CARDIOVASCULAR DISEASES RELATED TO DIABETES AND OBESITY

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CARDIOVASCULAR DISEASES RELATED TO DIABETES AND OBESITY

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EDITORIAL
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Editorial: Cardiovascular Diseases Related to Diabetes and Obesity

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Editorial on the Research Topic

Cardiovascular Diseases Related to Diabetes and Obesity

Worldwide, the prevalence of obesity continues to rise unabated due to the rapid urbanization in the developed and developing countries and poor lifestyle habits. Obesity is also attributed to the development of major cardiovascular diseases (CVD), diabetes, arthritis, behavioral changes, depression, cancers and hepatic diseases. Therefore, it is of paramount significance that the development of obesity is thwarted by introducing behavioral changes (aimed at decreasing the consumption of high calorie food and inculcating the habit of regular physical activity). Ironically, these interventions do not benefit the majority of people who are morbidly obese. Furthermore, it is pertinent to note that emerging knowledge from clinical studies indicates that available pharmacological modes of therapy may not be sufficient to reduce the development of adverse CVD events in patients who are obese and have co-morbidities such as diabetes, hypertension or hepatic diseases such as non-alcoholic steatosis, non-alcoholic steatohepatitis or alcoholic steatohepatitis. Therefore, the objective of this special issue is to compile the recent clinical and pre-clinical studies which could expand our horizon in understanding the etiopathogenesis of these intertwined, multifactorial diseases.

Comprehensive reviews have highlighted the epidemiological forecasting of diabetes. Cheng et al., summarized that type 2 diabetes (T2DM) affected over 463 million people in 2019, and this number is expected to increase to 578 million by 2030 and 700 million by 2045; therefore, the priority objective is to identify all potential controllable risk factors for the development of diabetes with the purpose to have early screening and prevention. Based on results from the China Health and Retirement Longitudinal Study, increased serum uric acid is an important risk factor for the development of diabetes, in postmenopausal women. Venkatesan et al. tried to determine the prevalence of T2DM and estimate its heritability using family-based cohorts from three distinct Endogamous Ethnic Groups, representing Northern and Southern states of India. The heritability estimates of T2DM in these regions ranged from 30% to 82%. Other T2DM related traits (e.g., BMI, lipids, blood pressure) in these regions exhibited strong additive genetic influences, suggesting the high burden of T2DM in Indian Endogamous Ethnic Groups with significant and differential additive genetic influences on T2DM and related traits.

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An elegant review by Y. Wu et al. highlights the critical role of mitochondria in the pathogenies of diabetes. Mitochondrial-derived peptides (MDPs) are recognized as short peptides formed by transcription and translation of the open reading frame site in human mitochondrial DNA. During stress the cell can use MDPs as a new type of reverse signal molecule to retrograde pass the signals to the nucleus where gene transcription synthesis is turned on to exert anti-inflammatory, anti-apoptotic signals and bolster the mitochondrial physiology, thereby mitigating the development of diabetes and CVD. Studies have shown a difference in serum apelin levels between individuals with and without diabetic and/or obesity, supporting the role of apelin in diabetes and obesity development and also implying the potential use of apelin as a clinical biomarker for diabetes and obesity. Therefore, additional clinical and experimental studies clearly supporting the physiological and pathophysiological roles of the apelin–APJ system in glucose and lipid metabolism, particularly with its signaling pathways, is warranted (Li et al.).

In terms of diabetic complications, Chen et al. analyzed patients with acute myocardial infarction (AMI), admitted into a coronary care unit with follow-up of ≥1 year based on two cohorts of MIMIC-III (Medical Information Mart for Intensive Care III) and CIN (Cardiorenal Improvement Registry) in the United States and China, and found that AMI patients with diabetes have a significantly higher 30-day mortality and increased 1-year mortality than AMI patients without diabetes. Similarly, Du et al., reported that insulin resistance is associated with increased risk of CVD development in adolescent subjects with diabetes. L. Wu et al. described that Apolipoprotein E polymorphisms are associated with the development of CVD in patients with or without T2DM. By utilizing bioinformatic analysis tools and validation with db/db T2DM mouse heart tissue, Huang et al. revealed that calpain small subunit 1 (CAPNS1) is highly expressed in the heart of T2DM db/db mice and this was significantly decreased in the heart of T2DM patients with SARS-CoV-2 infection, suggesting a novel target in mitigating the adverse effects of SARS-Cov-2 infection. Although this is a novel and interesting hypothesis developed based on bioinformatic approach, the precise clinical significance is blurred and should be confirmed by additional preclinical and clinical studies.

Aberrant endothelial function in patients with T2DM is closely associated with the development of CVD. Flow-mediated dilation (FMD) is a noninvasive tool for evaluating endothelial function, which typically examines changes in the brachial artery diameter in response to ischemia using ultrasound Doppler. By evaluating FMD in patients with T2DM, Wang et al. performed a network meta-analysis to explore the improvement of endothelial function with antidiabetic drugs and found that glucagon-like peptide-1 receptor (GLP-1R) agonists, sodium glucose co-transporter-2 inhibitor and thiazolidinedione exhibited favorable effects in improving the endothelial function in T2DM patients. Consistently, Dardano et al. also reported the efficiency and safety of GLP-1R agonist in mitigating adverse effects of CVD in a T2DM patient living with HIV. In addition, the pre-clinical study indicated the protective effects of GLP-1R agonists treatment in mitigating the development of diabetic cardiomyopathy (El-Shafey et al.). Last, the meta-analysis by Sun et al. revealed that sesamin dietary supplementation improved blood pressure and serum lipid profiles, and postulated sesamin as a supplement therapy or health supplement to prevent the development of CVDs.

In sum, the aforementioned articles published in this Research Topic highlight the multifaceted etiopathogenic aspects of closely connected diseases such as obesity and diabetes. It also sets the stage for delving further in deciphering the crucial link between obesity and diabetes. This could also help in unraveling the selective and specific biomarkers and/or pharmacological targets, aiding in the management of these debilitating chronic diseases.

AUTHOR CONTRIBUTIONS

MR and LC wrote the first draft. MR, JY, YX, and LC revised and approved the final submitted version.

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

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ORIGINAL RESEARCH
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Does Diabetes Mellitus Increase the Short- and Long-Term Mortality in Patients With Critical Acute Myocardial Infarction? Results From American MIMIC-III and Chinese CIN Cohorts

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Background: The harmful effect of diabetes mellitus (DM) on mortality in patients with acute myocardial infarction (AMI) remains controversial. Furthermore, few studies focused on critical AMI patients. We aimed to address whether DM increases short- and long-term mortality in this specific population.

Methods: We analyzed AMI patients admitted into coronary care unit (CCU) with follow-up of ≥1 year from two cohorts (MIMIC-III, Medical Information Mart for Intensive Care III; CIN, Cardiorenal ImprovemeNt Registry) in the United States and China. Main outcome was mortality at 30-day and 1-year following hospitalization. Kaplan-Meier curves and Cox proportional hazards models were constructed to examine the impact of DM on mortality in critical AMI patients.

Results: 1774 critical AMI patients (mean age 69.3 ± 14.3 years, 46.1% had DM) were included from MIMIC-III and 3380 from the CIN cohort (mean age 62.2 ± 12.2 years, 29.3% had DM). In both cohorts, DM group was older and more prevalent in cardio-renal dysfunction than non-DM group. Controlling for confounders, DM group has a significantly higher 30-day mortality (adjusted odds ratio (aOR) (95% CI): 2.71 (1.99-3.73) in MIMIC-III;

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aOR (95% CI): 9.89 (5.81-17.87) in CIN), and increased 1-year mortality (adjusted hazard ratio (aHR) (95% CI): 1.91 (1.56-2.35) in MIMIC-III; aHR (95% CI): 2.62(1.99-3.45) in CIN) than non-DM group.

Conclusions: Taking into account cardio-renal function, critical AMI patients with DM have a higher 30-day mortality and 1-year mortality than non-DM group in both cohorts. Further studies on prevention and management strategies for DM are needed for this population.

Clinical Trial Registration: clinicaltrials.gov, NCT04407936.

Keywords: acute myocardial infarction, critical, diabetes mellitus (DM), short-term mortality, long-term mortality

INTRODUCTION

The prevalence of diabetes mellitus (DM) ranges from 20-30% in acute myocardial infarction (AMI) patients (1, 2), and the burden of DM has been heavy and keeps increasing among AMI patients (3). The Thrombolysis in Myocardial Infarction (TIMI) study, a multi-center, maximus sample study, indicated that ST-segment elevation myocardial infarction (STEMI) patients with DM have a 40% higher 30-day mortality and 22% higher 1-year mortality than those without DM in a cohort of 62,036 patients from 55 countries (4). To date, however, numerous studies have observed no significant association between DM and short- or long-term mortality (5, 6). Recently, an Italian cohort study also reported DM is not significantly associated with short-term mortality in STEMI patients, possibly due to underlying cardio-renal dysfunction in patients with DM (7). Therefore, the independent harmful effect of DM on short- and long-term mortality in AMI patients may be inconsistent. Furthermore, most studies on the relationship between DM and prognosis were conducted among the unselected AMI population (5, 6), while limited studies focused on AMI patients first admitted to CCU that are more prone to hemodynamic instability, multiple organ dysfunction and sudden death.

To provide reliable evidence, we investigated the possible harmful effect of DM on short- and long-term mortality following critical AMI in the American Medical Information Mart for Intensive Care (MIMIC-III) cohort and Chinese Cardiorenal ImprovemeNt (CIN) cohort.

MATERIALS AND METHODS

Data Sources and Study Population

We analyzed two existing datasets to examine the significance of DM on 30-day and 1-year mortality in patients with critical AMI. In the American cohort, we analyzed a coronary care unit (CCU) cohort from the MIMIC-III database describing CCU admissions to the Beth Israel Hospital (Boston MA, USA) from 2001 to 2012 (8). To access the database, we completed the National Institutes of Health's web-based course Protecting Human Research Participants (certification number 36478705). In the Chinese cohort, we analyzed CCU admissions to an advanced teaching hospital, Guangdong Provincial People's Hospital (Guangdong, China). The CIN study (ClinicalTrials.gov NCT04407936) enrolled consecutive patients undergoing coronary angiography (CAG) or percutaneous coronary intervention (PCI) from January 2007 to December 2018 in Guangdong Provincial People's Hospital, Guangdong, China.

This study included patients with acute myocardial infarction (AMI) who had been admitted to the CCU on admission. Patients with missing information on diabetes diagnosis and cardiac and renal function were excluded. All patients were followed for at least 1 year. Eventually, 1774 patients were included in the MIMIC-III, and 3380 patients were included in the CIN (**Figure 1**). All traceable personal identifiers were removed from the analytic dataset to protect patients' privacy. The study was approved by the local ethics committee and was performed according to the Declaration of Helsinki.

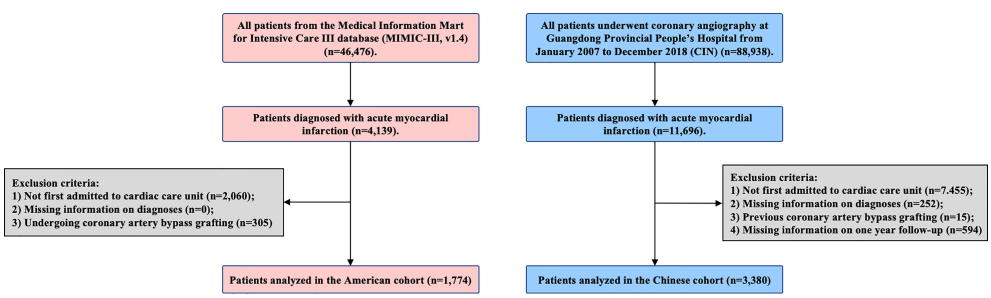

FIGURE 1 | Patient flow diagram of MIMIC-III and CIN.

Data Collection

MIMIC-III (1.4 version) database includes information from 2002 to 2011. Hourly physiologic readings from bedside monitors, validated by CCU nurses, were recorded. The database also contains records of demographics, laboratory results, and other clinical variables. International Classification of Diseases, Ninth Revision (ICD-9) codes was also documented for specific diseases by hospital staff on patient discharge. This information is extracted from the database based on Structure query language (SQL) (website: https://github.com/MIT-LCP/mimic-code). The follow-up was started from the date of admission and ended at death.

The data of CIN were extracted from the electronic clinical management records system of the Guangdong Provincial People's Hospital. We examined all primary and secondary care records. The baseline information included demographic characteristics, coexisting conditions, laboratory examinations, and medications at discharge. The follow-up information was matched from the Guangdong Provincial Public Security based on the electronic Clinical Management System of the Guangdong Provincial People's Hospital records.

Endpoint and Variable Definition

The primary endpoint was 30-day and 1-year mortality. DM was diagnosed if this disease and/or antidiabetic treatment, including oral agents or insulin, were recorded in the medical record. Anemia was defined as a hematocrit ≤39% for males or ≤36% for females. The estimated glomerular filtration rate (eGFR) was calculated by the Modification of Diet in Renal Disease (MDRD) formula, and chronic kidney diseases (CKD) were defined as eGFR <60 mL/min/1.73m^2 (9). Congestive heart failure (CHF) was defined as New York Heart Association (NYHA) class > 2 or Killip class > 1 (10). Acute myocardial infarction (AMI), hypertension and atrial fibrillation (AF) were defined using ICD-10 codes.

Statistical Analysis

Baseline characteristics were presented as means ± SDs for continuous variables, and proportions for categorical variables. The differences of baseline characteristics between groups were compared using Student t-test for continuous variables and chi-square tests for categorical variables. Time-to-event data among groups are presented graphically using Kaplan-Meier curves and compared by the log-rank test. In addition, 30-day and 1-year mortality were compared in critical AMI patients with or without DM in MIMIC-III and CIN cohort. Multivariable Logistic and Cox regression models were used to examine the relationship between DM and 30-day and 1-year mortality, respectively. The factors used to construct the Multivariable Logistic and Cox regression models in this study included: age, sex, hypertension, CKD, CHF, PCI, AF and anemia. Three models were sequentially constructed with or without adjustment for covariates: 1) unadjusted; 2) CHF and CKD; 3) age, sex, PCI, comorbidities (CHF, CKD, AF, hypertension and anemia). Presented tests were 2-tailed for all, and a p-value <0.05 was considered statistically significant. All statistical analyses were performed using R (ver. 4.0.3).

RESULTS

A total of 1774 patients (mean age 69.3 ± 14.3 years, 46.1% had DM) were included in the American cohort and 3380 (mean age 62.2 ± 12.2 years, 29.3% had DM) in the Chinese cohort. In the MIMIC-III cohort, 37.8% were female, 43.6% and 43.3% had CHF and CKD. In the CIN cohort, 19.6% were female, 24.0% and 25.0% suffered from CHF and CKD.

In both cohorts, DM group tended to be older than non-DM group. In the American cohort, no significant difference in DM prevalence was observed in gender, while female patients were more in DM group compared with non-DM group (26.4% vs. 16.8%) in the CIN cohort (p<0.001). In both cohorts, the prevalence of CKD and CHF was significantly higher in DM group than in non-DM group. Among patients with DM, the American cohort had a higher prevalence of CHF, CKD, and lower use of PCI than the CIN cohort at baseline. AMI patients undergoing PCI were less in DM group compared with non-DM group (90.5% vs. 92.7% in the CIN cohort; 64.06 vs. 75.73 in MIMIC-III cohort, respectively). More details of the baseline information of both cohorts are listed in **Table 1**.

Thirty-Day and 1-Year Mortality

At 30-day follow-up, a total of 288 patients (16.2%) in MIMIC-III cohort and 102 (3.0%) in CIN cohort died. At 1-year follow-up, 473 (26.7%) and 230 (6.8%) died in MIMIC-III and CIN cohort (**Figure 2**). As Kaplan-Meier curves showed (**Figure 3**), the short- and long-term mortality of DM in critical AMI patients was significantly higher compared to non-DM group in both MIMIC-III and CIN cohort (Log-rank test, p<0.0001). Controlling for confounding variables, DM group had a higher risk of 30-day mortality than non-DM group in both cohorts (adjusted odds ratio (OR) (95% CI): 2.71 (1.99-3.73) in MIMIC-III; aOR (95% CI): 9.89 (5.81-17.87) in CIN) (**Table 2**); and had a significantly higher 1-year mortality in both cohorts [(adjusted hazard ratio (aHR) (95% CI): 1.91 (1.56-2.35) in MIMIC-III; aHR (95% CI): 2.62 (1.99-3.45) in CIN)] (**Table 3**).

DISCUSSION

To our knowledge, this is the first study to examine the harmful effect of DM on short- and long-term mortality simultaneously in critical AMI patients from American and Chinese cohorts. In our two cohorts, DM is very common among critical AMI patients (nearly half and one-third, respectively). Even after taking into account cardio-renal function, both the American and Chinese cohorts suggest that critical AMI patients with DM have 1.71- and 8.89-fold higher 30-day mortality, and 0.91- and 1.62-fold higher 1-year mortality than non-DM group, respectively. Accordingly, the death burden of DM in critical AMI patients is heavier in the Chinese cohort than in the American cohort.

Notably, the prevalence of DM is very high in patients with critical AMI. In this study analyzing an American cohort and a Chinese cohort, 46.1% and 29.3% respectively of patients with critical AMI had comorbid DM. Previously, Atman et al. have

TABLE 1 | Baseline characteristics of critical AMI patients with and without DM in American MIMIC-III and Chinese CIN databases.

Characteristics	MIMIC-III (N=1774)			CIN (N=3380)		
	Non-DM N=956	DM N=818	P-value	Non-DM N=2388	DM N=992	P-value
Demographic characteristics						
Age, year	67.7 ± 15.3	71.1 ± 12.7	<0.001	61.4 ± 12.3	64.1 ± 11.5	<0.001
Female, n (%)	355 (37.13)	316 (38.63)	0.549	400 (16.75)	262 (26.41)	<0.001
Complication						
Hypertension, n (%)	450 (47.07)	376 (45.97)	0.676	1060 (44.39)	607 (61.19)	<0.001
CHF, n (%)	340 (35.56)	434 (53.06)	<0.001	484 (20.27)	327 (32.96)	<0.001
CKD, n (%)	290 (30.95)	463 (57.59)	<0.001	502 (21.02)	344 (34.68)	<0.001
Stroke, n (%)	24 (2.51)	50 (6.11)	<0.001	115 (4.82)	83 (8.37)	<0.001
PCI, n (%)	724 (75.73)	524 (64.06)	<0.001	2214 (92.71)	898 (90.52)	0.038
Anemia, n (%)	509 (54.32)	491 (61.15)	0.005	777 (34.83)	421 (44.32)	<0.001
AF, n (%)	192 (20.08)	217 (26.53)	0.002	46 (1.93)	27 (2.72)	0.187
Medications						
β blocker, n (%)	774 (80.96)	685 (83.74)	0.143	1993 (83.46)	723 (83.29)	0.954
Statins, n (%)	765 (80.02)	693 (84.72)	0.012	2260 (94.64)	815 (93.89)	0.462
ACEI or ARB, n (%)	673 (70.40)	566 (69.19)	0.618	1615 (67.63)	528 (60.83)	<0.001
DAPT, n (%)	729 (76.26)	642 (78.48)	0.289	2119 (88.74)	775 (89.29)	0.705

ACEI, angiotensin converting enzyme inhibitor; AF, atrial fibrillation; AMI, acute myocardial infarction; ARB, angiotensin receptor blocker; CHF, congestive hearts failure; CKD, chronic kidney disease; DAPT, dual antiplatelet therapy; PCI, percutaneous coronary intervention.

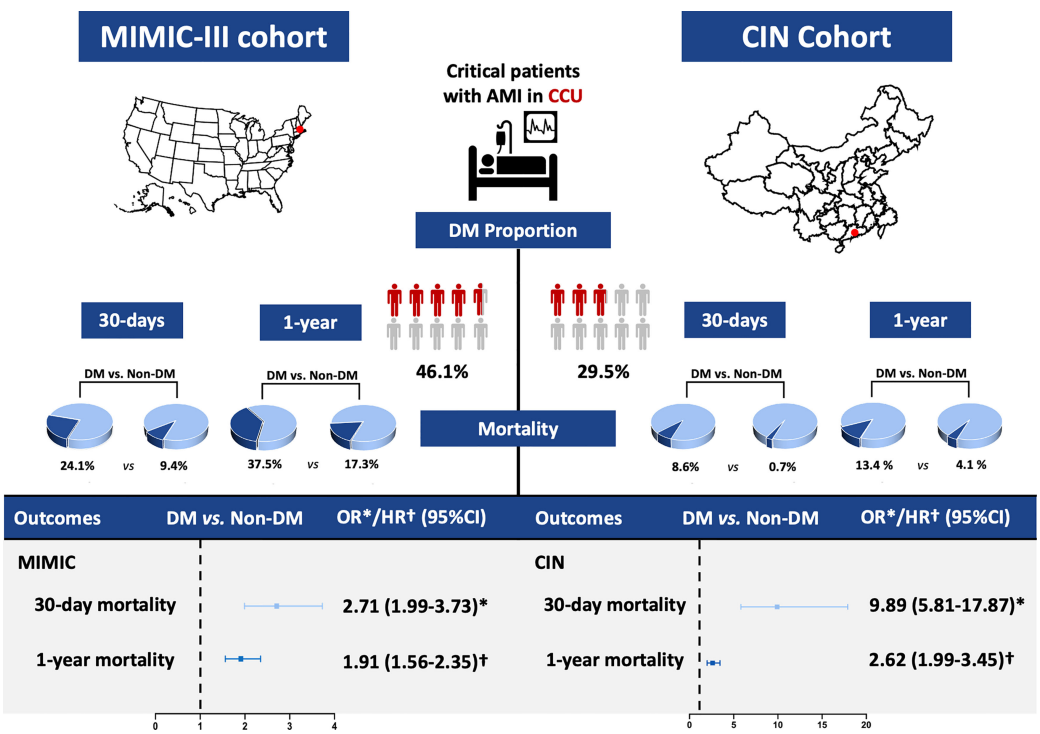

FIGURE 2 | Summary graph of the role of DM in patients with critical AMI in the MIMIC-III and CIN cohort. *Odds ratio. †Hazard ratio.

reported a high prevalence of DM (54%) in patients with critical AMI admitted into CCU in a 1,598 Qatar cohort (11). Possible explanations for the even higher prevalence compared with our two study cohorts could be due to the country itself has a high overall prevalence of diabetes in Qatar, and could be related to the highly prevalent consanguinity in marriages in the society (11). Similar to our results, in the INTERHEART study including 12,431 AMI patients admitted to CCU or equivalent cardiology ward from 52 countries, the 3,030 Chinese AMI participants had a lower rate of self-report DM at admission compared with 9,431 AMI patients in

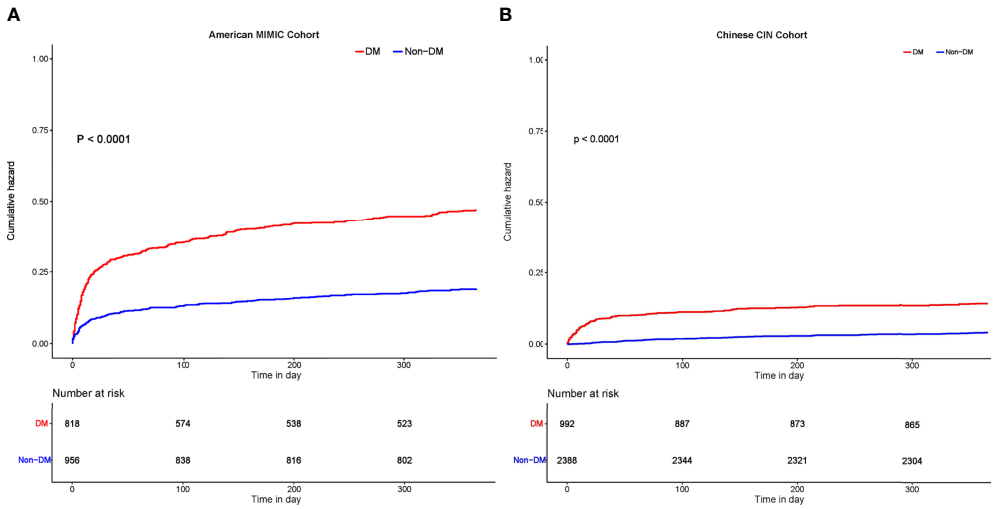

FIGURE 3 | Kaplan-Meier curves for all-cause mortality in patients with critical AMI with and without DM. **(A)** 1-year mortality among patients with critical AMI with and without DM in MIMIC-III. **(B)** 1-year mortality among patients with critical AMI with and without DM in CIN.

TABLE 2 | Logistic regression analyses of DM to predict 30-day mortality in patients with critical AMI in American MIMIC-III and Chinese CIN databases.

Database		Model 1		Model 2		Model 3	
		OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
MIMIC-III	**DM vs. Non-DM**	3.07 (2.35-4.04)	<0.001	2.52 (1.87-3.44)	<0.001	2.71 (1.99-3.73)	<0.001
CIN		13.07 (7.93-22.87)	<0.001	10.15 (6.11-17.88)	<0.001	9.89 (5.81-17.87)	<0.001

Model 1: unadjusted.
Model 2: adjusted for chronic kidney disease, congestive hearts failure.
Model 3: adjusted for age, sex, hypertension, chronic kidney disease, congestive hearts failure, percutaneous coronary intervention, atrial fibrillation, anemia.

TABLE 3 | Cox proportional hazards analyses of DM to predict 1-year mortality in patients with critical AMI in American MIMIC-III and Chinese CIN databases.

Database		Model 1		Model 2		Model 3	
		HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
MIMIC-III	**DM vs. Non-DM**	2.49 (2.06-3.01)	<0.001	1.82 (1.48-2.23)	<0.001	1.91 (1.56-2.35)	<0.001
CIN		3.55 (2.73-4.62)	<0.001	2.76 (2.12-3.61)	<0.001	2.62 (1.99-3.45)	<0.001

Model 1: unadjusted.
Model 2: adjusted for chronic kidney disease, congestive hearts failure.
Model 3: adjusted for age, sex, hypertension, chronic kidney disease, congestive hearts failure, percutaneous coronary intervention, atrial fibrillation, anemia.

other countries (13% vs. 23%) (12). Noticeably, both the MIMIC-III cohort and CIN cohort were substantially higher in the prevalence of DM than the INTERHEART study (18.5%) among AMI patients (13), probably because the INTERHEART study relied only on a self-report history of DM, underestimating the DM prevalence in its study population. Another reason for the difference in the prevalence of DM is that the INTERHEART study was conducted from 1999 to 2003 and predated both MIMIC-III and CIN cohorts by years, and the burden of DM had increased rapidly throughout those years (14). As is shown above, despite the overall improvement in healthcare service and therapies throughout the years, DM co-morbidity remains high in AMI patients and substantially higher in critical AMI patients.

Noteworthily, the prevalence of DM in patients with critical AMI in China seems lower compared to the United States. The reason for this phenomenon may be that physicians in the United States and China have different diagnostic standards for diabetes. The National Glycohemoglobin Standardization Program standardized the vast majority of assays used in the United States in 2000 (15, 16), and an HbA_{1c} level ≥6.5% was later supplemented into the American diagnostic criteria for diabetes besides fasting and 2-hour blood glucose levels (17).

However, in China, physicians have been using the 1999 World Health Organization criteria to diagnose DM until 2020, only utilizing a fasting blood glucose ≥7.0 and 2-hour blood glucose of ≥7.8 mmol/l. Since less severe cases were not recognized as DM under the stricter diagnostic criteria, the prevalence of DM could be underestimated in China. Meanwhile, it may explain why the mortality risk of DM in critical AMI patients seems relatively higher in China. Nonetheless, new cases of DM among adults significantly decreased from 2008 to 2018 in the US (18), while China is experiencing a rapid increase in the burden of DM (19, 20).

Another noticeable difference between two cohort are the 30-day and 1-year mortality, regardless of DM status. There are some reasons accounting for the higher mortality of patients in the MIMIC-III cohort. The patients were older, had higher prevalence of comorbidities and worse cardio-renal function. Moreover, PCI was an effective means of treatment for AMI, but the use of PCI was distinctly less than that in CIN cohort. In addition, the enrollment period differs between the two study cohorts (American registry vs. Chinese registry, 2001 to 2012 vs. 2007-2018, respectively), with the continuous improvement of medical technology, the survival rate of patients was improving.

DM remains a good prognostic predictor even in critical AMI patients who have a higher risk of death. Our study reported DM is associated with a nearly 2 to 9-fold higher risk of 30-day mortality and increased risk of 1-year mortality in patients with critical AMI. Few studies have investigated the prognostic effect of DM in critical AMI patients. Several studies evaluated the impact of DM after AMI on excess mortality and indicated that DM was associated with over 50% increase in short- and long-term mortality following unselected AMI, even after adjusting for confounders (5, 6, 21). However, some studies reported conflicting results. In a nationwide cohort of 2,018 DM and 19,547 Non-DM patients with a first hospitalized AMI in the Netherlands, DM was not independently associated with increased risk of 28-day mortality, but the result was not fully adjusted due to limited data, including other risk factors, comorbidity and treatment (22). In addition, Syed et al. reported that no difference was seen in the 1-year major cardiac event (MACE) or mortality between those with and without DM, who presented with AMI and were treated with drug-eluting stents, after adjusting co-morbid conditions, but the DM population still had a higher unadjusted mortality, although the sample size was relatively small (161 DM and 395 Non-DM patients) (23). Additionally, DM may be associated with mortality but not independent of other variables. Marenzi et al. reported that the increased in-hospital mortality of DM patients with STEMI was mainly driven by their underlying cardio-renal dysfunction in an Italy cohort (7). Consistent with another cohort of elderly adults with non-ST-elevation ACS (NSTEACS) from Italy (24), DM and hyperglycemia on admission were associated with higher mortality, mostly because of preexisting cardiovascular and renal damage. Furthermore, previous studies suggested that DM with concomitant renal insufficiency or cardiovascular co-morbidities independently or synergistically was related to an increase in worse outcomes after AMI (25, 26). Similarly, in two cohorts of our study, CKD and CHF were independent risk factors of mortality. Thus, DM may act as both an independent and synergistic factor to aggravating disease progression in patients with critical AMI.

Consistently, patients with DM have a greater atherosclerosis burden, with more diffuse and more multivessel coronary artery disease (25). Besides, patients with DM and AMI present more rapidly accumulate micro-and macrovascular complications, which may contribute to their worse outcomes (21). DM independently increases short- and long-term mortality in patients with AMI, and some pathophysiological mechanisms have been proposed to account for the adverse influence, including abnormalities in the endothelial, vascular smooth muscle cell, and platelet function; decreased bioavailability of nitric oxide; increased oxidative stress; pro-inflammatory/thrombotic state (27).

All findings based on two cohorts from the United States and China support that clinician need to pay specific attention to critical AMI patients who are diagnosed with DM. Early DM screening and treatment for critical AMI patients may be an effective means to reduce short-term deaths. Appropriate relaxation of the diagnostic criteria for DM may help distinguish potentially high-risk patients, so as to provide timely management and treatment for better ourcomes. In China, HbA1c levels should be incorporated in the diagnosis of DM according to the latest guidelines to better identify DM patients. Clinicians should be aware of effective glycemic control (recommended target <180 mg/dL while avoiding hypoglycemia) (28) and stress blood glucose monitoring during follow-up. In addition, comprehensive systematic care after the event is needed in order to improve survival in critical AMI patients, incorporating intensification of care at various levels: aggressive management of multiple cardiovascular risk factors, strengthening guideline-recommended treatments, and importantly, patients' education and support (29). Moreover, a reduction of mortality in critical AMI patients with DM may be achieved through cardio- and renal-protective therapies (7). Recently, sodium-glucose cotransporter 2 inhibitors (SGLT2i) were shown to provide cardiovascular protection and prevent renal function deterioration, moreover, SGLT2i can also inhibit platelet activation and may act synergistically with anti-platelet therapies in the setting of acute myocardial infarction (30–32), and could be of clinical benefit to this vulnerable population. Further research is needed on the development of novel interventions to reduce mortality and improve survival quality in critical AMI patients with DM.

Limitations

First, because the current study was the retrospective pattern, our inferences did not reflect direct causality, and some relevant information has not been collected completely. To make up for the disadvantage, we conducted the study simultaneously analyzing two existing high-quality cohorts and observed consistent effects, which to some extent enhanced the reliability of our results. Second, the enrollment period differs

between the two study cohorts, which may potentially influence both the short- and long-term outcomes of the enrolled patients (American registry vs. Chinese registry, 2001 to 2012 vs. 2007-2018), and may contribute to the noticeable difference between mortality of two cohorts. Third, the baseline status of the patients with critical AMI in the two countries is different, but for this reason, the relationship between DM and prognosis that we have verified is more applicable among critical AMI patients with DM. Fourth, the diagnostic criteria for DM was different between two countries. But it still manages to reflect a high prevalence of DM, and DM was proven independently associated with mortality in each cohort. Lastly, we only explored all-cause mortality for a single endpoint and did not have a more comprehensive assessment of the prognosis of critical AMI patients with DM, because neither cohort registered specific information about cause of death and other follow-up information. Nonetheless, for these critical AMI patients, cardiogenic death is the main cause of death.

CONCLUSION

Both American and Chinese cohorts suggest that DM is very common among critical AMI patients (nearly half and one-third, respectively). Even after taking into account cardio- and renal function, critical AMI patients with DM have 1.71- and 8.89-fold higher 30-day mortality, and 0.91- and 1.62-fold higher 1-year mortality than non-DM group in MIMIC and CIN cohorts, respectively. Further studies are needed on prevention and treatment strategies on DM among critical AMI patients with DM, especially in China.

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 Guangdong Provincial People's Hospital ethics committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

The authors' responsibilities were as follows—Research idea and study design: SC, ZH, LC, JL, and YL. Data acquisition: XZ, YK, WL, XL, YZ, YH, HH, QL, YL, NT, and JC. Data analysis/interpretation: JL and SC. Statistical analysis: ZH. Supervision and mentorship: YL. Writing guidance: YL. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions on the accuracy or integrity of any portion of the work are appropriately investigated and resolved. The authors declare that there is no competing interest. All authors contributed to the article and approved the submitted version.

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Mediating Effect of Body Mass Index and Dyslipidemia on the Relation of Uric Acid and Type 2 Diabetes: Results From China Health and Retirement Longitudinal Study

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Objective: This study assessed temporal relationships of serum uric acid (SUA) with blood glucose and determine the mediating effects of body mass index (BMI) and dyslipidemia on the relation of SUA and risk of type 2 diabetes.

Methods: Participants aged ≥ 45 years were participated in 2011 and followed up until 2015. Cox proportional hazards regression with a robust variance estimator was performed to explore the association of SUA with the risk of diabetes, and crosslagged path analysis was introduced to examine the temporal relationships between SUA and blood glucose. A mediation analysis was finally used to identify the mediating effect of BMI and dyslipidemia on the relation of SUA and the future risk of diabetes.

Results: A total of 9,020 participants were included with an average age of 58.59 years at baseline in 2011, and 53.6% of them were women. Linear dose–response relationship was identified by restricted spline cubic analysis between baseline SUA and follow-up blood glucose (the non-linear trend for fasting plasma glucose (FPG): $\beta_2 = -0.71$, $p = 0.52$; for HbA1c: $\beta_2 = 0.05$, $p = 0.07$; for risk of diabetes: $\beta_2 = 0.12$, $p = 0.39$). Additionally, compared with the lowest quartiles of SUA, the adjusted risk ratios of diabetes were 1.00 (95% *CI*: 0.82–1.23), 1.08 (95% *CI*: 0.89–1.31), and 1.37 (95% *CI*: 1.11–1.96) for quartile 2–4 (p-trend < 0.01), respectively. Further additional adjustments for BMI or dyslipidemia, these ratios were not statistically significant. In addition, a unidirectional relationship from baseline SUA to follow-up FPG ($\rho_1 = 0.24$, $p = 0.03$) was further confirmed using crosslagged path analysis. After stratifying by genders, the above results were only significant in the women subgroup, and we thus conducted a mediation analysis in women and found that the BMI and dyslipidemia partially mediated the effect of SUA on diabetes with a 23.05 and 18.82% mediating effect, respectively.

Conclusions: These findings provide strong evidence that hyperuricemia preceded diabetes, and the effect of baseline SUA on follow-up type 2 diabetes was more pronounced among middle-aged and elderly Chinese women, especially in

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postmenopausal women, and this effect is partly mediated by BMI and dyslipidemia at baseline.

Keywords: diabetes, BMI, uric acid, dyslipidemia, mediation effect

INTRODUCTION

Type 2 diabetes is a metabolic disease characterized by insulin resistance, which affected over 463 million people in 2019, and this number is expected to increase to 578 million in 2,030 and 700 million in 2045 (1). Identifying all potential controllable risk factors for the incidence and development of diabetes is essential for its early screening and prevention.

As the main component of metabolic syndrome, diabetes, hyperglycemia, and hyperlipidemia interconnect and influence each other, forming a complex framework of chronic diseases (2). The link between hyperuricemia and diabetes has been well documented in the previous studies (3–6), some of them demonstrated that for every 1 mg/dL increase in serum uric acid (SUA) concentration, the risks of type 2 diabetes were increased by 6–11% (6), and these studies also showed differences between genders. Meanwhile, the epidemiological and clinical evidence supports a strong significant positive association between SUA and obesity in the adult population of China, Japan, India, Pakistan, and Iraq (7, 8). About 44% of diabetes cases are overweight or obese (9), and adults with body mass index (BMI) $>$ 35 kg/m^2 are 20 times as likely to develop type 2 diabetes than those with a BMI between 18.5 and 24.9 kg/m^2. Also, some researchers reported that the relation of SUA and diabetes is largely decreased or eliminated when additional adjusting BMI (10, 11). The possible mechanism is that hyperuricemia can cause obesity by accelerating liver and peripheral fat production (12). In addition, dyslipidemia is a common comorbidity in patients with diabetes (13), and over 70% of them have one or more lipid abnormalities. Low levels of high-density lipoprotein cholesterol (HDL-C) are often associated with elevated triglyceride levels, the most prevalent form of dyslipidemia in patients with diabetes (14). Studies illustrated that SUA can inhibit the synthesis of adiponectin in adipocytes by reducing the production of nitric oxide in arterial endothelial cells, disrupting the tricarboxylic acid cycle and the oxidation of fatty acid β, and finally promoting the oxidative activity of cells (15).

Besides, available data suggest that uric acid is not necessarily an antioxidant and, depending on the chemical milieu, may become a prooxidant (16, 17). This partly explained the U- or L-shaped association between SUA and blood glucose reported in previous cross-sectional studies (18, 19). What is more, Rodriguez and his colleagues identified that individuals with prediabetes are at a higher risk of developing gout, but once they develop diabetes, their risk drops to a lower level than that of non-diabetic individuals, and diabetes may reduce the future risk of gout through the uricosuric effect of glycosuria or the impaired inflammatory response (20).

To clarify the complex interaction between metabolic syndrome components and formulate reasonable diabetes control measurements, this study collected data from a nationally representative database, the China Health and Retirement Longitudinal Study (CAHRLS), to initially explore whether the uric acid level is independently related to the future risk of diabetes and then introduced a restricted cubic spline function to identify whether there is a non-linear relationship between baseline uric acid and follow-up blood glucose; further used crosslagged path analysis to determine the temporal relation of blood uric acid and blood glucose. Once the temporal relationship is established, we would investigate the mediating effect of BMI and dyslipidemia on the relation of uric acid and risk of diabetes.

MATERIALS AND METHODS

Study Population

The China Health and Retirement Longitudinal Study (CHARLS) takes the mainland of China as the sampling frame, the community (in the city) or village (in urban) as the sampling unit, and uses probability proportionate to size sampling (PPS) as the sampling technology, to investigate the information on health and retirement of middle-aged and elderly people aged 45 or over, with no upper age limit (21). The CHARLS data can be freely downloaded from the official website (http://charls.pku.edu.cn/index/zh-cn.html). So far, CHARLS yields four waves of data in 2011, 2013, 2015, and 2018 and two blood test data in 2011 and 2015. Information about demographics, biomedical measurements, socioeconomic status, and self-reported health status and functioning was measured by trained health workers (21), and all participants were asked to take venous blood on fasting overnight (22). The CHARLS is a large-scale interdisciplinary research project sponsored by the National Development Institute of Peking University. Ethical approval for all the CHARLS waves, therefore, was granted from the Institutional Review Board (IRB) at Peking University, including anthropometrics (IRB00001052-11015) and biomarker collection (IRB00001052-11014).

In this study, at baseline (2011), a total number of 17,708 respondents completed a face-to-face computer-assisted personal interview, and the exclusion criteria were as follows: (1) without blood samples; (2) missing value of SUA, fasting blood glucose (FPG), hemoglobin A1c (HbA1c), blood urea nitrogen (BUN), estimated glomerular filtration rate (eGFR); (3) with cancer; and (4) diagnosed with type 2 diabetes. A number of 9,431 participants were remained at baseline after exclusion criteria. In 2013 and 2015, 8,545 and 8,323 subjects were successfully followed, and 68 and 762 of them were diagnosed with diabetes, respectively. A number of 9,020 participants were finally followed up, and 795 of them were diagnosed with type 2 diabetes. More details are shown in **Figure 1**.

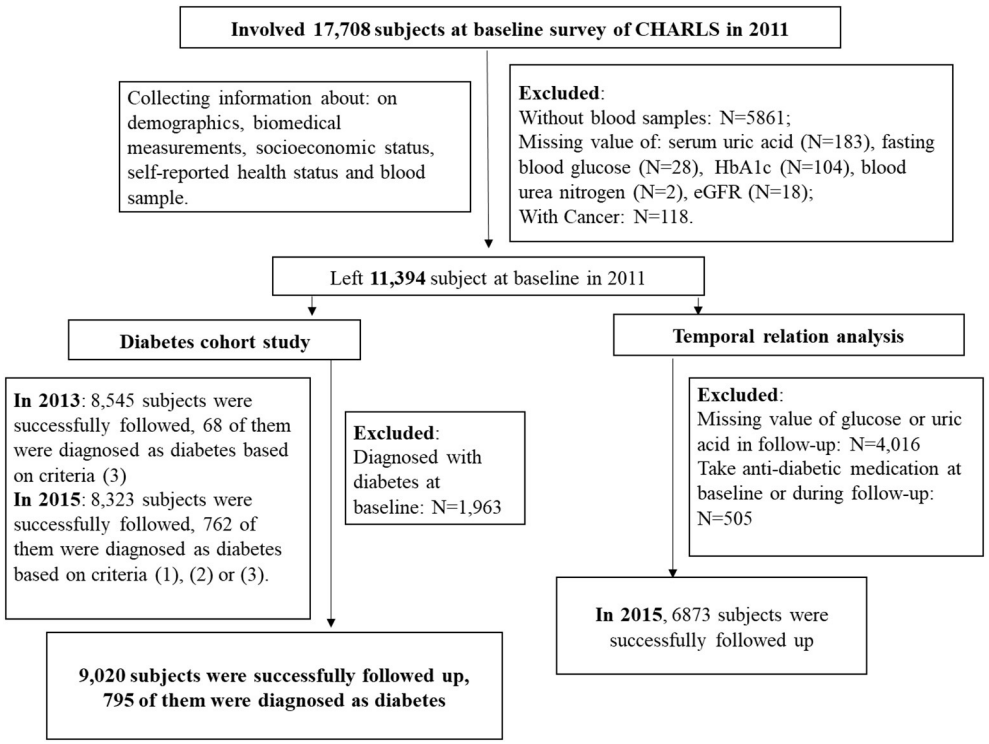

FIGURE 1 | Flow diagram of patients included in this study.

