TyG index, sample size, follow-up duration, and study design.

Results: About 12 studies involving 28,795 patients with CAD were finally taken into the quantitative analysis. Our findings showed that there was a 2.14-fold higher risk of MACEs among CAD populations in the highest TyG group compared with those in the lowest TyG group (HR: 2.14, 95% CI: 1.69–2.71, $P < 0.001$). A greater risk of MACEs was observed in participants with higher BMI than those with lower BMI ($P = 0.03$ for interaction). In the analysis of secondary outcomes, we also observed a markedly increased risk of MI, stroke, and revascularization in the highest TyG group compared with the lowest TyG group. No evidence of a significant association between TyG index and CV mortality or all-cause mortality in patients with CAD was identified.

Conclusions: The elevated TyG index is a promising predictive factor of adverse cardiovascular events in patients with CAD.

Systematic Review Registration: https://www.crd.york.ac.uk/PROSPERO, identifier: CRD42021228521.

Keywords: triglyceride-glucose index, insulin resistance, coronary artery disease, adverse cardiovascular events, meta-analysis

INTRODUCTION

Despite the guideline-directed therapy, coronary artery disease (CAD) is still the primary cause of death globally (1, 2). Although optimal treatments including drug therapy and revascularization effectively decrease the incidence of chest pain, individuals with CAD remain at an increased risk of recurrent adverse cardiovascular events (3). Therefore, it is crucial to identify the CAD population with a high risk of future cardiovascular events, which may contribute to the optimization of clinical management.

Insulin resistance (IR) is characterized by metabolism disorders, especially abnormal glucose, and lipid metabolism, leading to aggravation of atherosclerosis in the coronary artery (4, 5). Recently, a novel convenient biomarker of IR, Triglyceride-glucose (TyG) index calculated by the formula ln [fasting triglycerides (mg/dl) × fasting plasma glucose (mg/dl)/2], has elicited the interests of researchers because of its superior performance in the estimation of IR than a homeostatic model assessment of IR (HOMA-IR) (6–9). Certain studies in humans showed that TyG index was positively correlated with coronary artery calcification (10), coronary plaque progression (11), and subclinical myocardial injury (12), providing plausibility of the prognostic significance of TyG index in CAD. As a product of triglycerides and plasma glucose, the TyG index may provide additional information for the risk assessment of adverse cardiovascular events. Consistent clinical data have indicated that elevated TyG index was associated with a higher incidence of CAD (13–15). However, there are conflicting data regarding the predictive capacity of the TyG index for adverse events in patients with CAD (16–18). Thus, we conducted a meta-analysis to investigate the relationship between the TyG index and poor prognosis among patients with CAD.

METHODS

Search Strategy and Eligibility Criteria

This meta-analysis has been registered in PROSPERO (https://www.crd.york.ac.uk/PROSPERO). The registration number is CRD42021228521. We performed this meta-analysis in accordance with the recommendations of the Meta-analysis of Observational Studies in Epidemiology group (19). In June 2021, to identify studies investigating the relationship of the TyG index with the risk of adverse outcomes in CAD, two reviewers independently conducted a systematic literature search of four databases (PubMed, Scopus, EMBASE, and Web of Science). On October 5, 2021, a repeat literature retrieval was conducted to supplement the latest research. Search terms included: "triglyceride-glucose index" OR "TyG index" AND "coronary artery disease" OR "coronary heart disease" OR "ischemic heart disease" OR "myocardial infarction" OR "stable angina" OR "unstable angina." The literature retrieval was restricted to human studies published in English. A detailed search method of PubMed is presented in **Supplementary File 1**.

The inclusion criteria were: (1) observational studies (cohort study or nest case-control study); (2) participants were diagnosed with CAD (≥18 years old); (3) participants were exposed to different levels of TyG index at baseline; (4) the outcomes of interest were composite cardiovascular events, all-cause death, CV death, MI, stroke, and revascularization; (5) Adjusted hazard ratio (HR) with 95% CI from multivariate Cox regression models was available. The exclusion criteria were: (1) follow-up duration < 3 months; (2) non-English works of literature; (3) duplicate reports, if studies including the same participants or overlapping participants were published, the study with the largest sample size was chosen.

Data Extraction and Quality Evaluation

Two investigators independently abstracted the following data from each enrolled study: (1) basic characteristics of eligible study: name of the first author, publication year, country origin, sample size, and follow-up duration; (2) baseline demographic and clinical characteristics of subjects: age, sex, diabetes status, body mass index (BMI), low-density lipoprotein cholesterol (LDL-C) as well as the highest TyG index group, and lowest TyG index group; (3) outcomes: adjusted HRs for all-cause death, cardiovascular death (CV death), myocardial infarction (MI), stroke, revascularization, and a composite of cardiovascular events. In instances where there was insufficient information, we contacted the corresponding author. If disagreements occurred, an expert (WH Duan) in the field was consulted and made a judgment.

To assess the methodological quality of each selected study, we adopted the Newcastle-Ottawa-Scale (NOS) (20) designed for case-control study and cohort study. The NOS is mainly comprised of three dimensions: selection of participants, comparability among groups, as well as an outcome assessment. The NOS score ranges from 0 to 9 stars. Articles rated as 7 stars or above are of high quality; articles rated as 4 to 6 stars are of fair quality; articles rated as <4 stars are of low quality (21, 22).

Statistical Analysis

Our primary outcome was major adverse cardiovascular events (MACEs), including all-cause death, CV death, MI, stroke, hospitalization for unstable angina or heart failure, and revascularization. The secondary outcomes were all-cause death,

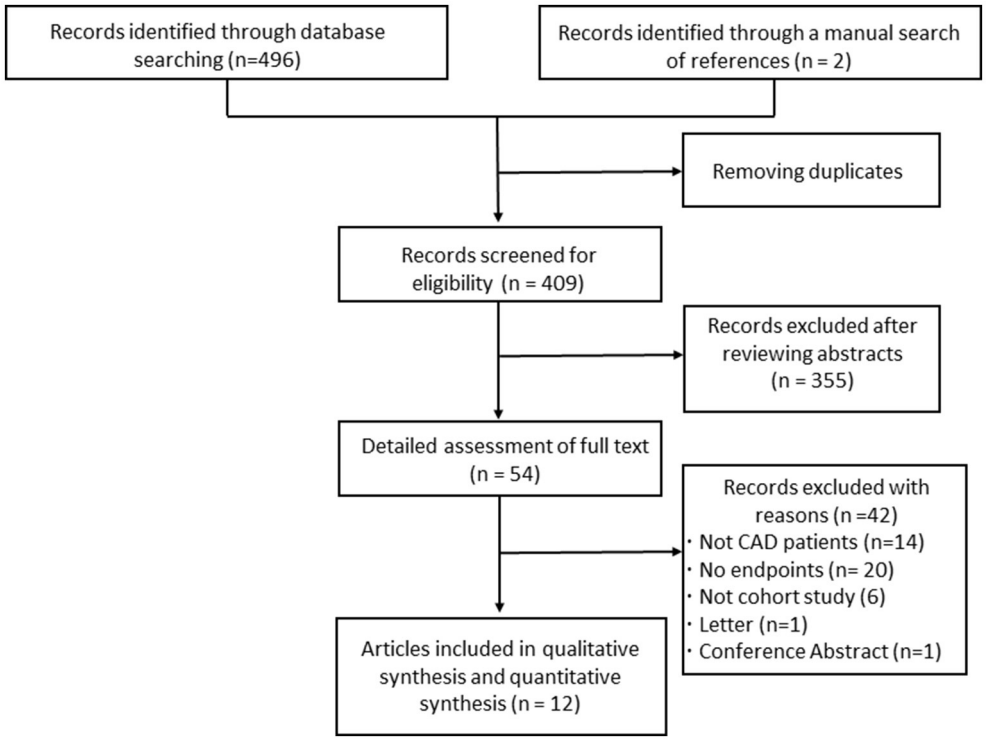

FIGURE 1 | Flowchart of the study selection process and results.

CV death, MI, stroke, and revascularization. To assess the relationship of the TyG index with the risk of future poor prognosis in individuals with CAD, we pooled the adjusted HRs and 95% CIs. If more than one model was used for the multivariate analysis, the most fully adjusted one was chosen. If outcomes were analyzed as relative risk (RR), we considered RR as an approximate HR in the meta-analysis (23). If the TyG index was evaluated as a categorical variable, the HR comparing the highest group of TyG index to the lowest group of TyG index was calculated. If the TyG index was evaluated as a continuous variable, the HR representing the risk of per 1-SD increment of the TyG index was calculated. I^2 statistic and Cochran's Q-test were used as a measure of heterogeneity across the included studies (24). If there was significant heterogeneity ($I^2 > 50\%$ or $P < 0.05$), sensitivity analysis, meta-regression analyses as well as subgroup analyses were conducted to explore the potential sources of heterogeneity. The random-effects model was used as the primary statistical analysis model in the estimation of pooled HR, even without significant heterogeneity, owing to the unavoidable clinical and methodological heterogeneity (e.g., demographics, medical history, medication, laboratory test of TyG index, and adjusted factors).

Sensitivity analysis by omitting one study at a time was conducted to assess the impact of every study on global HR. Meta-regression identifying the potential factors that might influence the pooled HR analyses were performed only if covariates were available in at least 10 studies. Moreover, we conducted subgroup analyses stratified by diabetes mellitus (DM) (with DM or without DM), age (<60 or ≥60), BMI (<26 or ≥26), LDL-C (<1.8 or ≥1.8), category of TyG index (median or tertiles), sample size (<2,000 or ≥2,000), follow-up duration (<36 or ≥36), study design (retrospective or prospective) and treatment (percutaneous coronary intervention, PCI). To assess the publication bias, we performed Begg's test or Egger's test (25, 26). $P < 0.05$ was considered as statistically significant. Statistical analyses were performed using STATA software version 12 (StataCorp LP, College Station, TX, USA).

RESULTS

Characteristics of the Included Studies

Figure 1 shows the literature selection process. About 12 studies (27–38) with a total of 28,795 patients with CAD were finally included in the qualitative and quantitative analysis. All the included studies were published within the last 5 years and were situated in China. Of the 12 studies, one (27) enrolled stable CAD, 10 (28–35, 37, 38) enrolled acute coronary syndrome (ACS), and one (36) enrolled both chronic coronary syndrome and ACS. The average age of 12 studies ranged from 55.7 to 66.3 years. The proportion of men ranged from 55.9 to 79.4%. The follow-up duration ranged from 12 to 48 months. Four studies (30, 31, 33, 34) included diabetic patients; three studies (32, 35, 36) included non-diabetic patients; five (27–29, 37, 38) studies included both diabetic patients and non-diabetic patients. Methodological quality assessment using NOS indicated the good quality of all enrolled works of literature (**Supplementary File 2**).

TABLE 1 | Characteristics of participants in the 12 included studies.