Demographic, Anthropometric, and Biochemical Parameters

According to the common classification in the existing literature, we divided the education level into the following four levels, including no formal education or illiterate, elementary or below, middle school, and high school or above. Based on the setting of the questionnaire, we simply divided marital status married or living with a partner, vs. others. Blood pressure was measured using an Omron HEM-7200 sphygmomanometer in the sitting position, three consecutive measurements were taken using the standard method, 45 s apart, and the average of the three results was taken as the final blood pressure. Hypertension means systolic blood pressure $\geq$ 140 mmHg or diastolic blood pressure $\geq$ 90 mmHg, and we also classified people into the hypertension group who had been diagnosed with hypertension by their doctor or currently using antihypertensive medication. A higher level of high-sensitivity C-reactive protein (hs-CRP) over 3 mg/dL indicates inflammation in the body (23). The estimated eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration equation (24).

Definition of Primary Variables

Participants who meet one of the following criteria are considered to have type 2 diabetes: (1) FPG $\geq$ 7.0 mmol/L (126 mg/dL); (2) random plasma glucose (without overnight fasting) $\geq$ 11.1 mmol/L (200 mg/dL); (3) HbA1c $\geq$ 48 mmol/mol (6.5%), (4) self-reported physician-diagnosed diabetes; and (5) currently taking antidiabetic medication.

The definition of hyperuricemia is different for men and women, for men with SUA $\geq$ 420 μmol/L, and for women with SUA $\geq$ 360 μmol/L (25). We further divided participants into four groups using SUA gender-specific quartiles.

Once participants met one of the following criteria, they were diagnosed with dyslipidemia: (1) total cholesterol (TC) $\geq$ 240 mg/dl, (2) high-density lipoprotein cholesterol (HDL-C) $<$ 40 mg/dl, (3) low-density lipoprotein cholesterol (LDL-C) $>$ 160 mg/dl, and (4) triglycerides (TG) $\geq$ 200 mg/dl (26).

Body mass index was calculated as dividing weight (kilogram) by height (meter) squared, and they were further divided into four categories (27), as follows: underweight (BMI $<$ 18.5 kg/m^2), normal weight (BMI $<$ 24 kg/m^2), overweight (BMI $<$ 28 kg/m^2), and obese (BMI $\geq$ 28 kg/m^2).

Statistical Analysis

Statistical analysis was conducted by STATA version 16.0 (StataCorp, College Station, TX, USA) and R 4.0.3 (R Project for Statistical Computing), and a p-value $<$ 0.05 (2-tailed) was considered statistically significant. To make this study represent the overall level of middle-aged and elderly people in China, we took blood weight published in the CHARLS database in 2011 as the initial weight, further introducing the jackknife method to conduct weighted analysis based on the PPS sampling design.

Baseline characteristics of study participants were reported by percentages for categorical variables or mean (standard error, SEM) for continuous variables. Groups were compared with one-way analysis of variance or the Kruskal–Wallis test for continuous variables, and Cochran–Mantel–Haenszel chi-square test for categorical variables.

Since new cases of diabetes were investigated at the 2013 or 2015 follow-up surveys, we were unable to estimate person-year accurately, in addition to the high incidence of diabetes in middle-aged and elderly people, prevented us from using odds ratios (*OR*) calculated by logistic regression to estimate relative risk (*RR*), since the use of *OR* instead of *RR* is artificially appropriate for rare events, Instead, we introduced the Cox proportional hazards regression with a robust variance estimator to estimate the *RR*, we set the follow-up time to 1, and we used the Breslow method to break ties (28).

In addition, our previous study has confirmed an L-shape association between SUA and blood glucose at the same measurement point (19), but we did not know whether the relation of baseline SUA and the risk of diabetes during follow-up is linear. So, we introduced restricted cubic spline models to examine the dose–response association of SUA (continuous) with follow-up blood glucose (continuous). If the above dose–response relationship was linear, we further conducted Cox proportional hazards regression to evaluate the association of SUA quartiles or continuous SUA levels (per 100 μmol/L elevations) or hyperuricemia (yes/no) with the risk of diabetes.

Further, the longitudinal changes of uric acid and blood glucose measured at two-time points are typically a crosslagged panel design (29). A regression residual analysis was initially used to identify the baseline and follow-up uric acid and blood glucose after adjusting all potential confounding factors, and then, values of adjusted residuals were standardized by Z-transformation (mean = 0, standard deviation = 1). The crosslagged path coefficients (ρ_1 and ρ_2) were estimated simultaneously based on the correlation matrix using the maximum likelihood method in R 4.0.3 (Package: "lavaan"). The validity of model fitting was indicated by the root mean square residual (RMR) and comparative fitness index (CFI) (30), and RMR $<$ 0.05 and CFI $>$ 0.90 indicate good fit to the observed data.

Finally, a Karlson–Holm–Breen (KHB) (31) method was constructed to examine the mediation effect of baseline BMI and dyslipidemia on the association between baseline SUA and follow-up risk of diabetes.

RESULTS

The Characteristics Regarding the Study Variables

Baseline demographic and clinical characteristics of the study population are given in **Table 1**. A total of 9,020 individuals (4,185 men and 4,835 women) were included in this study. At baseline, the mean BMI was 23.31 kg/m^2, the FPG level was 99.63 mg/dl, the uric acid level was 270.05 μmol/L, the HbA1c level was 5.08%, and the prevalence of dyslipidemia was nearly 39.55%. After 4 years of follow-up, BMI increased by 0.4 units and SUA levels increased by 20 umol/L. A total of 795 participants were diagnosed with new-onset diabetes, with a cumulative 4-year incidence of 8.81%. Compared with women, men were more likely to be current smokers, regular drinkers, with higher hs-CRP, BUN, and SUA levels at baseline. In contrast, never smoking, never drinking, being illiterate, with higher BMI, and eGFR level were more common in women.

Dose–Response Relation of Baseline Uric Acid and Incidence Diabetes

As shown in **Figure 2**, the β_2 coefficient of non-linear trend calculated by restricted cubic spline was not significant, suggesting a linear dose–response association of SUA with blood glucose [FPG (**Figure 2A**) and HbA1c (**Figure 2B**) as continuous variables, diabetes (**Figure 2C**) as a dichotomous variable, respectively].

The linear coefficients between SUA and diabetes are shown in **Table 2**. In the total samples, compared with the lowest sex-specific quartile of SUA levels, individuals in the higher quartiles of SUA had a higher incident risk of diabetes. The *RR*s of incident diabetes were 1.00 (95% *CI*: 0.82–1.23), 1.14 (95% *CI*: 0.94–1.39), and 1.37 (95% *CI*: 1.11–1.69) for individuals in Q2, Q3, and Q4, respectively (p-trend $<$ 0.01; basic model). Additionally, per 100 μmol/L of SUA level increase was significantly associated with 1.21 (95% *CI*: 1.09–1.36)-fold higher incident risk of diabetes in the basic model. Additionally, those participants diagnosed with hyperuricemia at baseline had a 41% increased risk of developing diabetes during follow-up (95% *CI*: 7–86%; $p <$ 0.01). The observed association attenuated and was no longer significant after further adjustment for baseline BMI levels (basic model + BMI) or baseline dyslipidemia levels (basic model + dyslipidemia), whether for SUA as a continuous or categorical variable.

Stratified analysis by sex found that SUA was not significantly associated with the risk of diabetes in the men subgroup. Whereas, in women, the basic model is similar to the total population, compared with the first sex-specific quartile of SUA levels, the *RR*s of diabetes were 1.08 (95% *CI*: 0.81–1.44), 1.34 (95% *CI*: 1.02–1.77), 1.64 (95% *CI*: 1.24–2.18) for quartile 2–4 (p-trend = 0.001, basic model), respectively. A 100 μmol/L increment of SUA was linked with 40% (95% *CI*: 21–62%) elevated risk of diabetes in women (basic model). The observed association decreased but remained significant after further adjustment for BMI or baseline dyslipidemia levels in women (basic model + BMI: p-trend $<$ 0.01; basic model + dyslipidemia: p-trend $<$ 0.01). For women, we further stratified by menopause status. As shown in **Table 3**, after adjusting for confounders, the relation of baseline SUA and the risk of diabetes in premenopausal women were not statistically significant. In the postmenopausal female subgroup, the results were similar to the overall analysis of all female patients, no matter took uric acid as a continuous variable or dichotomic variable, and the increase of uric acid can lead to the increased risk of diabetes in postmenopausal women.

TABLE 1 | Weighted characteristics of the study participants according to different gender.

	All	Men	Women	*p*-value
Baseline (2011)				
n	9,020	4,185	4,835	
Rural (%)	54.21	54.74	53.71	0.41†
Married or living with a partner (%)	82.74	86.80	78.77	<0.001†
Smoking status (%)				<0.001†
Never smoker	62.82	28.44	93.08	
Former smoker	9.09	17.48	1.71	
Current smoker	28.08	54.08	5.21	
Alcohol consumption (%)				<0.001†
Never drinking	58.71	59.31	83.90	
Former drinkers	7.20	7.26	3.79	
Occasional drinkers	8.61	8.69	5.64	
Regular drinkers	25.47	24.74	6.67	
Education levels (%)				<0.001†
No formal education	24.73	11.96	36.38	
Elementary or below	39.51	43.62	35.76	
Middle school	22.49	27.98	17.48	
High school or above	13.27	16.44	10.38	
Dyslipidemia (%)	39.55	40.37	38.79	0.38†
High hs-CRP (%)	17.50	19.58	15.60	<0.01†
Hypertension (%)	41.84	40.86	42.72	0.18†
Age (years)	58.59 (0.21)	59.16 (0.27)	58.07 (0.24)	<0.001§
BMI (kg/m^2)	23.31 (0.07)	22.86 (0.08)	23.74 (0.06)	<0.001§
Systolic (mmHg)	130.83 (0.36)	130.88 (0.43)	130.77 (0.36)	0.46‡
Diastolic (mmHg)	75.64 (0.22)	76.13 (0.31)	75.20 (0.24)	<0.001§
WC (cm)	83.81 (0.24)	83.63 (0.31)	83.97 (0.23)	<0.01§
BUN (mg/dl)	15.65 (0.09)	16.41 (0.10)	14.96 (0.10)	<0.001§
eGFR (ml/min/1.73 m^2)	85.56 (0.23)	84.13 (0.24)	86.89 (0.28)	<0.001§
HDL-C (mg/dl)	50.75 (0.33)	49.95 (0.33)	51.49 (0.26)	<0.001§
LDL-C (mg/dl)	115.54 (0.56)	112.80 (0.64)	118.04 (0.65)	<0.001‡
T-cho (mg/dl)	189.77 (0.69)	185.75 (0.69)	193.44 (0.72)	<0.001§
TG (mg/dl)	123.79 (1.74)	119.68 (2.23)	127.54 (1.85)	<0.001§
SUA (μmol/L)	270.05 (1.27)	300.87 (1.81)	241.85 (1.30)	<0.001§
FPG (mg/dl)	99.63 (0.23)	99.64 (0.30)	99.62 (0.25)	0.65§
HbA1c (%)	5.08 (0.02)	5.08 (0.01)	5.07 (0.01)	0.55‡
Follow-up (2015)				
BMI (kg/m^2)	23.76 (0.05)	23.21 (0.06)	24.26 (0.06)	<0.001§
SUA (μmol/L)	298.11 (1.47)	331.94 (2.10)	268.57 (1.46)	<0.001§
FPG (mg/dl)	98.13 (0.35)	98.73 (0.50)	97.61 (0.44)	0.83§
HbA1c (%)	5.81 (0.01)	5.78 (0.01)	5.84 (0.01)	<0.001§
Diabetes (%)	8.81	8.48	9.10	0.30†

Unless indicated otherwise, data are given as the means (SEM) or as percentages.
† *Cochran –Mantel–Haenszel chi-square test.*
‡ *One-way analysis of variance (ANOVA).*
§ *The Kruskal–Wallis test.*

The Crosslagged Path Analysis of Uric Acid and Blood Glucose

As shown in **Figure 3A**, in all pathways, only the path coefficients (ρ_1) from baseline SUA to follow-up blood glucose ($\rho_1 = 0.24$, $p = 0.03$) were significant in the total population after adjusting for confounding factors, and the RMR and CFI were 0.009 and 0.998 for FPG, 0.002 and 0.999 for HbA1c, respectively, indicating a good fit to the observed data, suggesting that SUA was more likely to affect blood glucose and is a risk factor for diabetes. Further stratified by sex, we found that this path coefficient between baseline uric acid and follow-up blood glucose was significant only in the female subgroup (for

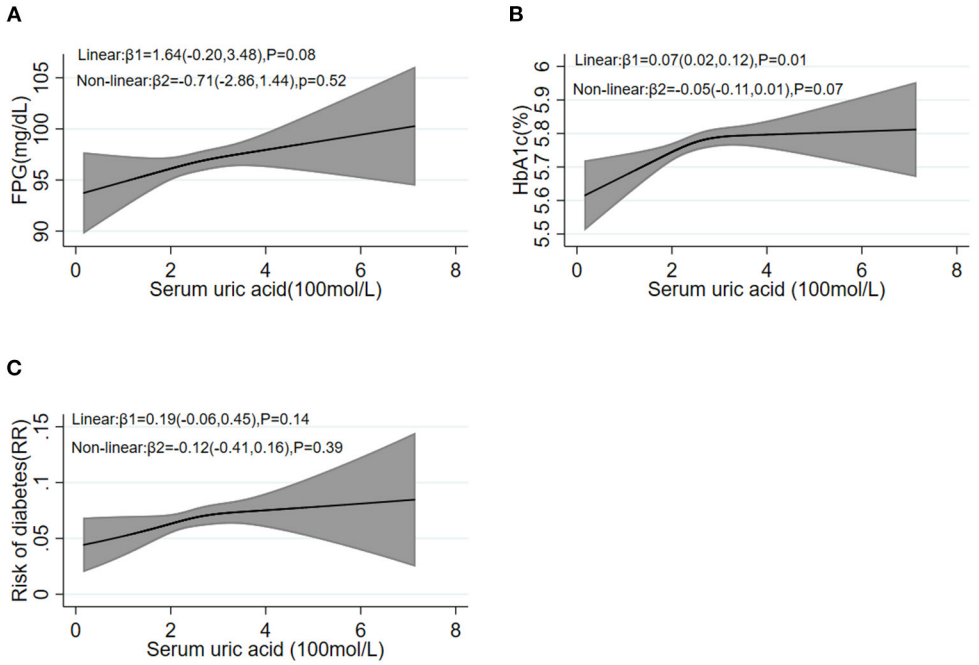

FIGURE 2 | Restricted cubic spline analysis between baseline SUA levels and follow-up FPG **(A)**, HbA1c **(B)**, and the risk of diabetes **(C)**. FPG, fasting plasma glucose; HbA1c, hemoglobin A1c.

FPG: $\rho_1 = 0.05$, $p < 0.01$; for HbA1c: $\rho_1 = 0.04$, $p = 0.03$; **Figure 3C**), and no longer had a statistical association in men (**Figure 3B**).

The Mediation Analysis in the Women Subgroup

Since the above statistical analysis confirmed that SUA was an independent risk factor for the development of diabetes in women in terms of both the strength of association (*RR*) and the temporal sequence (crosslagged path analysis), we analyzed the mediating effects of BMI and dyslipidemia in the female subgroup, respectively (**Figure 4**). After adjusting for all potential confounding factors in **Figure 4A**, the increased level of baseline SUA was positively correlated with baseline BMI ($\beta_1 = 0.12$, $p < 0.001$) or dyslipidemia ($\beta_1 = 0.07$, $p < 0.001$), and the change in baseline BMI or dyslipidemia could significantly increase the incidence of diabetes (for BMI: $\beta_2 = 0.38$, $p < 0.001$; for dyslipidemia: $\beta_2 = 0.57$, $p < 0.001$). Similar results were found in a subgroup of postmenopausal women who were assessed separately for BMI and dyslipidemia (**Figure 4B**). Multiple parallel mediation analyses (**Table 4**) showed a significant total effect of baseline SUA on diabetes risk ($\beta = 0.19$, 95% *CI*: 0.08–0.29, $p < 0.001$), the direct effect of SUA on the relation of diabetes accounted for 58.13% ($\beta = 0.11$, 95% *CI*: 0.01–0.22, $p < 0.001$) of the total effect, and the combined indirect effect of BMI and dyslipidemia explained residual 41.78% ($\beta = 0.8$, 95% *CI*: 0.06–0.10, $p < 0.001$) of the total effect. Additionally, in the stratified analysis, we confirmed this mediating effect in postmenopausal women.

DISCUSSION

This study represents a comprehensive examination of the longitudinal relationship between uric acid concentration and diabetes in a national-based population. Our results help reconcile conflicting evidence in the literature and demonstrate the following: (1) an unidirectional relationship between SUA and blood glucose was identified, and increased SUA is an independent risk factor for diabetes; (2) In men, the longitudinal association between SUA and diabetes was not significant after adjustment for confounders.; (3) In women, SUA is an important risk factor for the development of diabetes, especially in postmenopausal women, and this harmful effect of uric acid is partly mediated by BMI and dyslipidemia.

Since the sex-specific cutoff points for hyperuricemia have been widely used globally, it is of importance to identify whether there are sex-specific associations between SUA and diabetes. Previous studies reported that SUA increment was associated with increased risk for diabetes in women but not in men (32, 33). Consistent with this, we confirmed that for every 100 μmol/L increase in SUA in women, the risk of diabetes was 1.31-fold higher in women. Not only in its association with diabetes but also gender have differences also often been seen in other fields. In the Framingham Heart Study, levels of SUA were associated with an increased risk of cardiovascular death in women but not in men (34). Additionally, Ndrepepa et al.

TABLE 2 | Risk ratios (95% confidence intervals) of diabetes according to SUA.

	Univariate ***RR* (95% *CI*)**	**Basic model** ***RR* (95% *CI*)**	**Basic model + BMI** ***RR* (95% *CI*)**	**Basic model + Dyslipidemia** ***RR* (95% *CI*)**
Total participants				
Q1	Ref.	Ref.	Ref.	Ref.
Q2	1.01 (0.83, 1.23)	1.00 (0.82, 1.23)	0.96 (0.79, 1.18)	0.99 (0.81, 1.21)
Q3	1.16 (0.95, 1.40)	1.14 (0.94, 1.39)	1.08 (0.89, 1.31)	1.10 (0.90, 1.34)
Q4	1.43 (1.18, 1.72)***	1.37 (1.11, 1.69)**	1.23 (0.99, 1.52)	1.28 (1.04, 1.57)*
P for trend[a]	<0.001	<0.01	0.11	0.01
Normal uric acid	Ref.	Ref.	Ref.	
Hyperuricemia	1.50 (1.16, 1.93)**	1.41 (1.07, 1.86)*	1.32 (0.99, 1.74)	1.33 (1.01, 1.75)*
Uric acid (100 umol/l)	1.17 (1.08, 1.27)***	1.21 (1.09, 1.36)**	1.15 (0.03, 1.29)*	1.17 (1.05, 1.30)*
Male participants				
Q1	Ref.	Ref.	Ref.	Ref.
Q2	1.00 (0.76, 1.32)	1.01 (0.77, 1.34)	0.97 (0.74, 1.29)	1.00 (0.76, 1.32)
Q3	0.86 (0.64, 1.15)	0.88 (0.655, 1.18)	0.84 (0.63, 1.13)	0.86 (0.64, 1.16)
Q4	1.05 (0.79, 1.38)	1.05 (0.77, 1.43)	0.98 (0.72, 1.34)	1.02 (0.75, 1.38)
P for trend[a]	0.882	0.559	0.748	0.729
Normal uric acid	Ref.	Ref.	Ref.	Ref.
Hyperuricemia	1.43 (1.01, 2.04)*	1.42 (0.99, 2.01)	1.32 (0.99, 1.74)	1.41 (0.97, 2.16)
Uric acid (100 umol/l)	1.05 (0.91, 1.21)	1.07 (0.90, 1.26)	1.04 (0.88, 1.23)	1.05 (0.89, 1.24)
Female participants				
Q1	Ref.	Ref.	Ref.	Ref.
Q2	1.09 (0.82, 1.45)	1.08 (0.81, 1.44)	1.05 (0.79, 1.40)	1.08 (0.81, 1.44)
Q3	1.40 (1.07, 1.83)*	1.34 (1.02, 1.77)*	1.25 (0.95, 1.65)	1.27 (0.97, 1.67)
Q4	1.81 (1.40, 2.34)***	1.64 (1.24, 2.18)**	1.45 (1.09, 1.93)*	1.49 (1.13, 1.97)*
P for trend[a]	<0.001	0.001	0.006	0.001
Normal uric acid	Ref.	Ref.	Ref.	Ref.
Hyperuricemia	1.61 (1.11, 2.35)*	1.36 (0.91, 2.03)	1.23 (0.81, 1.85)	1.27 (0.86, 1.88)
Uric acid (100 umol/l)	1.46 (1.29, 1.65)***	1.40 (1.21, 1.62)***	1.31 (1.12, 1.52)*	1.32 (1.15, 1.53)**

*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.
The based model was adjusted for age (standardized), gender, education levels, marital status and place of residence, high-sensitivity C-reactive protein, blood urea nitrogen, smoking status, drinking status, and hypertension.
[a]*Test for linear trend was performed using the median SUA levels for each quartile as a continuous variable.*
SUA, serum uric acid; Q1, Q2, Q3, and Q4 were the quartiles of SUA; BMI, body mass index.

also demonstrated that hyperuricemia could predict an increased cardiovascular risk of mortality in both genders, with a stronger association in women (35). Possible mechanisms for gender differences include the following: first, there were differences in BMI, hypertension, WC, BUN, eGFR, and blood lipid between genders, which partly explained the different reactivities of uric acid and blood glucose in men and women; second, a reduction in estrogen levels after menopause in women may result in dysregulation of blood glucose and lipid metabolism (36), and of the 4,835 women included in this study, about 68% were postmenopausal, and our subgroup analysis proved that elevated uric acid increased the risk of diabetes only in postmenopausal women. Besides, a genome-wide association study reported a significant association between the SLC2A9 and urate concentrations, whereas the proportion of the variance of SUA concentrations explained by expression levels was 3.5% in men and 15% in women (37), which possibly suggests a genetic basis for the sex differences.

The mediating effect is based on the premise that the causal relationship between independent and dependent variables holds. Previous studies have identified uric acid as a risk factor for diabetes (18, 19, 38); however, Lu et al. used an animal model which confirmed that hyperuricemia could accelerate but do not cause diabetes by inhibiting islet β-cell survival (39); only male mice were used in their study, which may impede the extrapolation from animal experiments to human populations. Meanwhile, several Mendelian randomized (40–42) studies also do not support a causal role of SUA for the development of diabetes and limit the expectation that UA-lowering drugs will be effective in the prevention of diabetes (39); whereas the genetic risk score in above Mendelian randomization studies only explained 2.9% of SUA variation, the strength

TABLE 3 | Risk ratios (95% confidence intervals) of diabetes according to SUA in women subgroup analysis.

	Univariate ***RR* (95% *CI*)**	**Basic model** ***RR* (95% *CI*)**	**Basic model + BMI** ***RR* (95% *CI*)**	**Basic model + Dyslipidemia** ***RR* (95% *CI*)**
Without menopause (*N* = 1,545)				
Q1	Ref.	Ref.	Ref.	Ref.
Q2	1.16 (0.67, 2.03)	1.16 (0.66, 2.03)	1.14 (0.65, 2.00)	1.15 (0.66, 2.02)
Q3	1.51 (0.88, 2.58)	1.40 (0.80, 2.44)	1.35 (0.78, 2.33)	1.31 (0.75, 2.29)
Q4	2.20 (1.34, 3.61)**	1.44 (0.83, 2.50)	1.30 (0.75, 2.62)	1.28 (0.73, 2.22)*
P for trend[a]	<0.01	0.557	0.698	0.700
Normal uric acid	Ref.	Ref.	Ref.	Ref.
Hyperuricemia	1.66 (0.71, 3.85)	1.38 (0.56, 3.41)	1.29 (0.52, 3.20)	1.27 (0.51, 3.15)
Uric acid (100 umol/l)	1.57 (1.25, 1.99)***	1.25 (0.95, 1.65)	1.18 (0.89, 1.57)	1.16 (0.88, 1.54)
Menopause (*N* = 3, 290)				
Q1	Ref.	Ref.	Ref.	Ref.
Q2	1.05 (0.75, 1.47)	1.04 (0.74, 1.47)	1.00 (0.72, 1.42)	1.05 (0.75, 1.48)
Q3	1.31 (0.96, 1.79)	1.28 (0.93, 1.76)	1.18 (0.85, 1.62)	1.23 (0.89, 1.69)
Q4	1.64 (1.22, 2.21)**	1.57 (1.15, 2.16)**	1.39 (1.01, 1.93)*	1.44 (1.05, 1.97)*
P for trend[a]	<0.01	0.02	0.02	0.04
Normal uric acid	Ref.	Ref.	Ref.	Ref.
Hyperuricemia	1.55 (1.03, 2.37)*	1.21 (0.76, 1.92)	1.08 (0.67, 1.75)	1.11 (0.71, 1.73)
Uric acid (100 umol/l)	1.40 (1.21, 1.62)***	1.35 (1.15, 1.58)***	1.26 (1.07, 1.49)**	1.28 (1.10, 1.49)**

*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.
The based model was adjusted for age (standardized), gender, education levels, marital status and place of residence, high-sensitivity C-reactive protein, blood urea nitrogen, smoking status, drinking status, and hypertension.
[a]*Test for linear trend was performed using the median SUA levels for each quartile as a continuous variable.*
SUA, serum uric acid; Q1, Q2, Q3, Q4 were the quartiles of SUA; BMI, body mass index.

TABLE 4 | Multiple parallel mediation effect of baseline BMI and dyslipidemia on the relation of baseline SUA and risk of diabetes in women.

	Mediators	**Effect**	**SE**	**95% *CI***	***P***	**Proportion**
Total women (*N* = 4,835)						
Direct effect		0.11	0.05	(0.01, 0.22)	<0.001	58.13%
Indirect effect	BMI+ Dyslipidemia	0.08	0.01	(0.06, 0.10)	<0.001	41.87%
	BMI	0.04	0.01	(0.03, 0.07)	<0.001	23.05%
	Dyslipidemia	0.04	0.01	(0.02, 0.06)	<0.001	18.82%
Total effect		0.19	0.05	(0.08, 0.29)	<0.001	100%
Postmenopausal women (*N* = 3,290)						
Direct effect		0.12	0.06	(−0.01, 0.23)	0.06	60.68%
Indirect effect	BMI+ Dyslipidemia	0.07	0.01	(0.05, 0.10)	<0.001	39.32%
	BMI	0.04	0.01	(0.03, 0.07)	<0.001	23.05%
	Dyslipidemia	0.03	0.01	(0.02, 0.06)	<0.001	18.82%
Total effect		0.19	0.06	(0.07, 0.31)	<0.001	100%

The mediation model is based on the Karlson–Holm–Breen (KHB) method. All analysis was adjusted age, marital status, education background, smoking, drinking, high-sensitivity C-reactive protein, and blood urea nitrogen.
The total effect is the effect of SUA on diabetes without considering BMI or dyslipidemia; the direct effect is the effect of SUA on diabetes when controlling for BMI and dyslipidemia; the indirect effect is the effect of SUA on diabetes through BMI or dyslipidemia; proportion: mediation effect by BMI or dyslipidemia is calculated by indirect effect/total effect × 100.

of the evidence may be insufficient. Taking the above results into consideration, we further explored this chicken-and-egg question by crosslagged path analysis, which is a powerful statistical approach in dissecting a causal relationship between intercorrelated variables (29). In our results, the path coefficient from baseline SUA to follow-up blood glucose was statistically significant in the total population and the female subgroup, providing statistical evidence that uric acid is a possible cause of blood glucose; therefore, we only explore the potential mediation effect in women.

Similar to our findings, some research pointed out that the association between SUA and the risk of diabetes is largely

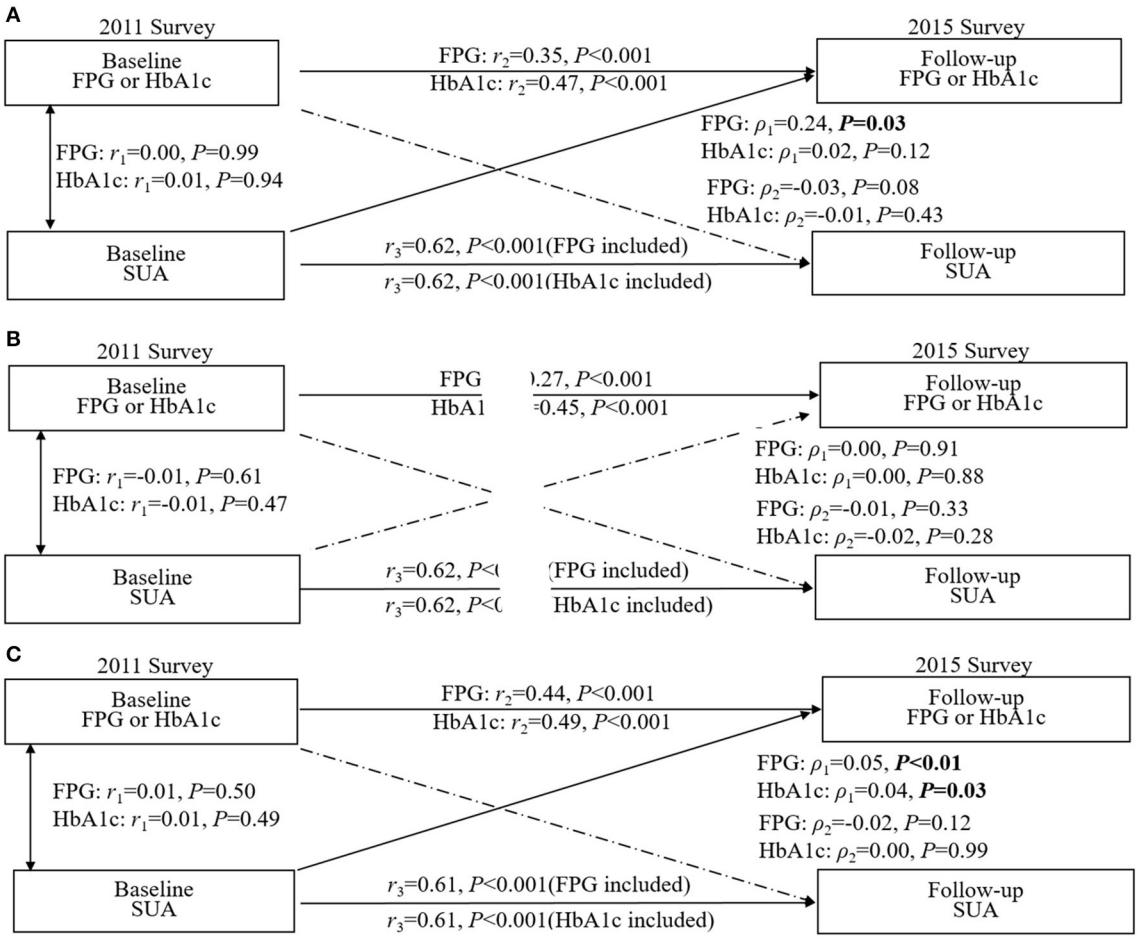

FIGURE 3 | Crosslagged path analysis models for the association of fasting plasma glucose (FPG) and HbA1c with SUA. **(A)** included all participants (n = 6,873); **(B)** included male participants (n = 3,162); **(C)** included female participants (n = 3,711). All results were adjusted for age, sex, marital status, education background, waist circumference, smoking, drinking, hypertension and high-sensitivity C-reactive protein. ρ_1, crosslagged path coefficient from baseline SUA to follow-up blood glucose (FPG and HbA1C); ρ_2, crosslagged path coefficient from baseline blood glucose (FPG and HbA1C) to follow-up SUA. r_1, represents synchronous correlations; r_2 and r_3 represent tracking correlations.

decreased or eliminated after adjusting for BMI (10, 11). UA can affect adipocytes by inducing upregulation of proinflammatory factors and downregulation of the insulin sensitizer and antiinflammatory factor adiponectin (43). Adiponectin is negatively associated with BMI and body fat (44) since low levels of adiponectin are associated with the development of insulin resistance (45), and it could be speculated that adiponectin is part of the link between UA and insulin resistance (46). Additionally, data coming from the National Health and Nutrition Examination Survey (NHANES) demonstrated a significant association between elevated SUA levels and the increased prevalence of abdominal obesity, hypertriglyceridemia, and hyperglycemia (47), and some prospective studies also showed that elevated SUA levels may increase the risk of hypertriglyceridemia (48). Hypertriglyceridemia is known as a dominant lipid abnormality in insulin resistance by inducing elevated levels of free fatty acids, which plays an important role in the development of diabetes (49). The interactive effects of increased TG and the LDL-C/HDL-C ratio suggest that dyslipidemia might exaggerate the risk of diabetes in hypertensive patients (50). This study demonstrated that nearly 42% total effect of UA on diabetes is mediated by BMI and dyslipidemia in women. This provides a theoretical basis for the designation of preventive measures. For middle-aged and older women, especially those with higher uric acid levels, reasonable diet control and physical exercise can keep them in the normal BMI range and reduce the probability of dyslipidemia, which can help us avoid developing diabetes in the future.

Strengths of this study include a wealth of sociodemographic, clinical, and laboratory variables collected by medical professionals in a standardized manner with a low rate of missing data. In addition, the confirmation of multiple mediating roles of BMI and dyslipidemia helps to clarify the pathogenetic pathway of uric acid to diabetes. What is more, CHARLS adopts probability proportional to size sampling, so the results can represent the current level of China and have

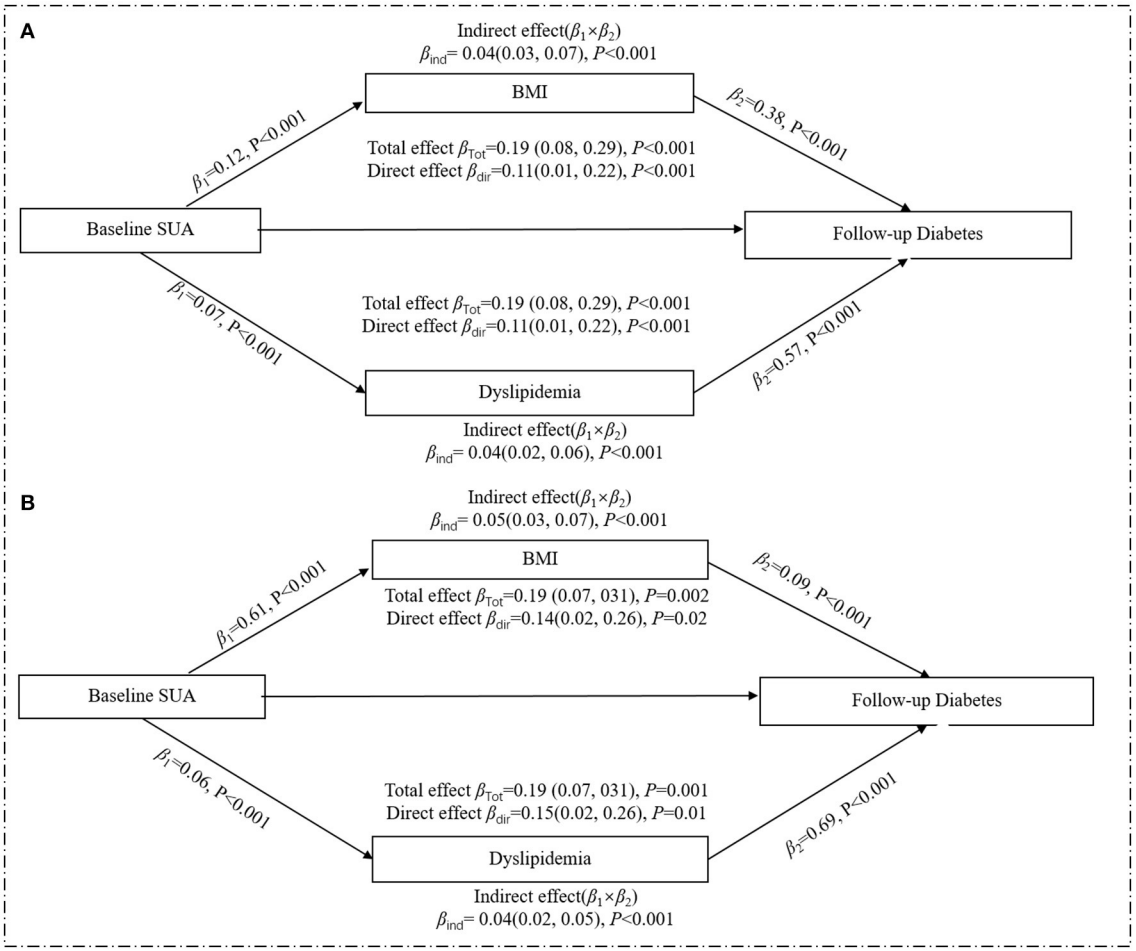

FIGURE 4 | Mediation effect of baseline obesity on the relation of baseline SUA and risk of diabetes in the women. **(A)** included all women (n = 4,835); **(B)** included the postmenopausal female subgroup (n = 3,290). All analyses were adjusted age, marital status, education background, smoking, drinking, high-sensitivity C-reactive protein, menopausal status, and eGFR. The total effect is the effect of SUA on diabetes without considering BMI or dyslipidemia; the direct effect is the effect of SUA on diabetes when controlling for BMI and dyslipidemia; the indirect effect is the effect of SUA on diabetes through BMI or dyslipidemia. Mediation effects by BMI or dyslipidemia are calculated by indirect effect/total effect × 100. β, regression coefficients.

good extrapolation. Study weaknesses include our follow-up period which was only 4 years, which was comparatively shorter than other cohort studies, and despite adjustment for a range of potential confounders, the possibility of residual and unmeasured confounders may not be ruled out, such as diet, drugs, and genetic information. Besides, as in any observation study, causality cannot be determined by the strength of relation and temporal relation alone, and more definitive basic studies are warranted to confirm causality between UA and diabetes.

CONCLUSION

In conclusion, using a sample drawn from the China Health and Retirement Longitudinal Study, this study extended the findings of the previous literature by confirming that SUA and risk of type 2 diabetes are only significant in middle-aged and elderly Chinese women, and further quantified the mediating proportion of BMI and dyslipidemia in the relationship between SUA and risk of type 2 diabetes in women. For middle-aged and elderly Chinese women, especially those with high uric acid, in addition to corresponding measures to reduce uric acid, integrally targeted interventions and strategies that can alleviate BMI and dyslipidemia should be combined to reduce the risk of 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: the CHARLS data can be freely downloaded from the official website (http://charls.pku.edu.cn/index/zh-cn.html).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CHARLS is a large-scale interdisciplinary research project sponsored by the National Development Institute of Peking University. Ethical approval for all the CHARLS waves, therefore, was granted from the Institutional Review Board (IRB) at Peking University, including anthropometrics (IRB00001052-11015) and biomarker collection (IRB00001052-11014). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HJ designed the study. FC performed the statistical analyses, drafted the manuscript, and created the figures. YL, HZ, and LT interpreted the data and edited the manuscript. All authors listed have approved the manuscript that is enclosed and the corresponding author would take final responsibility for the paper.

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frontiers in Endocrinology

REVIEW
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Mitochondrial-Derived Peptides in Diabetes and Its Complications

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The changes of mitochondrial function are closely related to diabetes and its complications. Here we describe the effects of mitochondrial-derived peptides (MDPs), short peptides formed by transcription and translation of the open reading frame site in human mitochondrial DNA (mtDNA), on diabetes and its complications. We mainly focus on MDPs that have been discovered so far, such as Humanin (HN), mitochondrial open reading frame of the 12S rRNA-c (MOTS-c) and Small humanin-like peptides (SHLP 1-6), and elucidated the role of MDPs in diabetes and its major complications stroke and myocardial infarction by improving insulin resistance, inhibiting inflammatory response and anti-apoptosis. It provides more possibilities for the clinical application of mitochondrial derived peptides.

Keywords: mitochondrial-derived peptides (MDPs), humanin, MOTS-c, SHLPs(1-6), stroke, myocardial infarction, diabetes

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INTRODUCTION

Mitochondria, as the integration center of key signals regulating bioenergy metabolism and regulating the initiation and execution of oxidative balance protein apoptosis, can sense cellular stress and help cells adapt to the challenges of microenvironment (1, 2). Mitochondria are important organelles involved in glucose metabolism and the main source of ROS in cells, and their functional changes are closely related to blood glucose level, which can cause oxidative stress when hyperglycemia occurs due to excessive production of peroxide in mitochondrial electron transport chain (3). Oxidative stress is widely believed to play a key mediating role in the development and progression of diabetes and its complications due to the increased production of free radicals and impaired antioxidant defense ability (4).

As a one of the world's fastest growing disease, diabetes and its complications is a major cause of death in diabetes. The body long carbohydrate metabolism disorder can cause multiple system damage, lead to eyes, kidneys, nerves, heart, blood vessels and other tissues and organs of chronic progressive lesions. Common complications of diabetes mainly include Cardiovascular complications, Diabetic nephropathy, Diabetic foot, Diabetic retinopathy, etc. (5–7) among which cardiovascular diseases and neurological diseases (8, 9) are the main causes of disability and death in diabetic patients. Mitochondria are related to the occurrence and development of diabetes and its complications (10, 11). Hyperglycemia can cause increased generation of mitochondrial ROS (12), and then affect diabetic complications such as ischemic stroke (8) myocardial infarction. In addition to the above mentioned regulation of diabetic hyperglycemia

by affecting glucose metabolism, current studies have found that open reading frame sites contained in human mitochondrial rRNA can encode and form polypeptides called mitochondrial-derived peptides (MDPs). MDPs (13, 14) can be used as a new type of reverse signal molecule, the cell will retrograde pass the signals to the nucleus during stress, the regulation of gene transcription synthesis, thereby exert anti-inflammatory antiapoptotic and promote the synthesis of mitochondrial biological effect and so on, which affect the development of diabetes and its complications. We mainly discuss MDPs and the correlation of diabetes, we found a retrospect of the polypeptide function and their relationship with diabetes mellitus and related complications, especially the two more studied HN and MOTS-c.

A MITOCHONDRIAL-DERIVED PEPTIDE TYPES AND FUNCTIONS

Humanin

Humanin was first isolated and discovered by Japanese researcher Hashimoto (15) in the context of the protective factor of Alzheimer's disease. It is composed of 24 amino acids encoded in the 16S rRNA region of mtDNA and transcribed by the mitochondrial multi-cistron gene MT-RNR2. Replacing Ser at position 14 with Gly produces a potent form of HN-derived S14G-humanin (HNG), which is more than 1000 times more active than naturally sourced HN (16). The mRNA of HN peptide contains 21 amino acids for mitochondrial translation and 24 amino acids for cytoplasmic translation (17), both of which have similar biological functions and share the same essential functional domains in HN secretion and cell protection. HN exists not only in circulating body fluids, such as blood and cerebrospinal fluid, but also in metabolically active organs and tissues, such as heart, liver, and kidney, as well as neurons and skeletal muscles (16, 18, 19). The HN has three regions, including the negatively charged C-terminal (PVKRRA), the positively charged N-terminal (MAPR), and a central hydrophobic region (GFSCLLLLTSEIDL) (20) that can bind hydrophobic pockets of proteins to form alpha helix (21). HN acts by activating formyl peptide-like receptors such as (FPRL) (22) and heterotrimeric humanin receptor (23, 24) composed of gp130, ciliary neurotrophic factor receptors (CNTFR), and WSX-1. HN binding to extracellular formylpeptide receptor-like 1/2 (FPRL1/2) induces increased Ca^{2+} flux and cascading activation of extracellular signal-regulated kinases (ERK 1/2) and downstream signals, resulting in anti-apoptotic effects, and thus improved cell survival. HN binds to Gp130 WSX-1 and CNTFR receptors, and trimerization of the receptors induces activation of Janus kinases (JAK1 and JAK2), which in turn activate signal transduction factors and transcriptional activator 3 (STAT3). Dimerized STATS translocations to the nucleus to regulate target gene transcription and play a protective role in cells (25). HN also co acts with insulin-like growth factor binding protein 3 (IGFBP-3) (26) to inhibit IGFBP-3-induced apoptosis (27). Currently, HN is mainly derived from exogenous sources. In retinal pigment epithelium (RPE) cells, the exogenous HN is located in mitochondria and can promote the secretion of endogenous HN (28). The current study has found that HN, as a retroactive signal peptide molecule produced by mitochondria, has certain anti-inflammatory (29), and anti-apoptotic (30, 31), effects, promotes mitochondrial biosynthesis, enhances signal molecules in insulin-mediated Akt signaling pathway and fatty acid metabolism signaling pathway, and regulates metabolism related to aging (**Figure 1**).

MOTS-c

MOTS-c sequences, which were discovered by Lee J et al. (14), are highly conserved, especially for the first 11 residues, and are the first clear example of a reverse signal peptide molecule that can enter the nucleus and affect the stress transcriptional response. Studies have found that MOTS-c, as a reverse signal transgenic molecule-regulating gene transcription, translocates into the nucleus and binds to DNA under stress, and works with other transcription factors such as ARE to regulate the transcription of stress response genes, enhance cellular resistance and maintain homeostasis *in vivo* (32–35). MOTS-c is mainly extranuclear and co-localized in mitochondria under resting conditions (14, 32). However, during metabolic or oxidative stress, MOTS-c can be rapidly translocated to the nucleus in an AMPK-dependent manner within 30 min (32). MOTS-c entry into the nucleus requires a hydrophobic core, and in the nucleus, MOTS-c is able to bind chromatin through its hydrophobic and positive regions, as well as adaptive stress response transcription factors, including NFE2L2,Nrf2 and activating transcription factor 1 (ATF1) (32). MOTS-c is expressed in various organs and tissues of rodents and human skeletal muscle, myocardial kidney, and circulating plasma. MOTS-c has been shown to inhibit *de novo* purine synthesis, activate AMPK, and regulate fatty acid metabolism *in vivo* (14). MOTS-c also prevents coronary endothelial dysfunction by inhibiting NF-κB and reducing the release of pro-inflammatory cytokines and adhesion molecules. MOTS-c also has a regulatory effect on aging, insulin resistance caused by glucose metabolism disorders, and other aspects (**Figure 1**).

SHLP (1-6)

SHLPs are 20-38 amino acid long peptide sequences encoded by mitochondrial 16S rRNA, which are divided into six types, SHLP1-SHLP6.Of these SHLP2 and SHLP3 are widely studied and have similar protective effects to Humanin. SHLP2 is mainly found in liver. The expression of SHLP3 is high in the kidneys and muscles, while SHLP3 is mainly high in the brain and spleen (36). Studies have shown that SHLP2 can increase the signal of the insulin-mediated Akt pathway and fatty acid metabolism signaling pathway, thus maintaining the homeostasis of glucose metabolism and fatty acid metabolism. SHLP2 can also increase the number of pancreatic cells, improve mitochondrial bioenergy, and participate in a chaperon-like effect (36, 37). It also reduces apoptosis by downregulating the effect of caspase family on age-related macular degeneration cells (31). With the increase in age, SHLP2 level in blood circulation gradually decreases, suggesting that it is related to the progression of age-related diseases (36). SHLP3 can inhibit ROS production, mediate ERK signal transduction, and promote adipose cell differentiation. In addition, SHLP2/SHLP3 can play an

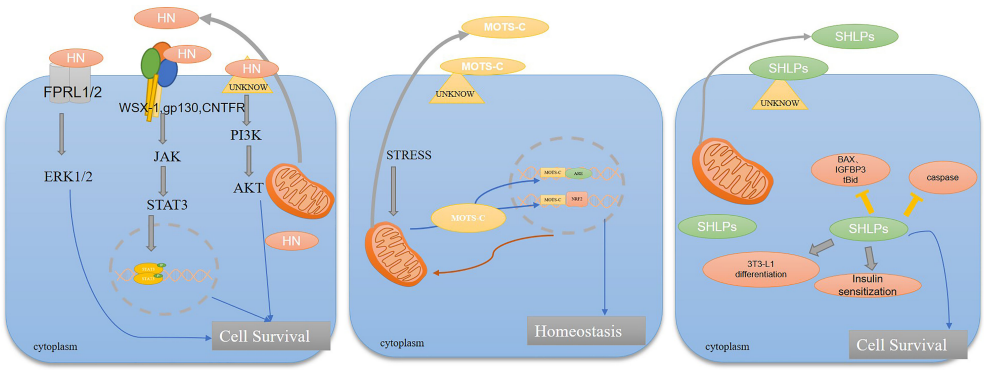

FIGURE 1 | Role of mitochondria derived peptides in cells and their regulation of related diseases. HN acts by activating formylpeptide-like receptors (FPRL) and heterotrimeranthroinin receptors composed of GP130 ciliated neurotrophic factor receptor (CNTFR) and WSX-1. In addition, HN promotes phosphorylated STAT3 dimer entry into the nucleus to regulate gene transcription and activates the FPRL1/2-ERK1/2 pathway to play a protective role in cell. MOTS-c translocates to the nucleus in a 5' -adenosine monophosphat-activated protein kinase (AMPK) -dependent manner after metabolic stress. MOTS-c regulates a wide range of genes in response to glucose restriction, including genes with antioxidant response elements (ARE), and interacts with stress response transcription factors that regulate ARE, such as the nuclear factor erythrocyte 2-associated factor 2 (NFE2L2/NRF2), to enhance mitochondrial function and thus maintain cellular homeostasis. SHLPs can enhance insulin sensitivity, promote adipocyte 3T3-L1 differentiation, inhibit the expression of caspase and ROS, and play a cellular protective role.

insulin sensitization role *in vitro*, enhance the ability of insulin to inhibit glucose production in the liver, promote the peripheral disposal of glucose, and play a role in regulating glucose metabolism homeostasis. SHLP2 and SHLP3 can also enhance cell viability and reduce cell apoptosis, while SHLP6 can do the opposite (36). In addition, SHLP2 and SHLP3 can block mitochondrial membrane damage induced by staurosporine (STS) and activation of caspase-3, thus playing a protective role (38) (**Figure 1**).

MITOCHONDRIAL-DERIVED PEPTIDES AND DISEASE

Mitochondrial-Derived Peptides and Diabetes

Diabetes mellitus (DM) (39, 40) is mainly divided into type 1 diabetes mellitus with absolute insulin deficiency caused by the destruction of pancreatic beta cells, type 2 diabetes mellitus with insulin resistance (41), and other special types of diabetes mellitus, according to clinical manifestations, pathophysiology, and etiology. Current treatments for diabetes include hyperglycemic drugs such as oral hypoglycemic agents, insulin, exercise therapy, and surgery. In metabolic tissues with insulin resistance (42), abnormal mitochondrial morphology is often found, the number of mitochondria and their oxidase is reduced, and the production of ATP is reduced. The accumulation of high circulating free fatty acids in these tissues also reduces glucose processing in response to insulin stimulation.

Present studies have demonstrated that, by targeting skeletal muscle, MDPs have a mitigating effect on insulin resistance and induce glucose uptake into the pentose phosphate pathway to avoid hepatotoxicity caused by drugs such as metformin (43) or methotrexate. Among them, Humanin has the ability to bind insulin-like growth factor binding protein 3 (26). Humanin's entry into the ventricle leads to increased insulin sensitivity in the liver and muscle, resulting in reduced glucose production in the liver and increased insulin mediated Akt signaling and fatty acid metabolism signaling. Humanin also enhances peripheral glucose uptake and inhibits liver glucose production (44, 45). Han et al. showed that HNG may improve insulin resistance by reducing Ser636 phosphorylation of insulin receptor substrate 1 (IRS1) in the hippocampus. In addition, SHLP2 and SHLP3 can also improve insulin response, enhance the ability of insulin to inhibit glucose production in the liver, and promote peripheral glucose processing. In 2016, Cobb LJ et al. (36) found the insulin sensitization effect of SHLP2 and SHLP3 *in vitro* and *in vivo*. In response to insulin, SHLP2 and SHLP3 both accelerated the differentiation of 3T3-L1 cells in mouse pre-adipose cell lines and enhanced insulin sensitivity. Compared with SHLP3, SHLP2 improved insulin responsiveness, enhanced insulin's ability to inhibit hepatic glucose generation (HGP), and promoted glucose access to peripheral tissues. MOTS-c targets skeletal muscle, and thus can enhance systemic insulin sensitivity, improve glucose processing rate, and promote AMPK activation and GLUT4 expression through muscle. In 2015, Lee et al. found that MOTS-c can promote AMPK activation and GLUT4 expression under high-fat diet (HFD), enhance systemic insulin sensitivity through muscle, and increase the glucose processing rate of insulin stimulation (14). Lu et al. demonstrated for the first time that MOTS-c treatment can prevent ovariectomy-induced insulin resistance, fat deposition and inflammatory response in mice (46). After oophorectomy, estrogen deficiency increases fat load and disrupts normal fat function, thus forcing insulin resistance. MOTS-c regulates fat metabolism by increasing energy consumption and inhibiting fat bulge, thus alleviating diabetes caused by insulin resistance. In addition, Zhai et al. observed that in mice infected with methicillin-resistant

Staphylococcus aureus (MRSA), MOTS-c enhanced phagocytosis and bactericidal capacity of macrophages by inhibiting MAPK, enhancing expression of negative regulator of inflammation AHR and phosphorylation of STAT3. At the same time, the levels of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β decreased, and the levels of anti-inflammatory cytokine IL-10 increased (47, 48). Thus, MDPs provides a new direction for the treatment of insulin resistance associated with inflammation.

Mitochondrial-Derived Peptides and Stroke

Stroke is a major complication of diabetes. As an acute cerebrovascular disease, it is a group of diseases caused by brain tissue damage due to sudden rupture of blood vessels in the brain or inability of blood flow to the brain due to vascular obstruction, including ischemic and hemorrhagic stroke (49). In ischemic stroke, tight junction proteins of vascular endothelial cells are degraded, and changes in BBB permeability lead to the activation of immune cells, which then penetrate into endothelial cells and infiltrate brain tissue, thereby triggering an inflammatory cascade that leads to neuronal damage and cell death. Recent studies have confirmed that HN (50) plays a protective role against ischemic brain injury. HN can pass the blood-brain barrier (BBB) and regulate NF-κB (25) PI3K-Akt, JAK-Stat3, and other pathways (49), or regulate the expression of apoptotic related proteins to inhibit neuronal apoptosis, thus playing a protective role against ischemic brain injury. Peng et al. (49) conducted *in vivo* experiments on mice with *in vivo* middle cerebral artery occlusion (MACO) model and *in vitro* studies on Bend3 cells treated with hypoxia and glucose deficiency, showing that HNG, a reverse signaling molecule, can reduce inflammatory response *in vivo* by inhibiting the activation of NF-κB signaling pathway factors IKK, and IKB, and reducing the accumulation of P65 in the nucleus. For example, HN can inhibit the production of cytokines such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), while at the same time inhibiting vascular adhesion molecules in cortical tissues such as VCAM-1 and ICAM-1. It was speculated that the disorder of BBB endothelial cells in the brain of MCAO mice might promote the passage of HNG through BBB. Moreover, in a 2016 study, Kim et al. (23) found that in neuronal cell lines, HNG can activate Akt Erk1/2 and Stat3 signaling pathways through the glycoprotein 130kDa (GP130/IL6ST) receptor complex to play a protective role in nerve cells. HNG can inhibit oxidative stress ROS production by activating the JAK2/STAT3 signal and the mitochondrial pathway-related apoptosis induced by Bax and caspase3 (25). HN inhibits BAX-mediated neuronal apoptosis mainly through two pathways (51). First, HN prevents the translocation of Bax from cytoplasm to mitochondria. Second, HN interacts with the mitochondrial membrane bound to Bax to prevent the recruitment of cytoplasmic Bax and its oligomerization in the membrane.

Mitochondrial-Derived Peptides and Myocardial Infarction

Another major complication of diabetes, myocardial infarction (52), refers to acute myocardial ischemic necrosis, mostly on the basis of coronary artery lesions, the coronary artery blood supply is sharply reduced or interrupted, resulting in severe and lasting acute ischemia of the corresponding myocardium. Current treatments include drug therapy, interventional medicine and surgery. Nevertheless, the traditional method of treatment has been unable to meet the needs of clinical patients. Therefore, it is necessary to seek efficient and reliable treatment. Current studies have found that as an important peptide for regulating and maintaining mitochondrial function, MDPs (53) can be involved in the pathological changes in cardiovascular disease (CVD) through different mechanisms. The heart is an organ with high internal oxygen consumption, and ROS are mainly produced by cardiac mitochondria. To be specific, the complex of the electron transport chain (ETC) is the main source of ROS produced by cardiac mitochondria (54). ROS are multipotent, and in relatively high concentrations (pathology) cause oxidative stress, but at a lower level (physical) act as a signal molecules. The increase of ROS will lead to changes in mitochondrial membrane potential and ATP level of cardiomyocytes. At the same time, oxidative stress can trigger mitochondria and endoplasmic reticulum stress mediated apoptosis pathways, causing cell damage.HN can protect cells and mitochondria through antioxidant stress and endoplasmic reticulum stress. Savitree and his colleagues demonstrated that HNG can reduce mitochondrial damage caused by complex I and reduce oxidative stress caused by H_2O_2 and ROS production (55). Moreover, HNG was more effective than cyclosporine A (CsA, MPTP inhibitor) in reducing mitochondrial ROS and increasing ATP production. It has been found in a series of studies (56) that high dose HNG (252 ug/kg) can increase the HN level of damaged myocardium and reduce arrhythmias, area of myocardial injury and mitochondrial dysfunction. In addition, Laura E. Klein et al. (57) also found that the reduction of intracellular ROS after HNG-treatment was dependent on the activation of a pair of non-receptor tyrosine kinases C-ABL and arginine. Their results provide mechanistic insights into the observed HNG-mediated cardiac protection *in vivo* (58). Glutathione (GSH) is an important component of mitochondrial antioxidant defense system. Matsunaga et al. (59) showed that HN can restore mitochondrial GSH synthesis by increasing the catalytic subunit of rate-limiting glutamylcysteine ligase and inhibiting the production of superoxide, thus protecting against various ER stress-induced apoptosis. In addition, Muzumdar RH et al. (60) found that HNG may activate AMPK-eNOS -mediated (endothelial nitric oxide synthase) signal transduction during myocardial ischemia-reperfusion injury (MI-R model) in mice. Activating AMPK, increasing the phosphorylation level of eNOS, and decreasing the expression of the apoptotic factor Bax can help reduce the myocardial infarction area, enhance cardiac function, improve the survival rate of myocardial cells, and play a cardiac protective role in a dose-dependent manner (HNG,2 mg/kg is the best) in the MI-R mouse model. Yuan et al. (61) investigated the effects of MOTS-c on cardiac function and structure of rats during chronic aerobic exercise by intraperitoneal injection of MOTS-C, and detected echocardiography by HEMO dynamics with HE staining. After analyzing cardiac function, it was found that MOTS-c could

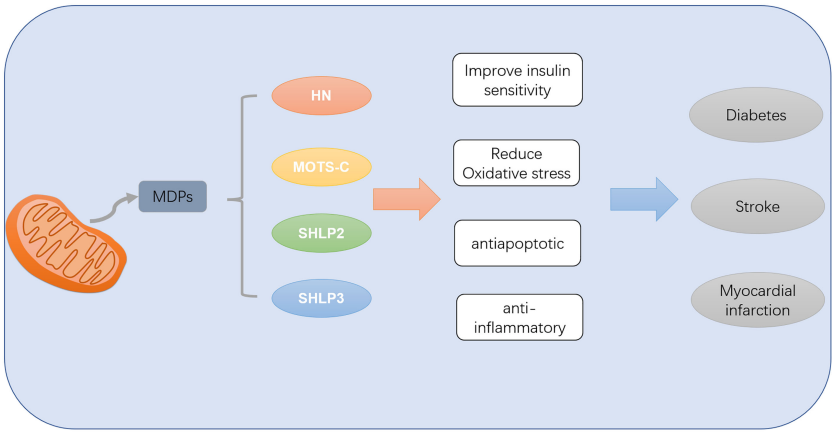

FIGURE 2 | MDPs can improve diabetes mellitus and its complications of myocardial infarction and stroke by improving insulin sensitivity and reducing oxidative stress inflammation and apoptosis.

improve cardiac mechanical efficiency, enhance cardiac systolic function, and have a tendency to improve diastolic function, thus improving cardiac function.

CONCLUSION

Mitochondria are the energy metabolism center in the body, while brain and heart are the most metabolically active organs in the body. Therefore, changes in mitochondrial function will affect the process of diabetes and its complications in the heart and brain tissues (62). ROS produced by mitochondria in the process of metabolism is considered to be the main cause of diabetic microangiopaxia caused by mitochondrial mutation damage to aging tissues. MDPs encoded by mitochondrial genes can regulate diabetes and its cardiovascular and cerebrovascular complications through anti-inflammatory and anti-apoptosis promotion of mitochondrial biosynthesis, etc. (**Figure 2**). HN (23) can reduce ROS interference with BAX translocation and recruitment through activation of PI3K-AKT, JAK2-STAT3, NF-κB, AMPK-eNOS and other pathways. MOTS-c (58) can regulate glucose and lipid metabolism by targeting specific activation of AMPK in skeletal muscle, regulate coronary endothelial function, enhance cardiac systolic function, improve coronary artery microvascular disorders, etc. SHLP2 and SHLP3 can also improve insulin sensitivity and glucose metabolism *in vivo* and *in vitro*, thus contributing to the efficacy of diabetes and its complications.

Mitochondrial derived peptides mainly play a role in the regulation of diabetic nervous system complications by HN, while other MDPs have not been fully reflected in the study of neuroprotection. In addition, most of the mitochondria derived peptides used in the current research are exogenous. As protein polypeptides, they can be quickly cleared by tissues in the body, so how to make them play a role in the body for a long time is also one of the problems that need to be solved.

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Dissecting the Association of Apolipoprotein E Gene Polymorphisms With Type 2 Diabetes Mellitus and Coronary Artery Disease

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Background: Apolipoprotein E (*APOE*) gene mediates lipoprotein clearance and is one of the most studied candidate genes for type 2 diabetes mellitus (T2DM) and coronary artery disease (CAD). This study was performed to determine the association between *APOE* polymorphisms and T2DM with and without CAD, and its effect on plasma lipid levels in a Chinese population.

Methods: A total of 1,414 subjects involving 869 patients and 545 health individuals were recruited. These patients were categorized into three distinct groups: 264 in T2DM group, 401 in CAD group, and 204 in T2DM+CAD group. Logistic regression analysis was used to obtain odds ratio (OR) and 95% confidence interval (CI) in predicting the risk probability of *APOE*. Besides, a meta-analysis was preformed to integrate an evaluation index to evaluate their associations.

Results: Genotype frequency ratio of genotype ϵ3/4 and allele ϵ4 among the CAD patients with or without T2DM was obviously increased. Compared with ϵ3/3 genotype, the ϵ3/4 genotype had a significant increased risk of CAD (adjusted OR = 1.90, 95% CI = 1.30–2.77) and T2DM+CAD (adjusted OR = 1.95, 95% CI = 1.24–3.08). In the meta-analysis, four studies were included and provided a strong evidence for the *APOE* ϵ4 mutation elevating the risk of CAD in patients with T2DM (ϵ3/ϵ4+ϵ4/ϵ4 *vs*. ϵ3/ϵ3, OR = 1.51, 95% CI = 1.13–2.02). In the T2DM group, the plasma levels of low-density lipoprotein cholesterol (LDL-C) showed significant difference among the three APOE isoforms. The high-density lipoprotein cholesterol (HDL-C) levels of CAD patients with ϵ4-bearing genotypes were lower than those with ϵ3/3 genotype.

Conclusions: Our results indicate that *APOE* gene polymorphisms are related to CAD with or without T2DM and have influence on lipid profiles in both T2DM and CAD patients.

Keywords: apolipoprotein E, polymorphism, type 2 diabetes mellitus, coronary artery disease, meta-analysis

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a common chronic metabolic disease characterized by high levels of sugar in the blood and is prevalent throughout the world. The incidence of T2DM is rising at an alarming rate attributed to changing dietary patterns, increasing life expectancy, and westernization of lifestyles in developing countries (1). T2DM frequently coexists with various complications such as hypertension and dyslipidemia and is also known as a major independent risk factor for coronary artery disease (CAD) (2). Cardiovascular disease including CAD is increased in T2DM subjects, which is associated with significant morbidity and mortality. Patients with T2DM have two- to fourfold greater risk of developing CAD compared to individuals without diabetes (3). The development of CAD in the setting of T2DM due to a complex combination of various risk factors plays important role in the beginning and the evolution of atherosclerosis (4). The inherited aspect of risk factors is most often a number of genes interacting with each other or with the environmental factors. Therefore, managing genetic risk factors for T2DM and CAD may improve the understanding of these disease and result in better clinical management.

Apolipoprotein E (*APOE*) gene maps in the long arm of chromosome 19 at position q13.32, which encodes a multifunction glycoprotein containing 299 amino acids (**Figure 1A**). It acts as cholesterol carrier and is involved in mediating the transportation and metabolism of lipids (5). As shown in **Figure 1B**, two single-nucleotide polymorphisms (SNPs) in *APOE*, namely, rs429358 (T>C) and rs7412 (C>T), gives rise to three major alleles: ϵ2 (rs429358-T, rs7412-T), ϵ3 (rs429358-T, rs7412-C), and ϵ4 (rs429358-C, rs7412-C). Therefrom, the three alleles yield six different genotypes, of which three are homozygous, namely, ϵ2/ϵ2, ϵ3/ϵ3, and ϵ4/ϵ4, and three are heterozygous, namely, ϵ2/ϵ3, ϵ2/ϵ4, and ϵ3/ϵ4. Besides, these variants encode three different protein isoforms: APOE2 (ϵ2/ϵ2, ϵ2/ϵ3; Cys112/Cys158), APOE3 (ϵ2/ϵ4, ϵ3/ϵ3; Cys112/Arg158), and APOE4 (ϵ3/ϵ4, 4/ϵ4; Arg112/Arg158, **Figure 1C**) (6). Previous studies have shown that variants of APOE could govern the metabolism of lipoproteins. Allele ϵ4 is associated with lower plasma high-density lipoprotein cholesterol (HDL-C) level but had higher plasma levels of low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), and triglycerides (TG), when compared with ϵ3 allele. Meanwhile, the presence of ϵ2 is usually coupled with lower plasma levels of LDL (7, 8). Referring to those previous studies, evidence suggests that a functional interaction between *APOE* polymorphisms and LDL receptor (LDL-R) influences the risk of CAD and T2DM, and ϵ4 allele has higher affinity to LDL-R than other alleles (9, 10). It is thus likely that the effects of APOE ϵ4 are due to overproduction of LDL or fewer LDL-R, overwhelming the limited ability of mediating the clearance of lipoproteins (10, 11). In contrast, Larifla et al. has reported that the lack relationship between *APOE* polymorphisms and CAD in Afro-Caribbean people (12). Besides, according to a recent meta-analysis including 13 eligible studies, *APOE* gene ϵ4 allele had a significant increased risk for CAD patients with T2DM, whereas the ϵ2 variation had null association (13).

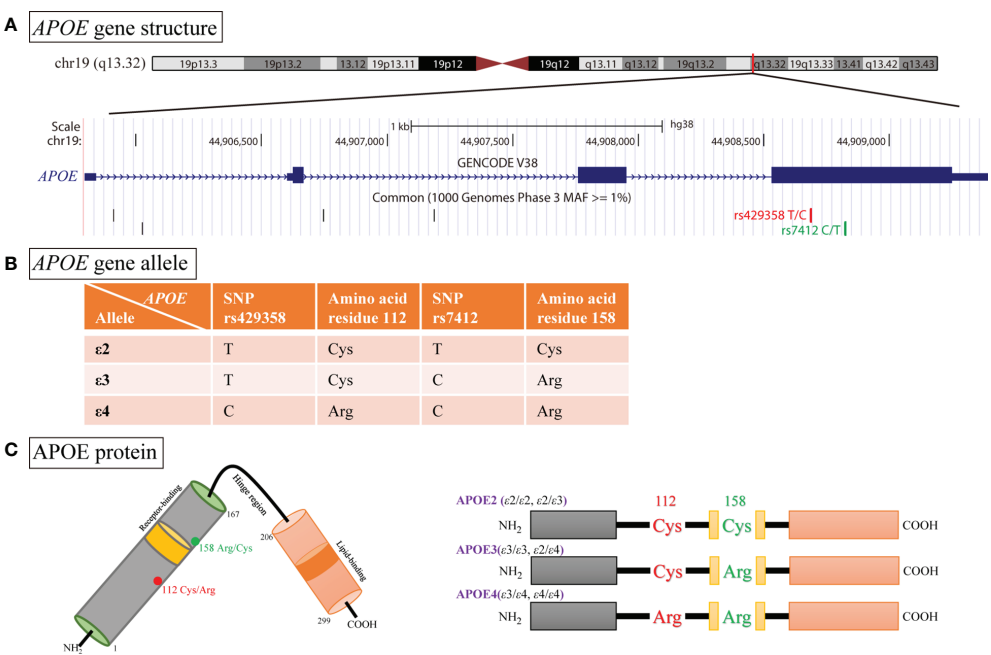

FIGURE 1 | Schematic illustration of *APOE* genotype and APOE isoform. **(A)** Functional single nucleotide polymorphisms (SNPs) in the *APOE* gene viewed in UCSC genome browser. **(B)** Two SNPs (rs429358 and rs7412) in exon 4 of *APOE* generate three major allelic variants (ϵ2, ϵ3, and ϵ4). **(C)** APOE protein is a polypeptide chain with 299 amino, and the APOE2, APOE3, and APOE4 isoforms differ from one another at amino acid residues 112 or 158.

Consequently, we conducted a case–control study to investigate the association of *APOE* polymorphisms with T2DM and CAD in a Chinese population and its potential role in lipid metabolism.

METHODS

Study Population

The studied subjects were recruited from The First Affiliated Hospital of Nanjing Medical University (Nanjing, China) from January 2018 to December 2019. Our study was approved by Ethical Committee of The First Affiliated Hospital of Nanjing Medical University, and all donors signed a written informed consent before enrollment. Only Chinese subjects aged 18 years or above were recruited. Questionnaires were used to collect the information of age, sex, genetic family history, medical history, and lifestyle habits. Other clinical and biochemical data such as blood pressure, dyslipidemia, and blood glucose were obtained from clinical and laboratory examinations. Dyslipidemic or hyperlipidemic feature matches the following conditions: TC > 5.17mmol/L (200 mg/dl), TG > 1.69 mmol/L (150 mg/dl), LDL-C > 3.38 mmol/L (130 mg/dl), or HDL-C < 1.03 mmol/L (40 mg/dl). A fasting plasma glucose (FPG) level ≥7.0 mmol/L (126 mg/dl) or a 2-h plasma glucose ≥11.1 mmol/L (200 mg/dl) meets the threshold for the diagnosis of diabetes.

According to the above criteria, studied subjects were classified into four groups. First is the T2DM group that included 264 subjects that fulfilled the diabetes diagnostic criteria of FPG ≥7.0 mmol/L or were under treatment with oral antidiabetic drugs. Second is the CAD group that consisted of 401 subjects with at least 50% stenosis in a major coronary artery or one of their branches defined by coronary angiography. Third is the T2DM +CAD group included 204 subjects diagnosed to have diabetes complicated with coronary artery disease. Exclusion criteria included type 1 diabetes mellitus, malignant tumors, liver and kidney diseases, metabolic disorders, and autoimmune diseases. Fourth is the control group that included 545 healthy individuals without hyperlipidemia, hypertension, cardiovascular diseases, and diabetes.

APOE Genotyping

Genomic DNA was isolated from leukocytes of the peripheral blood using a commercial kit following the manufacturer's protocols (Sinochip, Zhuhai, China). DNA concentration and purity were estimated by 260 and 280 nm (optical density, OD) light absorbance on a Nanodrop 2000^{TM} spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Two *APOE* SNPs (rs429358 and rs7412) were genotyped using a detection kit (GeneChip Assay, Sinochip, Zhuhai, China). All samples were amplified according to the manufacturer's instructions, and then, the amplified products were assayed by the fully automated GeneChip detection system (Sinochip, Zhuhai, China).

Lipid Profiles

After an overnight fast of at least 12 h, venous blood samples were collected from these patients. Plasma TC, TG, HDL-C, LDL-C, and FPG were quantified using the Beckman biochemical assembly line.

Systematic Review and Meta-analysis

We conducted a literature search for all studies that evaluated the association of *APOE* polymorphisms with T2DM and CAD in the PubMed database up to December 2021. The following key terms were used in the search: "apolipoprotein E" or "*APOE*," "polymorphism," "CAD," and "T2DM." References cited in each retrieved article were also manually scanned to discover additional eligible studies. Articles were recruited for this meta-analysis if they fitted the following criteria: (1) investigated the associations between *APOE* polymorphisms and CAD in patients with T2DM, (2) sufficient data for estimating the odds ratios (ORs) and 95% confidence intervals (CIs), and (3) published in English. Exclusion criteria were (1) duplication of previous data and (2) not using coronary angiography to confirm CAD.

Statistical Analysis

Continuous data such age and lipid profile were compared using Student's *t*-test or Wilcoxon test for two groups and Kruskal–Wallis test for more than two groups. Categorical variables (sex and *APOE* genotypes) were expressed as frequency and compared using Pearson's χ^2 test or Fisher's exact test. Hardy–Weinberg equilibrium was conducted to evaluate the allele and genotype difference among groups. The associations between *APOE* polymorphism and diseases were estimated by computing crude or adjusted ORs and 95% CIs from unconditional logistic regression. All the statistical analyses were done with R 4.0.1, and two-sided p-value <0.05 was considered statistically significant.

RESULTS

Characteristics and Clinical Features of Subjects

The demographic and clinical features of 1,414 included individuals are summarized in **Table 1**. The data from normal controls were used as a reference to compare with the data obtained from three observation groups consisted of patients with T2DM, CAD, and T2DM+CAD. A significant difference in age was found between control group (mean age, 67.2 years) and the observation groups (T2DM, 69.5 years; CAD, 65.3 years; T2DM+CAD, 70.5 years), implying a higher risk of developing T2DM with increasing age. Gender was equally distributed in CAD group with 111 female patients and 290 male patients and in T2DM+CAD group with 65 female and 139 male patients compared to control group with 206 female and 339 male subjects. There was also significant sex difference between CAD patients and the controls (p = 0.001) due to the high prevalence of male patients among CAD compared to control group (72.3% vs. 62.2%). Besides, T2DM and T2DM+CAD groups had significantly higher levels of TG than the normal control group. Patients with CAD or T2DM had lower levels of HDL-C than the controls. Therefore, the TG/HDL-C ratio was

TABLE 1 | Clinical characteristics and genotype distribution of APOE gene in different groups.

Variables		Control	T2DM		CAD		T2DM+CAD	
		n = 545	n = 264	p	n = 401	p	n = 204	p
Age (years)		67.2 ± 13.4	69.5 ± 12.7	**0.022**	65.3 ± 12.3	**0.029**	70.5 ± 13.0	**0.003**
Sex								
Female		206 (37.8%)	85 (32.2%)	0.120	111 (27.7%)	**0.001**	65 (31.9%)	0.132
Male		339 (62.2%)	179 (67.8%)		290 (72.3%)		139 (68.1%)	
Lipid profile (mmol/L)								
TC		4.25 ± 0.96	4.30 ± 1.23	0.974	4.33 ± 1.13	0.635	4.29 ± 1.34	0.385
TG		1.42 ± 0.91	1.72 ± 1.30	**<0.001**	1.49 ± 0.89	0.238	1.86 ± 1.45	**<0.001**
HDL-C		1.15 ± 0.33	1.05 ± 0.28	**<0.001**	1.09 ± 0.26	**0.032**	1.02 ± 0.27	**<0.001**
LDL-C		2.58 ± 0.71	2.65 ± 0.89	0.506	2.69 ± 0.86	0.173	2.70 ± 1.03	0.794
TG/HDL-C ratio		1.38 ± 1.12	1.87 ± 1.88	**<0.001**	1.49 ± 1.10	**0.032**	2.08 ± 2.13	**<0.001**
***APOE* genotypes**								
APOE2	ϵ2/ϵ2	6 (1.10%)	3 (1.14%)	1.000	1 (0.25%)	0.249	0 (0%)	–
	ϵ2/ϵ3	87 (15.96%)	47 (17.80%)	0.509	52 (12.97%)	0.198	27 (13.24%)	0.355
APOE3	ϵ2/ϵ4	6 (1.10%)	2 (0.76%)	1.000	3 (0.75%)	0.741	4 (1.96%)	0.473
	ϵ3/ϵ3	387 (71.01%)	171 (64.77%)	0.072	268 (66.83%)	0.169	132 (64.71%)	0.096
APOE4	ϵ3/ϵ4	58 (10.64%)	39 (14.77%)	0.090	75 (18.70%)	**<0.001**	39 (19.12%)	**0.002**
	ϵ4/ϵ4	1 (0.18%)	2 (0.76%)	1.000	2 (0.50%)	0.577	2 (0.98%)	0.182
***APOE* alleles**								
ϵ2		105 (9.63%)	55 (10.42%)	0.621	57 (7.11%)	0.052	31 (7.60%)	0.222
ϵ3		919 (84.31%)	428 (81.06%)	0.101	663 (82.67%)	0.340	330 (80.88%)	0.112
ϵ4		66 (6.06%)	45 (8.52%)	0.066	82 (10.22%)	**<0.001**	47 (11.52%)	**<0.001**

Data are presented as mean ± SD, or numbers (N) and percentage. p-value: comparison between T2DM/CVD/T2DM+CAD group and control group. Groups were compared using Student's t-test or Wilcox test (for continuous variables) and Pearson's χ^2 test or Fisher's exact test (for categorical variables). Bold values denote statistical significance at the p < 0.05 level.

calculated, and this index was significantly higher among the observation groups.

APOE Genotype and Allele Frequencies of Subjects

Our extracted genomic DNA was of good quality with an OD260/OD280 ratio between 1.7 and 2.0. The genotype distributions of all included groups were in Hardy–Weinberg equilibrium (p > 0.05), exhibiting group representation. The most frequent genotype was ϵ3/ϵ3 in our subjects, followed by ϵ3/4 and ϵ2/3 genotypes, while ϵ2/2, ϵ2/4, and ϵ4/4 genotypes were the lowest, indicating that there was a significant difference in the distribution frequency of genotype ϵ3/4 among these groups. Compared with the control group, the genotype ratio of ϵ3/4 obviously increased in the CAD and T2DM+CAD groups (p < 0.001 and p = 0.002, **Table 1**). The differences in ϵ4 allele frequency distribution between the CAD and T2DM+CAD groups and the control group were considered statistically significant (both p < 0.001).

Association of *APOE* Polymorphism With Diseases

Logistic regression was performed to evaluated the correlation between *APOE* polymorphism and T2DM or CAD. As shown in **Table 2**, when the ϵ3/3 genotype was used as the reference, the ϵ3/4 genotype had a significant increased risk of CAD and T2DM+CAD (adjusted OR = 1.90, 95% CI = 1.30–2.77 for CAD; adjusted OR = 1.95, 95% CI = 1.24–3.08 for T2DM +CAD). Furthermore, *APOE* allele ϵ4 appeared to increase the risk of developing CAD without or with T2DM, with adjusted OR of 1.72 (95% CI, 1.22–2.42) and 1.97 (95% CI, 1.32–2.93), respectively, compared with the allele ϵ3. However, allele ϵ4 was not found to be associated with the risk of CAD in T2DM (ϵ4 *vs.* ϵ3, OR = 1.36, 95% CI = 0.88–2.09). These results encouraged us to conduct a meta-analysis to explore the association between *APOE* allele ϵ4 and T2DM complicated with CAD. According to the above search criteria, three articles (14–16) and our study were included in the meta-analysis (**Supplementary Figure S1**). **Figure 2** displays that ϵ3/4 + ϵ4/4 genotype increased the risk of developing CAD in T2DM patients, with a pooled OR of 1.51 (95% CI, 1.13–2.02), compared to the ϵ3/3 genotype. Besides, no heterogeneity was detected, indicating that the pooled results of this meta-analysis were statistically steady and robust.

Relationship Between APOE Polymorphism and Lipid Profiles

We analyzed the blood lipid profiles in subjects with different APOE genotypes. In the control and T2DM group, the levels of LDL-C showed significant difference among APOE2, APOE3, and APOE4 individuals; in the CAD group, APOE4 patients had lower levels of HDL-C than APOE3 and APOE2 patients. However, there was no significant difference in TG/HDL-C ratio across different APOE genotype groups (**Supplementary Table S1**). The frequencies of the ϵ4 allele might contribute to the difference in APOE distribution between observation groups and control group. Therefore, we analyzed the correlation between ϵ4-bearing genotypes (ϵ2/ϵ4, ϵ3/ϵ4, and ϵ4/ϵ4) and plasma HDL-C expression. As shown in **Figure 3**, ϵ3/ϵ3 genotype as a reference, ϵ4-bearing genotypes had significant decreased levels of HDL-C in CAD group (p = 0.016) but not in the other three groups. We also examined the association between ϵ4-bearing genotypes and TG/HDL-C ratio, and yet,

TABLE 2 | Comparison of APOE genotypes and alleles frequency between the control group and the case group.

Variables	Control	T2DM			CAD			T2DM+CAD		
APOE genotypes	**n**	**n**	**OR (95% CI)**	**OR (95% CI)[a]**	**p[a]**	**n**	**OR (95% CI)**	**OR (95% CI)[a]**	**p[a]**	**n**	**OR (95% CI)**	**OR (95% CI)[a]**	**p[a]**
ϵ2/ϵ2	6	3	1.13 (0.28-4.58)	1.19 (0.29-4.83)	0.813	1	0.24 (0.03-2.01)	0.22 (0.03-1.82)	0.158	0	–	–	–
ϵ2/ϵ3	87	47	1.22 (0.82-1.82)	1.22 (0.82-1.82)	0.334	52	0.86 (0.59-1.26)	0.86 (0.59-1.25)	0.427	27	0.91 (0.57-1.46)	0.90 (0.56-1.46)	0.673
ϵ2/ϵ4	6	2	0.75 (0.15-3.78)	0.77 (0.15-3.86)	0.748	3	0.72 (0.18-2.91)	0.69 (0.17-2.81)	0.605	4	1.96 (0.54-7.03)	1.84 (0.5-6.72)	0.357
ϵ3/ϵ3	387	171	Reference	Reference	–	268	Reference	Reference	–	132	Reference	Reference	–
ϵ3/ϵ4	58	39	1.52 (0.98-2.37)	1.45 (0.93-2.27)	0.102	75	**1.87 (1.28-2.72)**	**1.90 (1.30-2.77)**	**0.001**	39	**1.97 (1.26-3.1)**	**1.95 (1.24-3.08)**	**0.004**
ϵ4/ϵ4	1	2	4.53 (0.41-50.25)	5.18 (0.46-57.94)	0.182	2	2.89 (0.26-32.01)	2.23 (0.20-24.85)	0.515	2	5.86 (0.53-65.19)	6.27 (0.56-70.17)	0.136
Alleles													
ϵ2	105	55	1.13 (0.80-1.59)	1.13 (0.80-1.60)	0.484	57	0.75 (0.54-1.06)	0.74 (0.53-1.04)	0.081	31	0.82 (0.54-1.25)	0.82 (0.53-1.25)	0.346
ϵ3	919	428	Reference	Reference	–	663	Reference	Reference	–	330	Reference	Reference	–
ϵ4	66	45	1.46 (0.99-2.18)	1.42 (0.96-2.12)	0.082	82	**1.72 (1.23-2.42)**	**1.72 (1.22-2.42)**	**0.002**	47	**1.98 (1.34-2.94)**	**1.97 (1.32-2.93)**	**< 0.001**

[a]Adjusted for age and sex. Bold values denote statistical significance at the p < 0.05 level.

the difference was not statistically significant (**Supplementary Figure S2**).

DISCUSSION

T2DM, a chronic condition disease, induced by a genetic predisposition together with environmental factors, is a well-established risk factor for CAD. T2DM and its related cardiovascular complications propose specific challenges at diverse stages of the life. *APOE* polymorphisms have been reported to significantly associate with risk for T2DM and CAD, which were considered as the most influential genetic risk factors. Here, we carried out several experiments to evaluate the association between the *APOE* ϵ2/ϵ3/ϵ4 polymorphisms with the risk of T2DM and CAD. When combined with the analysis the polymorphism of *APOE* and blood lipid levels, these results provided new understanding on the correlation between *APOE* gene and T2DM patients with CAD.

Our study provided evidence for the significant correlation between *APOE* ϵ3/ϵ4 genotype and an elevated risk of CAD without or with T2DM. After adjusting for age and sex, logistic regression analysis showed that ϵ4 allele increased the risk of CAD by 1.72 times, compared with ϵ3 allele. Besides, T2DM patients carrying ϵ4 allele had 1.97-fold higher risk of CAD as compared to the controls. Our data indicated that ϵ4 allele was an independent risk factor for CAD but not for T2DM. Previous studies have investigated the probable associations between *APOE* polymorphisms and patients with T2DM or CAD. Chaudhary et al. reported that the ϵ4 allele was significantly higher in both T2DM and CAD as compared with controls (16). The independent predictor of individuals carrying ϵ4 allele remained significantly associated with both CAD (adjusted OR = 2.32, 95% CI = 1.17–4.61) and T2DM (adjusted OR = 2.04, 95% CI = 1.07–3.86). El−Lebedy et al. found that the frequencies of ϵ3/ϵ4 genotype and ϵ4 allele were increased in both T2DM patients and cardiovascular disease (CVD) patients as compared with controls but were significant only in CVD patients (17). Diabetic patients who carried ϵ3/ϵ4 genotype had 2.4-fold increased risk of developing CVD (95% CI, 1.14–5.19), and the ϵ4 allele was associated with 2.23-fold higher CVD risk (95% CI, 1.09–4.59). A recent meta-analysis including 13 studies provided evidence that there were significant associations between ϵ4 allele and the risk of CAD in patients with T2DM (ϵ3/ϵ4 vs. ϵ3/ϵ3, OR = 1.69, 95% CI = 1.38–2.08; ϵ4/ϵ4 vs. ϵ3/ϵ3, OR = 2.72, 95% CI = 1.61–4.60) (13). Combined with our own data and setting of strict inclusion and exclusion criteria, our meta-analysis found that ϵ4 mutation could elevate the risk of CAD in patients with T2DM. Therefore, we may conclude that ϵ3/4 genotypes and ϵ4 allele of *APOE* contributed to CAD in T2DM patients.