Study	Country	Study design	Subjects	Sample size	Male (%)	Mean age (years)	Follow-Up (months)	Diabetes (%)	BMI (kg/m^2)	The highest TyG index group vs. the lowest TyG index group	Mean LDL-C(mmol/L)
Jin et al. (27)	China	Prospective nested case-control	Stable CAD	1,740	72.1	59.5	36	26.9	25.75	<8.40 vs. >9.17	2.51
Mao et al. (28)	China	Prospective cohort study	NSTE-ACS	438	67.4	62.5	12	32.6	24.33	≤8.805 vs. >8.805	2.66
Hu et al. (29)	China	Retrospective cohort study	ACS after PCI	9,285	75.3	59.9	17.4	43.9	26.2	≤8.92 vs. >8.92	2.4
Wang et al. (30)	China	Retrospective cohort study	ACS	2,531	55.9	66.3	36	100	25.9	≤8.848 vs. ≥9.383	2.88
Ma et al. (31)	China	Retrospective cohort study	ACS after PCI	776	72.2	61	30	100	26.1	<8.80 vs. ≥9.29	2.4
Zhao et al. (32)	China	Retrospective cohort study	NSTE-ACS after PCI	1,510	73.7	59.7	48	0	25.8	≤8.72 vs. >8.72	2.57
Zhao et al. (33)	China	Retrospective cohort study	NSTE-ACS	798	68.3	60.9	36	100	26.7	<9.18 vs. ≥9.18	2.45
Zhang et al. (34)	China	Retrospective cohort study	AMI	1,932	68.5	65.4	26.8	100	25.8	≤8.91 vs. >9.54	2.58
Zhang et al. (35)	China	Retrospective cohort study	ACS	1,010	72.8	65	35.6	0	25.5	<8.33 vs. ≥8.33	1.5
Yang et al. (36)	China	Prospective cohort study	CAD after PCI	5,489	79.4	57.2	29	0	25.7	≤8.52 vs. ≥8.92	2.49
Zhao et al. (37)	China	Prospective cohort study	NSTE-ACS after PCI	2,107	72	60.02	24	34.2	26.08	≤8.87 vs. >8.87	2.52
Gao et al. (38)	China	Prospective cohort study	MINOCA	1,179	73.7	55.7	41.7	15.9	25.47	<8.52 vs. ≥8.99	2.29

ACS, acute coronary syndrome; AMI, acute myocardial infarction; NSTE, non-ST-segment elevation; PCI, percutaneous coronary intervention; BMI, Body mass index; MINOCA, myocardial infarction with non-obstructive coronary arteries; eGFR, estimated glomerular filtration rate; LDL-C, low-density lipoprotein cholesterol.

The main characteristics of 12 studies are shown in **Table 1**. The definition of MACEs and adjusted covariates in the individual enrolled studies are presented in **Supplementary File 3**.

Primary Outcome

In the meta-analysis of the TyG index evaluated as a categorical variable, 10 studies (28–35, 37, 38) reported the association between the TyG index and the risk of MACEs in patients with CAD. As illustrated in **Figure 2**, the pooled HR of MACEs comparing the highest TyG index group to the lowest TyG index group was 2.14 (95% CI: 1.69–2.71, $P < 0.001$). Nevertheless, we detected obvious heterogeneity across the 10 studies (I^2 =82.7%, $P < 0.001$). The approximately symmetric funnel plot indicated a low risk of publication bias which was consistent with the Egger's test ($P = 0.273$) (**Supplementary File 4**). Similarly, in the meta-analysis of TyG index evaluated as a continuous variable, a 1-SD increment in TyG index was associated with 70% higher risk of MACEs (HR: 1.7, 95% CI: 1.37–2.1, $P < 0.001$; heterogeneity: I^2 = 85.6%, $P < 0.001$; **Supplementary File 5**).

Secondary Outcomes

Compared with the lowest TyG index group, the highest TyG index group was associated with an increased risk of **MI** (HR: 1.87, 95% CI: 1.46–2.46, $P < 0.001$), **revascularization** (HR: 2.6, 95% CI: 1.76–3.84, $P < 0.001$), and **stroke** (HR: 1.56, 95% CI: 1.06–2.28, $P = 0.02$) in patients with CAD. However, no statistic difference was observed in the analysis of risk of **all-cause death** (HR: 1.33, 95% CI: 0.82-2.17, $P = 0.245$) **or CV death** (HR: 1.87, 95% CI: 0.9–3.88, $P = 0.094$). Evidence of publication bias was detected in the analysis of the risk of all-cause death (Egger's test: $P = 0.04$), but not in the analysis of the risk of MI, revascularization, or stroke by Begg's test or Egger's test (all $P > 0.05$). **Table 2** summarizes the HRs for secondary outcomes. Forest plots of secondary outcomes are presented in **Supplementary File 6**.

Sensitivity Analyses, Meta-Regression Analyses, and Subgroup Analyses

In the sensitivity analysis of primary outcome, we found that removing each study did not remarkably reduce the heterogeneity except the study of Zhang et al. (35) which slightly reduced the heterogeneity ($I^2 = 66.1\%$, $P = 0.003$). It should be noted that the study of Zhang et al. (35) differs from others in LDL-C below 1.8 mmol/L. Besides, sensitivity analysis of primary outcome revealed that the removal of any one study did not substantially alter the overall estimation of HR for MACEs. In the sensitivity analysis of secondary outcomes, removing anyone study did not dramatically decrease the heterogeneity among studies, nor did it markedly alter the pooled HR.

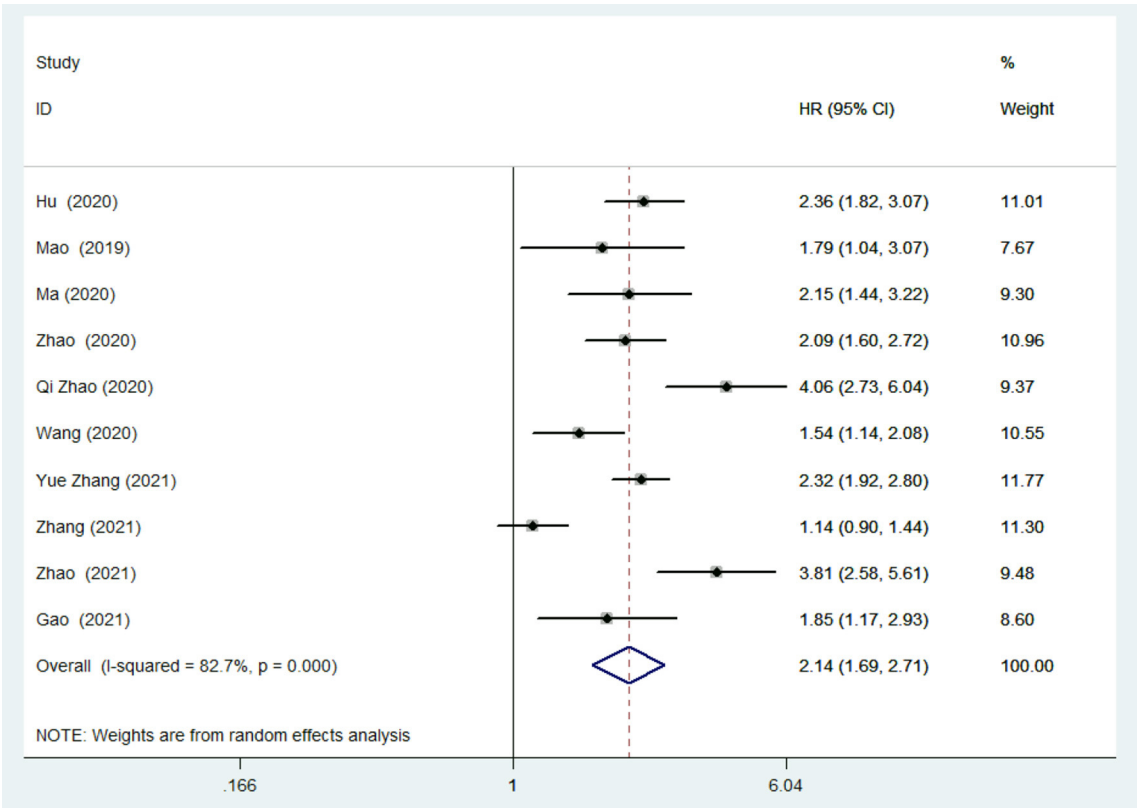

FIGURE 2 | Risk of major adverse cardiac events (MACEs) in the highest Triglyceride-glucose (TyG) index group compared with the lowest TyG index group.

TABLE 2 | Association between the Triglyceride-glucose (TyG) index and cardiovascular events.

Outcomes	No. of studies	HR (95%CI) and *P*-Value	Heterogeneity
Primary outcome			
MACEs	10	2.14 (1.69, 2.71), $P < 0.001$	82.7%, $P < 0.001$
Secondary outcomes			
All cause death	5	1.33 (0.82, 2.17), $P = 0.245$	65.4%, $P = 0.021$
CV death	3	1.87 (0.90, 3.88), $P = 0.094$	68.2%, $P = 0.043$
MI	5	1.89 (1.46, 2.46), $P < 0.001$	0%, $P = 0.96$
Revascularization	4	2.60 (1.76, 3.84), $P < 0.001$	71%, $P = 0.016$
Stroke	4	1.56 (1.06, 2.28), $P = 0.02$	0%, $P = 0.845$

Meta-regression analyses of primary outcome did not detect any correlations between HR of MACEs with sample size ($P = 0.781$), age ($P = 0.245$), proportion of male ($P = 0.654$), follow-up duration ($P = 0.611$), and BMI ($P = 0.074$).

In subgroup analyses of the primary outcome, we separated studies according to diabetes status (with DM or without DM), age (<60 or ≥60), BMI (<26 or ≥26), LDL-C (<1.8 or ≥1.8), category of TyG index (median or tertiles), sample size (<2,000 or ≥2,000), follow-up duration (<36 or ≥36) and study design (retrospective or prospective) and focused on the patients after PCI. As demonstrated in **Table 3**, there was a more pronounced risk of MACEs in populations with higher BMI than in those with lower BMI ($P = 0.03$ for interaction). No significant difference was identified across other subgroups (both $P > 0.05$ for interaction). Notably, the heterogeneity within the subgroup remarkably reduced in patients younger than 60 years old ($I^2 = 0\%$, $P = 0.624$) and moderately reduced in patients after PCI ($I^2 = 55.8\%$, $P = 0.079$) as well as studies in which TyG index was reported as tertiles ($I^2 = 44.7\%$, $P = 0.143$), meaning that age, treatment of PCI and category of TyG index might be the factors contributing to heterogeneity. In the subgroup analyses of secondary outcomes, we stratified studies according to diabetes status. As demonstrated in **Table 4**, there was no evidence of statistical heterogeneity between diabetic patients and non-diabetic patients in the risk of all-cause death, MI, revascularization, and stroke associated with elevated TyG index (both $P > 0.05$ for interaction).

DISCUSSION

This meta-analysis shows that the elevated TyG index is associated with increased risk of MACEs, MI, revascularization,

TABLE 3 | Subgroup analyses of the primary outcome.