One outcome from this work is the levels of HDL-C in CAD patients with ϵ4-bearing genotypes (ϵ2/ϵ4, ϵ3/ϵ4, and ϵ4/ϵ4) were lower than patients with ϵ3/ϵ3 genotype. It was well-known that the HDL-C levels were important factors of blood lipid levels. The relationship between ϵ4 allele and lipid profile remained controversial. Li et al. found that the *APOE* ϵ4 carrier had a lower HDL-C than the ϵ2 allele but not for ϵ3 allele in a Chinese

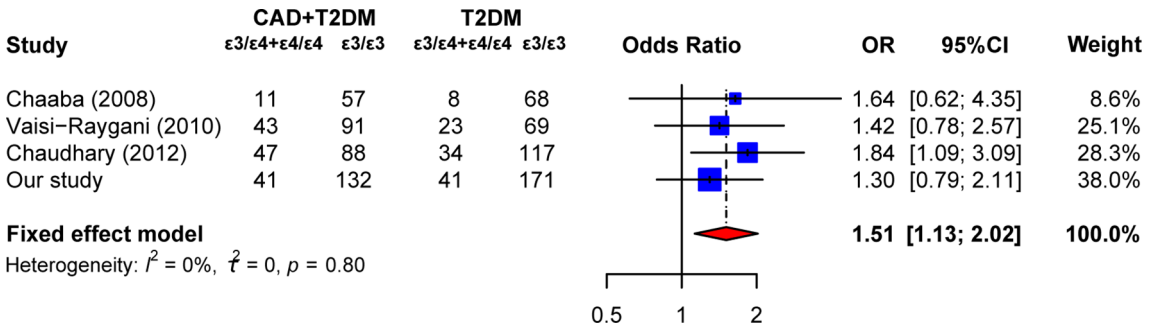

FIGURE 2 | Summary estimates of the association between the *APOE* ϵ4 mutation (ϵ3/ϵ4+ϵ4/ϵ4 vs. ϵ3/ϵ3) and the risk of coronary artery diseases in type 2 diabetes patients. Each study is displayed by a square whose center represents the odds ratio (OR), the area of the square is proportional to the weight of studies, and the horizontal line indicates the 95% confidence interval (CI).

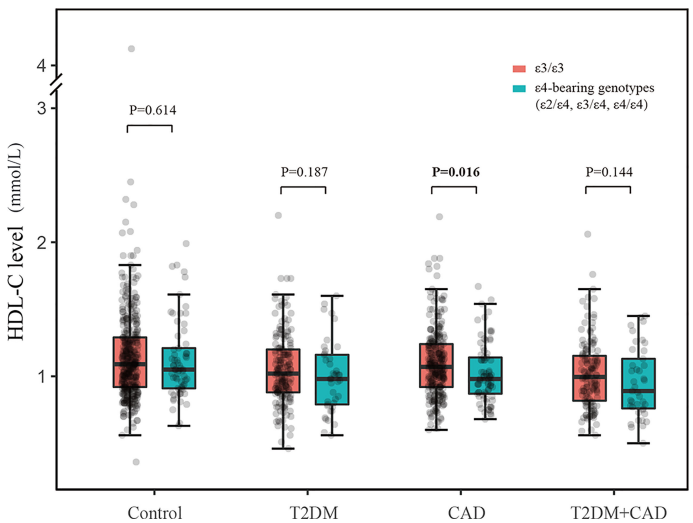

FIGURE 3 | Correlation between plasma HDL-C level values and *APOE* ϵ4-bearing genotypes (ϵ2/ϵ4, ϵ3/ϵ4, and ϵ4/ϵ4).

population of CAD (18). In a recent study on Kashmiri population, the CAD patients carrying ϵ4 allele had significantly lower HDL-C levels (19). Chaaba et al. found that the ϵ4 allele was only associated with elevated LDL-C concentration and with CAD in type 2 diabetic men in Tunisian population, showing that gender interacted with the effects of *APOE* polymorphism (14). Therefore, these inconsistent findings might be complicated by considerable differences in the allele frequency distributions among different ethnic populations.

Current evidence showed that APOE is a versatile glycoprotein that plays a central role in lipoprotein metabolism (20). Although the three APOE isoforms differ in only one or two amino acids, these slight changes affect the structure and alter the affinity to lipoproteins. APOE3 binds preferentially to HDL, whereas APOE4 shows an enhanced lipid bind ability of VLDL particles, which impairs their lipolytic processing in the circulation, resulting in a more pro-atherogenic lipoprotein–cholesterol distribution. Therefore, abnormalities of lipoprotein metabolism may partly explain that the plasma HDL-C levels were lowest in APOE4 patients compared to APOE3 and APOE2 patients in CAD group. Besides, excessive amounts of circulant lipids may affect systemic inflammation and insulin resistance (IR) (21, 22). Previous studies reported the TG/HDL-C ratio as predictor of IR (22). A higher ratio indicated a large amount of circulant lipids. Our study showed that the T2DM+CAD group had the highest TG/HDL-C ratio than the control, T2DM, and CAD groups. Then, we assessed whether the APOE isoforms were associated with IR, but no association was observed. Confirming results of previous studies, the relationship between *APOE* allele frequencies and IR was controversial (23–25).

There are several limitations that should be pointed out as follows. First, this case–control study was hospital based, and the selection bias was inevitable. Therefore, we adjusted for potential confounding factors such as age and sex to minimize the bias in logistic regression. Second, adult populations showed specific mutations not only owing to genetic background. Life habits,

diets, climate, pollution, and even pandemic might influence it. As this study recruited subjects aged 18 years or above, subgroup analysis for age (children and adults) could not be performed. Meanwhile, we did not have the detailed information of some risk factors (smoking, diet, and physical activity) in the development of CAD and T2DM, and the gene–environment interactions analysis was not conducted. Third, there was no significant association between *APOE* alleles and T2DM in our study, which was not consistent with previous study (26, 27), and the results from our single-center study required external validation in other training programs. A final comment on the low number of individuals in some subgroups (control, T2DM, CAD, and T2DM+CAD for ϵ2/ϵ2, ϵ2/ϵ4 and ϵ4/ϵ4) should be taken up with care and caution.

In conclusion, although the *APOE* ϵ4 allele was not found to be associated with T2DM, it increased the risk of CAD and related to the development of T2DM with CAD. Different APOE isoforms were also linked to variations in lipid and lipoprotein levels in circulation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of The First Affiliated Hospital of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TX and LW designed the research. LW, YZ, HZ, GR, PH, FW, and TX conducted research. LW and YZ analyzed data. LW wrote the initial draft of the manuscript. FW and TX revised the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.838547/full#supplementary-material

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Identification of Crucial Genes and Key Functions in Type 2 Diabetic Hearts by Bioinformatic Analysis

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Type 2 diabetes (T2D) patients with SARS-CoV-2 infection hospitalized develop an acute cardiovascular syndrome. It is urgent to elucidate underlying mechanisms associated with the acute cardiac injury in T2D hearts. We performed bioinformatic analysis on the expression profiles of public datasets to identify the pathogenic and prognostic genes in T2D hearts. Cardiac RNA-sequencing datasets from *db/db* or BKS mice (GSE161931) were updated to NCBI-Gene Expression Omnibus (NCBI-GEO), and used for the transcriptomics analyses with public datasets from NCBI-GEO of autopsy heart specimens with COVID-19 (5/6 with T2D, GSE150316), or dead healthy persons (GSE133054). Differentially expressed genes (DEGs) and overlapping homologous DEGs among the three datasets were identified using DESeq2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes analyses were conducted for event enrichment through clusterProfile. The protein-protein interaction (PPI) network of DEGs was established and visualized by Cytoscape. The transcriptions and functions of crucial genes were further validated in *db/db* hearts. In total, 542 up-regulated and 485 down-regulated DEGs in mice, and 811 up-regulated and 1399 down-regulated DEGs in human were identified, respectively. There were 74 overlapping homologous DEGs among all datasets. Mitochondria inner membrane and serine-type endopeptidase activity were further identified as the top-10 GO events for overlapping DEGs. Cardiac *CAPNS1* (calpain small subunit 1) was the unique crucial gene shared by both enriched events. Its transcriptional level significantly increased in T2D mice, but surprisingly decreased in T2D patients with SARS-CoV-2 infection. PPI network was constructed with 30 interactions in overlapping DEGs, including *CAPNS1*. The substrates *Junctophilin2* (*Jp2*), *Tnni3*, and *Mybpc3* in cardiac calpain/CAPNS1 pathway showed less transcriptional change, although *Capns1* increased in transcription in *db/db* mice. Instead, cytoplasmic JP2 significantly reduced and its hydrolyzed product JP2NT exhibited nuclear translocation in myocardium. This study suggests *CAPNS1* is a crucial gene in T2D hearts. Its transcriptional upregulation leads to calpain/CAPNS1-

associated JP2 hydrolysis and JP2NT nuclear translocation. Therefore, attenuated cardiac *CAPNS1* transcription in T2D patients with SARS-CoV-2 infection highlights a novel target in adverse prognostics and comprehensive therapy. CAPNS1 can also be explored for the molecular signaling involving the onset, progression and prognostic in T2D patients with SARS-CoV-2 infection.

Keywords: type 2 diabetes, acute cardiac injury, differentially expressed genes, bioinformatics, calpain small subunit 1 (capn4), COVID-19, self-protective role

INTRODUCTION

Type 2 diabetes (T2D) as a chronic medical condition is one of the most important risk factors for adverse outcomes of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1–4). The available data has supported that increased susceptibility in patients with T2D to SARS-CoV-2 hospitalizations (5, 6). Moreover, an acute cardiovascular manifestation of COVID-19 often presents as an acute cardiac injury with cardiomyopathy, ventricular arrhythmias, hemodynamic instability and cardiogenic shock, in the absence of obstructive coronary artery disease (7, 8). Putative mechanisms contributing to increased susceptibility for COVID-19 in patients with T2D have been concluded (3, 5, 9). The initial step is that SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) expressing in key metabolic organs and tissues (10). SARS-CoV-2 directly infects cardiomyocytes *in vitro* or in T2D patients in an ACE2-dependent manner (6, 11). ACE2 cascade also associates with diabetic cardiomyopathy in *db/db* T2D mice either (12). Furthermore, SARS-CoV-2 may cause pleiotropic alterations of glucose metabolism that could complicate the pathophysiology of preexisting diabetes or lead to new mechanisms of disease (10). That means the increased susceptibility of hearts themselves in T2D patients is directly involved in the adverse outcomes of COVID-19, although SARS-CoV-2 infection alone could also cause clotting issues in the coronaries. Therefore, acute cardiac injury in T2D appears as one of the leading causes of severe disease and death in patients with COVID-19 (4, 9). To date, the significance of important mediating mechanism for the acute COVID-19 cardiovascular syndrome in T2D patients has not been fully illuminated.

Genome-wide molecular profiling is able to reveal molecular changes in disease occurrence and progression and has proved to be a high-efficient way to identify key genes (13–15). Recently, a transcriptomic analyses has been selected to identify the synergistic effect of SARS-CoV-2 infection and idiopathic pulmonary fibrosis patients in lung epithelium cell datasets (16, 17). Meanwhile, autopsy specimens from the patients with SARS-CoV-2 infection have also been analyzed using a combination of different RNA and protein analytical platforms to characterize inter-patient and intra-patient heterogeneity of pulmonary virus infection (18).

Given that both animal and human with diabetic cardiomyopathy share the same potential biomarkers (19), in the present study, we employ the transcriptomics analyses and key gene validation for both *db*/*db* T2D mice *vs* control (GSE161931) and autopsy specimens of T2D patients with SARS-CoV-2 infection (GSE150316) *vs* control (GSE133054) from NCBI-Gene Expression Omnibus (NCBI-GEO) (20, 21). We identify differentially expressed genes (DEGs) to characterize the overlapping homologous DEGs (16, 22). And also perform functional enrichment analyses of DEGs, identify crucial genes and validate molecular mechanism in T2D hearts, respectively (16). Our results reveal that attenuated cardiac *CAPNS1* transcription in T2D patients who succumbed to SARS-CoV-2 infection highlights a novel event in adverse prognostics, and provide for a more detailed molecular mechanism underlying the acute cardiac injury of occurrence and progression in T2D patients with SARS-CoV-2 infection. Therefore, cardiac CAPNS1 can be explored for the molecular signaling involving the onset, progression and prognostic in T2D patients with SARS-CoV-2 infection and holding promise for using as a biomarker and potential therapeutic target in anti-SARS-CoV-2 comprehensive therapy.

MATERIALS AND METHODS

Dataset and Identification of DEGs

The gene expression profile datasets of heart samples (GSE161931) from 7 *db/db* T2D mice and 9 BKS mice as wild-type control were sequenced through Illumina NextSeq 500 platform (Novogene, Tianjing, China), and updated to NCBI-GEO in the present study (https://www.ncbi.nlm.nih.gov/geo/) (20, 21). The profile datasets of cardiac gene expression from 6 autopsy specimens patients (5/6 with T2D) who succumbed to SARS-CoV-2 infection (GSE150316), and 8 autopsy specimens from dead healthy persons (free from any major disease) (GSE133054) were obtained from GEO (https://www.ncbi.nlm.nih.gov/geo/) (20, 21, 23). The age range distributed from 30-years-old to 80-years-old of T2D patients with COVID-19 (18). Identification of DEGs was performed using DESeq2 packages based on the R programming language (version 3.12). Since R package can assist in the process of generating publication quality figures of DGE results files from *Cuffdiff*, *DESeq2* and *edger*, in order to integrate of these functions in a user-friendly way (22). Batch effect between GSE150316 and GSE133054 was removed by R package sva (version 3.38.0). The adjusted *P*-values (adj *P*-value) were adopted to avoid the occurrence of false-positive results (14). Genes with fold change (FC) larger than1.5 or less than 1/1.5 (24) and *P*-value less than 0.05 were

taken as DEGs between T2D hearts and control samples. The datasets were examined both intra-groups and inter-groups to obtain overlapping DEGs and subsequently identify the genes unique to T2D hearts. Ggplot2 and Venn Diagram packages of R were applied to generate volcano plot and Venn diagram, for the visualization of identified DEGs and overlapping homologous DEGs, respectively (14).

Functional Enrichment Analysis

Gene Ontology (GO) function (25) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the candidate DEGs were performed through clusterProfiler package (versions3.0.4) (14). GO or KEGG analyses were presented in dot or ridge plot format, respectively.

Protein-Protein Interaction Network Construction and Module Analysis

The DEG-encoded proteins and their interactions amongst each other were established through the Retrieval of Interacting Genes Database (STRING, version 11.5; https://string-db.org/cgi/input.pl), visualized by Cytoscape software and further analyzed by Molecular Complex Detection (MCODE) algorithm (26, 27). Subsequently, the PPI network for overlapping homologous DEGs was constructed with a confidence score ≥ 0.7. The advanced options set as degree cut-off = 2, K-Core = 2, and Node Score Cut-off = 0.2.

Animals and Tissue Samples

Eight male LepR$^{db/db}$ (*db/db)* T2D mice (eight-weeks-old) and 11 male C57BLKS/J (BKS) mice (eight-weeks-old) were purchased from Institute of Biomedical Research, Nanjing University (Nanjing, Jiangsu, China). Mice were housed in a suitable environment (23 ± 1°C and 70% humidity) with a 12-hour light-dark cycle and had free access to water and standard chow food for further 8 weeks. All studies were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and approved by the Animal Care and Ethics Committee of Zhejiang University (№ ZJU-2015-435-01), and the entire *in vivo* study protocols were approved by the 2nd Affiliated Hospital Research Ethics Committee of Zhejiang University, China (Protocol #2020-1151). Heart tissues were obtained from mice (16-weeks-old). Briefly, mice were anaesthetized using 10% chloral hydrate. The whole heart was removed immediately, frozen with liquid nitrogen and stored at −80°C for further study. Tissue sections were prepared as reported (28), and cut into 5-μm continuous sections. For scanning electron microscope (SEM), the apex area of heart was fixed in chilled 2.5% glutaraldehyde and preceded to following steps.

Quantitative Real-Time RT-PCR

Total RNA was extracted from heart tissues by Trizol reagent (Invitrogen). Then, 2 μg of RNA underwent reverse transcription using Reverse Transcription (RT) kit (TAKARA, Dalian, China) according to the manufacturer's instructions. Amplifications of cDNA were performed using SYBR premix extaq kit (TAKARA) in Bio-Rad CFX 96 (Bio-Rad, California, USA). The forward and reverse primers were as shown in **Supplementary Table 1**. Measurement was normalized to *Gapdh* for *Capns1*, *Jp2* (*Junctophilin2*), *Tnni3* (troponin 1 type 3), and *Mybpc3*. The relative gene expression was presented by comparative CT method.

Western Blot Analysis

Western blot was performed as previously described (29). Briefly, sample lysates were resolved in SDS-PAGE and transferred onto PVDF membrane (Merck Millipore, Billerica, MA, USA). The PVDF membranes were incubated with the primary antibody JP2 (ab110056, Abcam, MA, USA, 1:1000) and GAPDH (HA-ET1702-66-200, HUABIO, Hangzhou, China), respectively. Then samples were incubated with HRP-conjugated secondary antibodies (LK-GAM007, LK-GAR007, LK-RAG007, MULTISCIENCES, Shanghai, China). Blots were developed using enhanced chemiluminescence reagents (Cat#. 32106, Pierce™, IL, USA).

Analysis of Cardiac Ultrastructure

Left ventricular tissue was dissected into 3-mm^3 pieces and fixed in 4% glutaraldehyde and 1% osmic acid in turn. The samples were dehydrated by ascending concentrations of acetone, embedded with EPON812, stained with toluidine blue, cut into slices of 70 nm and double-stained with uranyl acetate and lead citrate. Ultrastructure was examined by transmission electron microscopy (TEM) (H7500TEM, Hitachi, Japan). The ultrastructures of mitochondria and myocardium were assessed, respectively. The visible image of 5 randomly selected areas per slice was photographed at 30000× magnification.

Echocardiography Analysis

Mice were anaesthetized with isoflurane (1–2% in oxygen gas mixture). After shaving hair carefully on the left chest, cardiac geometry was measured from the parasternal long axis view using a small animal color ultrasonic diagnostic apparatus (Visual Sonic Vevo 2100, Toronto, ON, Canada) with a probe frequency of 30 MHz. A clear image of the left ventricular area was recorded using M-type echocardiography. Left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were then calculated based on the mean values from 6 cardiac cycles. All the echocardiographic images were analyzed using Vevo 2100 software.

Immunofluorescence Image Analysis

All immunostaining was conducted as previously described (29). Briefly, samples were probed with the primary antibodies as follows: cardiac troponin T (cTnT, ab8295, Abcam, MA, USA.1:100), JP2 and its N-terminal fragment (NT-JP2) (ab110056, Abcam, MA, USA, 1:100). Then samples were incubated with DyLight488 goat anti-mouse secondary antibody (GAM4882, Multisciences, (Lianke) Biotech Co., Ltd. Hangzhou, China.1:200) and 4′, 6-Diamidino-2-phenylindoledihy-drochloride (DAPI, ab188804, Abcam, MA, USA. 2 μg/ml). Images were acquired under laser scanning Confocal microscopes from Leica Microsystems (Leica Microsystems Inc., Buffalo Grove, IL, USA). Relative ratio of

JP2-NT nuclear translocation was analyzed by ImageJ software (National Institutes of Health, USA).

Statistical Analysis

Data were reported as means ± SD. Statistical analysis was performed through GraphPad Prism (version 8, San Diego, CA) software. Student's *t*-tests were utilized for the comparison of two sample groups. Differences were considered as statistically significant when P <0.05.

RESULTS

Identification of DEGs in T2D Hearts

The gene expression profiles in public datasets from NCBI-GEO were analyzed to identify DEGs in the heart tissues between T2D mice and control, as well as T2D patients who succumbed to SARS-CoV-2 infection (age range from 30-years-old to 80-years-old) (18) and control (**Figure 1A**). Upon setting the cut-off criterion as genes with FC > 1.5 (or <1/1.5) and P < 0.05, we identified 1027 DEGs (542 up-regulated and 485 down-regulated) in GSE161931, and 2210 DEGs (811 up-regulated and 1399 down-regulated) in GSE150316 *vs* GSE133054 showed by volcano plots, respectively (**Figure 1B** and **Supplementary Tables 2, 3**). The overlapping homologous DEGs among the three datasets were further identified. Venn diagram made all the 74 overlapping DEGs visualization (**Figure 1C** and **Supplementary Table 4**), including 23 down-regulated in T2D patients but up-regulated in T2D mice, 24 down-regulated in both patients and mice, 8 up-regulated in patients but down-regulated in mice, and 19 up-regulated in both patients and mice. These results indicated that SARS-CoV-2 infection did affect the transcriptions of some genes in T2D hearts. The overall statistics are shown in **Supplementary Tables 2, 3**.

Functional Enrichment and Module Analysis of DEGs

Given that gene ontology enrichment is significant to elucidate the mechanisms of DEGs, we further performed GO function and KEGG pathway enrichment analyses to investigate the biological functions of DEGs from the three datasets through clusterProfiler package. The DEGs were examined and based on the *P*- value, the

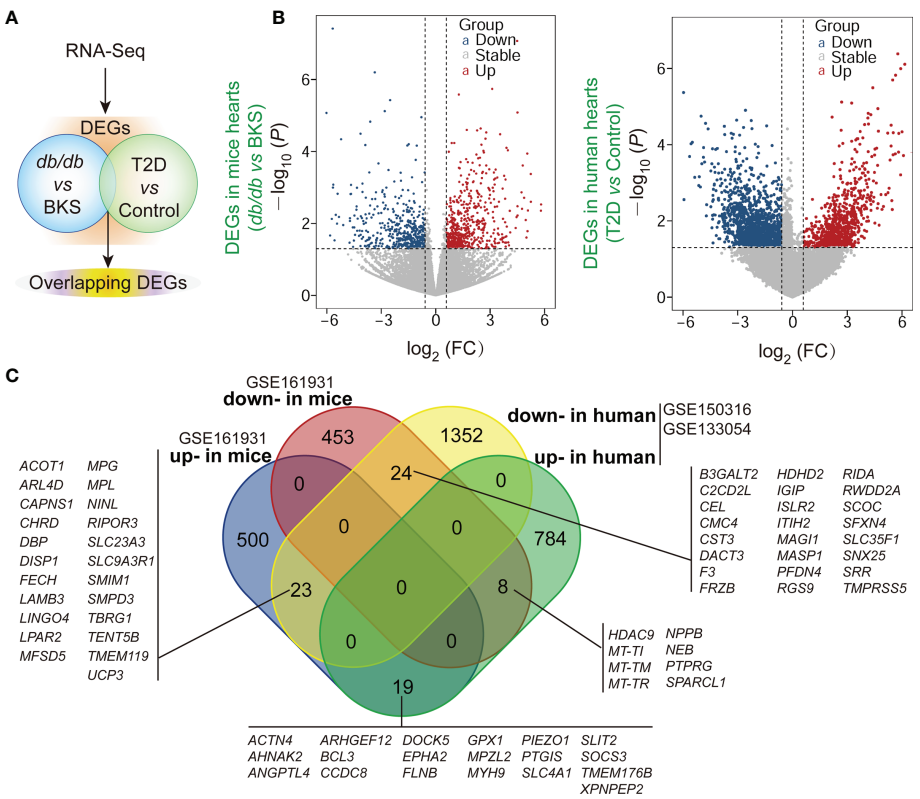

FIGURE 1 | | Identification of differentially expressed genes (DEGs) and overlapped homologous DEGs in T2D or non-T2D hearts with or without SARS-CoV-2 infection. **(A)** Scheme of the experimental procedure. The gene expression profiles in public datasets from NCBI-GEO were analyzed to identify DEGs in the heart tissues between T2D mice and control, as well as T2D patients who succumbed to SARS-CoV-2 infection and control. **(B)** Respective volcano plots of sifted out DEGs for datasets in the accordance with public database GSE161931(mice), GSE150316 *vs* GSE133054 (human) of NCBI-GEO. Blue and red plot represent up- and down-regulated genes, respectively. Gray plot represent the remaining genes with no significant difference. **(C)** The Venn diagrams of the overlapping homologous DEGs among the three datasets. Threshold was set to be FC > 1.5 (or <1/1.5) and P < 0.05.

top 10 best ranked gene annotations were considered for further analysis for three sub-ontologies as follows, biological process, molecular functions and cellular components (30). The functions of DEGs were mainly enriched and described by dot plots as the top-10 GO events (**Figures 2A, B** and **Supplementary Figures 1A-C**). As could be seen, several dot plots showed overlapping homologous DEGs. The frequency of the top-10 GO events containing overlapping homologous DEGs was subsequently explored. Both serine-type endopeptidase activity in molecular function module and mitochondrial inner membrane in cellular components module contained more overlapping homologous DEGs than the others in the top-10 GO events (**Figures 2A, B**). There are seven crucial genes in T2D hearts according to the gene abundance grades in single event among the top-10 GO events, including *Capns1*, *F3*, *Mosp1*, *Tmpress5* in molecular function module and *CAPNS1*, *FECH*, *SFXN4*, *UCP3* in cellular components module. KEGG analyses were also presented in ridge plot format (**Supplementary Figure 1D**), both dot plot and ridge plot displayed the significantly altered events in T2D hearts. In the case of SARS-CoV-2 infection, mitochondria exhibited the most significant ranked gene annotations in cellular components of GO function enrichment in T2D hearts. Following the findings, we further observed that a unique gene *CAPNS1*/*Capna1* (calpain small subunit 1) was significantly enriched in serine-type endopeptidase of molecular function module and mitochondrial inner membrane of cellular components module in T2D hearts (**Figure 2C**). Owing to setting the cut-off criterion as genes with FC > 1.5 (or <1/1.5) and $P < 0.05$ to identified DEGs between T2D

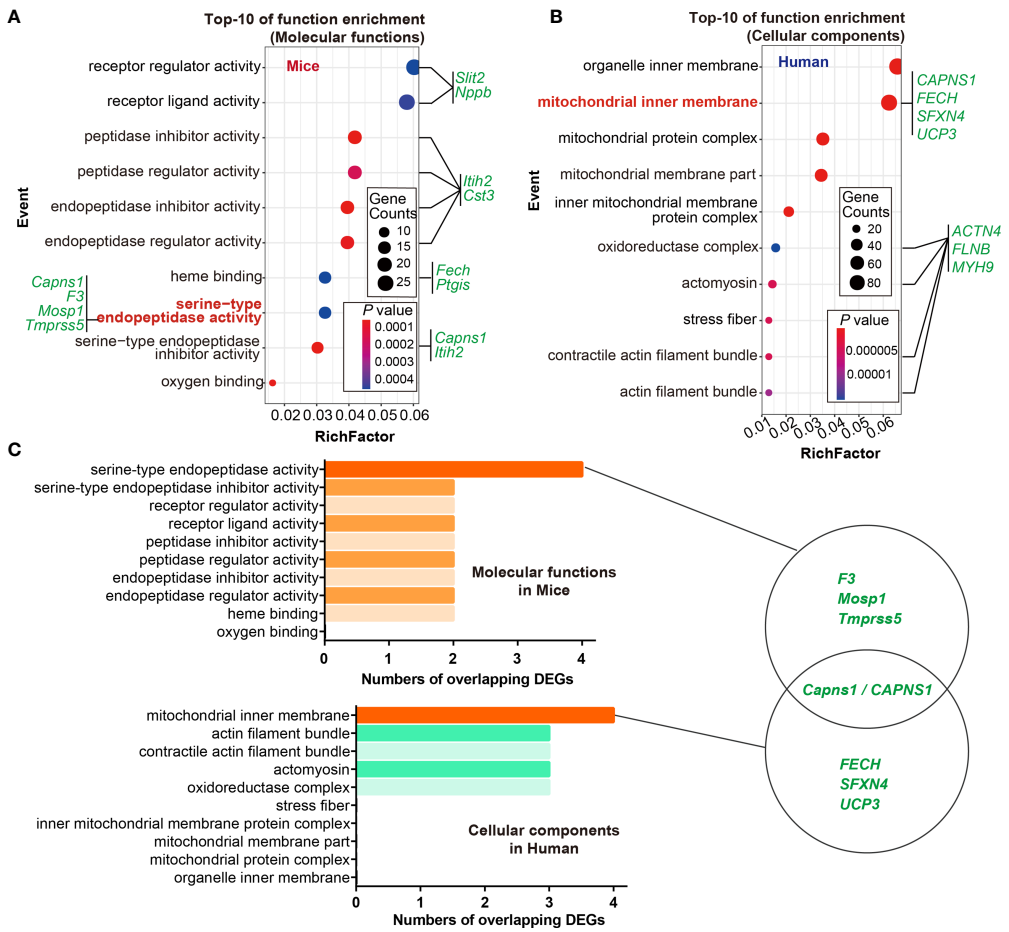

FIGURE 2 | GO functional enrichment and module analysis of DEGs in the three datasets. **(A, B)** Bubble maps showed the top-10 GO events associated with DEGs of molecular functions module and cellular components module. Significantly enriched functions of two modules were indicated in *Y*-axis. Rich factor in the *X*-axis represented the enrichment levels. The larger value of Rich factor represented the higher level of enrichment. Color of the dot stands for the different *P*-value and size of the dot reflected the number of target genes enriched in the corresponding functions. Green letters in both modules indicated the overlapping DEGs. **(C)** Frequency of crucial overlapping DEGs (≥ 2 times) associated with top-10 events in both modules through GO analysis. There are seven crucial genes in T2D hearts according to the gene abundance grades in single event among the top-10 GO events, including *Capns1, F3*, *Mosp1*, *Tmpress5* in cellular components module, and *CAPNS1*, *FECH*, *SFXN4*, *UCP3* in molecular function module. $P < 0.05$. Schematics of GO analysis of overlapping DEGs for datasets GSE150316, GSE133054, and GSE161931 from NCBI-GEO (left panel). The richest DEGs enriching in corresponding functions showed in circles, and *CAPNS1* was the unique crucial gene shared by both modules (right panel).

hearts and control samples, parts of DEGs included positive or negative regulation in transcription for the same gene in the heart samples of T2D (**Figure 3A**). The transcription level of *CAPNS1*/*Capna1* in hearts significantly increased in T2D mice, but surprisingly decreased in T2D patients who succumbed to SARS-CoV-2 infection (**Figures 3A, B**). The results suggested that SARS-CoV-2 infection did cause the transcriptional changes of gene in T2D hearts.

PPI Network Construction and Module Analysis

The DEG-encoded proteins and their interactions among each other can provide a valuable clue (31). The PPI network was constructed with DEGs from all the three public datasets of NCBI-GEO described above (**Supplementary Figures 2A, B**). PPI analysis revealed there were 30 pairs of interactions in the overlapping homologous DEGs (**Supplementary Figure 2C**). These proteins were selected based on a combined score ≥ 0.7 in STRING analysis. Consistently, molecule CAPNS1 shared by molecular function module and cellular components module in transcription were also displayed in the interaction of PPI network (**Supplementary Figure 2C**). The results supported that the most significant function of DEGs were also enriched in their encoded proteins and their interactions to each other in T2D hearts, respectively.

Enhanced Cardiac *Capns1* Transcription Association With JP2 Proteolysis in *db/db* Mice

Calpain proteolysis contributes to the pathogenesis of heart failure. CAPNS1 was a regulatory subunit of calpain, both functional enrichment of DEGs and PPI analysis revealed that calpain/CAPNS1 pathway might serve as a crucial target in T2D hearts. To explore whether proteolysis catalyzed by calpain/CAPNS1 was involved in the pathological process of T2D hearts, we first observed cardiac *Capns1* mRNA level in *db/db* mice at the age of 16 weeks (late stage of T2D heart progress). As showed in **Figure 4A**, *Capns1* transcription significantly enhanced in the hearts of *db/db* mice, reached 2.29 fold of that in control. We then examined the transcription levels of the substrates, *Jp2*, *Tnni3*, and *Mybpc3*, catalyzed by calpain/CAPNS1 in the hearts of *db/db* mice. Compared to that in

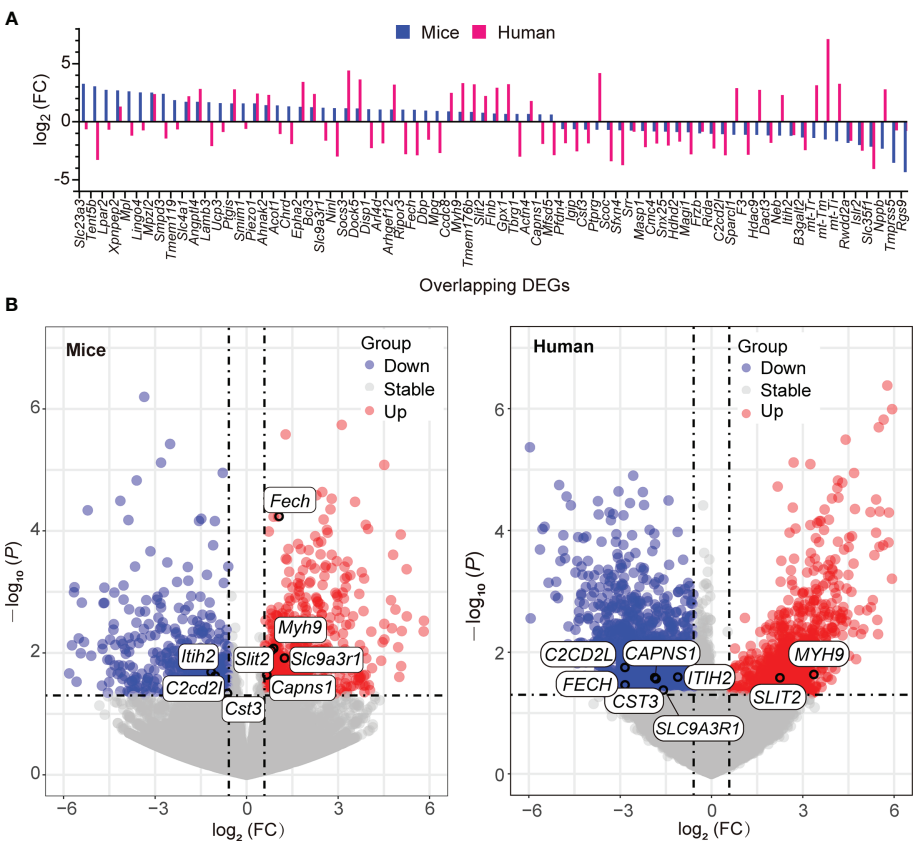

FIGURE 3 | Transcription of the overlapping homologous DEGs with significance in T2D hearts. **(A)** Spectrum of the up- and down-regulation levels of the overlapping homologous DEGs in the datasets GSE161931 (mice), GSE150316 *vs* GSE133054 (human). **(B)** Distributions of the up- and down-regulated overlapping DEGs among the top-10 GO events in respective volcano plots.

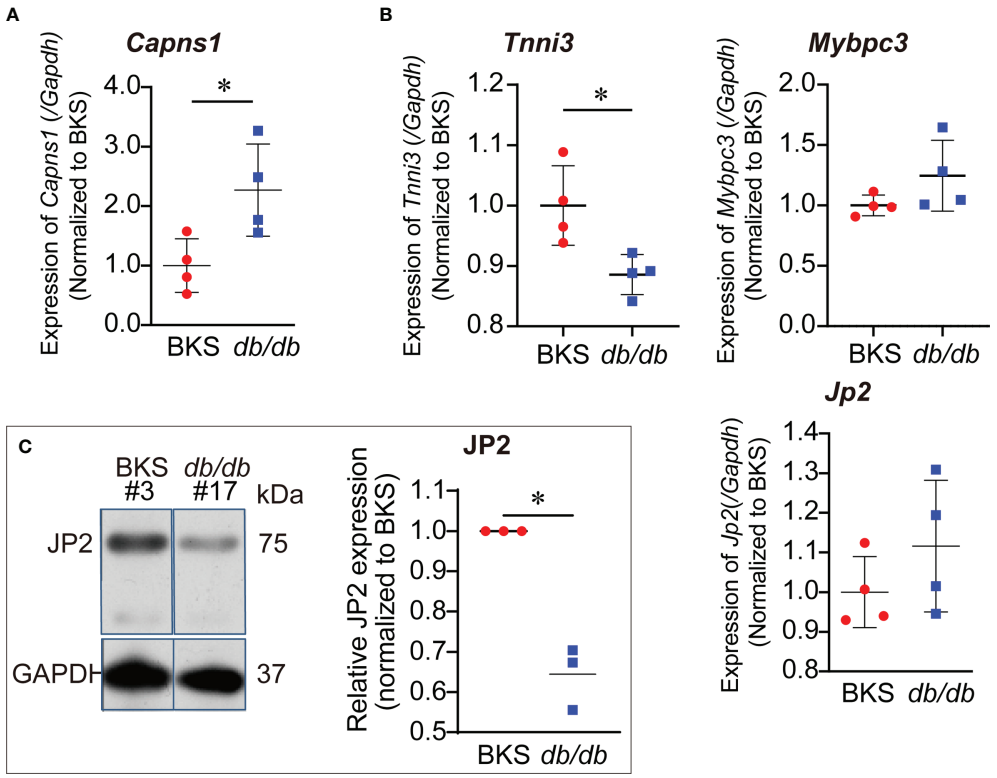

FIGURE 4 | Increased transcription of *Capns1* in the hearts of *db/db* mice. **(A)** Cardiac *Capns1* transcription enhanced in *db/db* mice (16-weeks-old). **(B)** The transcription levels of substrates *Jp2*, *Tnni3*, and *Mybpc3* in cardiac calpain/CAPNS1 pathway were not reduced (n=4, each sample was repeated three times). **(C)** Representative blotting showing JP2 protein decreased in expression in T2D hearts (n=3, each sample was repeated three times, #3 and #17 were original lab codes of animals). *$P < 0.05$.

control, *Jp2* and *Mybpc3* did not change in transcription, while *Tnni3* showed significant decrease (**Figure 4B**). It suggested that the transcription of substrates *Jp2* and *Mybpc3* to calpain/CAPNS1was stable. The attenuation of troponin 1 type 3 (*Tnni3*) in T2D heart appeared as early as in transcriptional step in a non-enzymatic catalytic manner. Furthermore, the relative expression of JP2 declined to 32.2 ± 3.9% compared to control by Western blot assay (**Figure 4C**). These results indicated that substrate JP2 of calpain/CAPNS1 did undergo a proteolysis process.

Abnormalities of Mitochondrial Ultrastructure in *db/db* Mice

Considering that in the case of SARS-CoV-2 infection, mitochondria exhibited the most significant ranked gene annotations in cellular components of GO function enrichment in T2D hearts, we further examined cardiac mitochondrial ultrastructure in *db/db* mice at the age of 16 weeks by transmission electron microscopy. Qualitative analysis of electron micrographs showed disorganized mitochondrial cristae (inner membrane) and diminished cristae density in the hearts of *db/db* mice in comparison with those in controls (**Figure 5A**). Meanwhile, echocardiographic analysis demonstrated left ventricular dysfunction in *db/db* mice. Compared to control, both left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were significantly decreased in the T2D hearts (**Figure 5B**). Taken together, in the case of left ventricular dysfunction in T2D progress, *CAPNS1* located in the worse cardiac mitochondria cristae might enhance in transcription, thereby contributing to trigger substrate JP2 proteolysis.

JP2NT Nucleus Translocation in the Hearts of *db/db* Mice

To explore whether or not substrate JP2 proteolysis took a role in T2D hearts, we used immunofluorescence confocal image to analyses the myocardium of *db/db* mice. We focused on the abundance and distribution of JP2 in cardiomyocytes of T2D heart. The sarcomere structure of cardiac troponin T (cTnT) seriously damaged and dramatically reduced. Compared to that in control, the distribution of JP2 displayed completely separating from cTnT in T2D hearts (**Figure 5C**, left panel). On the other hand, hydrolyzed N-terminal fragment of JP2 (JP2-NT) imported into the nuclei of myocardium in *db/db* mice, which characterized by the overlay of JP2-NT with DAPI in cTnT positive cells (**Figure 5C**, arrows in left panel). The ratio of JP2-NT localized in nuclei was significantly increased in T2D hearts compared with control (**Figure 5C**, right panel).

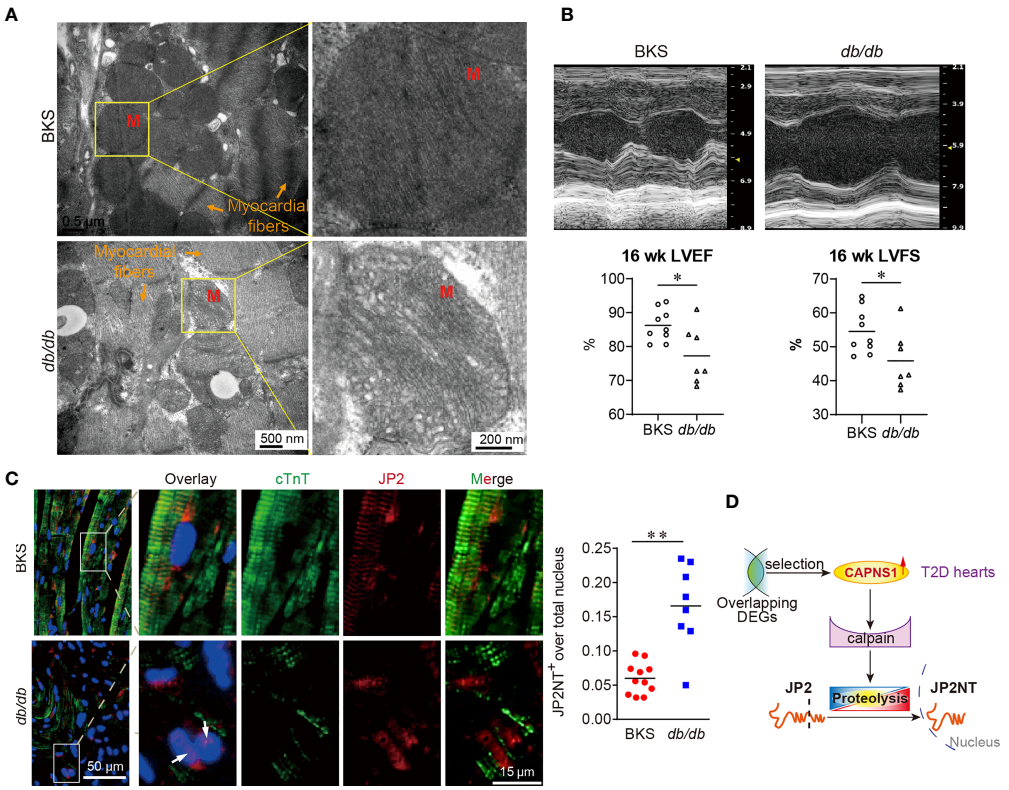

FIGURE 5 | Mitochondrial cristae disruption and hydrolyzed JP2 nuclear translocation in myocardium of *db/db* mice. **(A)** Electron micrographs showed the decreased areas of cristae organization and density of mitochondria (M) in *db/db* mice compared with controls. Scale bar: 500 nm and 200 nm. **(B)** Representative images of echocardiography. Both left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) decreased. $^{*}P < 0.05$, n=9 (BKS mice), n=7 (*db/db* mice). $^{*}P < 0.05$, $^{**}P < 0.01$. **(C)** The abundance and distribution of JP2 (red) changed in cardiomyocytes (green, cTnT) of T2D heart. The colocalization of JP2NT (red) with nuclei (blue, DAPI) appear in cardiomyocytes of T2D heart (arrow: nuclear JP2NT). **(D)** In the case of cardiac mitochondrial ultrastructure lesions, and cardiac dysfunction in T2D hearts, the hydrolyzed product JP2NT import into the nucleus of myocardium. Scale bar: 50 μm and 15 μm (left panel). Ratio of JP2NT existed in nuclei were showed in dot chart (right panel) (n=8~11, 4 random fields for each sample).

In the present study, we revealed a novel regulating mechanism in the late stage of T2D heart progress. *CAPNS1* increased in transcription at the mitochondria cristae, which favored the substrate JP2 to calpain/CAPNS1 hydrolysis and nuclear translocation.

DISCUSSION

The available data have supported that increased susceptibility in patients with T2D to SARS-CoV-2 hospitalizations (5, 6). Moreover, an acute cardiovascular manifestation of COVID-19 often presents as an acute cardiac injury in the absence of obstructive coronary artery disease (7, 8). SARS-CoV-2 directly infects cardiomyocytes *in vitro* or in T2D patients in an ACE2-dependent manner (6, 11). But the underlying mechanism of how SARS-CoV-2 damages the heart remains to be elucidated. The present study attempts to identify genomic differences between T2D- and non-T2D hearts and also signify transcriptomic effects of SARS-CoV-2 infection on the hearts through a number of bioinformatics approaches.

The primary principle in R package is more suitable for our datasets. Since the R package provides a straight forward method for visualizing DGE result files that from the most commonly used DGE tools: DESeq2, edger and Cuffdiff. Nine functions are provided, including six distinct visualizations with three matrix options. In R package, the regularly updated tools can provide continuous support in the long run. DESeq2 is designed for differential gene expression analysis of RNA-seq data, especially for those experiments with small numbers of replicates, and allow a more general, data-driven parameter estimation (22).

In the current study, we perform bioinformatics analysis on the expression profiles of public datasets and identify a pathogenic and prognostic gene *CAPNS1* in T2D hearts. We reveal that T2D hearts themselves originally over-transcribe *CAPNS1*, a gene of regulatory subunit of calpain. Using bioinformatics analysis including GO function enrichment analysis, KEGG pathway analysis, and PPI network analysis, we find that serine-type endopeptidase activity in molecular function module, and mitochondria inner membrane in cellular component module are the major GO enriched events for the overlapping homologous DEGs. The upregulated and

downregulated genes suggest that the expression and/or transcription of genes in T2D hearts undergo robust changes under the SARS-CoV-2 infection condition. In the present study, there are seven crucial genes in T2D hearts according to the gene abundance grades in single event among the top-10 GO events, including *Capns1, F*3, *Mosp1*, *Tmpress5* in cellular component module, and *CAPNS1*, *FECH*, *SFXN4*, *UCP3* in molecular function module. From the cellular components point of view, most crucial genes locate in mitochondria. The most valuable finding is that *CAPNS1* is the unique crucial gene shared by both molecular function module and cellular components module of the overlapping DEGs in T2D hearts. It means that transcriptional level of *CAPNS1* is extremely crucial for the T2D hearts. Transcriptional regulation is generally considered the mode of choice to adapt to chronic stimuli or diseases (6), markedly increase of *CAPNS1* mRNA does indicate its meaningful significance to adapt to the pathological function of T2D hearts. We therefore focus on the cardiac calpain/CAPNS1 pathway, and subsequently observe its biological function in *db/db* mice.

Calpain/CAPNS1 together form active μ-calpain and possess a mitochondrial targeting sequence in the N-terminal region of calpain (32). Calpain/CAPNS1 activation catalyzes substrates proteolysis. The present study has confirmed that cardiac *Capns1* increase in transcription in *db/db* T2D mice. As a regulate subunit, over-transcribed *CAPNS1* holds the more possibility to subsequently together with calpain, and favors μ-calpain formation in the mitochondria inner membrane of T2D hearts. JP2, Tnni3, and Mybpc3 are the substrates catalyzed by Calpain/CAPNS1 in the hearts. Mutations of *Jp2*, *Tnni3*, and *Mybpc3* are closely relevant to the heart diseases. Our results demonstrate that the transcription of *Jp2* and *Mybpc3* was stable in T2D hearts. The attenuation of *Tnni3* in T2D heart appeared as early as in transcriptional step in a non-enzymatic catalytic manner. The results suggest less effects of calpain/CAPNS1 on the transcription of its substrates. On the other hands, JP2 protein significantly decreases and its distribution exhibits separation from cTnT in cardiomyocytes of *db/db* mice, indicating that JP2 undergoes both quantitatively and functionally changes in cytoplasm. Hydrolyzed N-terminal fragment of JP2 (JP2NT) can trigger a pathway in the heart pathophysiology (33, 34). By this pathway, a self-protective mechanism that enables failing cardiomyocytes in the stressed myocardium to transduce mechanical information into salutary transcription reprogramming. But so far the upstream mechanism of this pathway is not elucidated. Current study further confirms that in the case of cardiac mitochondrial ultrastructure lesions, and cardiac dysfunction in *db/db* T2D mice, the hydrolyzed product JP2NT import into the nucleus of myocardium. Considering that *CAPNS1* is the unique gene not only shared by molecular function module and cellular component module of overlapped DEGs, but also involved in catalyzing JP2 hydrolysis, we suggest that T2D hearts should possess intrinsic CAPNS1-dependent self-protective mechanism. Based on our knowledge, this is the first time to reveal that cardiac *CAPNS1* overtranscription associated with JP2 hydrolysis might serve as a switch to initiate a compensatory role in the T2D hearts.

Given that both animal and human with diabetic cardiomyopathy share the same potential biomarkers (19), applying bioinformatic analysis to identify the potential pathogenic and prognostic DEGs are extremely valuable for the T2D heart samples of *db/db* mice and patients. The present study demonstrates the autopsy heart specimens of T2D patients display attenuated transcription of *CAPNS1* in the case of SARS-CoV-2 infection. Based on the findings, we suggested that attenuated cardiac *CAPNS1* transcription in T2D patients who succumbed to SARS-CoV-2 infection must decrease in the ability to hydrolyze JP2 and weaken self-protective mechanism, thereby leading to adverse prognostics (**Figure 6**). This finding is currently not included in the mechanisms for adverse outcomes of COVID-19. It surely takes an important contribution to deeply consummate the statement that SARS-CoV-2 directly infects cardiomyocytes *in vitro* and in T2D patients in an ACE2-dependent manner (6, 11). Based on our findings, acute cardiac injury should be one of the independent leading causes of adverse outcomes and death in T2D patients after SARS-CoV-2 infection, although COVID-19 often causes clotting issues in the coronaries of those sick. *Db/db* mice at 16-weeks-old are in the late stages of T2D progress. The current study identifies the overlapping homologous DEGs and conducts a series of bioinformatics analysis to screen the unique gene and pathway at a genome-wide scale through analyzing T2D datasets. SARS-CoV-2 infects vascular endothelium can trigger mitochondrial reactive oxygen species production and glycolytic shift (35, 36). S protein alone can damage vascular endothelial cells by down-regulating ACE2 and consequently inhibiting mitochondrial function (37). Mitochondrial dysfunction suggests that deleterious changes in mitochondria occurring in the heart in the context of T2D (38). Though mitochondrial redox state changes occur in the heart with obesity and diabetes, how it connects the remodeled energy metabolism with mitochondrial and cytosolic antioxidant defense and nuclear epigenetic changes remains to be determined (39). The underlying mechanism of SARS-CoV-2 infects cardiomyocytes mainly involves endoplasmic reticulum stress combined with mitochondria dysfunction and apoptosis (6, 11). Our findings in decreased transcription of *CAPNS1* in mitochondria inner membrane after SARS-CoV-2 infects and mitochondrial ultrastructure damage further support the putative mechanism. It may be attributable to poor mitochondrial condition triggering *CAPNS1* overexpression. In the case of SARS-CoV-2 infection, diminishing calpain/CAPNS1-associated self-protective mechanism could lead to T2D heart decompensatory.

This evidence implies a possibility that SARS-CoV-2 infection must diminish the self-protective mechanism by inhibiting *CAPNS1* transcription in the hearts of T2D patients. Based on described above, we suggest that lost cardiac *CAPNS1* gene should disturb JP2NT regulated salutary transcription reprogramming in the T2D severe cases with COVID-19. These results collectively suggest that a comprehensive

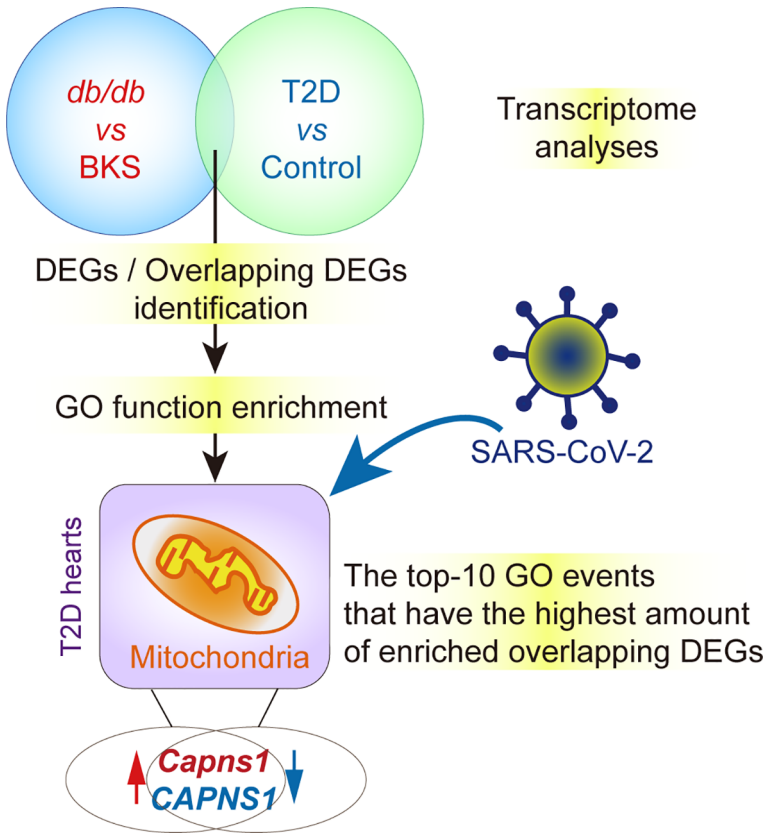

FIGURE 6 | | Hypothesis contributing to crucial gene and key functions in T2D hearts with and without SARS-CoV-2 infection. *Capns1/CAPNS1* is the unique gene shared by molecular function module and cellular components module of overlapped DEGs, and involved in catalyzing JP2 hydrolysis, T2D hearts should possess intrinsic CAPNS1-dependent self-protective mechanism. The autopsy heart specimens of T2D patients display attenuated transcription of *CAPNS1* in the case of SARS-CoV-2 infection. Attenuated cardiac *CAPNS1* transcription in T2D patients who succumbed to SARS-CoV-2 infection must decrease in the ability to hydrolyze JP2 and weaken self-protective mechanism, thereby leading to adverse prognostics.

investigation of these overlapping DEGs will facilitate our understanding of acute cardiac injury in T2D patients after SARS-CoV-2 infection. Therefore, *CAPNS1* gene may serve as a potential biomarker and therapeutic target for the onset, progression and prognostic of cardiovascular syndrome in T2D patients with SARS-CoV-2 infection, and a potential target of anti-SARS-CoV-2 comprehensive therapy, although it remains to be validated by further pre-clinical and prospective clinical studies (40).

Our transcriptomics based approach using T2D heart tissues of mouse and human (heart specimens with COVID-19) as a novel candidate progressive, prognostic and pharmacological target associated with T2D patients' anti-SARS-CoV-2 comprehensive therapy. However, there are several shortcomings of this study with regards to the role of CAPSN1 in the context of T2D hearts. First of all, we do not consider applying bottleneck method to explore hub genes, in this way analysis of the total amount of information probably will become easier. Otherwise we might be better predict the target and get a better representation. Next, given the fact that the heart specimens in T2D patients with COVID-19 are quite heterogeneous, further experiments and efforts to identify *Capns1* in mice mimicking SARS-CoV-2 infection appear well justified. The impact of inhibition of cardiac CAPSN1 protein function on T2D mice might also be needed. Although limitations of the current study, these are multiple research avenues to be further explored, for which the presented study highlights a novel starting point.

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 in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Ethics Committee of Zhejiang University, and 2nd Affiliated Hospital Research Ethics Committee of Zhejiang University, China.

AUTHOR CONTRIBUTIONS

XH conceived, designed the research, conducted the bioinformatics and molecular biological experiments, provided part of the financial and instrumental support, analyzed the data and wrote the manuscript. K-jZ and J-jJ, participated in the major collection of animal experiments, electron micrograph and analysis. S-yJ and J-bL performed the echocardiographic collection and analysis. Y-jL conducted part of the animal experimental design and provided part of the financial and instrumental support. 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/fendo.2022.801260/full#supplementary-material

Supplementary Figure 1 | | GO and KEGG enrichment analysis of overlapping DEGs. **(A, B)** Bubble maps showed the top-10 GO events associated with overlapping DEGs (green color). $P < 0.05$. The larger value of gene ratio represents the higher level of enrichment. The color of the dot stands for the different *P*-value, and the size of the dot reflects the number of target genes enriched in the corresponding event. **(C)** Volcano plots of sifted out DEGs for dataset GSE150316 *vs* GSE133054 (Human), and GSE161931 (Mice). **(D)** Ridge plots of KEGG analysis for DEGs. $P < 0.05$.

Supplementary Figure 2 | | Protein-protein interaction (PPI) network construction. **(A, B)** PPI network constructed with the DEGs from all the three public datasets of GEO. **(C)** The significant module identified from the PPI network of overlapping homologous DEGs using the molecular complex detection (MCODE) method with a score of ≥ 5.0.

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

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CASE REPORT
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Efficacy of Dulaglutide in a Patient With Type 2 Diabetes, High Cardiovascular Risk, and HIV: A Case Report

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Background: Type 2 diabetes (T2D) is a common comorbidity in people living with HIV (PLWH). Anti-hyperglycemic treatment in PLWH is still a challenge, and no randomized controlled studies using new glucose-lowering agents are currently available.

Case Description: A 55-year-old-women was admitted to our Diabetes Unit because of hyperosmolar hyperglycemic state (HHS) and sepsis. The medical history included HIV infection and insulin-treated diabetes. On clinical examination, the lady appeared dehydrated with dry buccal mucosa, tachycardia, altered mental status, genital infection, and fever. On admission, plasma glucose was 54.5 mmol/L, HbA1c 155 mmol/mol, osmolarity 389.4 mOsm/kg, bicarbonate 24.6 mmol/L with no detectable serum ketones. The patient was treated with i.v. fluid and insulin, and antibiotic therapy commenced. Upon HHS and sepsis resolution, a basal-bolus insulin therapy was implemented that was followed by significant improvement of daily glucose profiles and progressive reduction of insulin requirement until complete discontinuation. A low dose of metformin plus linagliptin was started. Since a severe atherosclerotic disease was diagnosed, a GLP-1 receptor agonist, dulaglutide, was added to metformin upon linagliptin withdrawal with maintenance of good glycemic control, treatment adherence and amelioration of quality of life and no side effects.

Conclusion: This case suggests that GLP-1 receptor agonist therapy may be effective and safe for treatment of T2D with high cardiovascular risk in PLWH, supporting the need of clinical trials directly assessing the safety and the efficacy of GLP-1 receptor agonist in these individuals.

Keywords: case report, GLP-1 receptor agonist, dulaglutide, type 2 diabetes, ASCVD, HIV infection

INTRODUCTION

In the last few years, introduction of antiretroviral therapy (ART) has much improved treatment of people living with HIV (PLWH) (1). Atherosclerotic cardiovascular disease (ASCVD) remains a main cause of morbidity and mortality among PLWH (2). Rates of myocardial infarction, heart failure, stroke, and other cardiovascular diseases (CVD) are greater in PLWH than in uninfected subjects (3). Moreover, PLWH are also more likely to develop type 2 diabetes (T2D) than people without HIV (4, 5) often due to diabetogenic effects of anti-HIV medications (6, 7). Currently, the management of T2D in people with HIV is still a matter of debate (8) and no dedicated trials have addressed the potential of novel anti-hyperglycemic agents.

We herein report the successful use of dulaglutide, a glucagon-like peptide-1 receptor agonist (GLP-1 RA), in a patient with T2D, HIV, and high atherosclerotic risk.

CASE DESCRIPTION

In April 2021, a 55-year-old woman was admitted to our Diabetes Unit because of hyperosmolar hyperglycemic state (HHS) and sepsis. Prior medical history included HIV infection, insulin-treated diabetes mellitus (both diagnosed at age 42), hypercholesterolemia, non-proliferative diabetic retinopathy, and chronic kidney disease. Home therapy included a three-drug combination ART (dolutegravir 50 mg, abacavir 600 mg, lamivudine 300 mg), arbitrarily stopped due to intolerance, atorvastatin (10 mg) and insulin therapy with poor adherence to treatment due to fear of hypoglycemia. On clinical examination, patient appeared dehydrated with dry buccal mucosa, tachycardia (120 beats per minute), altered mental status, fever (38.3°C), and ulcerated vulvar lichen sclerosus with signs of genital infection and purulent secretions. Her body weight was 48.5 kg (BMI 20.2 kg/m^2). The Malnutrition Universal Screening Tool (MUST) excluded malnutrition, undernutrition, or weight loss ≥5% in past 3-6 months. No drug addiction was apparent. Systolic blood pressure was 155 mmHg and diastolic blood pressure 90 mmHg. On admission, plasma glucose level was 54.5 mmol/L, HbA1c 155 mmol/mol, osmolarity 389.4 mOsm/kg, bicarbonate 24.6 mmol/L, and there were no detectable serum ketones (**Table 1**). Lipid levels were: total cholesterol 2.09 mmol/L; LDLc 0.57 mmol/L; HDLc 0.65 mmol/L, and triglycerides 1.51 mmol/L. Real time PCR confirmed the HIV-1 genoma with high HIV viral load (68.900 copies/ml). CD4 cell count was 362.6/μL (normal values: 410-1590/μL). Blood cultures were positive for methicillin-susceptible Staphylococcus aureus (MMSA). The patient was treated with i.v. fluid and insulin infusion and started on an empirical antibiotic therapy (piperacillin/tazobactam) that was subsequently switched to target MMSA (daptomycin and oxacillin). A transesophageal echocardiography excluded infective endocarditis. Upon HHS and sepsis resolution, basal-bolus insulin therapy was initiated yielding significant improvement of daily glucose profiles and progressive reduction of insulin requirement. Three weeks after initiation of insulin therapy, total daily insulin requirement was 0.3 unit per kg of body weight (approximately 15 U/day) with fasting capillary glucose levels ranging between 5.55-6.44 mmol/L and postprandial ones between 8.32-9.44 mmol/L with no hypoglycemic events. Pancreatic beta cell reserve was preserved as indicated by a fasting plasma C-peptide level of 0.5761 nmol/L. The search for anti-GAD and anti-IA2 autoantibodies was negative supporting a diagnosis of type 2 diabetes. **Table 1** shows changes in laboratory parameters at hospital admission and 21 days later when insulin therapy was stopped and low dose of metformin (500 mg twice daily) plus linagliptin (5 mg once daily) were started. On a vascular screening, bilateral carotid artery stenosis (50% in the left bulbar internal carotid artery) and bilateral hemodynamically significant renal artery stenosis (> 80% in the para-ostial district of the left renal artery and > 60% on the right) were identified. Magnetic resonance imaging of the brain showed chronic ischemic vasculopathy of the semi-oval centers and radiate crowns. An abdominal ultrasound (US) examination showed liver enlargement with rounded margins and inhomogeneous distribution of steatosis. In view of the patient's high cardiovascular risk linagliptin was discontinued and GLP-1 receptor agonist therapy in combination with metformin was initiated in agreement with current guidelines (9, 10). At discharge, the complete treatment plan for the patient

TABLE 1 | Lab test on admission and 21 day hospitalization.

	On admission	Day 21	Normal values
Fasting plasma glucose (mmol/L)	54.5	6.44	3.3-5.5 mmol/L
HbA1c (mmol/mol)	155	105	20-30 mmol/mol
Creatinine (μmol/L)	129.9	125.6	44-96.8 μmol/L
eGFR* (ml/min/1.73m^2)	38.8	41.5	> 90 ml/min/1.73m^2
Osmolality (mOsm/kg)	389.4	290.5	280-300 mOsm/kg
Sodium (mmol/L)	152	135	135-145 mmol/L
Bicarbonate (mmol/L)	24.6	27.2	22-30 mmol/L
Potassium (mmol/L)	4.01	3.82	3.4-4-5 mmol/L
Urea (mmol/L)	22.6	6.5	3.57-17.85 mmol/L
Blood urea nitrogen (mmol/L)	22.85	6.43	< 8.21 mmol/L
Chloride (mmol/L)	109	98	98-107 mmol/L
Calcium (mmol/L)	2.69	2.31	2.15- 2.55 mmol/L
C-reactive protein (mg/L)	312.1	37.1	< 5 mg/L
Procalcitonin (ng/L)	5080	80	< 500 ng/L

*CKD-EPI.

included dulaglutide (at starting dose of 0.75 mg once weekly), metformin (adjusted to 500 mg once daily to prevent pharmacological interaction with antiretroviral therapy), atorvastatin (10 mg once daily), aspirin (100 mg once daily), bisoprolol (1.25 mg once daily), amlodipine (5 mg once daily), and bictegravir/emtricitabine/tenofovir alafenamide (50 mg/200 mg/25 mg daily), as prescribed by the local infectious disease specialist, given the patient intolerance to previous antiretroviral therapy. Three months after starting dulaglutide, HbA1c was 51 mmol/mol, fasting glucose 7.3 mmol/L and self-monitoring of blood glucose showed a good glycemic control. The patient reported no hypoglycemia nor gastro-intestinal side effects ensuring a high level of adherence to therapy; body weight dropped from 48.5 kg to 45.5 kg with a final BMI of 18.9 Kg/m^2. At follow-up, an abdominal US no longer detected liver steatosis, although within the limit of inter-operator variability. Urinary albumin-to-creatinine ratio (from 346.7 to 92.1 mg/g) and creatinine value (from 129.9 to 96.4 μmol/L) improved as well and the HIV viral load was markedly reduced (29 copies/ml). **Figure 1** summarizes the effects of antihyperglycemic therapy on fasting plasma glucose, HbA1c and body weight.

METHODS

Plasma glucose was measured by the glucose oxidase reaction (Glucose Oxidase Analyzer). Plasma C-peptide was measured by a radioimmunoassay (Pantec Srl Turin, Italy). Anti-GAD and anti-IA2 autoantibodies were analyzed by a radioimmunoassay using a commercial kit (Medipan, Berlin, Germany). All other parameters were determined according to standard methods. Clinical laboratory data are reported in Standard International units. Reference values for healthy adults in our Laboratory are reported in **Table 1**.

DISCUSSION

To the best of our knowledge, the literature on the use of the newer classes of anti-hyperglycemic agents (i.e., sodium-glucose cotransporter 2 inhibitors, SGLT2i and GLP-1 RA) in people living with HIV and T2D is sparse. Only one prospective (24 weeks) observational study reported the experience with the SGLT2i canagliflozin in 8 HIV-infected diabetic subjects (11), and only three case reports have so far described the use of GLP-1 RA (12–14). Then, our case reporting on a lady with T2D and ART-treated HIV in whom the treatment with dulaglutide ensured clinically significant improvement of glycemic control, treatment adherence with no hypoglycaemia adds up to the existing literature to support the feasibility of using GLP-1 RAs in these subjects. Of interest, our case also had high cardiovascular (CV) risk as indicated by multidistrict atherosclerotic disease (carotid, renal and cerebral arteries), a condition where the use of a GLP1-RA with established CV benefit is highly recommended (9, 10). This is even more critical on the light that ASCVD remains a main cause of morbidity and mortality among PLWH (2) to the point that the American Heart Association has recently recommended considering HIV as a major CV risk factor (15). The reasons for an elevated risk of ASCVD among people living with HIV remains relatively poorly understood, but they are likely to be multifactorial (16). In the absence of randomized clinical trials or hypothesis-testing mechanistic studies, it is only possible to speculate that GLP-1 RAs can contribute to reduce the increased CV risk in subjects with HIV *via* "glycemic" and "extra- glycemic" actions including a beneficial effect on blood pressure, lipid profile, body fat, insulin resistance, and inflammation among the many ones (17).

On the other hand, to the best of our knowledge, cardiovascular outcome trials (CVOTs) investigating the safety and efficacy of GLP-1RAs did not specifically report HIV infection as an inclusion or exclusion criterion and no analyses or sub-analyses on this specific subgroup of patients have been reported out of these CVOTs (18–25). The management of T2D in people with HIV is still a matter of discussion (8) and subjects with HIV pose a special challenge because of possible drug interactions. Integrase strand inhibitors can increase the area under the curve of metformin. Though this may not be clinically significant (26) it may still require some caution (27). Of interest, a recent study showed that HIV-1 replication is suppressed by metformin in both primary human CD4 T cells and humanized

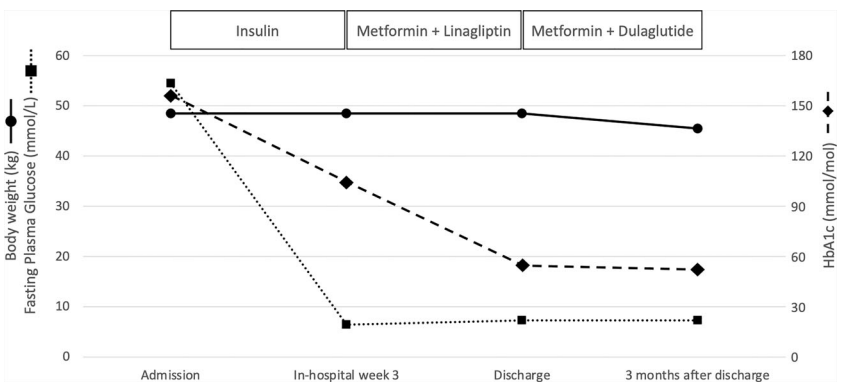

FIGURE 1 | Effects of antihyperglycemic therapy on fasting plasma glucose, HbA1c and body weight.

mice (28), which may open new opportunities in the future. Some protease inhibitors are CYP2C9 inducers and can decrease sulfonylurea levels (29, 30). Conversely, pioglitazone has been suggested to be the drug of choice in HIV-1-infected lipoatrophic adult individuals, although more data are needed on the overall safety of this compound (31). Moreover, when used along with CYP2C8 inhibitors (many protease inhibitors) its circulating levels may increase (32). Concern regarding gliptin use in HIV-infected individuals has been raised, as these agents have molecular targets on immune cells, although a small study showed no changes in CD4 or HIV RNA among treated HIV-infected subjects on sitagliptin (33). Of note, saxagliptin has been showed to interact with CYP3A4/5 inhibitors such as atazanavir, indinavir, ritonavir, and saquinavir (30, 32). No interactions between ART and SGLT-2 inhibitors are expected; however, UDP-glucuronosyltransferase enzyme inducers (e.g., ritonavir) may decrease the exposure to canagliflozin (32). To the best of our knowledge, no interactions between ART and GLP-1 RAs have been reported. In a patient like the one described here, the use of a GLP1-RA appears to be rational enough. SGLT2 inhibitors could have been a choice in our patient as well. However, the presence of a mycotic infection at hospital admission suggested a safer use of GLP-1 RA in this subject and dulaglutide proved to have a positive impact on the achievement and maintenance of glycemic control. Moreover, its weekly administration along with no occurrence of hypoglycemic events may have accounted for a good treatment adherence in a person who had in the past much trouble in managing a more complex insulin treatment. Also, the use of dulaglutide had no negative effects on liver or renal function. On possible matter of concern could have been the possible effect of delayed gastric emptying of GLP-1 agonists and the possible impact on orally administered oral agents. However, the gastric effect of long acting GLP-1 RAs like dulaglutide tends to vanish with time because of a tachyphylaxis. Moreover, dulaglutide has been shown not to alter bioavailability of many commonly used oral agents (34). As a possible downside, a 3 kg body weight reduction was recorded. Although this may be looked at with some concern, the reduction in body weight obtained with GLP-1 RAs, more evident within the first month of therapy, tends to plateau from the first to the 5th year of therapy (23) so that no further loss is expected with the prosecution of the therapy. Rather, this weight loss may have a favorable impact of ectopic fat and, in particular, on liver fat that tends to accumulate in response to antiretroviral therapy (35). Our subjects also had a renal artery stenosis and mild hypertension. With respect to this too, a GLP1-RA can be considered an interesting therapeutic option due to the known effect of these medications on blood pressure and because of pre-clinical data suggesting they could induce a vasodilation of the renal arteries (36).

In conclusion, our experience, although limited to a single case report, suggests that GLP-1 RA therapy may be a valid therapeutic opportunity in people living with HIV. Our observation calls for proper clinical studies exploring the potential use of GLP-1 RA in patients with special forms of diabetes or diabetes associated with special clinical condition such as HIV and its anti-viral treatment. To this extent, two randomized clinical trials are currently investigating the potential of GLP-1 RAs in these individuals. The first study will assess the efficacy of semaglutide as an adjunct to diet and exercise in achieving greater weight loss as compared to diet and exercise alone in HIV-1 infected patients ≥ 18 years with BMI ≥ 30 kg/m2 or BMI ≥ 27 kg/m2 and hypertension, dyslipidemia or type 2 diabetes (ClinicalTrials.gov Identifier: NCT04174755). The second trial will evaluate the efficacy of semaglutide vs placebo in treating lipohypertrophy among non-diabetic people living with HIV (ClinicalTrials.gov Identifier: NCT04019197).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval were not required for a case report study in accordance with the local legislation and institutional requirements. The patient provided the written informed consent to partecipate in this study.

AUTHOR CONTRIBUTIONS

AD conceived the case report description and wrote the first draft. AD and MA were involved in the clinical management of the patient. AD, GD, and RM made a critical literature review. SDP made a critical interpretation of the intellectual content of the article. AD is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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The Effects of Sesamin Supplementation on Obesity, Blood Pressure, and Lipid Profile: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Sun Y, Ren J, Zhu S, Zhang Z, Guo Z, An J, Yin B and Ma Y (2022) The Effects of Sesamin Supplementation on Obesity, Blood Pressure, and Lipid Profile: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Front. Endocrinol. 13:842152. doi: 10.3389/fendo.2022.842152

Aims: Sesamin, the main lignin constituent of sesame, plays a pivotal role in regulating physical state. Some studies have evidenced that the supplementation of sesamin may decrease cardiovascular disease risk. The goal of this systematic review was to summarize evidence of the effects of sesamin supplementation on obesity, blood pressure, and lipid profile in humans by performing a meta-analysis of randomized controlled trials.

Data Synthesis: Five databases (PubMed, Cochrane Library, EMBASE, Web of Science, and Scopus) were searched electronically from inception to July 2021 to identify randomized controlled trials that assessed the impact of sesamin on obesity, blood pressure, and lipid profile. Weighted mean difference (WMD) and standard deviation (SD) were used to present the major outcomes.

Conclusions: Seven trials (n = 212 participants) were included in the overall analysis. Results showed that sesamin supplementation caused a great reduction in TC (WMD: -10.893 mg/dl, 95% CI: −19.745 to −2.041, p = 0.016), LDL-c (WMD: -8.429 mg/dl, 95% CI: −16.086 to −0.771, p = 0.031), and SBP (WMD: −3.662 mmHg, 95% CI: −6.220 to −1.105, p = 0.005), whereas it had no effect on HDL-c, TG, DBP, or weight. Subgroup analysis showed that duration, parallel design, and unhealthy status can affect TC, LDL-c, and SBP evidently. We did not discover a strong link between indicators' changes and duration of supplementation. Sesamin can be used as an obtainable dietary supplement to improve blood pressure and blood lipids, and further as a health product to prevent cardiovascular diseases.

Keywords: sesamin, obesity, blood pressure, lipid profile, meta-analysis

1 INTRODUCTION

At the global level, the highest per capita cardiovascular disease (CVD) burden remains in the countries of Eastern Europe and Central Asia (1). Several published articles have mentioned that potentially modifiable risk factors, such as high blood pressure (BP), raised serum lipids, and obesity may play key roles in promoting the pathogenesis of CVDs (2–4). Blood profile levels are good indicators of cardiovascular risk and good predictors of coronary disease outcome (5). Dyslipidemia, defined as elevated levels of triglycerides and cholesterol (particularly LDL-c) and reduced levels of HDL-c, has been introduced as a strong risk factor for CVD (6, 7). High blood lipid levels can result in serious damage to systemic blood vessels and organs (8, 9). Several significant associations have been shown between CVD burden and circulati1ng levels of LDL-c, HDL-c, and triglycerides (10, 11). Hypertension (systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg) (12) and CVDs are inseparable, too.

Sesamin, which constitutes 1.5% of the weight of sesame seed (13), has been consumed as a health natural supplement. This nutrient is also present in several plants distributed in different genera, including camellia, magnolia, piper, sesamum, and virola (14). It has traditionally been believed to have health benefits in some East Asian countries for many years. In the recent decades, it has been shown that sesamin exhibits several physiological actions in animals, such as antiobesity, antihypertensive, and serum lipid–lowering effects (15–17). The animal experiment showed that sesamin has been used to decrease blood lipids and blood glucose levels in the aorta of rats with metabolic syndrome (18). Several animal studies have also confirmed that the supplementation of sesame seeds or sesamin could decrease cholesterol levels (19, 20).

Hirata et al. (21) have experimented with sesamin on human subjects, and the results were surprising. TC and LDL-c were significantly lower in the sesamin-treated group. Similarly, the results obtained by Mohammadshahi (22) also had an effect on TC and LDL-c. Up to now, randomized controlled trials (RCTs) have not reached a consistent conclusion about the effect of sesamin on blood pressure and lipid profile (23, 24).

Given the evidence that sesamin is related to a decreased risk of CVD, we carried out a systematic review and meta-analysis, which aims at determining whether sesamin intake has the potential to be used as an adjuvant therapy for persons who have cardiovascular disease.

2 MATERIALS AND METHODS

This study was executed based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (25) and registered in the International Prospective Register of Systematic Reviews (PROSPERO) database under the registration number CRD42021271145.

2.1 Search Strategy

Systematic literature retrieval was performed in the PubMed, SCOPUS, Cochrane Library, Embase, and ISI Web of Science databases from inception to July 2021 to determine a randomized controlled trial evaluating the effects of sesamin on obesity, blood pressure, and lipid profile. Medical subject heading terms (Mesh) were used: ("sesame" OR "sesamin" OR "sesamum") AND ("Blood Pressure" OR "Hypertension" OR "High Blood Pressure") AND ("HDL" OR "LDL" OR "Triglyceride" OR "Total cholesterol") AND ("BMI" OR "Weight") (**Supplementary Material**, which illustrates the search strategies). Then, the retrieved manuscripts were imported into EndNote software (version X9) to remove the duplicates. The inclusion and exclusion criteria are listed in **Table 1**. Two authors (YS and JR) independently and cooperatively determined suitable manuscripts for inclusion. Disagreements were discussed by the third author (SZ).

2.2 Data Extraction

Based on the pre-designed table, the important report data are listed as the following: publication information (first author's last name, the year published, study location), the details of the clinical trial (study design, intervention duration), the participants' characteristics (sample size, age, gender, health status), and all reported outcomes of interest. Standard deviation (SD), belonging to the category of descriptive statistics, was the experimental index to be captured. When SE was reported, we use the formula between SD and SEM (SD = SEM × sqrt (n); n = number of participants) to convert.

2.3 Assessment of Quality

Trials were assessed for bias risk using the Cochrane Bias Risk Tool (26) which includes sequence generation, allocation concealment, blinding, blinding of outcome assessment, incomplete outcome data, selective outcome reporting, and other bias. We ranked for "low", "high", or "unclear" risk of bias.

2.4 Quantitative Data Synthesis

All the analyses were performed using STATA version 11. Weighted mean difference (WMD), SD, and 95% CI were used as the effective measures for SBP, DBP, HDL-c, LDL-c, TG, TC, and weight. The net changes in them were equal to the post-intervention values minus the baseline values. The SD of the mean difference was calculated by the following formula:

$$\mathrm{SD} = \text{square root}\left[(\mathrm{SD}_{\text{pre-treatment}})^2 + (\mathrm{SD}_{\text{post-treatment}})^2 - (2R \times \mathrm{SD}_{\text{pre-treatmen}} \times \mathrm{SD}_{\text{post-treatment}})\right]$$

assuming a correlation coefficient (R) = 0.5 for both the pre-test/post-test (parallel groups) and the crossover designed studies. The heterogeneity index I^2 is used for quantitative analysis of heterogeneity, which ranges from 0% to 100%. There is no heterogeneity at 0%. The greater the I^2 value, the greater the heterogeneity. There was no statistical heterogeneity

Abbreviations: CI, confidence interval; TC, total cholesterol; TG, triglyceride; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; RCT, randomized controlled trial; SD, standard deviations; SE, standard error; WMD, weighted mean difference; CVD, cardiovascular diseases; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analysis.

TABLE 1 | PICOS criteria for inclusion of studies.

Parameter	Description
Population	Adult participants (healthy/unhealthy)
Intervention	I Sesamin administered for ≥2 weeks II Sesamin dosage is clearly indicated
Comparator	Placebo
Outcomes	Outcomes regarding at least one of the following markers: cholesterol, total cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides, triacylglycerol, VLDL, BMI, weight, blood pressure, diastolic blood pressure, systolic blood pressure
Study design	Randomized placebo-controlled clinical trial with a cross-over or parallel design

(I^2 < 50%) among the results of each study, and a fixed-effect model was used. If there was statistical heterogeneity (I^2 ≥ 50%) among the results, the random-effect model was used.

2.5 Meta-Regression Analysis

Meta-regression analysis was performed to calculate the duration–effect relationship between WMD and duration to explore potential explanations for heterogeneity.

2.6 Subgroup Analysis

We also conducted subgroup analysis studies including treatment duration (<42 days or ≥42 days), study design (crossover or parallel), and participants' health status (healthy or unhealthy). To evaluate the influence of individual study on the pooled-effect size, sensitivity analysis (leave-one-out) was conducted and p < 0.05 was considered as statistically significant.

2.7 Publication Bias

Begg's rank correlation and Egger's weighted regression statistics were used to evaluate potential publication bias (27, 28). p values less than 0.1 were considered statistically significant.

3 RESULTS

3.1 Flow and Characteristics of the Included Study

The initial search identified 527 papers for screening, of which 121 were removed because of duplication (**Figure 1**). After title and abstract screening, 365 records were excluded due to irrelevance to the inclusion criteria. The full texts of the remaining 41 articles were further screened, after which 34 studies were excluded for the following reasons: lack of sufficient information on the outcomes of interest (n = 6); the dosage of sesamin was not specified (n = 17); study not designed as an RCT (n = 6); and article published as meta-analysis or review (n = 5). Finally, 7 articles (15, 21–24, 29, 30) with 212 arms were enrolled in the present meta-analysis. The PRISMA flowchart of the study is shown in the following.

3.2 Characteristics and Quality of Included Studies

Characteristics of the included studies are shown in **Table 2**. Each of the included articles stated sesamin dosage, and four of the studies had dosages less than 200 mg/day. Four studies were on obesity, four studies were on BP, and five studies were on lipid profile. Included studies have been published between 1996 and 2016 and were conducted in 5 different areas: Japan, Taiwan, Australia, Thailand, and Iran. A total of 212 participants were enrolled in studies, and intervention duration ranged from 28 to 60 days. Four studies had a parallel design and three studies had a crossover design. Six trials were conducted in unhealthy individuals, and one trial was carried through healthy individuals.

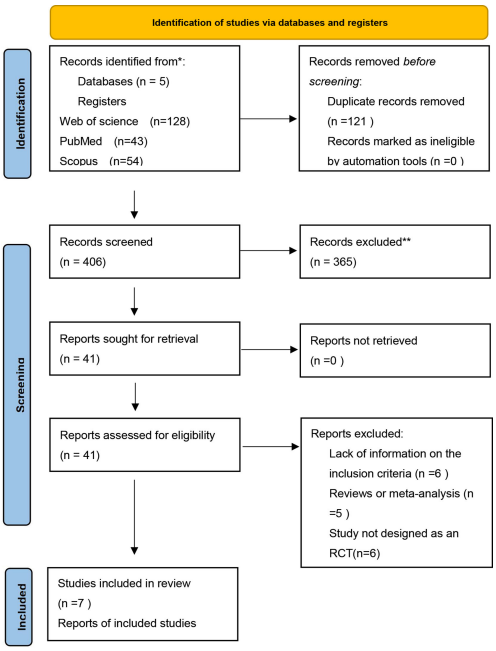

FIGURE 1 | The PRISMA flowchart.

3.3 Findings From Meta-Analysis

Meta-analysis showed that sesamin supplementation caused a great reduction in TC (WMD: -10.893 mg/dl, 95% CI: −19.745 to −2.041, p = 0.016), LDL-c (WMD: -8.429 mg/dl, 95% CI: −16.086 to −0.771, p = 0.031), and SBP (WMD: −3.662 mmHg, 95% CI: −6.220 to −1.105, p = 0.005) (**Figure 2**).

3.3.1 Effect of Sesamin Supplementation on Obesity Levels

Levels of obesity were reported in four of the included studies, including 145 participants. Sesamin ingestion showed a non-significant effect on mean body weight (WMD: -0.223, 95% CI: -3.766 to 3.321; p = 0.902) compared with control, without heterogeneity among the studies (I^2 = 0.0%, p = 0.976).

3.3.2 Effect of Sesamin Supplementation on BP Levels

The effect of sesamin on BP was reported in four treatments with 132 participants. Sesamin intake did significantly affect SBP (WMD: -3.662 mmHg, 95% CI: -6.220 to -1.105, p = 0.005; I^2 = 20.8%). However, the pooled result using a random-effect

TABLE 2 | Characteristics of study populations, type of interventions, and study designs in the included trials.

Reference	Study design	Journal	Country/ area	Sample size	Sex (M/F)	Target population	Mean age (y)	BMI (kg/m^2)	Intervention/ control	Duration	Main outcomes	Notes
Hirata (21)	R,PC,P	Atherosclerosis	Japan	12	12/0	Hypercholesterolemia	ns	ns	Placebo/ sesamin	8 weeks	TC TG LDL-c HDL-c	Mean SD
Wu (29)	R,PC,C	American Society for Nutrition	Taiwan	24	0/24	Healthy postmenopausal women	59	18-28	Placebo/ sesame	5 weeks	Weight TC VLDL-c LDL-c HDL-c TG VLDL-TG	Mean SD
Miyawaki (15)	R,DB,PC,C	J Nutr Sci Vitaminol	Japan	25	23/2	Mild hypertension	49.1	24.6	Placebo/ sesamin	4 weeks	SDP BDP BMI	Mean SE
Wu (23)	R,PC,C	Elsevier	Australia	33	18/15	Overweight men and post-menopausal women	54.7	30.8	Placebo/ sesame	5 weeks	Weight HDL-c LDL-c TC SBP DBP	Mean SD
Wichitsranoi (24)	R,PC,P	Nutrition Journal	Thailand	30	8/22	With prehypertension	49.8	26.1	Placebo/ sesame	4 weeks	SDP BDP	Mean SD
Helli (30)	R,DB,PC,P	Journal of the American College of Nutrition	Iran	44	0/44	Rheumatoid arthritis	55.49	32.8	Placebo/ sesamin	6 weeks	Weight BMI SBP DBP TG TC HDL-c LDL-c	Mean SD
Mohammadshahi (22)	R,DB,PC,P	J Babol Univ Med Sci	Iran	44	22/22	With type II diabetes	50.86	29.15	Placebo/ sesamin	60 days	Weight BMI total TC LDL-c HDL-c	Mean SD

C, crossover; CVD, cardiovascular disease; DB, double-blind; DBP, diastolic blood pressure; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; P, parallel; PC, placebo controlled; R, randomized; RA, rheumatoid arthritis; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

model showed a reduction in DBP (WMD = -2.304 mmHg, 95% CI: -5.596 to 0.988, p = 0.170) with sesamin intake. A high heterogeneity was also detected in DBP (I^2 = 62.4%).