Subgroups	No. of studies	HR (95%CI) and *P*-value	Heterogeneity within subgroup	*P* for interaction
Diabetes status				
With DM	6	2.49 (1.86, 3.34), $P < 0.001$	72.9%, $P = 0.002$	0.41
Without DM	4	1.95 (1.20, 3.17), $P < 0.001$	89.2%, $P < 0.001$	
Age				
<60	3	2.16 (1.82, 2.57), $P < 0.001$	0%, $P = 0.624$	0.93
≥60	7	2.17 (1.53, 3.06), $P < 0.001$	88.1%, $P < 0.001$	
BMI				
<26	6	1.74 (1.34, 2.26), $P < 0.001$	78.9%, $P < 0.001$	0.03
≥26	4	2.94 (2.15, 4.01), $P < 0.001$	66.9%, $P = 0.028$	
Category				
Median	6	2.30 (1.54, 3.42), $P < 0.001$	89.2%, $P < 0.001$	0.56
Tertiles	4	1.98 (1.60, 2.45), $P < 0.001$	44.7%, $P = 0.143$	
Sample size				
<2,000	3	2.05 (1.52, 2.76), $P < 0.001$	83.8%, $P < 0.001$	0.62
≥2,000	7	2.37 (1.48, 3.77), $P < 0.001$	85.0%, $P = 0.001$	
Follow-Up				
<36	6	2.11 (1.51, 2.94), $P < 0.001$	86.4%, $P < 0.001$	0.88
≥36	4	2.20 (1.49, 3.23), $P < 0.001$	80.1%, $P = 0.002$	
Study design				
Retrospective	7	2.06 (1.57, 2.71), $P < 0.001$	85.5%, $P < 0.001$	0.63
Prospective	3	2.38 (1.42, 3.99), $P = 0.001$	73.7%, $P = 0.022$	
LDL-C				
<1.8	1	1.14 (0.90, 1.44), $P = 0.28$		
≥1.8	9	2.32 (1.92, 2.79), $P < 0.001$	66.1%, $P = 0.003$	
PCI	4	2.45 (1.94, 3.14), $P < 0.001$	55.8%, $P = 0.079$	

TABLE 4 | Subgroup analyses of secondary outcomes.

Subgroups	No. of studies	HR (95%CI) and *P*-value	Heterogeneity within subgroup	*P* for interaction
All-Cause death				
With DM	3	1.64 (0.95, 2.84), $P = 0.08$	67.8%, $P = 0.045$	0.28
Without DM	2	0.93 (0.53, 1.64), $P = 0.79$	0%, $P = 0.457$	
MI				
With DM	3	1.93 (1.41, 2.64), $P < 0.001$	0%, $P = 0.837$	0.85
Without DM	2	1.82 (1.13, 2.93), $P = 0.01$	0%, $P = 0.653$	
Revascularization				
With DM	2	3.41 (1.68, 6.95), $P = 0.001$	81%, $P = 0.022$	0.31
Without DM	2	2.09 (1.58, 2.76), $P < 0.001$	0%, $P = 0.339$	
Stroke				
With DM	2	1.65 (1.01, 2.67), $P = 0.04$	0%, $P = 0.429$	0.75
Without DM	2	1.43 (0.77, 2.63), $P = 0.26$	0%, $P = 0.80$	

and stroke in patients with CAD. A greater risk of MACEs is observed among participants with higher BMI. Nevertheless, the current evidence suggests that the TyG index may not be an indicator of CV mortality or all-cause mortality in patients with CAD.

Consistent with a previous study (39), our findings demonstrated an association between IR the poor cardiovascular outcomes in patients with CAD. In the present study, individuals with a higher TyG index have a 2.14-fold greater risk for MACEs compared with individuals with a lower TyG index. Similarly, in the study of Uetani et al. (39), there was a 1.98-fold higher risk for cardiovascular events among subjects with higher HOMA-IR than those with lower HOMA-IR. Besides, we found a more significant risk of MACEs in patients with higher BMI, which is partly attributed to obesity-linked inflammation and abnormal metabolism (40). Previous works of literature had reported that metabolic disorders, including IR, diabetes, and prediabetes played an important role in the development of CAD (4, 5, 41). A meta-analysis including 129 studies showed that prediabetes was associated with poor prognosis in patients with and without baseline atherosclerotic cardiovascular disease (41). TyG index, a marker of IR, has been reported to be correlated with the development of prediabetes and diabetes mellitus (42, 43). Our subgroup analyses provided supportive evidence that the TyG index was a predictor of MACEs in patients with CAD and diabetes mellitus. Nevertheless, our subgroup analyses did not reveal significant differences across the participants with or without diabetes mellitus ($P > 0.05$ for interaction). Age, the concentration of LDL-C, and treatment of PCI are the suspect factors of heterogeneity. However, we did not detect a significant difference in the subgroup analyses stratified by age. We failed to compare the risk of MACEs between patients with LDL-C

below 1.8 mmol/L and patients with above 1.8 mmol/L, and between patients treated with PCI and patients not treated with PCI, due to the limited number of studies. Thus, further studies are needed to elucidate the effect of those suspect factors on the risk of MACEs associated with elevated TyG index in patients with CAD.

In this meta-analysis, five articles (30, 32–35) reported the relationship between the TyG index with all-cause mortality in CAD populations, and our findings showed that the TyG index was not associated with all-cause death in CAD regardless of diabetes status. An analogous result was obtained in the study of Drwila et al. (18) among non-diabetic patients with AMI. In contrast, numerous studies reported the increased risk of all-cause death associated with elevated TyG index. In a retrospective observational study among the general population, compared with the subjects in the lowest TyG index group, those in the highest TyG index group showed a 51% increased risk for all-cause death (44). Similarly, in a study of patients with ischemic stroke, compared with patients in the first quartile of TyG index, those in the fourth quartile of TyG index showed a 25% increase in all-cause mortality (45). Differences in comorbidities of participants and the corresponding drug therapies that improved survival, as well as the publication bias in the analysis of all-cause mortality may interpret this negative finding.

In this meta-analysis, only three studies (28, 34, 35) assessed the relationship between the TyG index with the risk of CV death in CAD populations. Our findings did not show the predictive value of the TyG index for CV death. Similarly, in a study of the general population (44), no significant increase in the occurrence of CV death was observed among subjects with the higher TyG index compared to subjects with the lower TyG index. In contrast, in patients with chronic heart failure and type 2 diabetes, Guo et al. (46) observed that the elevated TyG index was associated with higher CV mortality. These conflicting results might be partly attributed to the limited number of studies enrolled in our analysis and the heterogeneity of study populations. In the study by Liu et al. (44), a general population was analyzed. While in the study by Guo et al. (46), patients with chronic heart failure and type 2 diabetes were analyzed. Health status may affect the relationship of the TyG index with the risk of CV death. Moreover, for patients with CAD, the strengthened control of cardiovascular risk factors reduced the risk of CV death. Thus, changes in the TyG index over time should be examined and monitored.

In the present study, compared with the lowest TyG index group, patients in the highest TyG index group have a 1.89-fold risk and 2.6-fold risk for MI and revascularization, respectively. Similarly, in a prospective cohort study with 98,849 subjects, there was a 2-fold higher risk for MI in participants with a higher TyG index (47). Accumulating evidence indicated that the elevated TyG index was linked to accelerated atherosclerosis. The study of Won et al. (11) found that patients with elevated TyG index had a more remarkable increase in coronary plaque volume. The study of Lee et al. (48) demonstrated that there was a >3-fold increase in the incidence of coronary artery stenosis in subjects with elevated TyG index. Several cross-sectional observational studies among the Chinese and Korean populations illustrated that the TyG index was linked to arterial stiffness (49, 50). Moreover, an elevated TyG index was positively correlated with in-stent restenosis in patients after drug-eluting stent (51). These data supported that a high TyG index was closely related to accelerated progression of CAD, cumulatively leading to a high risk of revascularization.

As for the risk of stroke associated with elevated TyG index, an earlier meta-analysis showed that a higher TyG index was associated with a 26% increased risk of stroke among participants without atherosclerotic cardiovascular diseases at baseline (52). Similarly, in a cross-sectional study with a general population, the incidence of ischemic stroke increased by 22.8% for each additional SD in the TyG index (53). Our results are consistent with the earlier findings that elevated TyG index is related to the increased risk of stroke in patients with CAD. Nevertheless, this association was not observed in non-diabetic patients in our subgroup analyses. The difference between the two subgroups (with DM or without DM) was not statistically significant ($P = 0.75$ for interaction). To confirm the predictive role of the TyG index in non-diabetic patients with CAD, further studies are needed.

It has been reported that IR, prediabetes, and diabetes may mediate the development and progression of heart failure (54, 55). A meta-analysis comprising 28,643 participants showed that prediabetes was associated with worse outcomes in patients with heart failure (55). TyG index, a biomarker of IR, prediabetes, and diabetes, may be linked with the occurrence of heart failure. Indeed, a recent study demonstrated that the TyG index was positively associated with myocardial fibrosis as well as the risk of all-cause mortality and heart failure hospitalization in patients with heart failure (56). However, in this meta-analysis, the relationship between TyG index and heart failure in patients with CAD was not evaluated since only two studies (28, 38) reported the risk of heart failure. In the study by Mao et al. (28), no difference was observed in the occurrence of heart failure between the high TyG index group and the low TyG index group. The consistent result was shown in the study by Gao et al. (38) that the incidence of hospitalization for heart failure did not increase in parallel with the TyG index tertiles ($P = 0.08$). The predictive role of the TyG index for heart failure in patients with CAD needs further assessment.

There are several limitations in the present study. First, all the participates included in our study were from China. The prognostic role of the TyG index for CAD in other countries remains unclear. Second, there were a small number of studies assessing the relationship of the TyG index with the risk of CV death, revascularization, and stroke in individuals with CAD. Additionally, the predictive capacity of the TyG index for adverse events is not separately analyzed in different types of CAD due to a limited number of studies. A study on the prognostic significance of the TyG index in chronic coronary syndrome is lacking. Finally, our analysis only focused on the prognostic value of the baseline TyG index in CAD. The effect of longitudinal changes in the TyG index on the risk of MACEs in patients with CAD remains uncertain. Thus, one should be cautious to interpret the results of our study. More large-scale prospective studies

should be conducted to validate the prognostic significance of the TyG index for adverse cardiovascular outcomes in patients with CAD.

CONCLUSION

In this meta-analysis, the elevated TyG index was found to be closely associated with increased occurrence of MACEs, MI, revascularization, and stroke in patients with CAD, and a stronger risk of MACEs was observed in patients with higher BMI. These data provided a rationale to consider the elevated TyG index as a valuable predictor of cardiovascular events in CAD. Measuring the TyG index could contribute to risk identification and proper management in patients with CAD.

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/s.

AUTHOR CONTRIBUTIONS

J-WL, W-HD, and D-ZS conceived the idea of this meta-analysis. J-WL developed a protocol with the assistance of W-HD. J-WL and Y-QY performed the data analyses independently. J-WL and LS participated in the data interpretation and drafting of the manuscript. D-ZS and W-HD critically reviewed the manuscript. All the authors had browsed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.774781/full#supplementary-material

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Insulin Resistance Promotes the Formation of Aortic Dissection by Inducing the Phenotypic Switch of Vascular Smooth Muscle Cells

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Background: Insulin resistance (IR) plays a key role in the development of type 2 diabetes mellitus (T2DM) and is one of its most important characteristics. Previous studies have shown that IR and T2DM were independent risk factors for a variety of cardiovascular and cerebrovascular diseases. However, there are few studies on the relationship between IR and aortic dissection (AD). The goal of this research was to find evidence that IR promotes the occurrence of AD.

Methods: Through the statistical analysis, we determined the proportion of glycosylated hemoglobin (HbA1c) abnormalities (HbA1c $>$ 5.7) in people with acute thoracic aortic dissection (ATAD) and compared the difference of messenger RNA (mRNA) and protein expression of GluT1 in the thoracic aorta of normal people and those with ATAD to find evidence that IR is a causative factor in AD. The mouse model of IR and AD and the IR model of human aortic vascular smooth muscle cells (HA-VSMC) were established. Real time-PCR (RT-PCR) and Western blotting were used to study the mRNA and protein expression. Hematoxylin and eosin (H&E), Masson, and elastic fiber staining, and immunofluorescence were used to study the morphological structure.