3.3.3 Effect of Sesamin Supplementation on Lipid Profile Levels

Five trials with 157 participants measured the effect of sesamin supplementation on TC and LDL-c. Moreover, the results of TC (WMD = -10.893 mg/dl, 95% CI: -19.745 to -2.041, p = 0.016; I^2 = 49.8%) and LDL-c (WMD = -8.429 mg/dl, 95% CI: -16.086 to -0.771, p = 0.031; I^2 = 53.3%) are detected following sesamin supplementation. With random-effect models, the I^2 value of LDL-c was 53.3%, and the related p value was 0.073.

Four trials with 145 participants consuming sesamin affected HDL-c and TG. HDL-c (WMD = 1.644 mg/dl, 95% CI: -1.560 to 4.848, p = 0.314; I^2 = 0.0%) and TG (WMD = -2.034 mg/dl, 95% CI: -16.298 to 12.229, p = 0.780; I^2 = 0%) concentrations did not alter significantly following sesamin intake.

3.4 Risk-of-Bias Assessment

The quality of studies was evaluated by using the Cochrane collaboration's risk-of-bias assessment tool. Random sequence generation, allocation concealment, and blinding of outcome assessment of participants were low risk of bias in all included studies. Only one trial had a high risk of bias due to the incomplete outcome. Details of the quality of studies are shown in **Table 3**.

3.5 Meta-Regression Analysis

Results of meta-regression suggested no clear relationship between the duration and biomarkers we conducted (TC: coefficient = 0.377, p = 0.671; LDL-c: coefficient = 0.395, p = 0.686; HDL-c: coefficient = 1.043, p = 0.988; TG: coefficient = 2.039, p = 0.806; SBP: coefficient = 1.414, p = 0.921; DBP: coefficient = 1.575, p = 0.897; weight: coefficient = 0.987, p = 0.996; **Figure 3**).

3.6 Subgroup Analysis

Subgroup analyses showed no significant differences in the effect of sesamin on HDL-c, TG concentrations, and weight between subgroups, which are stratified by intervention duration (≥42 days vs.<42 days), study design (parallel vs. crossover), and health status (healthy vs. unhealthy) (**Table 4**). It indicated that reduction was greater in trials conducted with longer duration, as for TC (WMD = -21.363 mg/dl, 95% CI: -34.090 to -8.636, p = 0.001; I^2 = 0.0%) and LDL-c (WMD = -14.434 mg/dl, 95% CI: -24.929 to -3.939, p = 0.007; I^2 = 55.2%). However, the reduction was more remarkable in participants with shorter duration (<42 days), as for SBP (WMD = -3.824 mmHg, 95% CI: -6.588 to -1.060, p = 0.007; I^2 = 45.9%). In addition, the trials conducted in unhealthy participants showed a remarkable reduction in TC (WMD = -21.363 mg/dl, 95% CI: -34.09 to -8.636, p = 0.001; I^2 = 0.0%), LDL-c (WMD = -14.434 mg/dl, 95% CI: -24.929 to -3.939, p = 0.007; I^2 = 55.2%), SBP (WMD = -4.490 mmHg, 95% CI: -7.315 to -1.666, p = 0.002; I^2 = 0.0%), and DBP (WMD = -3.542 mmHg, 95% CI: -6.873 to -0.210, p = 0.037; I^2 = 46.0%). Subgroup analysis also suggested that trials with a

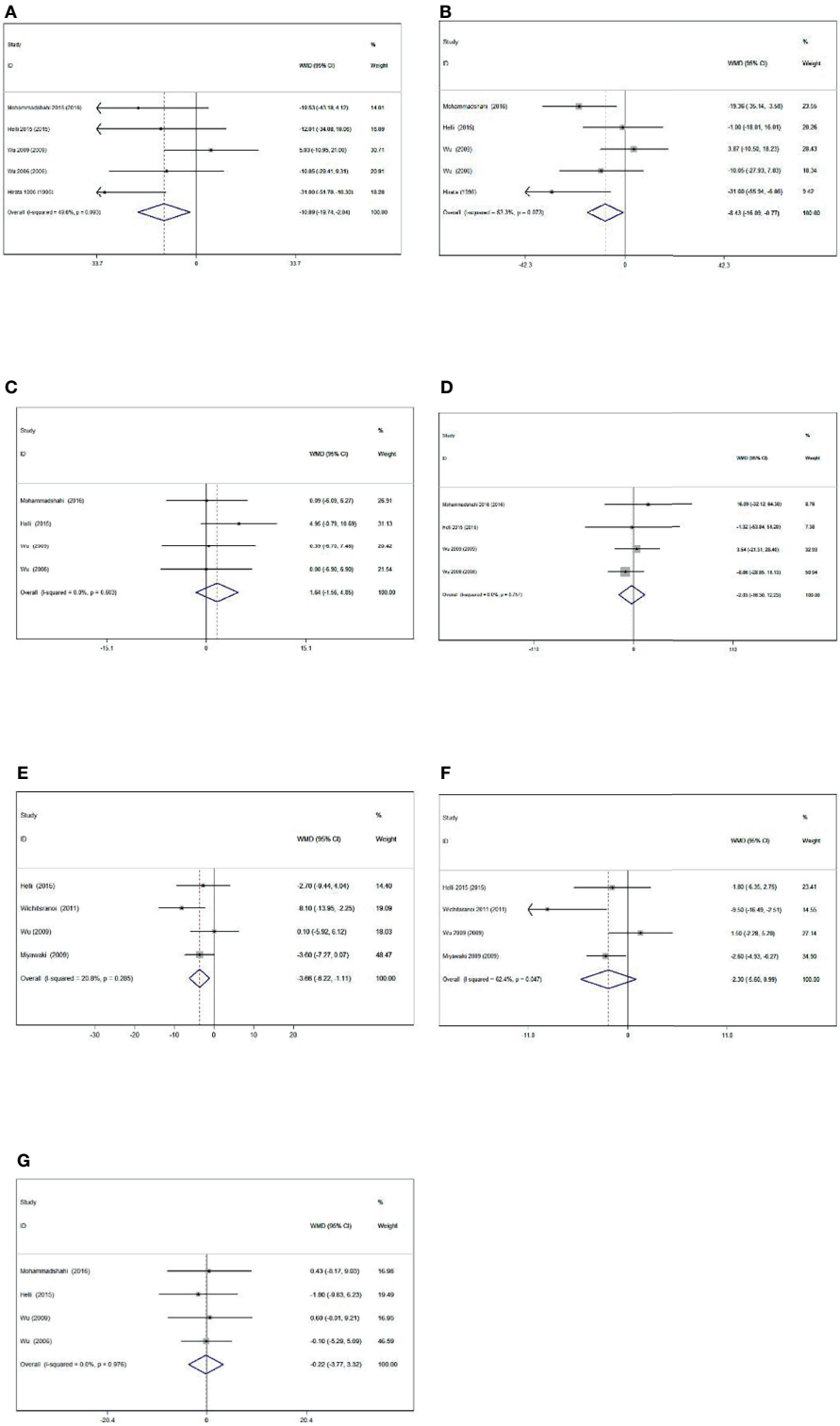

FIGURE 2 | Forest plot of the effect of sesamin supplementation on TC **(A)**, LDL-c **(B)**, HDL-c **(C)**, TG **(D)**, SBP **(E)**, DBP **(F)**, and weight **(G)**.

TABLE 3 | Assessment of risk of bias in studies included in the meta-analysis.

	Random sequence generation	Allocation concealment	Blinding of participants	Blinding of outcome assessment	Free of incomplete outcome	Free of selective reporting	Other bias
Hirata (21)	L	L	U	L	H	L	U
Wu (29)	L	L	U	L	L	L	L
Miyawaki (15)	L	L	L	L	L	L	U
Wu (23)	L	L	L	L	U	L	U
Wichitsranoi (24)	L	L	L	L	L	L	U
Helli (30)	L	L	L	L	L	U	L
Mohammadshahi (22)	L	L	L	L	L	L	L

L, low risk of bias; H, high risk of bias; U, unclear risk of bias.

parallel design were correlated with its reduction of TC (WMD = -21.363 mg/dl, 95% CI: -34.090 to -8.636, p = 0.001; I^2 = 0.0%), LDL-c (WMD = -14.434 mg/dl, 95% CI: -24.929 to -3.939, p = 0.007; I^2 = 55.2%), and SBP (WMD = -5.778 mmHg, 95% CI: -10.197 to -1.360, p = 0.010; I^2 = 28.9%).

3.7 Sensitivity Analysis and Publication Bias

There was no significant impact for any individual trial on the pooled effect sizes of meta-analyses results, so the results are reliable.

The funnel plots were asymmetric (**Figure 4**), indicating a possible publication bias in meta-analysis of the effects of sesamin on hemodynamics. However, the Begg's rank correlation test and Egger's linear regression test suggested no significant publication bias in this meta-analysis (all p>0.10) (**Table 5**).

4 DISCUSSION

Seven articles with 212 arms were enrolled in our present meta-analysis, which assessed that sesamin supplementation did not affect the levels of HDL-c, TG, DBP, or weight, but with a decrease in TC, LDL-c, and SBP. These changes varied substantially depending on the duration, study design, and health status. In a previous meta-analysis on dietary lignans, sesamin was mentioned, but which was not analyzed alone for an accurate result (31), as we did.

Compared to the current drug therapy, dietary supplements taking sesame for an example may potentially provide a rather safe, healthy, and low-cost way to prevent disease. A previous meta-analysis by Khalesi et al. showed that sesame affected the level of TG markedly (32), and its public health implication is bright. Sesame was widely applied for its heart protection (33) which may be because of its lignans such as sesamin and sesamolin (34, 35). Sesamin is composed of carbon, hydrogen, and oxygen, whose molecular formula is $C_{20}H_{18}O_6$, and its weight per mole is 354.35 g (36). Due to its health benefits, more and more animal and human experiments have been conducted in the past two decades.

4.1 Effects on Obesity

The rapid increase in obesity rates among adolescents and children around the world has shocked us (37). The status of obesity is not hopeful either, and the rise in adult obesity continues to rise (38). Since the outbreak of COVID-19, the interplay of obesity and COVID-19 has had devastating consequences with increased morbidity and mortality (39). Neidich et al. demonstrated that obese individuals were twice as likely to get the flu as healthy people who received the same vaccine (40). The health-related burden associated with obesity is estimated to have a substantial economic impact (41). Helli et al. have proved that intake of sesamin had a decrease in body weight (p = 0.001) (30). Sesamin increases lipolytic enzyme activity and decreases the activity of lipogenic enzymes, which also affects as an antagonist to liver X receptor (LXRα) and pregnane X receptor (PXR) ameliorating drug-induced hepatic lipogenesis (42, 43). However, our meta-analysis showed that the supplementation of sesamin had no association with reduction in body weight, so the effect of sesamin on weight could not be fully determined.

4.2 Effects on Blood Pressure

In the recent decade, hypertension is widely recognized to sharply increase the incidence of cardiovascular disease, which has dramatically increased the medical expenditure for patients around the world (44, 45). Through many *in vitro* and *in vivo* experiments, we now know that long-term effective antihypertensive therapy can avert hypertension-related mortality by nearly 50% (46). However, a systematic review written by Tadesse indicated that 45% of the subjects did not adhere to their antihypertensive medication, existing a low compliance (47). As dietary therapy becomes more and more accepted, sesamin has a good compliance prospect.

Our study revealed that a 4-week administration of 60 mg sesamin caused a decrease in BP with an average of 3.5 mmHg for SBP and 1.9 mmHg for DBP. Wu et al. proceeded a 4-week administration of sesamin and achieved a good antihypertensive effect that can greatly decrease SBP and DBP in mild-hypertensive participants (15). It was reported that patients with RA who consumed 200 mg/day sesamin showed a lower SBP level, but with no remarkable effect on DBP (30). According to Nakano (48), the mechanism of sesamin against high pressure is improving impaired endothelium-dependent vasodilatory responses. Our meta-analysis which covered both SBP and DBP levels indicated significant hypotensive effects of sesamin supplements, depending on study design, duration of treatment, and participants' health status. Our subgroup analyses indicated the antihypertensive function of sesamin in the experimental design type was parallel. What is more, patients who received shorter than 42 days of sesamin and had an unhealthy status can show more obvious effects. Kong et al. showed that sesamin inhibited the progress of eNOS uncoupling, coupled with the effect on p-eNOS increases NO biosynthesis to relieve hypertension. Sesamin also inhibits

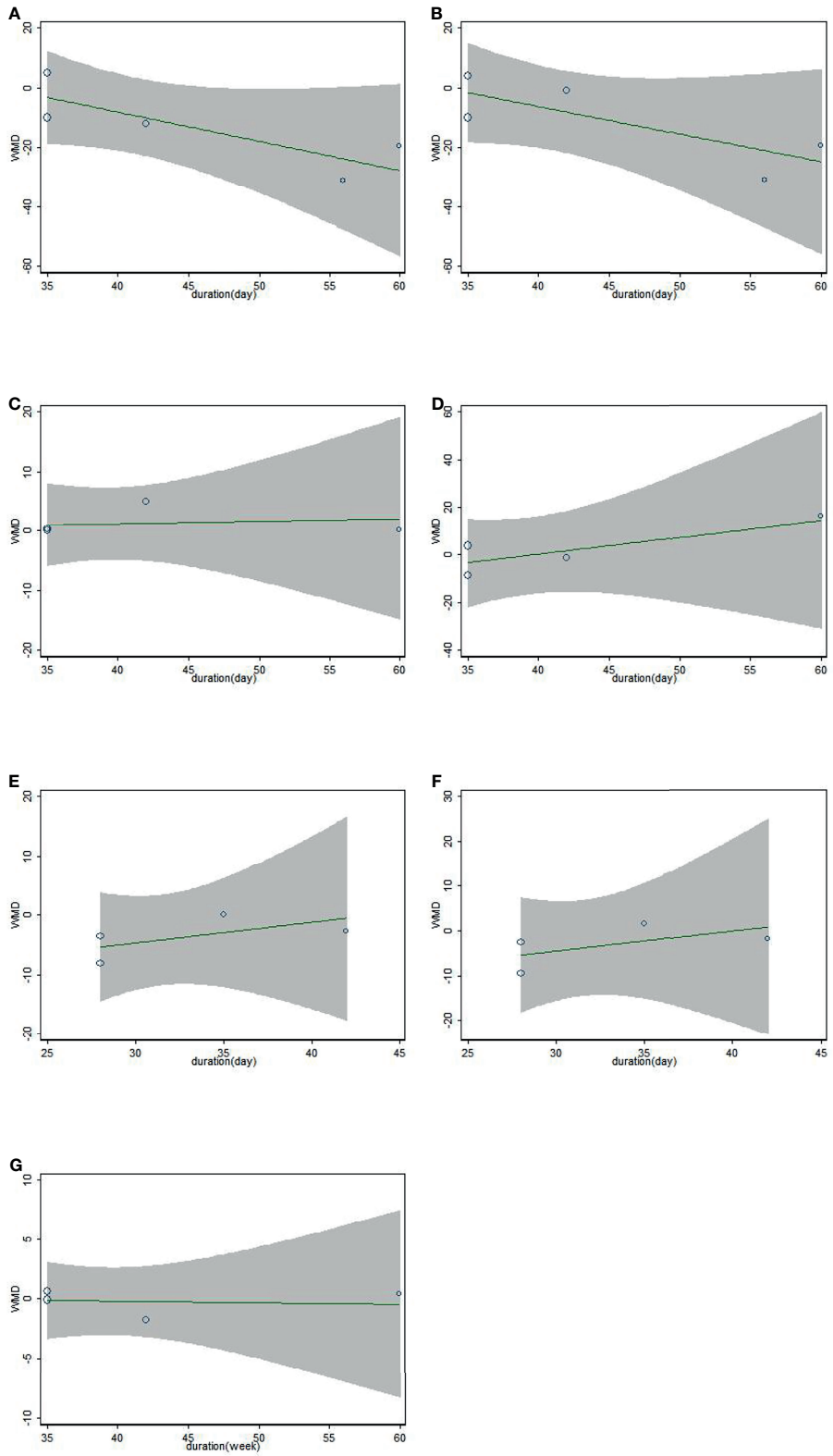

FIGURE 3 | Association between duration of study and effect size of sesamin supplementation on TC **(A)**, LDL-c **(B)**, HDL-c **(C)**, TG **(D)**, SBP **(E)**, DBP **(F)**, and weight **(G)**.

TABLE 4 | Subgroup analyses.

Variables[2]	Duration		Health status		Study design	
	≥42 days	<42 days	Healthy	Unhealthy	Parallel	Crossover
TC						
No. of treatment arms	3	2	2	3	3	2
WMD	-21.363	-1.082	-1.082	-21.363	-21.363	-1.082
(95% CI)	(-34.090 to -8.636)	(-13.402 to 11.238)	(-13.402 to 11.238)	(-34.090 to -8.636)	(-34.090 to -8.636)	(-13.402 to 11.238)
I^2 (%)	0	27.9	27.9	0	0	27.9
P	0.001	0.863	0.863	0.001	0.001	0.863
LDL-C						
No. of treatment arms	3	2	2	3	3	2
WMD	-14.434	-1.593	-1.593	-14.434	-14.434	-1.593
(95% CI)	(-24.929 to -3.939)	(-12.791 to 9.605)	(-12.791 to 9.605)	(-24.929 to -3.939)	(-24.929 to -3.939)	(-12.791 to 9.605)
I^2 (%)	55.2	29.3	29.3	55.2	55.2	29.3
P	0.007	0.780	0.780	0.007	0.007	0.780
HDL-C						
No. of treatment arms	2	2	2	2	2	2
WMD	2.697	0.188	0.188	2.697	2.697	0.188
(95% CI)	(-1.509 to 6.902)	(-4.758 to 5.135)	(-4.758 to 5.135)	(-1.509 to 6.902)	(-1.509 to 6.902)	(-4.758 to 5.135)
I^2 (%)	21.6	0	0	21.6	21.6	0
P	0.209	0.941	0.941	0.209	0.209	0.941
TG						
No. of treatment arms	2	2	2	2	2	2
WMD	8.129	-3.989	-3.989	8.129	8.129	-3.989
(95% CI)	(-27.385 to 43.644)	(-19.564 to 11.586)	(-19.564 to 11.586)	(-27.385 to 43.644)	(-27.385 to 43.644)	(-19.564 to 11.586)
I^2 (%)	0	0	0	0	0	0
P	0.654	0.616	0.616	0.654	0.654	0.616
SBP						
No. of treatment arms	1	3	1	3	2	2
WMD	-2.700	-3.824	0.100	-4.490	-5.778	-2.597
(95% CI)	(-9.438 to 4.038)	(-6.588 to -1.060)	(-5.922 to 6.122)	(-7.315 to -1.666)	(-10.197 to -1.360)	(-5.733 to 0.539)
I^2 (%)	0	45.9	0	0	28.9	5.4
P	0.432	0.007	0.974	0.002	0.010	0.105
DBP						
No. of treatment arms	1	3	1	3	2	2
WMD	-1.800	-2.695	1.500	-3.542	-5.174	-0.832
(95% CI)	(-6.348 to 2.748)	(-7.303 to 1.912)	(-2.283 to 5.283)	(-6.873 to -0.210)	(-12.662 to 2.314)	(-4.811 to 3.148)
I^2 (%)	0	74.9	0	46.0	69.5	69.4
P	0.438	0.252	0.437	0.037	0.176	0.682
WEIGHT						
No. of treatment arms	2	2	2	2	2	2
WMD	-0.762	0.087	0.087	-0.762	-0.762	0.087
(95% CI)	(-6.630 to 5.106)	(-4.359 to 4.532)	(-4.359 to 4.532)	(-6.630 to 5.106)	(-6.630 to 5.106)	(-4.359 to 4.532)
I^2 (%)	0	0	0	0	0	0
P	0.799	0.969	0.969	0.799	0.799	0.969

TC, total cholesterol; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure; WMD, weighed mean difference; CI, confidence intervals.

mg/dl for TC, LDL-c, HDL-c, TG; mmHg for SBP and DBP.

[2] is inherent to I2.

NADPH oxidase contributing to the suppression of hypertension in hypertensive rats (49).

4.3 Effects on Lipid Profiles

Hyperlipidemia, a life-threatening health condition endangering the life of most patients (50), represents as increasing TC/TG/LDL-c or decreasing HDL-c (51). With elevated LDL-c concentration as a risk factor, statins can effectively reduce the concentration to play an anti-hyperlipidemia role (52, 53). Although statins' primary application is to lower cholesterol (54), drug toxicity to the human body is still unavoidable. "Pharmacograde nutrients" may be a potentially safe, healthy, and cheap way to optimize lipid levels (55). Hirata et al. suggested that sesamin together with vitamin E can reduce the LDL-c level effectively ($p<0.05$) (21). Another research conducted by Mohammadshahi (22) declared that by supplementing a daily dose of 200 mg sesamin to patients with Type II diabetes, the level of TG, TC, and LDL-c became reduced. There is a biological basis of lipid lowering; Tai et al. (43)showed that sesamin might improve blood lipid by reducing hepatic steatosis and absorption of cholesterol through the intestine. Majdalawieh et al. (36) revealed that sesamin mainly played anti-hyperlipidemic roles by aiming at Δ5 desaturase, HMGCR,

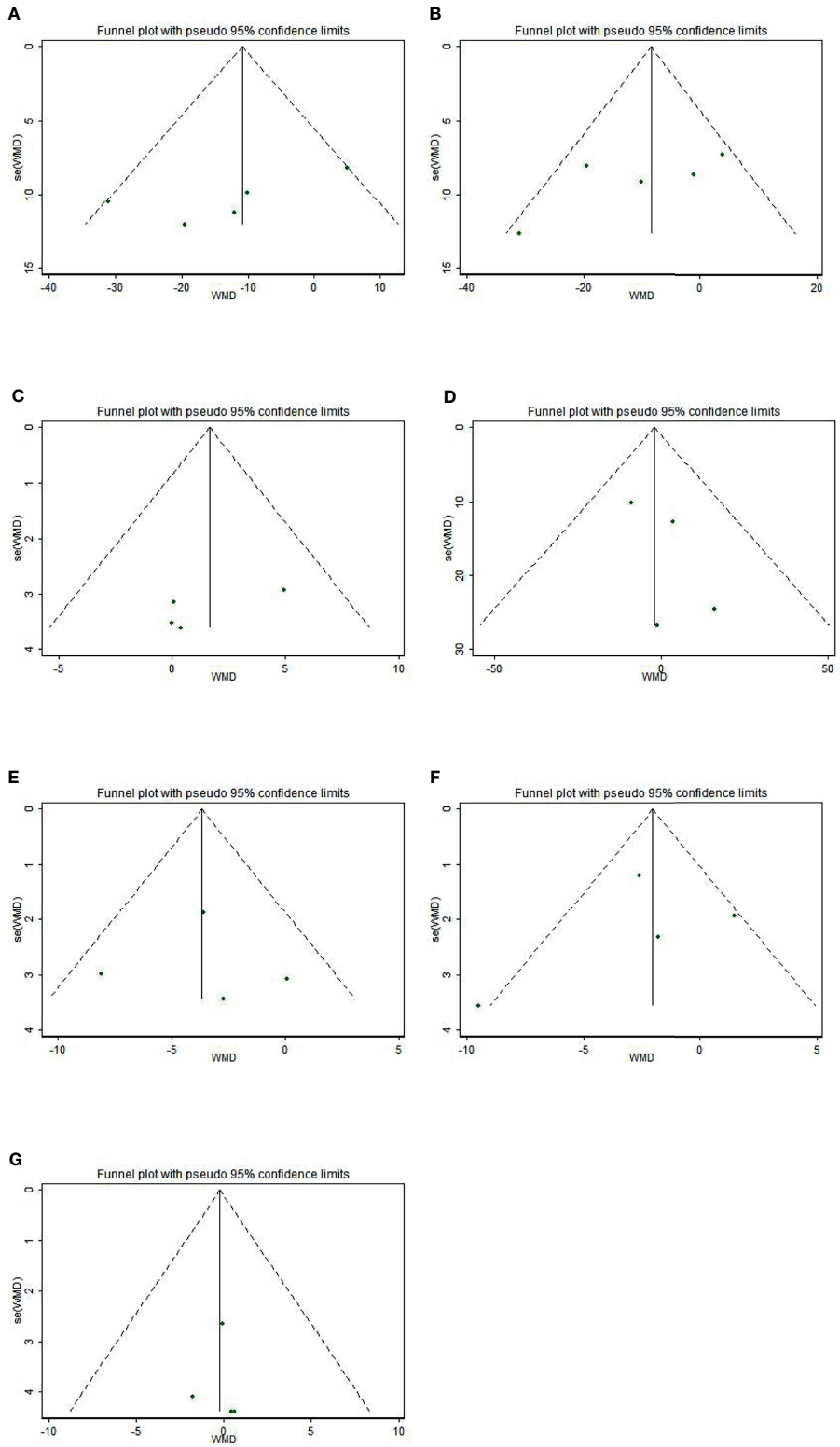

FIGURE 4 | Funnel plot of the effect of sesamin supplementation on TC **(A)**, LDL-c **(B)**, HDL-c **(C)**, TG **(D)**, SBP **(E)**, DBP **(F)**, and weight **(G)**.

TABLE 5 | Assessment of publication bias.

	Begg's rank correlation test			Egger's linear regression test		
	Kendal's score	Z value	P value	Intercept	95% CI	P value
TC	-6	-1.47	0.142	-7.052	(-17.595,3.490)	0.123
LDL	-6	-1.47	0.142	-5.415	(-15.558,4.728)	0.188
HDL	0	0	1.000	-6.138	(-21.603,9.327)	0.230
TG	2	0.68	0.497	0.956	(-2.388,4.299)	0.344
SBP	2	0.68	0.497	0.209	(-11.693,12.111)	0.947
DBP	-2	-0.68	0.497	-1.192	(-12.279,9.895)	0.689
Weight	4	1.36	0.174	-0.013	(-3.065,3.039)	0.987

ABCA1, and ABCG1 through PPARα, PPARγ, LXRα, and SREBP signaling pathways, which is important for the future supplement of sesamin.

Our meta-analysis showed that daily intake of sesamin was effective in improving LDL-c and TC levels but had no significant influence on TG and HDL-c. Long-term (≥42 days) sesamin intake is more effective than a short period of supplement. The reason may be that sesamin, as a food of nutrition, can make its accumulation in the body more obvious after long-term ingestion. The meta-regression showed LDL-c's trend becoming flat over time. Perhaps this is because LDL-c fluctuates in a dynamic range for different individuals, flattening out as its concentration nears a critical level. In the experiment conducted by Wu et al. (23), the duration was so short that sesamin played no role. Therefore, no significant influence on the indexes of lipid profile was indicated. Probably due to one crossover RCT with short-term included, findings also indicated that TC and LDL-c levels were decreased when the RCT was designed to be parallel-controlled.

Integrated data from numerous selected studies are the major superiority of our study, so the reliability and accuracy of our analysis are robust. The seven indicators we included were carefully selected and of great clinical guiding significance. What is more, the studies we included from various geographic regions around the world, so our conclusion has a wide range of application value for people in different cultural backgrounds. Additionally, our review not only provides a new food therapy target for people who are in an unhealthy status but also facilitates guidance for the development of dietary intervention therapy in the future.

The limitations of our study were self-explanatory. Firstly, a relatively small sample size of the included studies may cause a higher risk for publication bias whereas Begg's and Egger's tests suggested no significant publication bias in our meta-analyses. Secondly, the heterogeneity of LDL-c was relatively high, but we found that this may be caused by long duration, unhealthy status, or parallel-design trials. Thirdly, the gender ratio in the studies we included was different, and the influence of hormone level on obesity, lipid profile, and blood pressure could not be ignored.

5 CONCLUSION

Taken together, our results indicate that sesamin can be used as an easily obtainable dietary supplement to improve BP and blood lipids, and further as a health product to prevent cardiovascular diseases. In the future, large multinational prospective randomized controlled trials should help determine the ideal dose, duration, and formulation of the sesamin intervention specific to each individual patient's health status. More multi-geographical trials with sufficient subjects, involving more countries, hoped to be conducted for the ideal dose, for the appropriate duration, and for individual patients' fitting target.

In terms of implications for practice, the evidence from our meta-analysis suggests that unhealthy subjects taking sesamin may improve the lipid profile and BP more remarkably.

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

Substantial contributions to the conception and design of the meta-analysis were done by JYR and YXM. The literature search and data extraction were done by YTS, JYR, and SQZ. The data analyses were done by ZAZ, ZHG, JQA, and BWY. The manuscript and revision were done by YTS, JYR, and YXM. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of Apelin–APJ System in Diabetes and Obesity

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Nowadays, diabetes and obesity are two main health-threatening metabolic disorders in the world, which increase the risk for many chronic diseases. Apelin, a peptide hormone, exerts its effect by binding with angiotensin II protein J receptor (APJ) and is considered to be linked with diabetes and obesity. Apelin and its receptor are widely present in the body and are involved in many physiological processes, such as glucose and lipid metabolism, homeostasis, endocrine response to stress, and angiogenesis. In this review, we summarize the literatures on the role of the Apelin–APJ system in diabetes and obesity for a better understanding of the mechanism and function of apelin and its receptor in the pathophysiology of diseases that may contribute to the development of new therapies.

Keywords: apelin, diabetes, obesity, metabolic disorder, APJ

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1 INTRODUCTION

The prevalence of metabolic disorders is increasing worldwide in recent years. Diabetes and obesity are two of the most common health-threatening metabolic disorders. It is reported that there are 3.7 million and 2.5 million people who die every year globally due to type 2 diabetes mellitus and obesity, respectively. Metabolic disorders contribute to the development of insulin resistance, obesity, cardiovascular complications, and, eventually, multi-organ dysfunction (1). Diabetes is a state of hyperglycemia and is characterized by insulin resistance and/or insulin secretion dysfunction. Sustained hyperglycemia and insulin hyposensitivity lead to the dysfunction of various tissues and organs. Obesity is an important risk factor that contributes to the development of type 2 diabetes by dysregulating several metabolic and adipose tissue-derived factors. Adipose tissues secrete adipokines, which are involved in glucose and lipid metabolism, neuroendocrine function, insulin sensitivity, and other physiological processes. Therefore, an effective and novel therapeutic recommendation is needed to curb the incidence, morbidity, and mortality caused by metabolic diseases.

Apelin is a regulatory peptide as well as a ligand for G-protein-coupled receptor (APJ). It is widely present in the body, including in peripheral tissues and the central nervous system. There is a growing interest on the apelin–APJ system during the past decade due to its potential role in several physiological processes, such as homeostasis, body fluid management, cell proliferation, and energy metabolism (2–4). Apelin is produced and secreted by adipocytes; hence, it is called adipokine. Several studies have reported that the increased plasma apelin is related to metabolic pathologies. Evidences are in favor of the regulatory role of the apelin–APJ system in glucose and lipid

metabolism. The present review summarizes the current knowledge and literatures on the regulatory role of apelin, with emphasis on the regulation of glucose and lipid metabolism, that may provide novel therapeutic targets.

2 APELIN–APJ RECEPTOR SYSTEM DISCOVERY AND DEVELOPMENT

2.1 Apelin and Its Receptor APJ

In 1993, O'Dowd et al. isolated a special human G protein-coupled receptor and named it "APJ". It contains 380 amino acids and shares 54% sequence with the human angiotensin II receptor type 1 in the transmembrane (5). Subsequently, APJ receptor was also isolated in amphibians and rodents (6, 7). The APJ gene is located on chromosome 11 (11q12) without any intron in the coding region. However, angiotensin II does not interact with APJ receptor, and there was no APJ ligand identified until 1998, when apelin, a APJ endogenous ligand, was isolated by Tatemoto et al. from the bovine stomach (8). The apelin gene is located on Xq25-q26.3 chromosome. It encodes a 77-amino-acid prepropeptide that contains a secretory signal sequence and can be cleaved into different active forms such as apelin-36, apelin-17, apelin-13, and apelin-12 (8) (**Figure 1A**). Apelin-36 is the most widely expressed form, while apelin-13 is more potent and more abundant in the circulation (9). It has been demonstrated that there is a high sequence homology among human, bovine, and rodent preproapelin, particularly the last 22 C-terminal amino acids (8, 10). All isoforms of apelin could bind to APJ, but they have a different biological potency. Different organs have different apelin isoforms.

Apelin and APJ are widely expressed in many tissues and organs, including the brain, heart, lung, liver, kidney, gastrointestinal tract, endothelium and adipose tissues (11). Apelin-12 possesses a high affinity for APJ receptor, with cardioprotective properties of increasing the myocardial contractility and reducing the mean arterial pressure (12). Apelin-13 is the main neuroprotective peptide, with the highest abundancy in the plasma (13, 14). Previous research reported that apelin-13 participates in vasculopathy, energy metabolism, and humoral homeostasis (13, 15, 16). There was also a study that discovered the downregulation of apelin-13 in aging. The knockout of apelin-13 and APJ gene could accelerate aging in a mouse model, while the upregulation of apelin-13 restores vitality, a response to the stimuli and circadian rhythm (17). Glutamine cyclase can catalyze the translated N-terminal glutamine residues of apelin-13 to modify pyroglutamidation and produce the pyroglutamide form of apelin-13, called [pyr^1]-apelin-13. It can prevent apelin-13 from degradation by exopeptidase and exert long-term biological effects (4). Therefore, [pyr^1]-apelin-13 is considered a physiological ligand for APJ due to its higher anti-degradation properties (18). Apelin-17 was found to be the most potent inducer of APJ internalization, and the removal of a single amino acid at the C-terminus can abolish this process (19). Apelin-36 is mainly expressed in the lung, testis, and uterus (20). Apelin-13 and apelin-36 have been considered the most active isoforms with the greatest effect on the cardiovascular system (21). Apelin-13 and apelin-36 have different intracellular trafficking of APJ due to

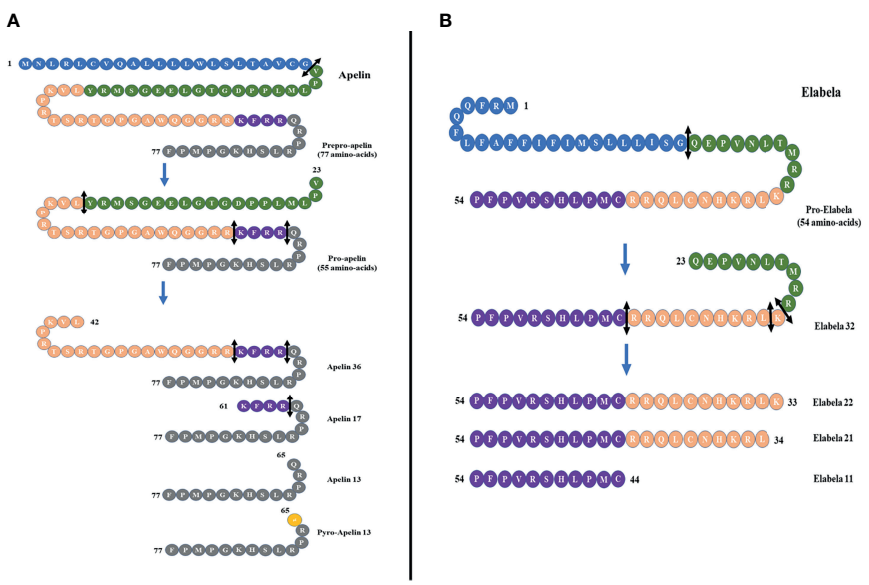

FIGURE 1 | **(A)** Different forms of apelin. The proteolysis of preproapelin at specific cleavage sites (double-headed arrows, black) leads to three fragments of 36, 17, and 13 amino acids named apelin-36, apelin-17, and apelin-13, respectively. The N-terminal glutamine residue of apelin-13 is pyroglutamylated, which produces the pyroglutamyl form of apelin-13 ([Pyr1]-apelin-13). **(B)** The proteolysis of proelabela at specific cleavage sites (double-headed arrows, black) leads to four fragments of 32, 22, 21, and 11 amino acids named elabela-32, elabela-22, elabela-21, and elabela-11, respectively.

different receptor binding affinities. In addition, the shorter apelin isoform seems to be the more potent activator of APJ than apelin-36 (22). It is worth noting that APJ knockout mice showed a disrupted cardiac development with rudimentary to absent heart resulting in prenatal mortality (23). On the contrary, apelin knockout mice had normal heart development (24). It indicates that there may be other ligands for APJ.

2.2 Novel APJ Ligand—Elabela/Toddler

Apelin was considered to be the only APJ ligand until 2013, when two different research groups discovered a second endogenous ligand for the apelin receptor in zebrafish (*Danio rerio*) during embryonic development, and it was named Elabela ("epiboly late because endoderm late") by Chng et al. and Toddler (referring to the loss of motogen properties when deleted) and by Pauli et al. (25, 26). Subsequently, the cDNA encoding Elabela was discovered in vertebrates and mammals (27). Recently, Elabela was found to be restrictedly expressed in human pluripotent stem cells and renal tissues. Elabela is a peptide of 54 amino acids with a secretory signal containing 32 amino acids (25) (**Figure 1B**). This precursor is cleaved to shorter sequences of 32, 21, and 11 amino acids (28) (**Figure 1B**), and all these short sequences can activate APJ as well as can be blocked by apelin receptor antagonists.

The discovery of this new ligand created several exciting possibilities. Evidences revealed the role of Elabela in homeostasis, cell signaling, energy metabolism, and cell aging. Serum Elabela is associated with several diseases, such as diabetes (29), myocardial infarction (30), hypertension (31), and kidney disease (32). Notably, Elabela level is also related to the prognosis of these diseases. It is reported that Elabela can ameliorate apoptosis *via* regulating the mitochondrial function (33). Recently, Elabela has been demonstrated to decrease kidney injury (34), and serum Elabela level in type 2 diabetes patients is closely related to the severity of renal injury (35). In addition, Elabela has been reported to improve endothelial cell function *via* the PI3K/Akt signaling pathway (36). Some literatures also showed that Elabela is a potent regulator of adipose cell metabolism. However, the effect of Elabela is still unclear, and further studies are required to clarify its role and significance.

3 EFFECT OF APELIN–APJ SYSTEM ON DIABETES

Nowadays, apelin is believed to assist in regulating glucose metabolism, and the apelin–APJ system has been demonstrated to be related with diabetes mellitus and diabetic complications. Apelin stimulates glucose uptake, increases insulin sensitivity, and regulates lipolysis and fatty acid oxidation (**Figure 2**). Diabetes-related diseases can be improved by apelin administration. Therefore, the apelin–APJ system is a potential therapeutic target in diabetes and its complications.

3.1 Apelin and Glucose Metabolism

Apelin is predominantly expressed in the beta and alpha cells of pancreatic islets, and APJ receptor is expressed in acinar cells and pancreatic ductal cells (37). Insulin is considered the prime regulator of apelin that stimulates its synthesis and release. Apelin is also affected by hypoxia and adiposity. In normal and insulin-resistant mice, apelin was noted to promote peripheral glucose uptake (38). Exogenous apelin administration was found to enhance glucose metabolism (39). Moreover, apelin-induced glucose uptake was also detected in isolated normal adipocytes and type 2 diabetic adipocytes (39, 40). These results indicate that apelin might act as an exogenous insulin sensitizer under high insulinemia.

However, in the beta cells of type 2 diabetes (T2D) db/db mice, the apelin level was detected to be upregulated. The administration of apelin-36 can inhibit glucose-stimulated insulin secretion (41). It is reported that apelin inhibits insulin secretion *via* stimulating the degradation of cAMP due to the activation of phosphodiesterase 3B activity, which subsequently results in the impairment of glucose elimination (42). Sorhede et al. found that insulin binds its receptor on adipocytes and promotes the expression of apelin. It provides negative feedback for insulin

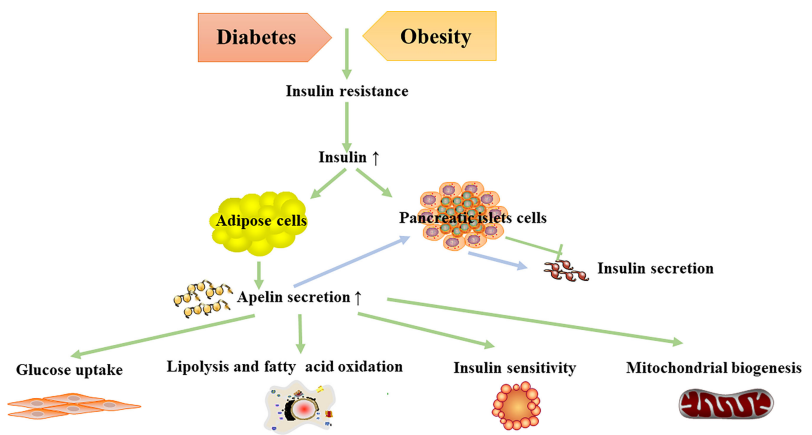

FIGURE 2 | Metabolic effects of apelin in diabetes and obesity.

secretion (41). On the contrary, Gao Z et al. reported that the administration of apelin-13 significantly reduces blood glucose and increases serum insulin level (43). Besides these, long-term apelin administration significantly improves pancreatic islet mass and insulin level in diabetes (44). These effects were related to the upregulation of PERK-IRE1a-CHOP signaling and the deactivation of AKT, ERK, and AMPK in the pancreas of diabetic mice (44, 45).

3.2 Apelin–APJ System and Diabetic Complications

Apelin has a significant role in regulating insulin secretion, oxidative stress, apoptosis, and angiogenesis; hence, it is involved in the pathogenesis of diabetic complications (**Figure 3**).

3.2.1 Cardiovascular Diseases

Both cardiomyocytes and microvascular dysfunction are critical in inducing diabetic cardiomyopathy. Apelin peptides are considered the most potent endogenous positive inotropic agent in the myocardium, and apelin-13, as the predominant apelin isoform in human myocardium, has been reported to play a crucial role in myocardial contraction, vascular relaxation, blood pressure regulation, and insulin sensitivity (46–48). Long-term apelin-13 administration can prevent pancreatic beta cell loss or dysfunction in type 2 diabetic rat models and reduce myocardial fatty acid uptake and oxidation through inhibiting the PPAR-α receptor (49). In addition, the upregulation of apelin inhibits apoptosis and oxidative stress *via* the PI3K and p38-MAPK pathways, resulting in protection from ischemia–reperfusion injury in diabetic myocardium (50).

SIRT3, a member of sirtuins family, is considered an essential transcription factor in the apelin-induced protection of diabetic cardiomyopathy. Previous studies showed that apelin increases SIRT3 expression, improves cardiac function, and ameliorates diabetic cardiomyopathy. Treatment with apelin increases the myocardial vascular density *via* upregulating SIRT3 (51) and VEGF/VEGFR2 expression (52), which also ameliorates diabetic cardiomyopathy and improves the echocardiography parameters of cardiac function. In post-myocardial infarction diabetic mice, the overexpression of apelin markedly upregulates SIRT3 and angiogenic growth factor expression (51, 53). Apelin gene therapy increases the expression of angiogenic growth factors and angiogenesis in endothelial progenitor cells, but these effects do not occur in SIRT3-knockout endothelial progenitor cells (51). Moreover, apelin treatment dramatically increases autophagy *via* upregulating SIRT3 and downregulating NF-κB-p65 expression in the myocardium of STZ-induced mice (53). Icariin has a cardioprotective effect in high-glucose-treated cardiomyocytes *via* upregulating apelin and SIRT3 expression, which can reverse diabetes-induced mitochondrial dysfunction; however, it did not affect the activity of SIRT3 in apelin silence samples (54). In addition, our previous research showed that Elabela can regulate the SIRT3-mediated inhibition of oxidative stress through Foxo3a deacetylation and prevent diabetic-induced myocardial injury (55). These results suggest that the apelin/SIRT3 signal pathway may be used as a novel therapeutic strategy for diabetes-related cardiovascular diseases.