Results: The proportion of HbA1c abnormalities in patients with ATAD was 59.37%, and the mRNA and protein expression of GluT1 were significantly lower than that in normal people. Fasting glucose concentration (FGC), serum insulin concentration (SIC), and the homeostasis model assessment of insulin resistance (HOMA-IR) of mice was obviously increased in the high-fat diet group and the protein expressions of Glut1 and GluT4 were reduced, indicating that the mouse IR model was successfully established. The incidence of AD was different between the two groups (IR: 13/14, Ctrl: 6/14), and the protein expression of MMP2, MMP9, and OPN were upregulated and SM22 and α-SMA were downregulated in mice. The expressions of mRNA and protein of GluT1 and SM22 in HA-VSMCs with IR were reduced and OPN was increased.

Conclusion: Combined results of clinical findings, mouse models, and cell experiments show that IR induced the phenotypic switching of vascular smooth muscle cells (VSMCs) from contractile to synthetic, which contributes to the occurrence of AD. It provides a basis for further research on the specific mechanism of how IR results in AD and a new approach for the prevention and treatment of AD.

Keywords: insulin resistance, phenotypic switch, aortic dissection, vascular smooth muscle cells, promotes

INTRODUCTION

Acute aortic dissection (AAD) is a life-threatening disease, and without treatment, the fatality rate rises by 1–2% per hour after the onset of symptoms (1, 2). With the development of diagnostic imaging technology, it can be identified and typed quickly and accurately. Surgical repair is the first choice for type A aortic dissection (AD), and type B is treated mainly by drugs or stent endovascular repair (3). However, the mortality in severe complications caused by vascular tear damage is still very high (4).

Previous studies showed that gender (5), age (4), hypertension (2, 6), aneurysm (7, 8), arteritis (9, 10), and atherosclerosis (11) were independent high-risk factors for acute thoracic aortic dissection (ATAD) and promote its occurrence and development. However, most clinical studies have shown that diabetes was negatively correlated with the occurrence of AD (12–15) and was also inversely related to the rupture of aneurysm and enlargement of the aneurysm volume (16–18).

Insulin resistance (IR) accompanies the occurrence and development of type 2 diabetes mellitus (T2DM), which is one of its most important features and is related to atherosclerotic diseases such as coronary artery disease, cerebrovascular disease, carotid artery stenosis, and peripheral vascular disease (19). Studies have shown that C-peptide, serum insulin concentration (SIC), and the homeostasis model assessment of insulin resistance (HOMA-IR) were significantly upregulated in patients with a larger aneurysm diameter (20), and HbA1c was a high-risk factor for AD (21). There have been few studies on the relationship between IR and AD. IR affects nutrient metabolism, inflammatory infiltration, and the release and activation of inflammatory cytokines, which is why we consider that IR is a causative factor of AD.

Through epidemiological investigations, we found that IR may be a high-risk factor for ATAD, and the discrepancies of the messenger RNA (mRNA) and protein expression of GluT1 in thoracic aortic smooth muscle cells (TASMC) between normal and patients with ATAD confirmed this. The IR models of mouse and human aortic vascular smooth muscle cells (HA-VSMCs) indicate that IR induced the phenotypic switching of vascular smooth muscle cells (VSMCs) from contractile to synthetic, which promotes the occurrence of AD. This provides evidence for further research on the definite mechanism and a new approach for the prevention and treatment of AD.

MATERIALS AND METHODS

Cell Culture and Establishment of the HA-VSMC IR Model

The HA-VSMC line T/G HA-VSMC (HTX2061) was maintained and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (PAN Biotech, Germany) and 1% penicillin/G-streptomycin sulfate in a 5% CO_2 humidified atmosphere with a constant temperature of 37°C. The T/G HA-VSMCs IR model was established by culturing cells in DMEM containing 10% FBS (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} mol/L), insulin, and 1% penicillin/G-streptomycin sulfate after 24 h of serum depletion in a 5% carbon dioxide (CO_2) humidified atmosphere with a constant temperature of 37°C. The glucose oxidase method (Glucose Determination Kit, Rubio, China) was used to detect the consumption of glucose in the culture medium, and the number of cells was detected by the Cell-Counting Kit-8 (Do Jindo, Japan). The glucose consumption of the same cells was used to screen out the insulin concentration and the action time of the highest state of IR.

Development of the Mouse Model

Three-week-old $AopE^{-/-}$ male mice (C57BL/6 background) were purchased from GenPharmatech Co. Ltd. (Nanjing, China). All mice were randomly divided into two groups and fed a regular diet [control group: normal diet; IR group: 60% high-fat diet (D12492)] and given beta-aminoproprionitrile (BAPN) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in drinking water (0.25%) for 4 weeks. At 7 weeks of

TABLE 1 | Antibodies used.

Antibody	Manufacturer	Catalog number	Source	Dilutions
GluT1	Immunoway	YT1928	Rabbit	1:1,000
GluT4	Immunoway	YT5523	Rabbit	1:1,000
SM22	Proteintech	60213-1-Ig	Mouse	1:5,000
α-SMA	Bioss	Bs-10196R	Rabbit	1:10,000
OPN	BOSTER	BM4208	Rabbit	1:1,000
MMP2	Proteintech	66366-1-Ig	Mouse	1:5,000
MMP9(anti-human)	Abcam	ab76003	Rabbit	1:2,500
MMP9(anti-mouse)	Abcam	ab228402	Rabbit	1:1,000
β-actin	BOSTER	BM0627	Mouse	1:10,000
Goat anti-mouse	Immunoway	RS0001	Goat	1:10,000
Goat anti-rabbit	Immunoway	RS0002	Goat	1:10,000

TABLE 2 | Primer sets.

Gene	Forward primer	Reverse primer
MMP2	5′-CTGTTGCTGCCCATCTGAAG-3′	5′-AGAATGATGGGCACTACCGTG-3′
MMP9	5′-TTGACAGCGACAAGAAGTGG-3′	5′-CCTCAGTGAAGCGGTACATAG-3′
OPN	5′-TGAGCATTCCGATGTGATTGAT-3′	5′-GGTCTACAACCAGCATATCTTCAT-3′
SM22	5′-AGAATGATGGGCACTACCGTG-3′	5′-CTGTTGCTGCCCATCTGAAG-3′
GluT1	5′-TCTGGCATCAACGCTGTCTT-3′	5′-CCGTGTTGACGATACCGGAG-3′
β-actin	5′-TGACGTGGACATCCGCAAAG-3′	5′-CTGGAAGGTGGACAGCGAGG-3′

age, osmotic mini-pumps (Alzet, Cupertino, CA, USA) delivering 1 ug/kg/min Ang-II (Sigma-Aldrich) were implanted subcutaneously, and the mice were euthanized 24 h after implantation. Fresh aortic tissue was obtained, liquid nitrogen was used to maintain the samples for protein verification, and 4% paraformaldehyde was used to fix specimens before paraffin embedding.

Fasting glucose concentration (FGC) was measured using reagent strips read in a glucose meter (YSI 2300-STAT), and SIC was measured using mouse insulin (INS) ELISA-kits (CSB-E05071m, CUSABIO, China) by the tail-cuff method after grouping and before implantation. The the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated to evaluate insulin resistance (HOMA-IR=[FGC*SIC/22.5]).

All studies in mice were approved by the Laboratory Animal Care and Use Committee of the School of Fujian Medical University (No. 2020-0089).

Epidemiological Investigation

We collected data on patients with ATAD in cardiac surgery from July 2017 to June 2020 in Fujian Medical University Union Hospital and excluded patients with Marfan syndrome, aortic arteritis, type 1 diabetes a history of syphilis, and excluded those who had not undergone HbA1c testing at the time of admission. In total, 3,185 participants were identified, and a total of 552 patients met the requirements.

Human Thoracic Aorta Tissues Samples

Normal thoracic aorta tissue from heart transplant donors was discarded during the operation. The thoracic aorta tissues of the ATAD groups were obtained from the tissues removed during thoracic aortic replacement surgery.

Samples of the intima and externa were removed and washed with cold phosphate buffer solution (PBS), then placed in liquid nitrogen, an RNA protection solution, and 4% paraformaldehyde, respectively, for subsequent protein and RNA extraction and paraffin embedding.

All procedures were approved by the Ethics Committee at the Fujian Medical University Union Hospital (No. 2021QH026).

Western Blot Analysis

Proteins were extracted from human aortic tissue, mouse aortic tissue, and HA-VSMCs using a radioimmunoprecipitation assay (RIPA) buffer containing phenylmethylsulfonyl fluoride (PMSF) (Bio Sharp, China). The total protein levels of each sample were quantified using the bicinchoninic (BCA) protein assay (Beyotime Biotechnology, China). Proteins were separated on SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) (Bio Sharp) membranes. Membranes were placed in 5% skimmed milk for 1 h at room temperature and then incubated with various primary antibodies (**Table 1**) overnight at 4°C. Subsequently, secondary antibody incubations were carried out at 37°C for 1 h. Signals were detected and quantified using the Chemiluminescent HRP Substrate (Millipore, USA) and the band density was quantified with Image J (NIH, USA).

RT-qPCR

Real-time- qPCR (RT-qPCR) was used to detect the gene expression levels of GluT1, GluT4, MMP-9, MMP-2, OPN, SM22, and α-SMA (**Table 2**). RNA was isolated using the TRIzol Reagent (Ambion, USA) and reverse transcribed using the reverse transcription kit (Takara, Japan) to determine these gene expression levels. β-actin was applied as an internal control for RNA-related experiments. The PCR primers are shown in **Table 1**. RT-qPCR was performed using SYBR green dye on the Biosystems 7500 Real-Time PCR System (Applied Biosystems, USA). Manufacturer protocols provided by Prime Script^{TM} RT-PCR Kit and the Fast Start Essential DNA Green Master (Roche, Germany) were used.

Immunofluorescence

Human and mouse thoracic aorta tissues were embedded with paraffin and sliced into 2.5-μm thick sections. Paraffin slides were treated in an oven (65°C) and soaked in xylene and alcohol with different concentration gradients for dewaxing, followed by 3% H_2O_2 for 20 min and blocked with goat serum. Further, the slides were immune-stained with antibodies against GluT1 (Immunoway, Catalog NO:YT1928, 1:100) overnight at 4°C. Subsequently, secondary antibody (Alexa Fluor555-labeled donkey anti-rabbit IgG, Beyotime, Catalog NO: A0453, 1:500) incubations were carried out at 37°C for 1 h. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) fluorescent stain was used to stain the nucleus for 15 min. These sections were mounted and visualized under a Nikon fluorescence microscope (Nikon ECLIPSE Ni).