Macrovascular injury is another important aspect of diabetes-related complications. Impairment of vascular contraction and dilatation is the hallmark of endothelial dysfunction, which is responsible for diabetic vascular pathophysiology. A previous research reported that the apelin–APJ system reduces vasodilatation and increases vasoconstriction in insulin resistance-related disorders, such as diabetes and cardiovascular dysfunction (56, 57). Zhong and his colleagues found that apelin plays a crucial role in vascular tone maintenance in diabetic mice by counteracting the vasoconstricting action of Ang II and potentiating the release of NO through the activation of the Akt-eNOS phosphorylation pathway (57). There is a high expression of apelin in the aorta of type 2 diabetes rat models (58). *In vitro*, apelin-13 can inhibit high-glucose-induced cell proliferation, migration, and invasion of aortic smooth muscle cells. Apelin-13 can also effectively attenuate high-glucose-induced calcification and dramatically suppress high-glucose-induced DNA injury through inhibiting reactive oxygen species (59).

In children with type 1 diabetes mellitus, the serum apelin level has a positive correlation with the carotid intima–media thickness, which indicates that serum apelin might be used as a predicting factor for atherosclerosis (60). However, in patients with type 2 diabetes mellitus, although the apelin level was lower in patients with diabetic complications than in patients without complications, there was no significant difference (61). In another clinical study,

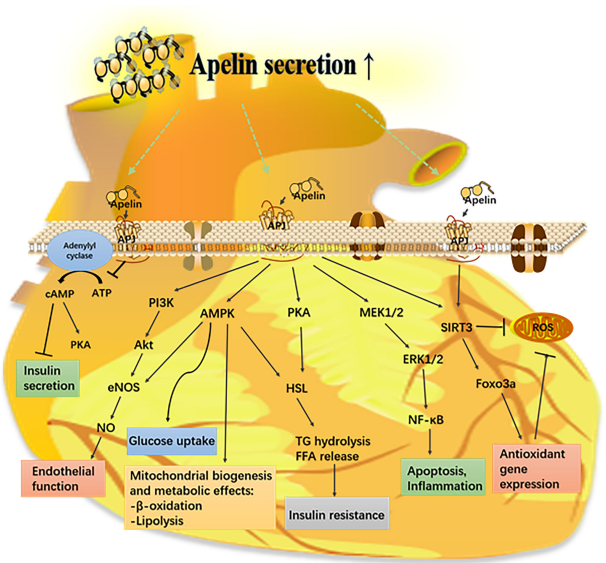

FIGURE 3 | Mechanism of the apelin–APJ system in diabetes and its complications. Apelin activates its receptor (APJ) and triggers various signaling pathways that have a protective effect on different organs from metabolic diseases. AMPK, AMP-mediated protein kinase; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular-regulated kinases 1/2; FFA, free fatty acid; Foxo3a, forkhead box protein O 3a; HSL, hormone sensitive lipase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; SIRT3, sirtuin 3.

patients with hypertension and/or type 2 diabetes have a lower serum apelin level, along with cardiac remodeling, and primarily concentric left ventricle hypertrophy; moreover, there is a negative correlation of apelin with cardiac structural parameters such as left ventricle remodeling and left atrial size (62). Another clinical research showed that physical training increases the apelin level, with a reduction of low-density lipoprotein cholesterol (LDL-C) and hsCRP levels, and insulin resistance, resulting in the decreased progression of the carotid intima–media thickness in patients with type 2 diabetes (63).

3.2.2 Diabetic Nephropathy

Diabetic nephropathy (DN) is characterized by glomerular, tubular, and tubulointerstitial injury caused by hyperglycemic status. The early stage of DN presents as glomerular hypertrophy and thickening of glomerular basement membrane. With the development of this disease, high glomerular filtration leads to proteinuria, eventually resulting in end-stage renal disease (64). APJ is expressed in the glomeruli and blood vessels of kidney, while apelin is expressed in renal vascular endothelial cells and is highly expressed in the inner stripe of the outer medulla (65). A previous study showed that serum apelin and its receptor APJ level were increased in DN patients, and a higher apelin and APJ level promoted the formation of blood vessels and induced the proliferation of glomerular capillaries, thus accelerating the development of DN (66). However, there are conflicting reports about the apelin level in diabetes. It was increased in some research (67, 68), while it was decreased in other research (69). It is speculated that a lower apelin level may be caused by apelin-regulated insulin sensitivity, which stimulates glucose utilization and enhances brown adipogenesis (70). In high-glucose-medium-cultured podocytes, APJ mRNA is upregulated when compared to the normal condition (71). It is reported that renal APJ expression is decreased in diabetic mice, but after apelin treatment, it is increased (72). Recent studies also found that apelin can control the reduction of podocyte proteasome activity *via* inducing endoplasmic reticulum stress and podocyte dysfunction through the regulation of the ERK, Akt, and mTOR-related pathways. These all contributed to the development of DN (73, 74).

Since Elabela is predominantly expressed in the kidney, the relationship between serum Elabela level and DN is also reported. Zhang et al. reported that the serum Elabela level was lower in T2D patients with albuminuria. Particularly, the serum Elabela level decreased progressively with the progression of DN; moreover, the serum Elabela level has a negative correlation with blood pressure, retinopathy, serum creatinine, and urinary albumin/creatinine ratio and has a positive correlation with the estimated glomerular filtration rate (29). A similar result was also confirmed by Erhan et al. They found that the Elabela level was higher in healthy individuals when compared with diabetic patients without microalbuminuria and even higher in diabetic patients without microalbuminuria when compared to patients with advanced albuminuria (35). These results suggest that Elabela may be an important clinical prognostic marker.

However, exogenous apelin and Elabela have been demonstrated to slow down the progression of DN. Apelin-13, the most active subtype of apelin, was paid more attention in DN. It was reported that Apelin-13 administration can reduce proteinuria, glomerular hypertrophy, mesangial expansion, and renal inflammation in type 1 and type 2 diabetic models (71, 75, 76). Hong et al. reported that apelin-13 treatment inhibits the hyperglycemia-induced elevation of inflammatory factors and histone hyperacetylation by upregulating histone deacetylase 1 (76). It was also reported that apelin-13 administration regresses DN by promoting the production of nitric oxide and alleviating renal fibrosis (73). Besides these, exogenous apelin inhibits the epithelial–mesenchymal transition of podocytes in diabetic mice *via* decreasing the expression of the immunoproteasome subunit β5i (77). Zhang Y et al. reported that the protective effect of Elabela in type 1 diabetes-induced podocyte injury might be related to the activation of the PI3K/Akt/mTOR pathway (78). However, further research is required to understand the mechanism of the apelin–APJ system in DN progression.

3.2.3 Vascular Effects, Endothelial Dysfunction, and Angiogenesis

Apelin has both vasodilation and vasoconstriction effects because the apelin–APJ system in the endothelium and smooth muscle cells exert an opposite action in regulating the vascular tone (2, 79). When apelin binds to endothelial APJ, it promotes the secretion of endothelium-derived relaxing factors, such as nitric oxide (NO) and prostacyclin, resulting in vasodilation (80–82). When apelin binds to smooth muscle APJ, it causes vasocontraction (83, 84). In a diabetic animal model, APJ expression was lower in the aorta and renal arteries, and Ang II-induced contractile responses were enhanced, but apelin administration reversed the abnormal vascular response to Ang II (57, 85).

The vascular endothelium behaves as an autocrine as well as paracrine organ in regulating vascular homeostasis. When it is impaired by hyperglycemia, vasoconstriction may occur and be accompanied with leukocyte adherence, platelet activation, oxidative stress, inflammation, thrombosis, and atherosclerosis (86). The apelin–APJ system is involved in diabetes-induced endothelial dysfunction and angiogenesis (**Figure 3**). It is reported that apelin might decrease apoptosis and the expression of adhesion molecules and increase proliferation and angiogenesis *via* APJ-activated NF-κB pathways, finally resulting in the protection of diabetes-induced endothelial dysfunction (87). In high-glucose-treated microvascular endothelial cells, apelin-12 suppressed apoptosis, inflammation, and oxidative stress *via* regulating the p-JNK and p-p38 pathways (88). Methylglyoxal, as a glycolytic metabolite, has been demonstrated to have a greater potential to stimulate endothelial dysfunction than glucose itself (89, 90). Previous studies have shown the impairment of endothelium-dependent vasorelaxation by methylglyoxal, mostly mediated by oxidative stress (90). Kim Sujin et al. demonstrated that apelin-13 can inhibit methylglyoxal-induced endothelial apoptosis and endoplasmic reticulum stress through the AMPK pathway (91). In diabetic $Lepr^{db/db}$ mice, apelin-36 restores the altered aortic vascular responsiveness to acetylcholine and Ang II by potentiating the phosphorylation of Akt and eNOS (57). In high-fat-diet-treated $Apoe^{-/-}$ mice, the loss of apelin results in

exacerbation of atherosclerosis, while apelin administration leads to a significant regression of atherosclerosis, which may be related to the activation of the nitric oxide pathway and inhibition of Ang II signaling (92).

In addition, apelin is confirmed as a potent angiogenic factor, especially in retinal endothelium. It is reported that the serum apelin-13 level has a positive correlation with proliferative diabetic retinopathy, which is unrelated with VEGF (93). Research by Yasir et al. and Wu et al. concluded similar results (94, 95). Li Yang et al. found that apelin can induce the proliferation, migration, and expression of the cytoskeleton and tight junction protein through the PI3K/Akt and MAPK/ERK pathways in human retinal pigment epithelial cells (96). Furthermore, in post-myocardial infarction diabetic mice, the overexpression of apelin mobilizes endothelial progenitor cells to ischemic regions and contributes to angiogenesis (51). Therefore, apelin may be a promising therapeutic target for diabetic angiogenesis-related diseases.

3.2.4 Central Nervous System

As mentioned above, apelin and APJ are widely expressed in the human central nervous system, more in the oligodendrocytes and neurons and less in astrocytes (97). The apelin–APJ system is also observed in the pituitary gland, indicating a role in the control of the hypothalamic–pituitary–adrenal axis (HPA). A study shows that APJ acts as a neuromodulator in modifying the HPA axis activity after acute stress stimuli (98). It was also reported that APJ-deficient mice, under hypoglycemic stress, had decreased ACTH release, confirming the role of central apelin in neuroendocrine functions. Recent studies have suggested that central apelin is involved in the transition from normal to diabetic state (99). These findings indicated that central apelin may control glucose release and glucose metabolism. Anne et al. found that the intracerebroventricular injection of apelin increases fasting blood sugar (99), which was related with the over-activation of the sympathetic nervous system, followed by liver glycogenolysis and gluconeogenesis (100). The over-expression of hypothalamic apelin was observed in obesity and diabetes (101). These results indicate that the apelin–APJ system in the central nervous system may be a new target for controlling glucose metabolism.

4 EFFECT OF THE APELIN–APJ SYSTEM ON OBESITY

Adipose tissue plays a central role in lipid and glucose metabolism (**Figure 2**) and is now considered a major endocrine organ. Apelin and its ligand are expressed in adipose tissue. Apelin is secreted by adipose tissue; thus, it is also called adipokine (102).

4.1 Apelin and Lipid Metabolism

Apelin, as an adipokine, is considered a crucial modulator of lipid metabolism. The plasma apelin level is lower in patients with elevated LDL-C when compared with the healthy controls (103). LDL-C reduction by statins is accompanied with an increase in serum apelin level in dyslipidemic patients (104). Apelin deficiency mice display increased adiposity and elevated circulating free fatty acids (105), whereas transgenic mice with over-expressed apelin is resistant to obesity (106). On the contrary, it was reported that cardiac apelin and APJ expression and serum apelin level were increased in obese rats, and downregulation of apelin and APJ expression alleviated insulin resistance and inflammation (107).

Chronic apelin treatment reduces fat mass and increases muscle oxidative capacity as well as mitochondrial biogenesis (108). Chronic apelin treatment showed a decrease of hepatic steatosis by reducing *de novo* lipogenesis in insulin-resistant mice (109). Apelin-13 can improve glucose and lipid metabolism and reduce the damage caused by oxidative stress and inflammation *via* the PI3K/Akt pathway in gestational diabetes mellitus mouse (110). Another animal experiment also showed that apelin-13 regulates the expression of PPARγto inhibit adipogenic differentiation and regulates the expression of perilipin to promote lipolysis, thereby reducing obesity (110). In a rat model of type 2 diabetes with a high-fat diet, apelin-13 reduces serum total cholesterol, triglyceride, and LDL-C and increases high-density lipoprotein cholesterol (HDL-C) (111). Moreover, apelin-13 promotes cholesterol efflux and decreases foam cell formation, which indicates its potential anti-atherogenic effect (112, 113). Besides these, Chun et al. found that apelin-13 administration abrogates angiotensin II-induced atherosclerosis in $ApoE^{-/-}$ mice through promoting NO production and inhibiting the angiotensin II intracellular pathway (92). Moreover, apelin-13 greatly ameliorates plaque stability *via* increasing the intraplaque collagen content and reducing the MMP-9 expression (114). In addition, apelin administration effectively diminishes the LDL-C/HDL-C ratio and the atherogenic index in Wistar rats with hypothyroidism (115). Hashimoto et al. found that the apelin–APJ system is the mediator of oxidative stress-linked atherosclerosis in blood vessels (116).

4.2 Apelin, Insulin Resistance, and Obesity

In obesity, adipocytes release more free fatty acids, which contribute to the development of insulin resistance. It is observed that insulin resistant individuals have a higher level of circulating free fatty acids (117). Apelin, as an adipokine, is upregulated in obesity. In clinical and experimental studies, serum apelin level or its adipose tissue expression is increased in obesity and insulin resistance status (68, 118). Patrick Yue et al. reported that apelin knockout significantly increases the serum concentration of glycerol, leptin, and free fatty acids, while exogenous apelin administration decreases these compounds (105). It is reported that supplementary apelin improves *in vitro* insulinotropic activity, glucose uptake by adipocyte, glucose elimination, and insulin release in obese mice (119). Bolus intravenous apelin administration improves glucose tolerance and insulin sensitivity during a hyperinsulinemic–euglycemic clamp in obese and insulin-resistant mice (38), which indicates that exogenous apelin is efficient despite an elevated plasma apelin level. After 28 days of apelin therapy, a marked improvement in insulin sensitivity and decrease in body fat have been observed in obese and insulin-resistant mice (108). However, administration for 4 weeks of Fc-

apelin-13 (apelin-13 fused with IgG Fc fragment) in obese mice significantly improves glucose tolerance, stroke volume, and cardiac output, while it decreases cardiac and hepatic fibrosis; but it does not affect food intake and body weight (120). When a chow diet was fed to 8-week-old apelin$^{-/-}$ mice, insulin level was significantly increased, and plasma adiponectin concentration and glucose intolerance were decreased. In addition, these mice also showed increased abdominal and epididymal fat without a difference in body weight (105). The apelin$^{-/-}$ mice were more glucose and insulin intolerant when they were fed with a high-fat diet and high-sucrose drinking water (121). Stable apelin-13 peptide analogues have shown promising short-term antidiabetic effects in mice with diet-induced obesity and diabetes (122).

Human studies revealed that Pyr1-apelin-13 injection in obese patients improves insulin sensitivity (123). However, a 6.5-year follow-up in overweight or obese children showed that the apelin level decreased significantly during pubertal development (124). In addition, Cavallo MG et al. reported that obese patients with T2D had a significantly higher apelin level than non-diabetic obese patients (125). Dayem et al. found that the apelin level has no influence on body mass index in diabetic patients (126). These results indicate that increased apelin level is directly associated with accompanying diabetes rather than obesity itself.

5 DRUGS TARGETING THE APELIN–APJ SYSTEM

The three-dimensional structure of APJ receptor is first reported by Langelaan and his colleagues (127). They presented a structure of the N-terminus and the first transmembrane segment of APJ (residues 1–55, AR55) that was comprised of residues essential for apelin binding in dodecyl phosphocholine micelles, which provided a new insight into the development of drugs. In recent years, some agonists and antagonists of APJ receptor have been discovered and synthesized, which have presented obvious therapeutic effects in animal models and patients. There are two natural endogenous ligands for APJ receptor, namely, apelin and Elabela. As mentioned above, apelin can be cleaved into different active forms such as apelin-36, apelin-17, apelin-13, apelin-12, and Pyr-apelin-13. Elabela, another APJ endogenous ligand, is also a strong agonist for APJ. In addition, there are some biosynthetic compounds targeting APJ receptor. E339-3D6 is the first nonpeptide APJ receptor agonist which was synthesized by Iturrioz et al. in 2010 (128). Then, other APJ receptor agonists were also synthesized, such as MM07, ML233, and CMF-019 (129–131). Meanwhile, several research on APJ antagonists were also conducted. Lee et al. generated an analog of apelin-13, called apelin-13 F13A, and found that it can block the hypotensive effect of apelin-13 (132). In addition, ALX40-4C is the first peptide antagonist for APJ receptor, which is a polypeptide comprised of nine arginine residues (133). ML221 is identified as the first non-peptide antagonist isoform of APJ, which blocks apelin-13-mediated cAMP production and β-arrestin recruitment for APJ (134). Further studies should focus on the beneficial effect of the compounds that target APJ and explain the therapeutic effect of novel synthetic ligands for APJ receptor in diabetes and obesity.

6 CONCLUSION

The apelin–APJ system is considered an emerging target with potential therapeutic properties in diabetes and obesity. Current literatures have suggested that apelin administration has effective protection in diabetic and/or obese mice. Current studies have shown a difference in serum apelin level between diabetic and/or obese patients and the control group, which supports the role of apelin in diabetes and obesity development and emphasizes the use of apelin as a clinical marker in diabetes and obesity. Despite numerous clinical and experimental studies clearly supporting the physiological and pathophysiological roles of the apelin–APJ system in glucose and lipid metabolism, the role of apelin in this complicated system is not yet fully elucidated, including relevant signaling pathways and biological adverse effects, especially in human. In the future, it will be important to identify the mechanism of action of apelin, such as the role of new receptors or regulating ligands or specific Elabela/apelin isoforms.

The apelin–APJ signaling system has emerged as an important biomarker and a novel therapeutic target against the development of metabolic diseases, especially diabetes and obesity. In this review, we summarized the literatures on the role of the apelin–APJ system in diabetes and obesity, and we hope for further research that will establish their role as a new diagnostic marker or therapeutic agent in diabetes and obesity.

AUTHOR CONTRIBUTIONS

CL reviewed the literature and drafted this review. HC reviewed the literature and corrected the figures. BA, SW, and NY reviewed the literature, gave critical comments, and revised the manuscript. WL gave critical comments and revised the manuscript. JS and YW reviewed the literature, gave critical comments, revised the manuscript, and took charge of project supervision and administration. All authors contributed to the article and approved the submitted version.

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Effects of Antidiabetic Drugs on Endothelial Function in Patients With Type 2 Diabetes Mellitus: A Bayesian Network Meta-Analysis

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Background: The changes of endothelial function in type 2 diabetes mellitus (T2DM) patients are closely associated with the development of cardiovascular disease (CVD). However, it is still unclear whether commonly used antidiabetic drugs can improve endothelial function. Flow-mediated dilation (FMD) is a noninvasive tool for evaluating endothelial function, which typically examines changes in the brachial artery diameter in response to ischemia using ultrasound. We performed a network meta-analysis (NMA) to explore the associations between changes in endothelial function and antidiabetic drugs by evaluating FMD in T2DM patients.

Methods: We systematically searched several electronic databases for randomized controlled trials (RCTs) published from inception until January 25, 2022 with no language restriction. The primary outcome was FMD change in all studies, and we performed subgroup analysis in T2DM patients without CVD. NMA was performed to calculate the mean differences (MDs) with 95% confidence intervals (CIs).

Results: From the 1,987 candidate articles identified in the initial search, 30 RCTs were eventually included in the analysis. In all studies, glucagon-like peptide-1 receptor (GLP-1R) agonists [MD = 3.70 (1.39–5.97)], TZD [MD = 1.96 (0.006–3.89)] produced improvement of FMD change compared to lifestyle intervention. GLP-1R agonists [MD = 3.33 (1.36–5.34) and MD = 3.30 (1.21–5.43)] showed significantly greater improvements in FMD change in pairwise comparisons with sulfonylureas and placebo. SGLT-2i also showed efficacy compared to sulfonylureas (MD = 1.89, 95% CI, 0.10, 3.75). In studies of T2DM patients without CVD, GLP-1R agonists [MD = 3.53 (1.24–5.76)], and TZD [MD = 2.30 (0.27–3.24)] produced improvements in FMD change compared to lifestyle treatment. GLP-1R agonists [MD = 3.25 (1.13–5.40), and MD = 3.85 (1.68–6.13)] showed significantly greater improvements in pairwise comparisons with sulfonylureas, and placebo.

Conclusion: In T2DM patients, both GLP-1R agonists, SGLT-2i and TZD have favorable effects to improve endothelial function in T2DM patients. In T2DM patients without CVD, GLP-1R agonists had a greater effect to improve endothelial function than sulfonylureas. These suggested that GLP-1R agonists are associated with significantly improved endothelial function in T2DM patients.

Keywords: antidiabetic drugs, endothelial function, flow-mediated dilation, type 2, diabetes, meta-analysis

INTRODUCTION

Cardiovascular diseases (CVDs) remain a leading cause of death and disability in patients with type 2 diabetes mellitus (T2DM) (1). In addition to their glucose-lowering effects, some antidiabetic drugs may improve cardiovascular outcomes. For example, the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial showed that glucagon-like peptide-1 receptor (GLP-1R) agonists significantly reduce major adverse cardiovascular events, such as cardiovascular death, nonfatal stroke, and nonfatal myocardial infarction (2). Other large clinical trials, such as the Empagliflozin Outcome Trial in Patients With Chronic Heart Failure With Reduced Ejection Fraction (EMPEROR-Reduced) (3) and the Dapaglifozin And Prevention of Adverse-outcomes in Heart Failure trial (DAPA-HF) (4), also showed that a newer class of antidiabetic drug, sodium glucose co-transporter-2 (SGLT-2) inhibitors, reduce the risk of hospitalization for heart failure or cardiovascular death. These two classes of antihyperglycemic agents are already recommended by the American Diabetes Association for the management of diabetes in patients with CVD or kidney disease (5).

However, the cardiovascular effects of different antidiabetic drugs are still controversial. Dipeptidyl peptidase-4 (DPP-4) inhibitors are another class of new oral antidiabetic drug that have been shown to have positive cardiac and vascular effects in preliminary studies. But the Cardiovascular and Renal Microvascular Outcome Study With Linagliptin (CARMELINA) trial, a placebo-controlled trial of the DPP-4 inhibitor, linagliptin, demonstrated non-inferiority and failed to prove cardiovascular benefits in T2DM patients with high cardiovascular risk over several years of observation (6). Similarly, the Acarbose Cardiovascular Evaluation (ACE) trial, which included 6,522 individuals with coronary heart disease and impaired glucose tolerance, showed that the α-glucosidase inhibitor acarbose was neutral with regard to major adverse cardiovascular events (7). Furthermore, a meta-analysis that included 1,325,446 diabetes patients suggested that another class of widely used insulin sensitizing drug, sulfonylureas, was associated with a significantly increased risk for cardiovascular death compared to other oral drugs for diabetes (8). In view of the inconsistent cardiovascular effects of antidiabetic drugs, we speculated that the effects of common antidiabetic drugs on vascular endothelial function in T2DM patients remained differences.

Endothelial dysfunction is closely associated with the development of CVDs involving inflammatory reactions and atherosclerotic progression in T2DM patients (9). The endothelium is highly responsive to various hemodynamic stimuli, namely, shear stress, circumferential stain, and wall strain (10). Endothelial function can be assessed non-invasively using the flow-mediated dilation (FMD) technique. FMD represents an endothelium-dependent, noninvasive tool for evaluating endothelial function, which typically examines changes in the brachial artery diameter in response to ischemia using ultrasound. A higher FMD reflects a better state of vascular elasticity (11). Several studies have demonstrated the prognostic value of brachial artery FMD for cardiovascular events: In the Multi-Ethnic Study of Atherosclerosis (MESA) trial, FMD was an independent predictor of cardiovascular events, and this inverse association remained significant after adjusting for multiple CVD risk factors (12). The Flow-Mediated Dilation Japan (FMD-J) study, a multicenter study that included 462 individuals from 22 university hospitals and affiliated clinics in Japan, also suggested that the decrease of FMD is closely associated with coronary events in patients with coronary artery disease after 3-year follow-up (13). The expert consensus statement of the European Society of Cardiology recommended FMD for examining the pathophysiology of CVD and possibly identifying subjects at risk for future cardiovascular events (14). However, it remains unclear how antidiabetic drugs affect FMD, as different studies have yielded conflicting results.

Several systematic reviews and meta-analyses have reported the impacts of specific classes of antidiabetic drugs on vascular function (15–17). However, there is still a lack of comprehensive evidence regarding the effects of traditional and newly developed antidiabetic drugs on endothelial function in T2DM patients based on FMD assessment. The utility of traditional pairwise meta-analysis is limited because it cannot evaluate the effects of interventions in head-to-head trials. The network meta-analysis (NMA) has overcome this limitation as it allows the comparison of the effects of two or more interventions through direct and indirect evidence (18). Therefore, we implemented NMA of randomized controlled trials (RCTs) to explore the effects of antidiabetic drugs on endothelial function in T2DM patients by FMD and to summarize the performance of these different drug treatments.

METHODS

Data Sources and Searches

Our study adhered to the Network Meta-analysis of the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA-NMA) and the Cochrane Handbook for Systematic Reviews of Interventions. Two reviewers (JW, HL) initially

screened titles and abstracts independently and the full texts of studies were perused to examine the suitability of potentially eligible articles. Any disagreements were resolved by two reviewers through discussion and an experienced professor was invited to judge the final set of standards if necessary. As all analyses were based on previous published studies, no ethical approval or patient consent was required. We searched the PubMed, the Embase, the Cochrane Central Register of Controlled Trials, the ClinicalTrials, the Wan Fang Database, the China National Knowledge Infrastructure Database, the Chinese Biomedical Literature Database, and the Chinese Scientific Journal Database from inception to January 25, 2022 for RCTs. The following Medical Subject Headings (MESH) terms and free text terms combined with Boolean operators were used in the search strategy with no language restriction: "metformin," "sulphonylureas," "glinides," "Thiazolidinedione", "α-glucosidase inhibitors," "dipeptidyl-peptidase IV inhibitors," "GLP-1RAs," "SGLT-2 inhibitors," "flow-mediated dilation," "FMD", "endothelial function," "endothelium", "endothelial dysfunction", "Hypoglycemic Agents" "type 2 diabetes," and "randomized controlled trials." In addition, we conducted a recursive manual search to retrieve full texts of studies from the bibliographies of relevant reports or similar systematic reviews to check for potentially eligible studies that may have been missed in the initial screen. The details of the search strategy are presented in the **Supplementary Material**. All citations were managed using Endnote X9 software (Thompson ISI Research Soft, Philadelphia, PA).

Study Selection

We included eligible RCTs based on the PICOS criteria, summarized below. Population: T2DM patients were diagnosed using appropriate clinical criteria, such as the American Diabetes Association Guidelines or the WHO-1999 criteria. The patients included in the studies were not limited to those with or without CVD. Intervention: Eight drug classes were included: metformin, sulfonylureas, glinides, thiazolidinedione, α-glucosidase inhibitors, DPP-4 inhibitors, GLP-1R agonists, and SGLT-2 inhibitors. Studies discussing agents that have been withdrawn, such as rosiglitazone, or those that are no longer available in clinical practice were excluded. Comparison: Interventions with eight classes of drug were compared to each other or with placebo and lifestyle intervention. Several studies designed control treatment as diet and (or) exercise without using drugs, we defined them as one class of intervention, lifestyle treatment to analysis (**Table 1**). Insulin treatment differs in type and dosage in T2DM patients; therefore, insulin intervention studies were also excluded (**Figure 1**). Outcome: The outcome was endothelial function assessed by FMD change from baseline to post-treatment with percentage (ΔFMD%) as standard of measurement in all studies. Study design: We confined our analysis to RCTs published without year and language restriction. Other studies, such as single-arm studies, were excluded.

Data Extraction and Quality Assessment

Two authors (YW, JW) independently extracted data to collect the relevant data from the included studies by following the Cochrane Consumers and Communication Review Group's data extraction template, namely, the name of the first author, the publication year, baseline characteristics (intervention, sample size, baseline age, baseline BMI, and baseline HbA1c level) and quality of the RCT. We extracted the mean, standard deviation (mean ± SD) and number of patients of experimental group and control group at baseline and at the last observation to calculate the change of FMD from baseline to post-treatment in each comparison. For effect sizes of FMD change, we present mean differences and 95% confidence intervals. The risk of bias was evaluated using the Cochrane Risk of Bias tool (**Supplementary Figure 1**). Any discrepancies of data extraction or quality assessment were resolved through discussion with the third author (HL).

Data Synthesis and Analysis

We first implemented a conventional pairwise meta-analysis to analyze the direct evidence from the included studies. Heterogeneity of treatment effects across trials was assessed by I^2 statistics. When the P-value was ≥0.1, and I^2 was ≤50%, it suggested that there was mild statistical heterogeneity. When the P-value was <0.1, and I^2 was >50%, we explored sources of heterogeneity by using subgroup analysis (**Supplementary Figure 5**). Comparison-adjusted funnel plots were drawn to determine the presence of publication bias. A network plot of each treatment was produced as a summary description to provide all of the available evidence for each treatment (**Figure 2**).

As the presence of effect sizes refers to continuous outcome, FMD change from baseline to post-treatment was calculated for each comparison. The 95% confidence interval (CI) and pooled mean difference (MDs) were calculated as measures of estimated uncertainty and pooled effect size, respectively. If the study for which SD of changes from baseline is not available, we use the formula "SD change = $[\text{baselineSD}^2 + \text{finalSD}^2 - (2 * \text{Corr} * \text{baselineSD} * \text{final SD})]^{1/2}$" to calculate SD change from baseline for experimental intervention and comparator intervention (19). If the mean and SD data to extract were presented in another form by calculating other available values, such as range or interquartile ranges (IQRs) to clarify SD, we use median to be a substitute for mean (19), Range/6 to estimate SD (20), and for estimating SD from IQR, we use formula SD ≈ (Q3−Q1)/1.35 (21).

To ensure sufficient similarity of various treatment comparisons and thus provide valid indirect inferences, two authors (JW, YW) independently evaluated the transitivity assumption by comparing and examining the clinical and methodological characteristics, such as the characteristics of participants, experimental design, and measurement of time frame, to ensure the clinical and methodological characteristics as comparable as possible for each included study before statistical analyses (22). If the study clinical characteristics is uncertain to meet the include criterion, consult three clinical experts to reach a consensus.

Based on the maximum likelihood and Bayesian estimation, the Markov chain Monte Carlo (MCMC) method with prior non-informative distributions was used in our NMA. We used the random-effect model with vague priors for multi-arm trials.

TABLE 1 | Baseline characteristics of all studies.

First Author, Year	Treatment		Sample size (EG/CG)	Age (years) (EG/CG)	Male (%)	Baseline HbA1c (%)	BMI (kg/m^2)	Treatment duration	Country	FMD (EG/CG Baseline, %)	ΔFMD (EG/CG,pre and post-treatment,%)	FMD measure timeframe	Study type
	Experience group	Control group		(mean±SD)		(mean±SD)	(mean±SD)	(week)		(mean±SD)	(mean±SD)	(First, second time)	(ITT/PP)
Li,2019 (50)	GLP-1 RA (liraglutide 1.2mg qd)	Metformin (0.75g bid)	50/50	67±2/67±2	0.6	NR	NR	12	China	3.2±1.0/3.2±1.6	2.0±1.0/-0.2±1.4	0,12 weeks	ITT
Shi,2020 (51)	SGLT-2 inhibitor (dapagliflozin 5mg qd)	Metformin (1-1.5g qd)	97/97	54±10/55 ±11	0.5	8.8±1.3/8.6±1.2	25.2±3.5/25.1±3.4	12	China	4.5±1.1/4.47±1.0	1.5±1.1/0.8±1.1	0,12 weeks	PP
Asnani,2006 (26)	TZD (pioglitazone 30mg bid)	Placebo	8/8	59±6/57±5	NR	10.0±2.3/8.7±2.3	NR	16	USA	10.1±4/8.3±7.1	4.5±4.0/-0.9±6.2	0,16 weeks	PP
Zhang,2002 (52)	Metformin (0.75g qd)	Placebo	27/28	58±7/57±6	0.6	NR	NR	24	China	4.6±2.5/3.4±2.9	4.5±2.5/0.6±3.4	0,12 weeks	PP
Deng,2015 (53)	DPP-4 inhibitor (vildagliptin 50 mg)	Metformin (0.5g)	32/33	NR	NR	8.5±0.5/8.8±0.6	NR	24	China	4.2±1.3/4.2±1.8	1.7±1.3/0.9±1.7	0,24 weeks	PP
Tang,2017 (54)	DPP-4 inhibitor (sitagliptin 100mg bid)	Glinides (repaglinide 1mg tid)	45/45	49±8/49±9	0.5	7.7±0.7/7.8±1.1	26.0±3.4/25.0±2.4	12	China	6.4±1.6/6.6±1.8	1.5±1.7/0.7±1.7	0,12 weeks morning	PP
Han,2014 (55)	DPP-4 inhibitor (saxagliptin 5mg qd)	Lifestyle treatment (diet and exercise)	40/40	47±8/46±10	0.6	8.8±1.0/8.9±0.8	22.6±2.2/22.5±2.2	12	China	4.8±0.8/4.6±0.9	5.2±1.0/1.9±1.1	0,12 weeks	ITT
Lambadiari,2018 (27)	GLP-1 RA (liraglutide 1.8mg qd)	Metformin (1g bid)	30/30	51±10/50 ±12	0.7	8.6±2.0/8.4±1.2	32.9±5.0/27.7±2.0	24	Greece	8.9±3.0/8.8±5.0	4.3±5.2/3.0±5.6	0,24 weeks	ITT
Suzuki,2012 (28)	DPP-4i (sitagliptin 50mg qd)	Lifestyle treatment (diet)	12/12	65±10/70±7	0.5	7.9±1.2/7.9±1.1	23.9± 2.4/23.0±4.2	12	Japan	3.7±2.3/3.4±1.9	1.7±2.2/0.06±2.1	0,12 weeks	PP
Suzuki,2014 (29)	GLP-1 RA inhibitor (liraglutide 0.9mg qd)	DPP-4i (sitagliptin 50mg qd)	24/16	59±16/56 ±15	0.6	9.8±2.2/9.1±1.6	28.2±7.2/26.3±7.2	24	Japan	6.4±1.6/6.4±1.6	2.1±1.8/0.2±1.4	0,24 weeks	PP
Tamura,2018 (30)	SGLT-2 inhibitor (empagliflozin 10mg qd)	Sulfonylureas (glimepiride 0.5mg qd)	30/28	59±9/54±12	0.6	6.9±1.1/6.6±0.7	26.1±3.7/25.9±5.4	12	Japan	5.5±2.1/5.5±2.2	-0.19±2.3/-0.37±2.8	0,12 weeks	PP
Sposito,2021 (31)	SGLT-2i (dapagliflozin 10mg qd)	Sulfonylureas (glibenclamide 5mg qd)	48/49	57±7/58±7	0.6	7.9±0.9/7.9±0.9	31.0±4.0/30.0±5.0	12	Brazil	1.6±3.9/1.2±3.0	3.3±6.1/-1.2±5.6	0,12 weeks	ITT
Kelly,2007 (32)	TZD (rosiglitazone 4mg bid)	Glyburide (10mg bid)	20/16	57±8/63±8	NR	7.8±1.1/7.3±0.7	NR	24	USA	10.1±4.0/8.3±7.1	2.0±3.8/-1±3.7	0,24 weeks	ITT
Zainordi,2020 (33)	SGLT-2i (dapagliflozin)	Placebo	36/36	57±8/58±7	0.8	9.7±1.9/9.3±1.6	27.5±4.1/29.9±4.2	12	Japan	11.2±8.3/11.5±5.8	0.2±10.4/-1.4±7.8	0,12 weeks	PP
Tsuchiya,2009 (34)	TZD (pioglitazone 18mg qd)	Lifestyle treatment (exercise)	20/21	58±10/60 ±11	0.5	9.0±1.1/7.3±1.0	26.2±4.3/27.3±4.3	12	Japan	5.5±1.6/5.5±2.2	2.0±2.0/0.9±2.4	0,12 weeks	PP
Papathanassiou,2009 (35)	TZD (Pioglitazone 30mg qd)	Sulfonylureas (glimepiride 4 mg qd)	14/14	63±7/64±7	0.2	7.7±0.7/7.4±0.8	33.9±7.0/31.9±5.5	24	Greece	2.2±2.4/2.3±1.8	2.0±2.6/0.1±1.7	0,24-25 weeks	ITT
Naka,2015 (36)	Metformin (0.85g bid)	Pioglitazone (30mg qd)	16/15	63±8/63±10	0.3	8.1±1.3/7.8±0.9	30.4±5.5/31.9±4.2	24	Greece	2.2±1.1/2.2±1.3	0.7±1.5/1.7±1.7	0,24 weeks	PP
Shigiyama,2016 (37)	DPP-4 inhibitor (linagliptin 5mg qd)	Metformin (1.5g qd)	26/29	60±9/60±12	0.6	6.9±0.6/6.9±0.7	25.3±4.4/26.2±4.0	16	Japan	4.9±2.7/5.3±2.4	1.3±3.5/1.3±3.3	0,16 weeks	ITT
Tripolt,2018 (38)	DPP-4 inhibitor (linagliptin 5mg qd)	Placebo	20/23	63±8	NR	6.8±2.7/6.8±3.0	NR	12	Japan	3.5±3.1/4.0±2.9	0.4±4.8/-0.5±3.0	0,12 weeks	ITT
Li,2016 (39)	DPP-4 inhibitors (saxagliptin 5mg qd)	Metformin (1.5g qd)	14/13	54±11/54 ±12	0.6	8.4±1.6/8.6±1.7	26.9±3.5/26.7±3.2	12	China	9.3±4.7/9.2±9.0	5.0±4.5/6.0±7.8	0,12 weeks 7– 9AM	PP
Kitao,2017 (40)	DPP-4 inhibitor (vildagliptin 100mg qd)	Metformin (1-1.5g qd)	48/48	62±11/60 ±14	0.6	7.3±0.5/7.2±0.6	25.7±4.1/26.1±4.7	12	Japan	5.5±2.0/6.1±3.0	-0.5±0.3/-0.58±0.3	0,12 weeks morning	ITT
Kim,2017 (41)	DPP-4 inhibitor (vildagliptin 50mg bid)	Sulfonylureas (glimepiride 2mg qd)	17/17	56±8/56±6	0.6	7.6±0.7/7.5±0.5	26.6±2.6/25.2±3.9	12	Korea	9.4±5.0/10.1±5.7	-1.4±4.7/-1.37±5.7	0,12 weeks	PP
Nomoto,2016 (42)	DPP-4 inhibitor (sitagliptin 50–100mg qd)	Sulfonylureas (0.5–2.0 mg qd)	48/55	62±11/60±6	0.6	7.4±0.4/7.4±0.3	25.7±3.9/25.2±3.5	26	Japan	5.6±2.8/5.6±2.2	0.002±2.0/0.43±2.0	0,26 weeks morning	PP
Maruhashi,2016 (43)	DPP-4 inhibitor (sitagliptin)	Lifestyle treatment (diet or exercise)	17/18	69±7/64±10	0.6	NR	26.8±3.3/27.2±5.0	96	Japan	4.3±2.6/4.3±2.4	0.1±2.5/0.8±2.3	0.96 weeks	PP
Nakamura,2014 (44)	DPP-4 inhibitor (sitagliptin 50–100mg qd)	α-Glucosidase inhibitor (voglibose 0.6mg qd)	24/31	67±12/68±9	0.5	NR	27.8±3.5/25.7±4.3	12	Japan	5.4±2.3/5.0±2.2	0.8±2.4/1.0±2.4	0,12 weeks	PP
Sawada,2014 (45)	Glinides (nateglinide 270mg qd)	α-Glucosidase inhibitors (miglitol 150mg qd)	46/47	69±10/70±9	0.8	7.0±0.4/6.9±0.5	24.1±4.0/25.0±3.7	16	Japan	3.0±1.7/3.3±1.3	0.29±1.8/2.0±1.8	0,16 weeks morning	PP
Irace,2015 (46)	SGLT-2 inhibitor (exenatide 5μg bid)	Sulfonylureas (4mg qd)	10/10	59±9/57±5	0.7	8.9±1.2/8.2±1.2	30.8±1.9/33.2±3.6	16	Italy	1.6±2.9/2.6±1.8	7.5±3.3/3.0±1.6	0,16 weeks	PP
Baltzis,2016 (47)	DPP-4 inhibitor (linagliptin 5mg qd)	Placebo	19/21	61±6/57±7	0.6	NR	32.4±4.9/36.1±9.2	12	USA	6.5±2.1/7.1±1.2	0.7±1.6/0.4±1.2	0,12 weeks	PP
Caballero,2003 (48)	TZD (troglitazone 200 mg tid)	Placebo	10/12	56±9/56±10	NR	NR	NR	12	USA	5.3±2/5.1±2.4	0.3±1.8/1.0±2.5	0, 8-12 weeks	PP
Widlansky,2017 (49)	DPP-4 inhibitor (sitagliptin 100 mg qd)	Placebo	16/14	63±9/62±10	0.3	6.9±0.8/6.8±0.2	32.6±6.3/32.1±6.6	8	USA	5.6±2.3/5.2±1.8	0.7±2.0/0.4±2.1	0,8 weeks	PP

EG, Experience Group. CG,Control Group.CVD, cardiovascular disease.GLP-1 RA, glucagon-like peptide-1 receptor agonist.SGLT-2i, sodium-glucose co-transporter 2 inhibitor. DPP-4 inhibitor,dipeptidyl peptidase-4 inhibitor. TZD, Thiazolidinedione. Lifestyle treatment,diet and/or exercise. NR,not report. ITT, Intention-to-treat analysis.PP, Per-protocol analysis. ΔFMD, FMD change from baseline to post-treatment.
Data are expressed as the mean±SD values.