The HA-VSMCs were seeded in six-well Nunc Lab-Tek Chamber Slide (Thermofisher, Catalog #177399) and cells were treated with DEMM and 10^{-7} mol/L insulin after 24 h of serum depletion. After treatment for 36 h, cells were washed with phosphate-buffered saline (PBS) and fixed with 4%

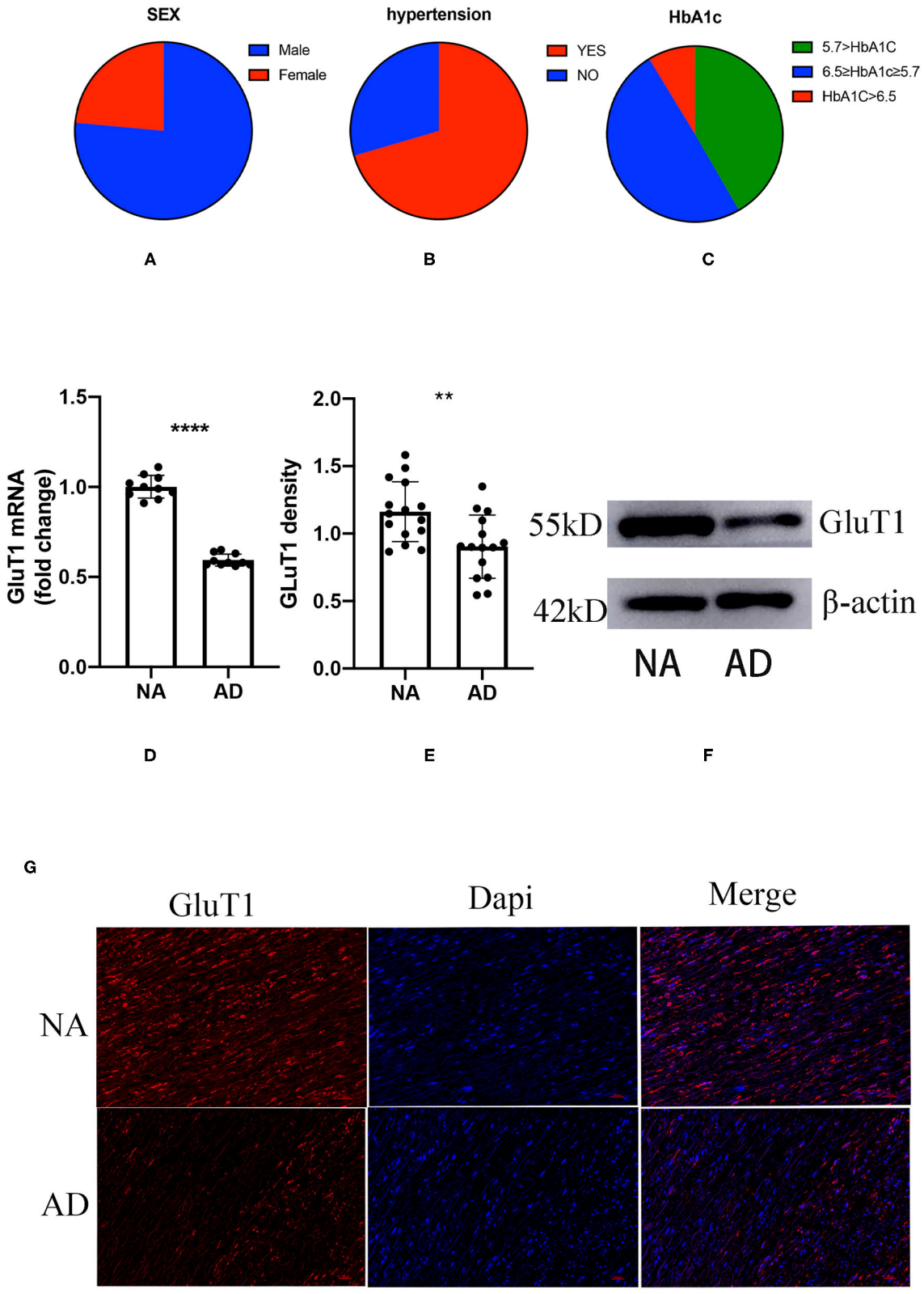

FIGURE 1 | The proportion of gender, hypertension, and glycosylated hemoglobin (HbA1c) in patients with acute thoracic aortic dissection (ATAD); the difference between aortic dissection (AD) and donors (NA) in the GluT1 expression in TAVSMCs. **(A)** M/F:76.45%:/23.55%; **(B)** hypertension/no-hypertension: 70.47%/29.53%; **(C)** HbA1c ≥ 6.5, 8.7%; 6.5 > HbA1c ≥ 5.7, 49.64%; HbA1c < 5.7, 41.67%; **(D)** qPCR analysis of GluT1mRNA levels, ****$P < 0.0001$. **(E,F)** WB analysis of GluT1

(Continued)

FIGURE 1 | protein levels, $^{**}P < 0.01$. **(G)** Immunofluorescence staining of GluT1 (red) in aortic human vascular smooth muscle cell (HVSMC) cells which from AD and NA, DAPI staining was used to visualize cell nuclei. Scale bar = 100 μm.

PFA, and permeabilized with 0.2% Triton-X 100 in 1X PBS for 15 min. Further, cells were immune-stained with primary antibodies (GluT1, Immunoway, Catalog YT1928, 1:100; SM22, Proteintech, Catalog 60213-1-Ig, 1:100; and OPN, BOSTER, Catalog BM4208, 1:50) overnight at 4°C and secondary antibodies (Alexa Fluor555-labeled donkey anti-rabbit IgG, Beyotime, Catalog No.: A0453, 1:500 and FITC-labeled goat anti-mouse IgG, Beyotime, Catalog No.:A0568, 1:500) incubations were carried out at 37°C for 1 h. DAPI was used to stain the nucleus for 15 min and samples were mounted and visualized by a Nikon fluorescence microscope (Nikon ECLIPSE Ni).

Histopathological Analysis

Complete gross and histopathological evaluations were performed with samples from the control and IR group mice. After euthanasia, aortic tissues were harvested from the thoracic aorta and were fixed in 4% paraformaldehyde, then were sectioned at 2.5-μm thickness after fixation and paraffin embedding, stained with hematoxylin and eosin (H&E) following standard procedures, and examined under light microscopy.

Elastin Staining

Elastin in the normal and dissected aortas was stained with Verhöeff elastic fiber dyeing liquid using an elastic fiber staining kit (Yuanye Bio-Technology Co. Ltd., Shanghai, China), according to the manufacturer's instructions.

Masson Staining

The collagenous fiber was stained with Masson's trichrome stain kit (Beijing Solar Bioscience and Technology Co. Ltd, Beijing, China), according to the manufacturer's instructions.

Statistical Analysis

All data are expressed as mean ± SEM. Unpaired *t*-tests were used to analyze the differences between the two groups, one-way ANOVA was applied to assess the differences among comparisons of more than two groups. Prism 8.0 software (GraphPad, San Diego, CA, USA) was used for statistical analysis. All data were analyzed using two-tailed tests and $P < 0.05$ were defined as statistically significant: $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***; $P < 0.0001$ ****.

RESULTS

Clinical Phenomenon and Human Thoracic Aortic Tissue Verification Results

The homeostasis model assessment of insulin resistance (HOMA-IR) is the most common index used for clinical evaluation of insulin resistance, but the patients with ATAD require emergency surgery, and it is difficult to collect FGC and SIC. However, HbA1c is not affected by diet, so we choose HbA1c as an indirect indicator of IR to reflect the extent of IR in patients with ATAD. Of our subjects, 76.45% were male, age 54.12 ± 12.13 years, and 23.55% were female, age 56.77 ± 11.18 years among the patients who met the criteria (**Figure 1A**). There were 70.47% with a history of hypertension (**Figure 1B**); HbA1c ≥ 6.5 8.7%, HbA1c < 5.7:41.67%, 6.5 > HbA1c ≥ 5.7:49.64% (**Figure 1C**). According to the diagnostic criteria for IR, we found that the majority of patients with AD simultaneously had IR.

To clarify the relationship between ATAD and IR, we used quantitative PCR (qPCR) to detect the expression of GluT1 in ascending aortic vascular smooth muscle. The results showed that the mRNA expression of GluT1 in VSMC of patients with ATAD was significantly reduced ($P < 0.0001$, **Figure 1D**)

GluT1 is a functional membrane protein that transports glucose into cells for metabolism. Using western blot technology, we found that the GluT1 of the TASMCs of patients with ATAD was significantly lower than that of normal people ($P < 0.01$) (**Figures 1E,F**). Moreover, we obtained a similar result using immunofluorescence that showed that GluT1 is reduced (**Figure 1G**). Based on these experimental results, we believe that IR is a high-risk factor for AAD.

Animal Studies

A High-Fat Diet Can Increase FGC, SIC, and HOMA-IR in Mice

After the ApoE$^{-/-}$ mice were quarantined and adapted, they were randomly divided into two groups (**Figure 2A**). The FGC was measured after 12 h of starvation and the tail vein blood was collected to obtain SIC. The HOMA-IR was calculated. There was no difference between the two groups in FGC, SIC, and HOMA-IR ($P = 0.7073$, $P = 0.945$, $P = 0.466$) (**Figures 2B–D**). After 4 weeks of feeding with different diets, it was found that FGC, SIC, and HOMA-IR in the high-fat diet group were significantly increased, all higher than in the ordinary diet group ($P = 0.0002$, $P = 0.0093$, $P = 0.0009$) (**Figures 2E–G**).

A High-Fat Diet Induces Decreased Expression of GluT1 and GluT4 Decline in the VSMCs in ApoE$^{-/-}$ Mice

To determine whether there was IR in mouse aortic tissue, we collected fresh aortic tissue of the mice, extracted the proteins, and used western blot to analyze the protein expression of Glut1 and GluT4 in the two groups. The results showed that the protein expression of Glut1 and GluT4 in the intervention group was significantly lower than that of the normal diet group ($P < 0.01$, $P < 0.01$) (**Figures 3A–C**), and results were further confirmed by immunofluorescence (**Figure 3D**).

The Incidence of AD in the IR Group Mice Was Higher Than in the Control Group

All mice had access to drinking water containing 0.25% BAPN, and the time of the occurrence of AD was recorded. The results

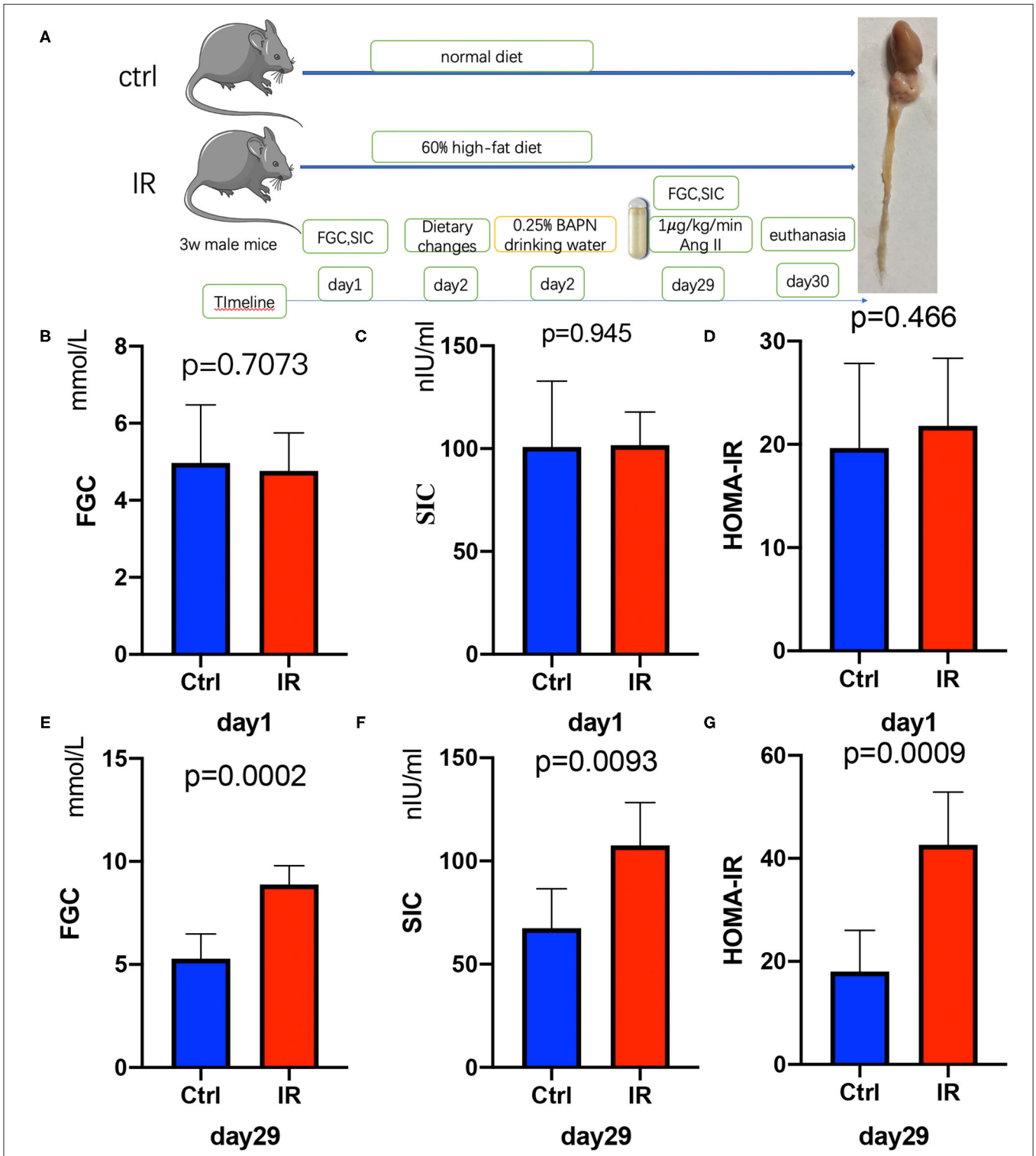

FIGURE 2 | Establishment of insulin resistance (IR) and AD model in ApoE$^{-/-}$ mice, Test fasting glucose concentration (FGC), serum insulin concentration (SIC), and calculate homeostasis model assessment of insulin resistance (HOMA-IR). **(A)** The flow diagram of animal experiments. **(B–D)** The FGC (P =.7073), SIC (P = 0.945), and HOMA-IR (P = 0.466) on day 1 in the two groups. **(E–G)** The FGC (P = 0.0002), SIC (P = 0.0093), and HOMA-IR (P = 0.0009) on day 29.

showed that the IR group of mice had AD in 13 of 14, and 8 of them died from AD before using Ang-II; 6 of 14 of the mice in the normal diet group were diagnosed as AD and 2 of them died from AD before implanting the Ang-II pump (**Figures 4A–C**).

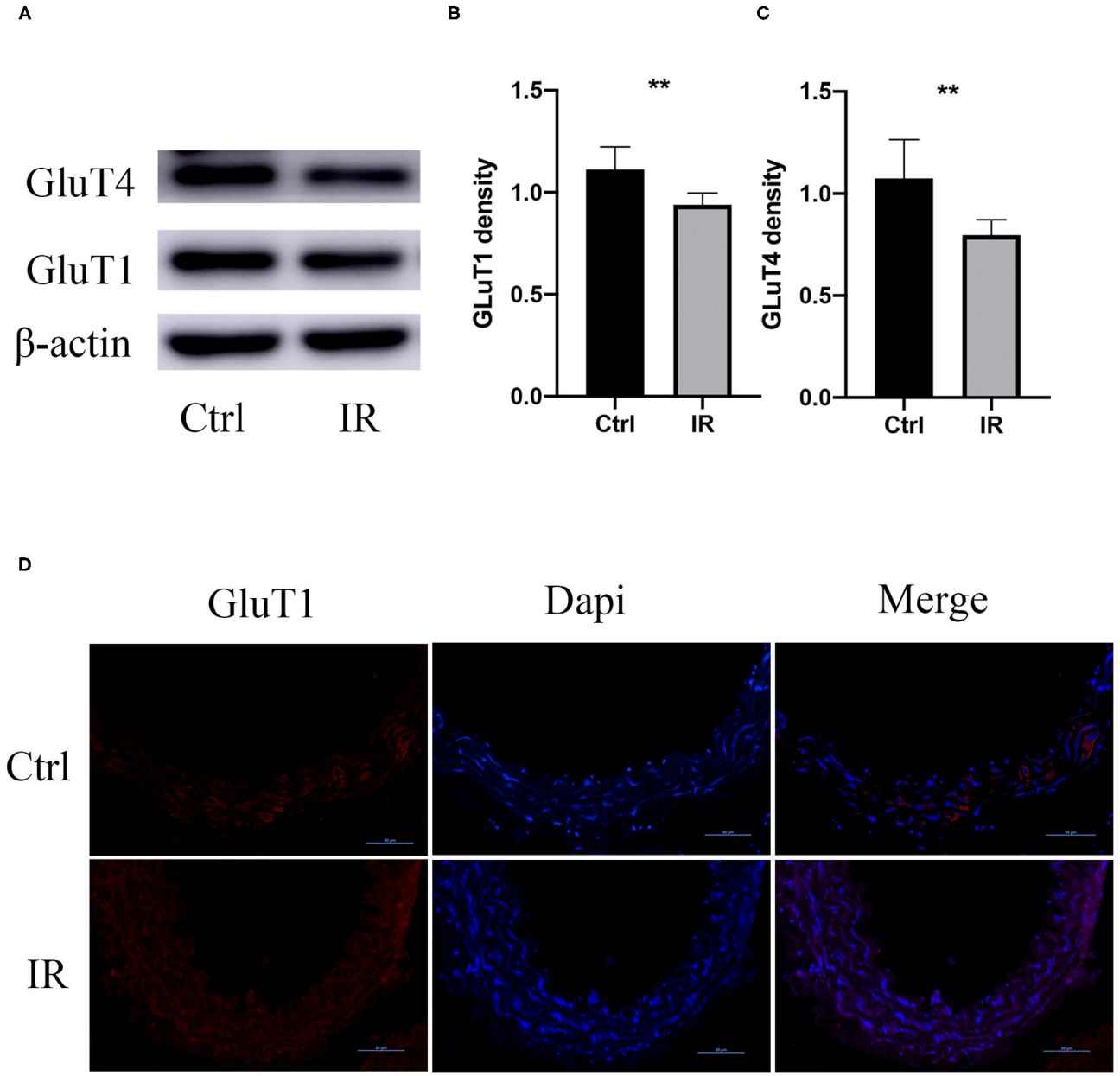

FIGURE 3 | GluT1 and GluT4 protein expression in mouse aortic tissue. **(A–C)** WB analysis of GluT1 and GluT4 protein levels, $^{**}P < 0.01$, $^{**}P < 0.01$. **(D)** Immunofluorescence staining of GluT1 (red) in aortic MSMC cells from IR and control groups. DAPI staining was used to visualize cell nuclei. Scale bar = 50 μm.

The mice aorta were embedded in paraffin after fixation with 4% paraformaldehyde and dehydration. The sections were stained with H&E, elastic fiber, and collagenous fiber for observation under the microscope. Compared with the control group of mice, the number of smooth muscle cells increased in the IR group, and the arrangement was disordered and the basement membranes thinner. The extracellular matrix was increased, the elastic fiber layer was thinner with smaller, broken fibers, and there were more collagen fibers (**Figures 4D–F**).

Phenotype Switch of AVSMCs and MMPs Is Upregulated in the IR Group

The phenotypic switch of aortic vascular smooth muscle cells (AVSMCs) is a characteristic of AD; MMP2 and MMP9 are the most common metalloproteinases that promote AD. We extracted tissue proteins from fresh mouse aortic tissue and used western blot to quantity the protein expression of SM22, α-SMA, OPN, MMP2, and MMP9. The results showed that the protein expression of OPN, MMP2, and MMP9 ($P < 0.05$, $P < 0.01$, $P <$

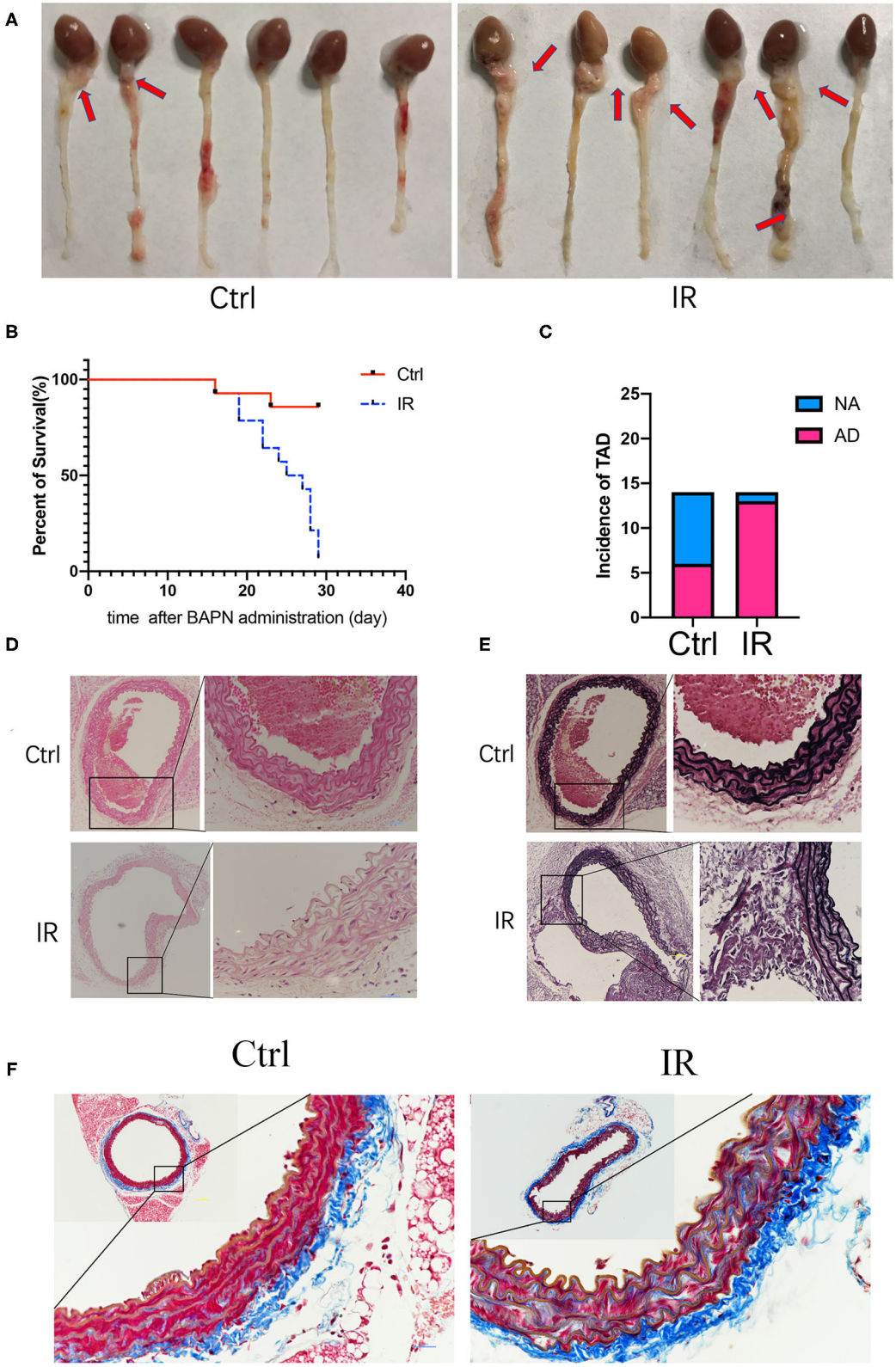

FIGURE 4 | Beta-aminoproprionitrile (BAPN) induced the formation of AD in ApoE$^{-/-}$ mice, reproducing major features of AD. **(A)** Representative images showing macroscopic features of isolated mouse aorta after different diets and BAPN treatment for 4 weeks; red arrow indicates the location of AD. **(B)** Kaplan-Meier survival curves. **(C)** The incidence of AD in the two groups. **(D)** Representative hematoxylin and eosin (H&E) staining showing the number of VSMCs was increased and the arrangement was disordered in the IR group. **(E)** Representative Verhöeff's staining shows that the elastic fiber layer becomes thinner and fibers smaller and broken in the IR group. **(F)** Masson staining showed that the collagen fibers increased in the IR group. Scale bars = 100 and 50 μm.

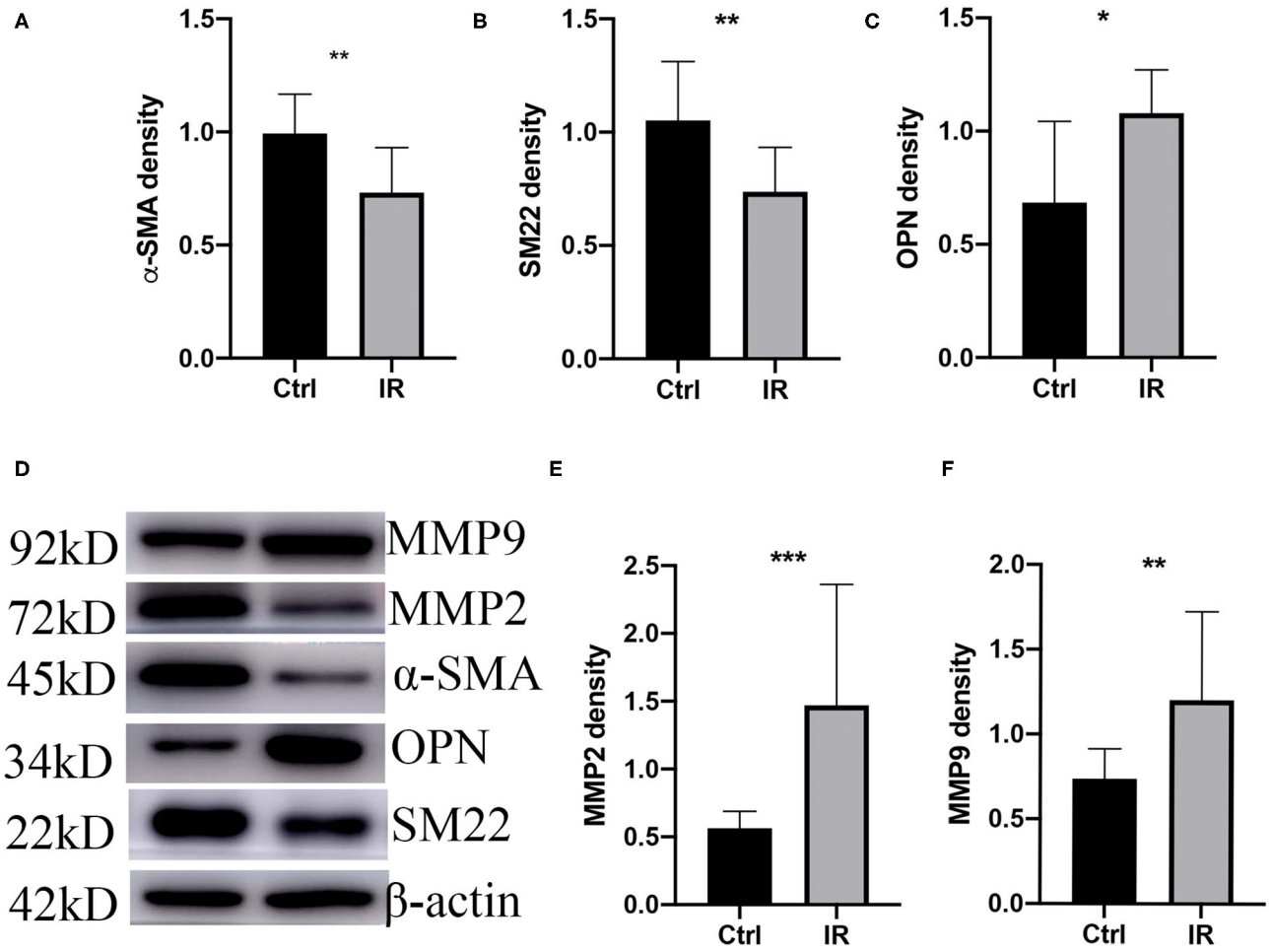

FIGURE 5 | Phenotype switch of vascular smooth muscle cells (VSMCs) and upregulation of MMPs in mice. **(A)** α-SMA. **(B)** SM22. **(C)** OPN. **(D)** Westen blot (WB) assay of MMP9, MMP2, α-SMA, OPN, and SM22. **(E)** MMP2 and **(F)** MMP9 western blot normalized intensity relative to β-actin staining. The *p*-value of fine representative bands between two group was $^{**}P < 0.01$, $^{**}P < 0.01$, $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{**}P < 0.01$, respectively.

0.001) was higher in the IR group, and that of SM22 and α-SMA ($P < 0.01$, $P < 0.01$) was lower (**Figures 5A–F**).

Cell Experiments

Insulin Can Downregulate Glucose Consumption of HA-VSMCs and Lead to IR

Adding different concentrations of insulin to the culture medium can reduce the glucose consumption of HA-VSMCs, the glucose consumption of HA-VSMCs is downregulated with different concentrations of insulin in the culture medium and the 10^{-7} mol/L is the most effective suppression, which is downregulated with 10^{-7} mol/L insulin in the culture medium in a different time and the 36 h is the most effective suppression time (**Figures 6A,B**). To determine whether insulin can cause HA-VSMCs IR, we detected the mRNA expression of Glut1 in HA-VSMCs by qPCR, found that the mRNA expression of GluT1 in co-cultured with insulin was reduced ($P < 0.0001$) (**Figure 6C**). Then, we performed a western blot to verify the protein expression of Glut1 protein compared to the control group. We found that it was reduced in the IR group ($P < 0.001$) (**Figures 6D,E**) and found the same trend with immunofluorescence (**Figure 6F**).

IR Leads to a Phenotypic Switch of HA-VSMCs

To clarify that IR causes HA-VSMCs phenotype switching, we choose specific SM22 and OPN as indicators, using qPCR to detect mRNA expression and western blot to detect protein expression. The results showed that both mRNA and protein expression of SM22 in the IR HA-VSMCs decreased ($P < 0.0001$, $P < 0.01$) and those of OPN were upregulated ($P < 0.01$, $P < 0.01$) (**Figures 7A–E**). Results were further confirmed by immunofluorescence (**Figure 7F**), which indicated that IR can induce phenotypic switching of HA-VSMCs from the contractile to the synthetic phenotype.

DISCUSSION

Our study found that IR mainly attenuates the uptake of glucose by diminishing the expression of GluT1 membrane protein, induces the phenotypic switching of VSMCs from the contractile

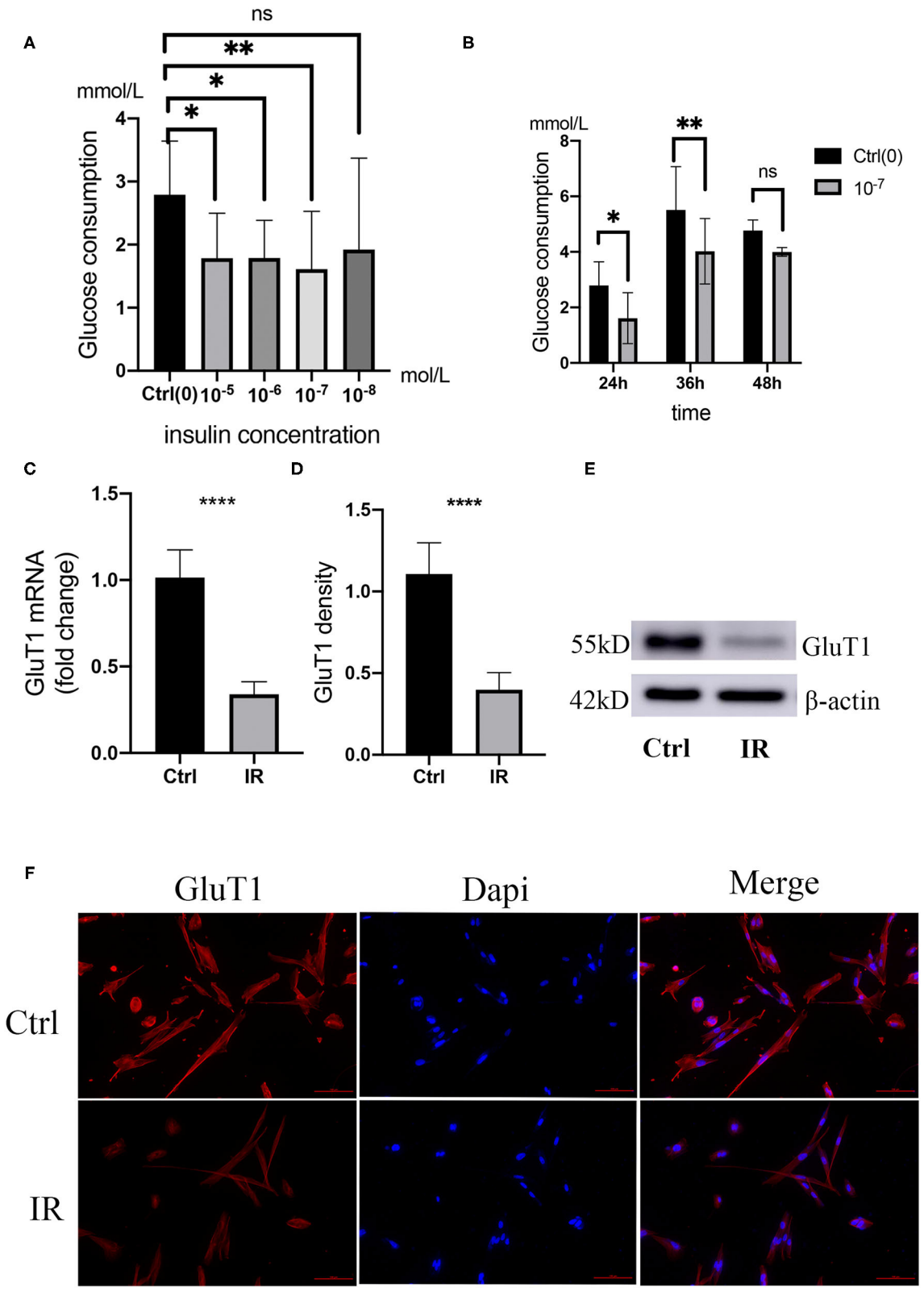

FIGURE 6 | Establishment and verification of IR model in human aortic VSMCs (HA-VSMCs). **(A)** Glucose consumption in HA-VSMCs is downregulated with co-cultivation with different concentrations of insulin. *$P < 0.05$, **$P < 0.01$, NS, Not Statistically Significant. **(B)** Glucose consumption in HA-VSMCs is downregulated by co-cultivation with 10^{-7} mol/L insulin at different times. **(C)** quantitative PCR (qPCR) analysis of GluT1 mRNA levels, ****$P < 0.0001$. **(D,E)** Western blots for GluT1

(Continued)

FIGURE 6 | and β-actin stain considering Ctrl and IR cells, normalized intensity relative to β-actin staining. The *P*-value of three representative bands between two groups was $^{**}P < 0.01$. **(F)** Immunofluorescence staining of GluT1 (red) in SMCs from the HA-VSMCs of the control and IR groups. DAPI staining was used to visualize cell nuclei. Scale bar = 100 μm.

to the synthetic phenotype, enhances the protein expression of MMP2 and MMP9, and contributes to the occurrence of AD. Combined with the results of clinical analysis, verification of the protein and mRNA levels in human thoracic aorta, animal model, and cell experiments, we believe that IR is a high-risk factor for AD. This provides a basis for in-depth research on the specific mechanism of IR that promotes AD and a new approach for prevention and treatment.

Insulin resistance reduces the sensitivity of target cells to physiological levels of insulin concentration, and higher concentrations of insulin are then needed to maintain the balance between the supply and demand of nutrients (22, 23) leading to hyperglycemia and dyslipidemia (24, 25) and disturbed energy metabolism as a result of the reduced expression and function of the membrane protein GluTs. There are many methods to assess insulin sensitivity, including direct methods such as the hyperinsulinemic euglycemic clamp (HEC) (26), insulin suppression test (IST) (27), tracer detection (28), and indirect detection methods such as homeostasis model assessment (HOMA-IR) and beta-cell function index (HOMA-beta) (29), quantitative insulin sensitivity check index (QUICKI), and Bennett insulin sensitivity index (BISI) (30) among others. These methods are complicated or time-consuming, However, the occurrence and development of AAD are likely to be fatal within a short period of time, so rescue treatment is the best and most effective way to save lives. Therefore, the above methods are not suitable for assessing insulin sensitivity in patients with ADD. HbA1c is a relatively ideal indirect evaluation index, with low sensitivity and high specificity (31–33) that is minimally affected by the time of food intake and stress. So, in our study, we chose HbA1c as an indicator for evaluating insulin sensitivity. Studies have found that C-peptide, SIC, and HOMA-IR are significantly upregulated in patients with larger aneurysm diameters (20) and that elevated fructosamine and HbA1c levels are high-risk factors for AD (21). One clinical study used HOMA-IR as an indicator of insulin sensitivity, and the results showed that the elasticity of the ascending aorta and its overall properties in IR patients changed with time, with increased stiffness and decreased elastic expansion; this deterioration in those with mild IR was more obvious in a short period of time (34).

Our study found that the abnormal level of HbA1c in patients with ATAD was higher, and combined with the sensitivity and specificity of HbA1c in the diagnosis of AD, we believe that AD is a high-risk factor for ATAD. Human vascular smooth muscle cells transport glucose into the cell for metabolism, which mainly relies on the membrane protein GluT1 (35, 36). IR may lead to a decrease in GluT1 expression. To further verify this clinical phenomenon, we used q-PCR, western blot, and immunofluorescence to analyze the mRNA and protein expression of GluT1 in the VSMCs of patients with ATAD, which were significantly reduced, indicating that IR contributes to the decreased expression of GluT1 and the occurrence of ATAD.

We changed the diet of $ApoE^{-/-}$ mice, monitored the FGC and SIC, assessed the insulin sensitivity of mice with HOMA-IR (37), and analyzed the protein expression of GluT1 in VSMC by western blot and immunofluorescence. We found higher HOMA-IR and lower protein expression of Glut1 in the high-fat diet group of mice, indicating that the animal model of IR was successfully established. We fed the mice 0.25% BAPN to induce AD (38). The results showed that the incidence of AD was significantly higher in the IR group. H&E, Masson, and elastin fiber staining were consistent with the pathology of the mouse model of AD investigated by others (39, 40). With protein analysis of aortic tissue in the two groups, we found that the expression of SM22 in VSMCs in the IR group was decreased and expression of OPN, MMP2, and MMP9 were increased, which showed that IR can promote the occurrence of AD and is a high-risk factor for AD.

We added different concentrations of insulin to the medium to establish insulin-resistant HA-VSMCs and determine the best insulin concentration and best action time for cellular IR (41, 42). We then verified the mRNA and protein expression of GluT1 by q-PCR and western blot, respectively. We found that the expression of SM22 was lower and OPN higher in insulin-resistant HA-VSMCs, which indicates IR caused the phenotypic switching of SMC from the contractile to the synthetic phenotype.

At present, most clinical studies have shown that diabetes has a protective effect against AD (43–45) and is negatively correlated with the enlargement and the rupture of the aneurysm (18, 46). Other studies showed that diabetes leads to hypertension and increases the frequency of AD (3), resulting in a higher frequency of hospitalization (47). Although a variety of oral hypoglycemic drugs that enhance insulin sensitivity have protective effects in reducing AD, insulin used to treat diabetes promoted the occurrence and development of AD (48). Through the analysis of clinical data, combined with the proportion of ATAD patients with abnormal HbA1c, it can be inferred that IR is a high-risk factor for ATAD. To clarify the relationship between IR and AD, it is necessary to carry out a related prospective clinical trial. We found that IR results in the phenotypic switch of VSMC, but the specific mechanism of action is still not clear, and further in-depth research is needed. This may provide a basis for reducing the occurrence and development of AD by actively treating IR, which may become a potential target for reducing the occurrence and treatment of AD and providing a new approach for its prevention and treatment.

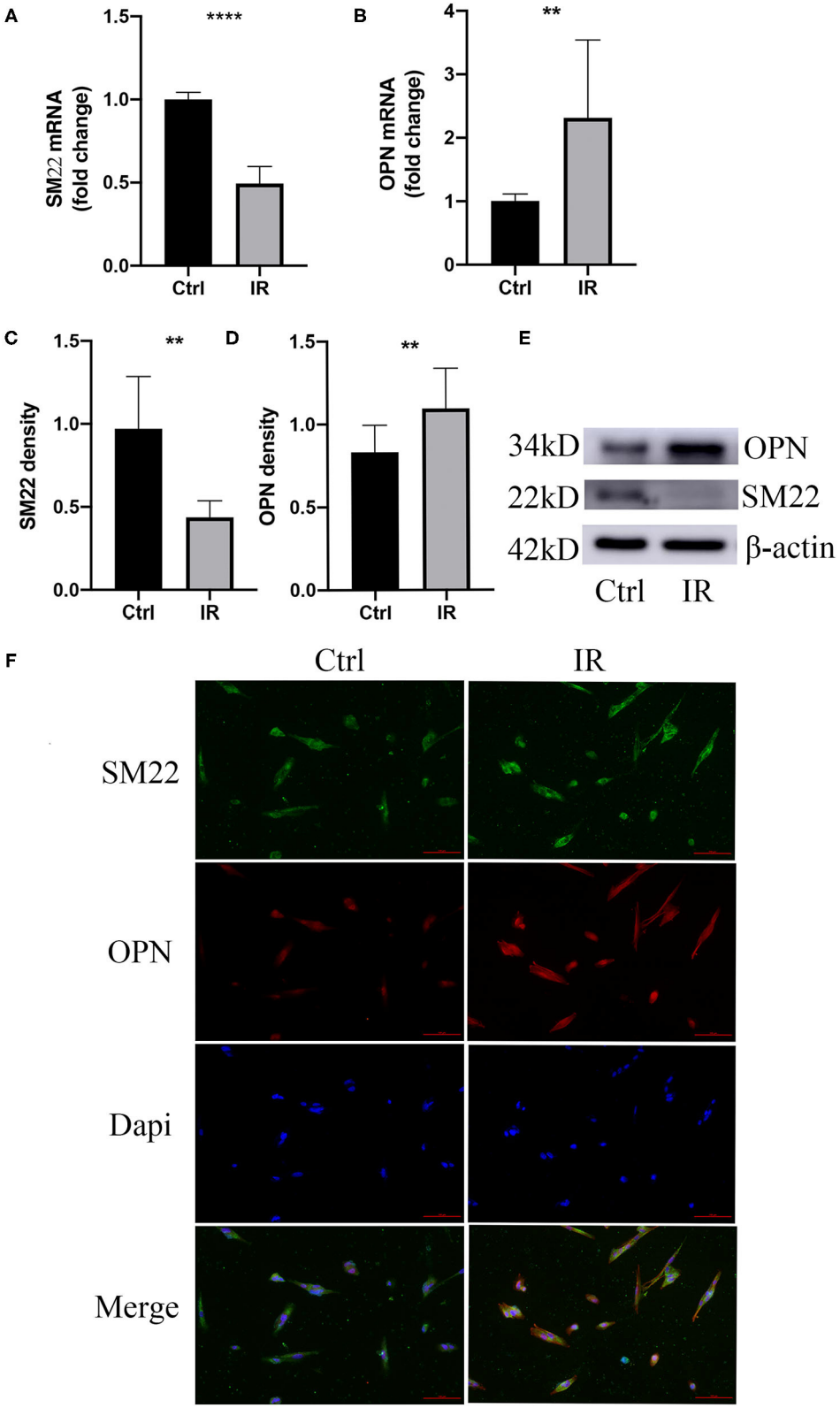

FIGURE 7 | Phenotype switching of VSMCs in the cell model. **(A,B)** qPCR analysis of SM22 and OPN messenger RNA (mRNA) levels, $^{**}P < 0.01$, $^{****}P < 0.0001$. **(C–E)** Western blots for SM22, OPN, and β-actin stain considering Ctrl and IR cells, this normalized intensity relative to β-actin staining, The *P*-value of three representative bands between the two groups was $^{**}P < 0.01$ and $^{****}P < 0.0001$, respectively. **(F)** Immunofluorescence staining of SM22 (green) and OPN (red) in SMCs from the HA-VSMCs of the control and IR groups. DAPI staining was used to visualize cell nuclei. Scale bar = 100 μm.

CONCLUSION

The results of this study show that IR induced phenotypic switching of SMCs from the contractile to the synthetic phenotype, which promotes the occurrence of AD. The study provides a basis for further research on the specific mechanism and a new approach for the active treatment of IR to reduce the occurrence of AD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession numbers can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee at the Fujian Medical University Union Hospital (No. 2021QH026). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. The animal study was reviewed and approved by the Laboratory Animal Care and Use Committee of School of Fujian Medical University (No. 2020-0089).

AUTHOR CONTRIBUTIONS

LC had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. HZ was responsible for the study concept and design, acquisition of data, and data analysis and interpretation. HZ and CW conducted the experiments, data analysis, and statistical analysis and drafted the manuscript. TC and JY provided technical support and editing. JH, YZ, and XW were responsible for the investigation, tissues samples, and clinical data collection. ZQ provided supervision. LZ and YL provided administrative, technical, or material support. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.732122/full#supplementary-material

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