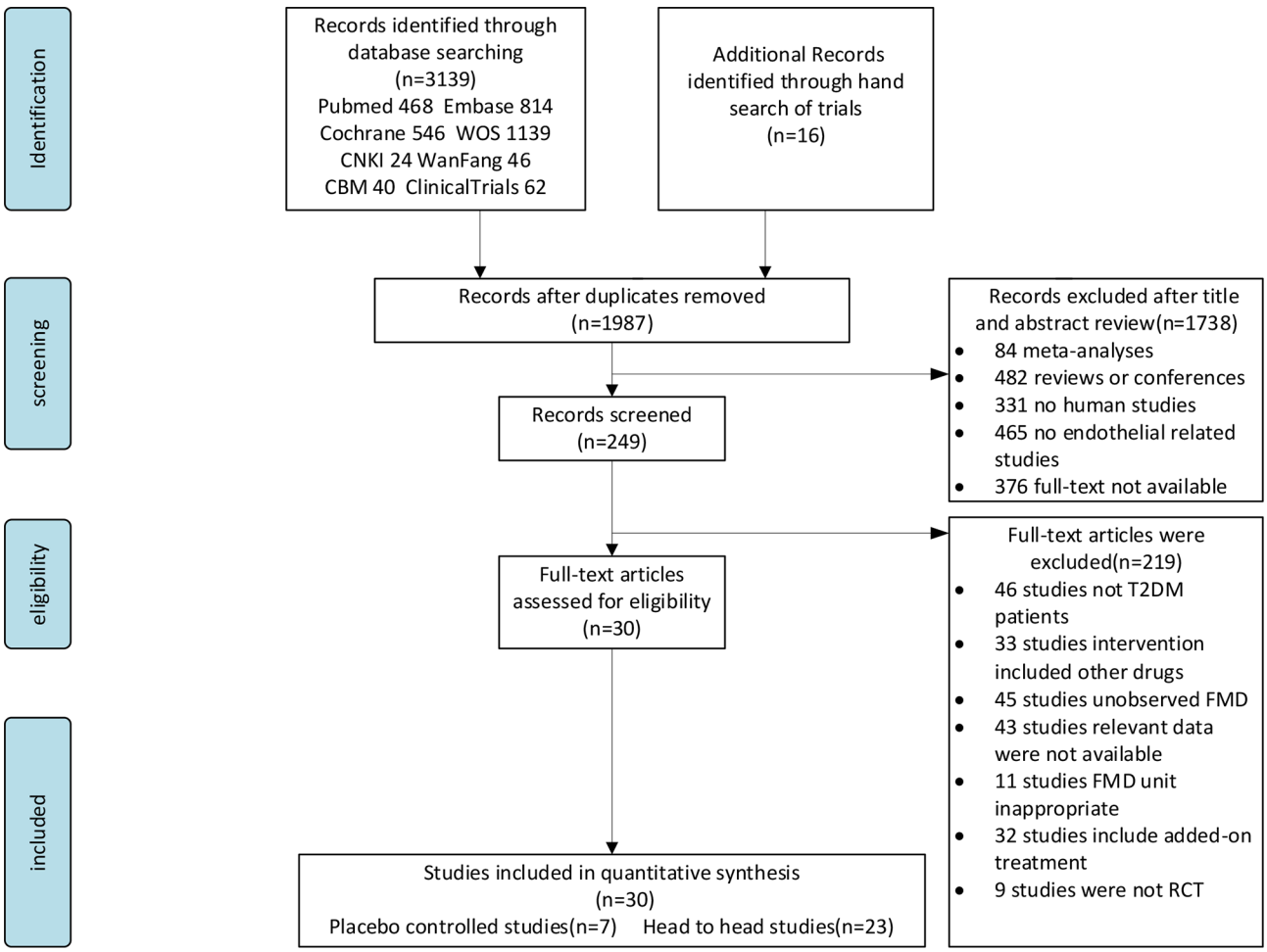

FIGURE 1 | Study selection flow chart.

Normal prior distributions with a mean of zero and a variance of 10^{-4} were used for all trial baselines and treatment effects, and uniform prior distributions with a mean of zero and a variance of 5 were used to calculate between-trial standard deviation. The estimation of posterior distribution is *via* Markov chain Monte Carlo method by using Gibbs Sampling. The model convergence was assessed by trace plots and Brooks–Gelman–Rubin plots. Three parallel Markov chains were used to check for convergence by starting analysis from different initial states, through stimulation to obtain the target distributions (23). A burn-in of 20,000 iterations was conducted to ensure the three chains had converged and the subsequent 80,000 iterations were sampled for the analysis.

The surface under the cumulative ranking curve (SUCRA) was presented as a simple numerical statistic cumulative ranking probability plot summarized to evaluate the change of FMD in each intervention. A higher SUCRA value (up to 1) indicates greater likelihood of the treatment being in the top rank or being highly effective, while lower values (down to zero) indicate that the treatment is worse (24, 25). The global inconsistency was used to explore the consistency of direct and indirect evidence in the network (**Supplementary Figure 3**), P <0.05 indicated the probability of inconsistency. All analyses were conducted in STATA, version 14.0 (Stata, Corp, College Station, TX, USA) and OpenBUGS, Version 3.2.3 (MRC Biostatistics Unit, Cambridge, UK).

RESULTS

Study Selection

Figure 1 shows the literature selection procedure. The initial search initially identified 1,987 candidate articles including sixteen additional articles that were found from relevant systematic reviews or meta-analyses by hand searching. A total of 1,168 were discarded due to duplication, 1,738 were removed after checking the title and abstract, and 219 were excluded at full-text stage according to our exclude criterion. Thirty articles were included after checking the full text. All records were discarded for various reasons as shown in **Figure 1**.

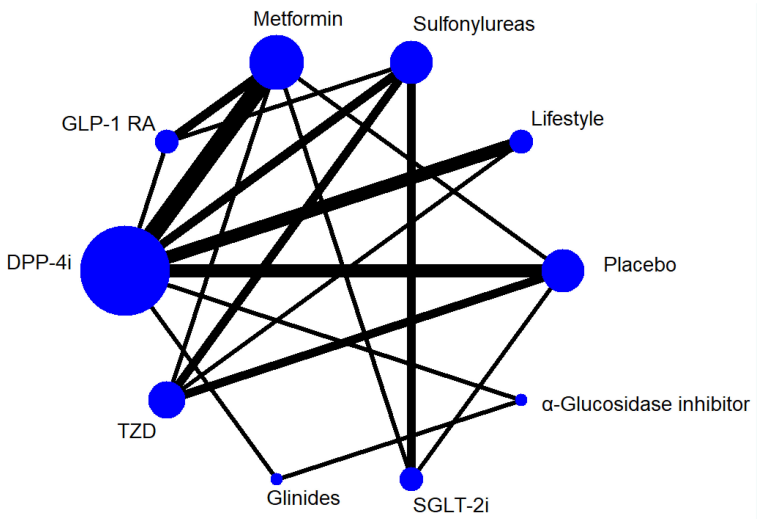

FIGURE 2 | Network Plot for all studies. GLP-1 RA, glucagon-like peptide-1 receptor agonist. SGLT-2i, sodium-glucose co-transporter 2 inhibitor. DPP-4i, dipeptidyl peptidase-4 inhibitor. TZD, Thiazolidinedione. Lifestyle,exercise and/or diet.

The 30 studies finally included in the analysis included 10 interventions; 24 studies were written in English (26–49) and six were written in Chinese (50–55). The studies were divided into the CVD group and non-CVD group according to CVD status of the participants. The CVD group consisted of five studies (31, 32, 37, 42, 45) in which participants were all diagnosed with CVD (hypertension, coronary atherosclerosis, or carotid atherosclerotic disease). The non-CVD group consisted of 25 studies (27–30, 33–36, 38–41, 43, 44, 46–49), none of whom were diagnosed with CVD. The participant characteristics and interventions in the studies are summarized in **Table 1**.

ROB Quality Assessment

We evaluated the quality of the included studies based on the Cochrane Collaboration tool for assessing the risk of bias (ROB). Ten studies were designed as open-label (28, 30, 31, 37, 39–43) or non-blind (29). Two studies (31, 42) were judged to have high risk of selective reporting bias because their data were extracted by transforming the original data manually. The details of ROB quality assessment for each RCT are shown in **Supplementary Figure 1**.

Primary Outcome

The primary outcome was FMD of all treatments in all 30 trials. **Figure 2** shows the network plot of interventions for all 30 trials. In terms of the outcome of FMD, two classes of drug showed significant benefits with regard to improving FMD in all T2DM patients compared to lifestyle treatment: GLP-1R agonists (MD = 3.70, 95% CI, 1.39, 5.97), TZD (MD = 1.96, 95% CI, 0.006, 3.89). In addition, GLP-1R agonists showed efficacy compared to sulfonylureas (MD = 3.33, 95% CI, 1.36, 5.34) and placebo (MD = 3.30, 95% CI, 1.21, 5.43), and SGLT-2i also showed efficacy compared to sulfonylureas (MD = 1.89, 95% CI, 0.10, 3.75) (**Table 2**).

The SUCRA curves showed the detailed ranking of each treatment (**Supplementary Figure 2**). Based on the SUCRA values, GLP-1R agonists (SUCRA 96.8%) showed the leading effect with regard to improvement of FMD, followed by SGLT-2 inhibitors (SUCRA 74.9%), α-glucosidase inhibitors (SUCRA 62.1%), metformin (SUCRA 60.6%) and thiazolidinedione (SUCRA 69.1%). The DPP-4 inhibitors (SUCRA 51.0%) are better than glinides (SUCRA 23.9%). The efficacy of sulfonylureas (SUCRA 22.7%) with regard to FMD improvement was lower than any other antidiabetic drug and nearly equivalent to placebo (SUCRA 23.3%). Lifestyle was the least effective treatment with SUCRA 15.1% (**Supplementary Table 1**).

Subgroup Outcomes

The non-CVD group included 25 studies, GLP-1R agonists (MD = 3.53, 95% CI, 1.24,5.76), and TZD (MD = 2.30, 95% CI, 0.27,3.24) produced improvements in FMD change compared to lifestyle intervention. GLP-1R agonists (MD = 3.25, 95% CI, 1.13, 5.40), and MD = 3.85, 95% CI, 1.68, 6.13) showed significantly greater improvements in FMD in pairwise comparisons with sulfonylureas, and placebo. Metformin also showed efficacy compared to placebo (MD = 1.71, 95% CI, 0.009, 3.55), and thiazolidinedione showed significantly greater improvements in FMD in pairwise comparisons with sulfonylureas (MD = 2.02, 95% CI, 0.18, 3.93) (**Table 2**).

The SUCRA value of non-CVD studies indicates that GLP-1R agonists (SUCRA 96.4%) had the highest probability of FMD improvement compared to seven other classes of antidiabetic drugs and lifestyle intervention. Thiazolidinedione (SUCRA 79.8%) also ranked highly among the 10 interventions, followed by metformin, α-glucosidase inhibitors, and DPP-4i with SUCRA 58.7, 57.4, and 54.6%. SGLT-2 inhibitors showed almost equal efficacy with these three drugs, with SUCRA 56.3%

TABLE 2 | Network meta-analysis results for ΔFMD in all studies (30 trials, left lower half) and non-CVD studies (25 trials, right upper half).

GLP-1 RA	-2.20 (-4.65 to 0.20)	-2.07 (-5.54 to 1.33)	-1.23 (-3.41 to 1.04)	2.14 (0.47 to 3.78)	-2.30 (-5.38 to 0.82)	-3.13 (-6.44 to 0.11)	**3.25 (1.13 to 5.40)**	**3.85 (1.68 to 6.13)**	**3.53 (1.24 to 5.76)**
-1.44 (-3.76 to 0.90)	**SGLT-2i**	0.11 (-3.56 to 3.78)	-0.96 (-3.45 to -0.94)	0.21 (-3.53 to 3.95)	0.20 (-4.07 to -0.57)	0.93 (-2.59 to 4.50)	1.06 (-1.15 to 3.18)	1.65 (-2.60 to 5.97)	1.33 (-1.31 to 3.84)
-1.93 (-4.92 to 0.98)	-0.50 (-3.61 to 2.55)	**α-Glucosidase inhibitor**	-0.96 (-3.45 to -0.94)	-0.05 (-2.11 to 1.92)	0.22 (-2.74 to 3.16)	1.05 (-2.98 to 5.17)	1.18 (-2.41 to 4.71)	1.53 (-0.70 to 4.09)	1.45 (-1.95 to 4.73)
-1.74 (-3.89 to 0.45)	0.30 (-1.90 to 2.48)	-0.20 (-3.11 to 2.62)	**TZD**	0.91 (-0.88 to 2.77)	1.07 (-5.32 to 1.34)	-1.90 (-5.23 to 3.23)	**2.02 (0.18 to 3.93)**	2.62 (0.46 to 4.97)	**2.30 (0.27 to 3.24)**
2.06 (0.36 to 3.74)	0.62 (-1.28 to 2.52)	0.13 (-2.58 to 2.76)	0.32 (-1.37 to 2.03)	**Metformin**	-0.16 (-1.45 to 1.05)	-0.98 (-4.07 to 2.00)	1.11 (-0.80 to 3.06)	**1.71 (0.009 to 3.55)**	1.39 (-0.52 to 2.73)
-2.38 (-4.16 to -0.65)	0.94 (-1.01 to 2.97)	0.45 (-1.94 to 2.80)	0.66 (-0.98 to 2.34)	-0.32 (-1.58 to 0.87)	**DPP-4i**	-0.82 (-3.61 to 1.91)	0.95 (-1.03 to 2.89)	1.55 (-0.05 to 3.21)	1.23 (-0.40 to 5.59)
-3.41 (-3.40 to -0.54)	1.98 (-1.04 to 5.05)	1.48 (-0.84 to 3.79)	-1.67 (-4.56 to 1.12)	-1.35 (-4.01 to 1.22)	-1.03 (-3.35 to 1.28)	**Glinides**	0.13 (-3.31 to 3.46)	0.72 (-2.46 to 3.97)	0.39 (-2.88 to 3.51)
3.33 (1.36 to 5.34)	**1.89 (0.10 to 3.75)**	1.40 (-1.41 to 4.25)	1.59 (-0.09 to 3.37)	1.27 (-0.37 to 2.98)	0.95 (-0.61 to 2.53)	-0.09 (-2.87 to 2.72)	**Sulfonylureas**	0.59 (-1.68 to 2.98)	0.27 (-2.05 to 2.54)
3.30 (1.21 to 5.43)	2.26 (-0.17 to 4.65)	1.76 (-1.12 to 4.56)	1.57 (-0.20 to 3.43)	1.64 (-0.28 to 3.53)	0.92 (-0.51 to 2.36)	-0.11 (-2.82 to 2.62)	0.07 (-2.49 to 2.60)	**Placebo**	0.32 (-1.80 to 2.61)
3.70 (1.39 to 5.97)	1.86 (-0.24 to 4.07)	1.37 (-1.38 to 4.14)	**1.96 (0.006 to 3.89)**	1.25 (-0.34 to 2.89)	1.32 (-0.30 to 2.83)	0.28 (-2.56 to 4.25)	0.68 (-1.66 to 3.02)	-0.39 (-2.37 to 1.69)	**Lifestyle**

Non-CVD studies

Significant comparisons

Treatments results are reported in league table.Significant pairwise comparisons of ΔFMD(FMD change from baseline to post-treatment) are highlighted in dark grey boxes and underlined. Treatments estimates are MDs (mean, val2.5pc to val97.5pc) of the column-defining treatment compared with the row-defining treatment for ΔFMD.Mean differences (MDs) more than 0 favor the column-defining treatment, MDs lower than 0 favor the row-defining treatment. GLP-1 RA, glucagon-like peptide-1 receptor agonist. SGLT-2i, sodium-glucose co-transporter 2 inhibitor. DPP-4i,dipeptidyl peptidase-4 inhibitor. TZD, Thiazolidinedione.

in non-CVD participants. Glinides and sulfonylureas (SUCRA 34.1 and 27.4%) showed the lowest efficacy among all included antidiabetic drugs (**Supplementary Table 1**).

Bias and Assessment of Inconsistency

No significant inconsistencies were identified as determined using the global inconsistency between direct and indirect estimates compared to all studies (P = 0.894, **Supplementary Figure 3**). Several factors may contribute to this inconsistency, for example, the sample size, study area, and study design.

The publication bias was investigated by visual examination of the funnel plot and several scatter plots were not symmetrical in the inverted funnel (**Supplementary Figure 4**).

DISCUSSION

A total of 30 RCTs were included in the NMA. Both GLP-1R agonists, SGLT-2i, and thiazolidinedione (TZD) significantly improved FMD change from baseline to post-treatment in specific comparisons, suggesting that these three drugs may have positive effects on endothelial function of T2DM patients. Furthermore, GLP-1R agonists had a greater effect than sulfonylureas in all T2DM patients. In non-CVD T2DM patients, GLP-1R agonists also had a favorable effect than sulfonylureas, placebo and lifestyle treatment on improvement of FMD change. These may suggest that GLP-1R agonists are the most beneficial drugs for improving endothelial function.

Endothelial dysfunction is characterized by increased vascular tone and increased production of procoagulant and proinflammatory factors, which are associated with progression of atherosclerosis (56). Patients with T2DM are more susceptible to endothelial function impairment due to insulin resistance, accumulation of advanced glycation end products, and a vascular inflammatory state (10), resulting in a significantly higher risk for CVD. Therefore, the improvement of endothelial function is important for T2DM patients. FMD is a potential indicator of vascular endothelial function, which is also a predictor of long-term cardiovascular events (14). Previous studies have explored the effects of several newer antidiabetic drugs on endothelial function, but the conclusions have been inconsistent. Therefore, we performed a systematic review to comprehensively explore the effects of different antidiabetic drugs on endothelial function by evaluating FMD in T2DM patients.

The Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) (2) and the Trial to Evaluate Cardiovascular and Other Long-term Outcomes with Semaglutide in Subjects with Type 2 Diabetes (SUSTAIN-6) (57) showed a reduced risk for major adverse cardiovascular events (MACE), supporting the possible beneficial cardiovascular effects of GLP-1R agonist-based treatments. With regard to endothelial function, GLP-1R agonists have been shown to significantly reduce arterial stiffness based on assessment of pulse wave velocity (16). Similar to these active outcomes of vascular protection, in our meta-analysis, GLP-1R agonists were the highest-ranking antidiabetic drugs with regard to improvement of FMD. This drug also significantly improved FMD in the non-CVD subgroup compared with metformin and sulfonylureas. This is the first study to indicate that GLP-1R agonists may be effective for improving vascular endothelial function in T2DM patients. The effects of GLP-1R agonists on vascular endothelium could be divided into direct and indirect effects based on *in vitro* studies and animal experiments. First, the AMPK-eNOS pathway was activated by GLP-1R agonists acting directly on endothelial

cells, promoting NO production, and mediating endothelial vasorelaxation (58). Second, GLP-1R agonists may protect the endothelium indirectly by having anti-inflammatory effects and improving lipid metabolism, slowing down the progression of atherosclerosis (59, 60). In addition, GLP-1R agonists are involved in the inhibition of platelet aggregation and thrombosis. The mechanism remains unclear whether this process is mediated by endothelial cells (61), but this effect on vascular endothelial function is positive in our analysis. On the other hand, the effects of GLP-1R agonists on FMD were more prominent in subgroup analysis of T2DM patients without comorbid CVD (i.e., the non-CVD group). We speculated that patients without comorbid CVD may be more sensitive to the effects of GLP-1R agonists because they have better vascular functional status than patients with CVD or at least the endothelial cell function is not severely impaired in this population.

DPP-4 inhibitors and SGLT-2 inhibitors are two newer classes of hypoglycemic agents, and DPP-4 inhibitors have been in use for longer time. The Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus-Thrombolysis in Myocardial Infarction (SAVOR-TIMI) 53 trial is one of the earliest completed trials of a DPP-4 inhibitor, which suggested that saxagliptin (a DPP-4 inhibitor antidiabetic drug) was related to increased hospitalization rates in patients with heart failure, raising concerns about the cardiovascular safety of this class of drug (62). In several subsequent clinical RCTs with MACE events as major endpoints, such as the Trial Evaluating Cardiovascular Outcomes with Sitagliptin (TECOS) and CARMELINA trials, there were no significant differences in the incidence of MACE events between DPP-4 inhibitor treatment and placebo (63). Reaven and his colleague reported the SAVOR-TIMI, TECOS and CARMELINA was primarily designed as non-inferiority trials, so they concluded these trials may have less sufficient power to assess the cardiovascular benefits of DPP-4 inhibitors (64). However, in our analysis, DPP-4 inhibitors did not show significant improvement on FMD better than the two new antidiabetic drugs, GLP-R against and SGLT-2 inhibitors. Moreover, two studies (42, 43) observed FMD from baseline to a medium-long time frame, the results indicated that sitagliptin, one class drug of DPP-4 inhibitor, did not significantly influenced endothelial function during the final observation. As the result of TECOS and CARMELINA trials indicated, DPP-4 inhibitors also did not perform significant effect to reduce the incidence of MACE events (63), this suggested that the protective effect of DPP-4 inhibitor on endothelial function remains limited. DPP-4 inhibitors are known to effect endothelial cells through GLP-1-dependent and GLP-1-independent pathways in animal models. The activation of the GLP-1R in endothelial cells could increase the phosphorylation of e-NOS and nitric oxide production (65), leading to vasodilation and endothelial repair. SDF-1α is a natural substrate of DPP-4 inhibitors, and is a major regulator of endothelial progenitor cells (EPCs) (66). SDF-1α can repair blood vessels by inducing EPC homing and migration (67). However, increased EPCs may have dual effects, because a sustained increase in number of EPCs is closely associated with the development of vascular stenosis and CVD, and this may have a detrimental effect on vascular endothelial function (68). Thus, the potential effects of the GLP-1-independent pathway may partially explain the differences between the effects of GLP-1R agonists and DPP-4 inhibitors on endothelial function. Therefore, the effects of DPP-4 inhibitors on FMD may represent the combined result of GLP-1-dependent and GLP-1-independent mechanisms, and this combined effect may not as effective as GLP-R against in terms of endothelial function.

Previous RCTs have confirmed the protective effects of SGLT-2 inhibitors on cardiovascular function. However, the specific mechanisms remain poorly understood. Recent meta-analyses based on several small RCTs have reported an ameliorative effect of SGLT-2 inhibitors on endothelial function by assessing FMD (16). In the present study, SGLT-2 inhibitors ranked better than traditional antidiabetic agents, such as metformin, glinides and sulfonylureas with regard to improvement of FMD, but as a new class of antidiabetic drug with certain cardiovascular benefits, SGLT-2 inhibitors still ranked behind the most effective drugs, GLP-1R agonists. It should be noted that SGLT-2 inhibitors, such as canagliflozin, may be associated with increased risk for limb amputation in patients with T2DM and show a close association with inadequate peripheral vascular perfusion (69). SGLT-2 inhibitors is proved to reduce blood volume in T2DM patients by increasing osmotic diuresis and reducing peripheral perfusion (70), which may significantly affect the change of FMD (71). In addition, previous clinical studies have suggested that SGLT-2 inhibitors may have cardiovascular protective effects by regulating renal blood flow and improving myocardial metabolism (72, 73). We speculated that the cardiovascular protective effects of SGLT-2 inhibitors may depend on their direct effects on cardiac and renal target organs. The hypoglycemic effects of SGLT-2 inhibitors may also improve endothelial function by reducing the hyperglycemic state of the vascular endothelium, but this effect seems to be limited. Further studies are required to confirm these speculations.

In the present study, sulfonylureas showed the lowest ranking among the classes of antidiabetic drug in terms of improving FMD. This suggests that the association between sulfonylureas and vascular endothelial function may not be strong. As a traditional hypoglycemic agent, the cardiovascular effects of sulfonylureas on patients with T2DM remain controversial (74). A network meta-analysis that included 18 studies using sulfonylureas showed that neither gliclazide nor glimepiride was associated with an increased risk for cardiovascular mortality, whereas glibenclamide showed a correlation with increased cardiovascular mortality risk (75). First-generation sulfonylureas are associated with cardiovascular mortality, which may be related to their blocking effect on ATP-sensitive K^+ channels (K_{ATP} channels) of cardiovascular smooth muscle cells. This effect impairs myocardial ischemic preadaptation, a physiological mechanism of protecting the myocardium against ischemic insult, which leads to reduction of coronary blood flow and increased peripheral vascular resistance (76). However, it is unclear whether this effect could affect FMD.

Furthermore, different sulfonylurea drugs show variable abilities to interfere with K_{ATP} channels, e.g., unlike glibenclamide, glimepiride has been shown to have no significant effect on vascular K_{ATP} channels (77), which may explain the inconsistencies of cardiovascular outcomes among previous meta-analysis. In the present study, the sulfonylureas mainly included two drugs, glibenclamide and glimepiride. As the effects of these two drugs on endothelial function were taken into account simultaneously, the ranking of sulfonylureas on FMD may need to be interpreted carefully.

The ranking of α-glycosidase inhibitors in the SUCRA table was prominent, and this effect should not be overlooked. A meta-analysis of five RCTs involving α-glucosidase inhibitor treatment showed that these drugs delayed the increase in CIMT in T2DM patients (78), suggesting that α-glycosidase inhibitors may have a positive effect on the vascular endothelium. Our network analysis of α-glycosidase inhibitors supports this conclusion. The postprandial peak in glycemic status induces oxidative stress, which directly impairs endothelial cell function (79). Therefore, α-glycosidase inhibitors may protect the vascular endothelium by effectively reducing postprandial hyperglycemia to attenuate injury due to oxidative stress. Although the completed RCT of α-glycosidase inhibitors, the Acarbose Cardiovascular Evaluation trial (ACE), did not find that these drugs reduced heart failure or cardiovascular death in patients with T2DM or impaired glucose tolerance, this study had several limitations, i.e., the study was performed only in China and the participants all had impaired glucose tolerance and coronary heart disease (80). Further relevant clinical studies are required to elucidate the effects of α-glucosidase inhibitors on vascular function in T2DM patients.

As traditional insulinotropic agents, the effects of thiazolidinediones (TZDs) on cardiovascular events are highly controversial and previous clinical trials have yielded inconsistent results. As rosiglitazone and troglitazone are no longer widely used in clinical practice, we only discuss the effects of one TZD drug, pioglitazone, which is still used in the clinic. In the SUCRA table, pioglitazone ranked better than the new class of drug, DPP-4 inhibitors, and metformin in improving FMD, suggesting that pioglitazone may have a positive effect on vascular function. In a study that used a hypertensive rat model, pioglitazone was shown to activate peroxisome-activated receptors (PPARs) by regulating endothelin-1 (ET-1) expression to attenuate the effects of oxidative stress on the vasculature. In addition, pioglitazone may improve vasodilatory function by increasing ET-1 receptor B (ETB) expression to release endothelial cell relaxing factors (81). The PROspective pioglitAzone Clinical Trial In macroVascular Events (PROactive trial) was the first large RCT to evaluate the effects of pioglitazone monotherapy on cardiovascular outcomes, and the results showed that pioglitazone reduced the risks of all-cause mortality, nonfatal heart attack, and stroke in patients with T2DM with macrovascular disease (82). The results of a meta-analysis showed a beneficial effect of pioglitazone on the risk for recurrent cardiovascular events in patients with established CVD (83). However, it is not clear whether the effects of pioglitazone on improving vascular endothelial function could have a beneficial effect on future CVD events, particularly as side effects, such as edema and fluid retention, are associated with increased risk for heart failure in T2DM patients (84). Therefore, the effects of pioglitazone on endothelial function must be interpreted carefully in the context of other complications.

In our network, metformin also had a significant positive effect on FMD change in non-CVD subgroup analysis. As an insulin sensitizer, metformin is still the drug of first-line choice for T2DM treatment which shown multiple beneficial effects against CVD. A long-term clinical trial compared to placebo shown that the metformin added treatment significantly reduced levels of several endothelial function biomarker which are associated with the risk of CV morbidity in T2DM patients (85). *In vitro* evidence also shown that metformin may represent the result of multiple mechanisms involving AMPK activation, endothelium-dependent vascular response, and oxidative stress on endothelial protection (86). These suggest that metformin may improve endothelial function through different pathways, our analysis also supported the potential beneficial effect of metformin in endothelial function especially on T2DM patients without CVD.

This meta-analysis analyzed the effects of several common antidiabetic drugs on FMD. We found that several antidiabetic drugs have positive effects on endothelial function while simultaneously contributing to blood glucose control, and these effects may explain their specific benefits for the risk of future CVD outcomes in T2DM patients. Though from the analysis we didn't find lifestyle change to be an effective treatment to benefit the endothelial function compared to other antidiabetics drugs, however, the effect of lifestyle on vascular function needs more evidence to prove. Therefore, the potential effects on endothelial function should be taken into consideration when choosing suitable treatments for patients with T2DM.

As far as we know, this is the first study to indicate that GLP-1R agonists may be effective for improving vascular endothelial function in T2DM patients. As FMD is recommended as a reproducible and practical technique to be used for different term pharmacological interventions (87), several factors such as the vascular condition of the patient, measure time frame, and laboratory experience should be noticed to evaluate the effect of FMD (88). Patient under different vascular condition may reflect the response of FMD measurement, our subgroup analysis result indicated that more than two antidiabetic drugs may have positive effect on endothelial function in T2DM patients who under better vascular condition. For studies under different measure time frame, we mainly analyzed studies measured FMD at medium to long time frames to focus on its stable effect on CVD prediction value. Studies should perform FMD according to guidelines which are crucial to ensure valid conclusions and clinical evaluation.

Limitations

This study was a comprehensive assessment of representative antidiabetic drugs for treatment of T2DM, and we found that their effects on vascular function as measured *via* FMD varied

considerably. The novel antidiabetic drugs GLP-1R agonists may have unique advantages in improving vascular function in T2DM patients. However, this study had some limitations that should be taken into account when interpreting our findings. First, in our network, we extracted the mean, SD, and sample size at baseline and post-treatment to calculate the change of FMD. The data extraction and transformation process of two studies (31, 42) may lead to follow-up bias. Second, eight classes of antidiabetic drug were analyzed. However, there may have been discrepancies due to different drugs within a single class. In addition, several factors may have contributed to the inconsistencies observed in this study. The duration of diabetes ranged from newly diagnosed to more than 3 years, there were ethnic and regional differences in participants (20 study populations were from Asia, 3were from Europe, 5 were from North America, 1 was from South America, and 1 was from Oceania) included in the analysis. In addition, even if twelve of the included studies clearly stated that assessing FMD was performed by professional ultrasound physicians in a blinded manner, there may have been measurement error.

Some potential confounders affecting FMD were not controlled in our analysis. There were several factors that may have affected FMD assessment in studies, namely, age, sex, BMI, HbA1c and measure timeframe. The influence of these factors should be considered when interpreting the results of this meta-analysis. Many studies were not designed as RCTs, and so the evidence that could be used for network analysis was limited. In addition, we only included published studies, and so cannot exclude the possibility of publication bias.

Conclusions

Three classes of antidiabetic drug, GLP-1R agonists, TZD, and SGLT-2i inhibitors, may have positive effects on endothelial function in T2DM patients. Among antidiabetic drugs of the present network meta-analysis, GLP-1R agonists were superior to other antidiabetic drugs in endothelial function improvement. Thus, GLP-1R agonists have potential as novel therapeutics to protect endothelial function and reduce CVD outcomes in T2DM patients.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

YHW designed the study, performed statistical analysis, and interpreted the data for analysis. MYY wrote the first draft. JCW and HZL conducted the database search, screened and extracted data. XLZ, LZ, and XDH contributed to the discussion and editing. ZHL designed the study and critically revised the draft manuscript. HXG had full access to the data and had final responsibility for the decision to submit for publication. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.818537/full#supplementary-material

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Role of Dapagliflozin and Liraglutide on Diabetes-Induced Cardiomyopathy in Rats: Implication of Oxidative Stress, Inflammation, and Apoptosis

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The current study aims to assess the protective effects of dapagliflozin (Dapa; a sodium-glucose cotransporter-2 inhibitor) and/or liraglutide (Lira; a glucagon-like peptide 1 agonist) in an experimental model of diabetic cardiomyopathy (DCM). A single dose of streptozotocin (STZ) was administrated to male Sprague–Dawley rats by intraperitoneal injection at a dose of 50 mg/kg to induce diabetes mellitus (DM). Dapa (1 mg/kg, orally), Lira (0.4 mg/kg, s.c.), and Dapa–Lira combination were administrated for 8 weeks once-daily. Blood samples were evaluated for glucose level and biochemical markers of cardiac functions. Cardiac tissue was dissected and assessed for redox homeostasis (malondialdehyde (MDA), glutathione (GSH), and catalase (CAT)), pro-inflammatory mediators (NF-κB and tumor necrosis factor-α (TNF-α)), and apoptotic effectors (caspase-3). Moreover, the effect of treatments on the cardiac cellular structure was studied. Dapa and/or Lira administration resulted in significant improvement of biochemical indices of cardiac function. Additionally, all treatment groups demonstrated restoration of oxidant/antioxidant balance. Moreover, inflammation and apoptosis key elements were markedly downregulated in cardiac tissue. Also, histological studies demonstrated attenuation of diabetes-induced cardiac tissue injury. Interestingly, Dapa–Lira combination treatment produced a more favorable protective effect as compared to a single treatment. These data demonstrated that Dapa, Lira, and their combination therapy could be useful in protection against DM-accompanied cardiac tissue injury, shedding the light on their possible utilization as adjuvant therapy for the management of DM patients.

Keywords: dapagliflozin (PubChem CID: 9887712), liraglutide (PubChem CID: 16134956), diabetes risk, cardiomyopathy, rats

INTRODUCTION

Diabetes mellitus (DM) is a complex chronic metabolic disease whose incidence is escalating globally (1, 2). Type 1 DM (T1DM) is caused by progressive T cell-mediated immune damage of pancreatic β cells resulting in persistent hyperglycemia (3). On the other hand, type 2 DM (T2DM) is associated with deficient insulin secretion due to compromised β-cell function accompanied by peripheral insulin secretion. However, recent reports proposed β-cell as a key contributor to the pathogenesis of T1DM *via* evading the immune attack, highlighting the contribution of β-cell stress responses to disease onset (4). Thereby, several therapeutic interventions that aimed at improving glycemic control and ameliorating pressure exerted on β cells in T2DM have been evaluated in the context of T1DM (5). Among these strategies, glucagon-like peptide-1 (GLP-1) analogs and sodium-glucose cotransporter-2 (SGLT2) inhibitors have recently shown some benefit as adjuvant therapy with insulin in the treatment of patients with T1DM (6). Further, intensive insulin therapy in T1DM increases the occurrence of abdominal obesity, dyslipidemia, and hypertension, putting them at higher risk of cardiovascular disorders. Hence, antihyperglycemic classes including GLP-1 analogs and SGLT2 inhibitors hold promise as additional adjunctive therapy options that complement insulin efficacy, reduce the risk of weight gain, and improve overall glycemic control (7).

Dapagliflozin (Dapa) is an SGLT2 inhibitor that prevents renal glucose reabsorption in proximal tubules. Thus, it reduces the blood glucose level only when it exceeds a reduced renal threshold, decreasing the incidence of hypoglycemia. Additionally, urinary loss of glucose helps weight reduction (8). Liraglutide (Lira) is a synthetic long-acting GLP-1 receptor agonist that has a high structural similarity to human GLP-1. It acts by reducing glucagon secretion, suppressing appetite, slowing gastric emptying, and helping weight loss (9).

Several clinical trials were conducted to assess the utility of using SGLT2 inhibitors and GLP-1 receptor agonists as adjuncts to insulin therapy for T1DM patients. However, the results were not conclusive. This can be explained by the small sample size and the unsatisfactory glucose-lowering efficacy of these agents (10). Dapa has now been licensed for clinical use as adjuvant therapy to insulin in Europe and Japan. However, it has not been approved in the United States due to the increased risk of diabetic ketoacidosis (DKA) (11). However, a risk mitigation strategy has been developed for reducing DKA in T1DM patients treated with SGLT2 inhibitors (12). On the other hand, the use of Lira in combination with insulin resulted in a smaller HbA1c decrease; therefore, it was not considered for a license. Nevertheless, using various combinations of inhibitors of SGLT2 and agonists of GLP-1 receptor is currently under investigation to test whether this approach would yield better therapeutic outcomes in T1DM patients. In this context, a clinical trial conducted by Kuhadiya et al. demonstrated significant improvement in glycemic control and body weight when Dapa was added to Lira and insulin for the treatment of T1DM patients (13). However, two patients developed DKA. With the reported suppressive effect Lira on ketogenesis (14), using a lower dose of SGLT2 inhibitors in combination can achieve the needed balance between clinical benefit and increased risk of DKA. Indeed, the combined beneficial effects of SGLT2 inhibitors and GLP-1 receptor agonists on metabolic indices and vascular complications need further investigations for further establishment of this combination in the treatment of T1DM patients.

Uncontrolled DM is accompanied by a lot of complications that affect various body organs. Among diabetes-associated organ complications, diabetic cardiomyopathy (DCM) is a major leading cause of death in diabetic patients (15). It is characterized by diastolic and systolic dysfunction and pathological cardiac remodeling that may end in heart failure. Various preclinical studies delineated multiple intracellular pathways that are implicated in the pathogenesis of DCM including endoplasmic reticulum stress, oxidative stress, impaired calcium handling, increased lipid utilization, and activation of inflammatory pathways (16). The use of SGLT2 inhibitors and GLP-1 receptor agonists in T2DM has improved associated disorders in various preclinical (17), and clinical studies (18). However, the effect of these antidiabetic drugs on organ injury in T1DM needs further investigation. The present study aimed to study the protective effects of Dapa and/or Lira in an experimental model of DCM. Further, the potential underlying molecular mechanisms have been evaluated.

MATERIALS AND METHODS

Experimental Design and Treatments

All used protocols were approved by the guidelines of the Ethics Committee at the Faculty of Medicine, Mansoura University, Mansoura, Egypt. Fifty male Sprague–Dawley (SD) rats were obtained from the laboratory animal unit of the Urology and Nephrology Center, Mansoura, Egypt, and kept under required conditions of temperature (21°C ± 2°C), humidity (50% ± 10%), and light (12 h light/dark cycle) with *ad libitum* access to distilled water and a standard rat diet. After 1-week acclimatization, a single intraperitoneal injection of streptozotocin (STZ) at a dose of 50 mg/kg was used to induce T1DM as previously described (19). Hyperglycemia incidence was confirmed 3 days later *via* assessment of blood glucose level using a glucometer (Accu-Check, Roche, Mannheim, Germany). Rats were considered diabetic when blood glucose level was greater than 250 mg/dl. The diabetic rats were further randomly assigned into subgroups (n = 10 for each group), including a saline group, a Dapa group (FORXIGA, AstraZeneca, Mississauga, ON, Canada, 1 mg/kg/day, orally), a Lira group (VICTOZA, Novo Nordisk, Bagsværd, Denmark, 0.4 mg/kg/day, s.c.), and a Dapa+Lira group. Each rat was given treatment for 8 weeks. An additional group of rats (n = 10) was used as the normal control group and received saline.

At the end of the experimental period, blood glucose level was assessed as previously mentioned. Animals were sacrificed under anesthesia, and blood samples were withdrawn from the retro-orbital plexus and centrifuged at 3,000 rpm for 10 min to separate the serum, which was further used for biochemical analysis. The hearts were rapidly isolated. One part was fixed in

10% formalin for histopathological examination and immunohistochemistry (IHC) analysis. Another part was washed with ice-cold saline, rapidly frozen in liquid nitrogen, and stored at −80°C for protein and RT-PCR assays.

Biochemical Measurements

The serum biochemical profiles, including insulin (Cloud-Clone Corp., Houston, TX, USA), creatine kinase-MB (CK-MB) (bioMérieux Diagnostics, Milan, Italy), lactate dehydrogenase (LDH; Spectrum Diagnostic Company, Cairo, Egypt) were evaluated according to the manufacturers' instructions.

Histological Studies

Dissected organ specimens were fixed and dehydrated in ascending grades of ethanol. Thereafter, cardiac specimens were embedded in paraffin wax. Then, 5-μm-thick sections were prepared from paraffin blocks. Sections were further stained with H&E to be assessed for histopathological alterations. The examination was performed by a qualified observer without the identification of the experimental groups. All records were performed using Olympus light microscope equipped with a digital camera (Tokyo, Japan). For morphometric analysis, semiquantification of myocardial injury was performed (20, 21),. Each slide was inspected for cardiac pathological changes in three high-power fields using the following scoring system for grading the cardiomyopathy severity: 0 = no damage, 1 = mild lesion, 2 = moderate lesion, and 3 = severe lesion, with (1+) for the presence of myocardial fiber swelling and interstitial edema, (1+) for disorganization of myocardial fiber with or without fibroblastic proliferation, (1+) for perinuclear vacuolization or myocardial fiber vacuolization, (1+) for myocardial fibers myocytolysis/necrosis, and 0 when there was no damage noted.

Preparation of Cardiac Tissue Homogenate and Assessment of Oxidative Stress

Cardiac tissue was homogenized in phosphate-buffered saline (PBS) to prepare 10% (w/v) homogenate using Omni-125 handheld homogenizer (Omni International, Kennesaw, GA, USA). The homogenates were further spun at 5,000*g* for 15 min at 4°C. Oxidative stress biomarkers were then assessed in freshly prepared supernatants. Levels of malondialdehyde (MDA), a marker of lipid peroxidation, and the antioxidant reduced glutathione (GSH) in addition to the activity of the antioxidant enzyme catalase (CAT) were measured in prepared tissue homogenates assayed using commercially available kits by Bio Diagnostic (Giza, Egypt) according to the manufacturer's protocols.

Assessment of Cardiac Inflammatory Cytokine Levels by ELISA

Levels of interleukin-1β (IL-β) and interleukin-6 (IL-6) in cardiac tissue homogenates prepared from different experimental groups were assessed by the ELISA method according to the manufacturer's instructions (Cloud-Clone Corp., Houston, TX, USA).

Real-Time PCR

Total RNA was extracted from cardiac tissues from all experimental groups using Direct-zol RNA Miniprep Plus (ZYMO RESEARCH CORP., Irvine, CA, USA, Cat# R2072). Extracted RNA was assessed for quantity and quality by spectrophotometry using Beckman dual spectrophotometer (Brea, CA, USA).

Extracted RNA was then utilized for reverse transcription into complementary DNA (cDNA) using the SuperScript IV One-Step RT-PCR kit (Thermo Fisher Scientific, Waltham, MA, USA, Cat# 12594100). cDNA amplification was performed using a 48-well plate StepOne instrument (Applied Biosystems, Foster City, CA, USA). The thermal profile included the following: reverse transcription for 10 min at 45°C, RT inactivation and initial denaturation by 40 cycles of 10 s at 98°C, and an amplification step for 10 s at 55°C followed by 30 s at 72°C. Data were then expressed in cycle threshold (Ct) for the housekeeping gene and the target genes. ΔΔCt method was used to normalize variation in the target genes expression by referring to the expression value of mean critical threshold (CT) of a housekeeping gene. Primer sequence for tumor necrosis factor-α (TNF-α) gene was forward 5′-TAC TGA ACT TCG GGG TGA TTG GTC C-3′ and reverse 5′-CAG CCT TCT CCC TTG AAG AGA ACC-3′, for caspase-3 gene was forward 5′-ATGGACAACAACGAAACCTC-3′ and reverse 5′-TTAGTGATAAAAGTACAGTTCTT-3, and for β-actin housekeeping gene was forward 5′-CTAAGGCCAACCGTG AAAAG-3′ and reverse 5′-GCCTGGATGGCTACGTACA-3′. The relative quantitation (RQ) of each target gene is performed based on the calculation of the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry

Sections were dewaxed, rehydrated, and washed, followed by incubation in 3% hydrogen peroxide (H_2O_2, 3%) for 10 min and then blocking with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS). Thereafter, immunolocalization was performed by incubation with antibodies for NF-κB/p56 (Thermo Fisher Scientific Inc., Waltham, MA, USA), TNF-α (sc-52746, Santa Cruz, Paso Robles, CA, USA), and cleaved caspase-3 (GB11532, Wuhan Servicebio Biotechnology, Wuhan, China) at 4°C overnight. Then, sections were washed in TBS 3 times, followed by incubation with secondary antibodies. After washing in TBS, diaminobenzidine/peroxidase was used for development and hematoxylin for counter-staining. The sections were then mounted and examined using Olympus light microscope equipped with a digital camera (Tokyo, Japan). For IHC quantitative assessment, an immunoreactive score (IRS) was used. It provides a scale of 0–12 representing IRS index (0–1 = negative, 2–3 = mild, 4–8 = moderate, and 9–12 = strongly positive). IRS is obtained by multiplication between staining intensity grading (0–3) and positive cells proportion grading (0–4) (22) and quantified using the QuPath program (0.1.2) (23).

Statistical Analysis

Analysis and graphical representation of data were accomplished by GraphPad prism statistical software (version 8, USA). Results are expressed as mean ± SE, and statistical analysis was

performed using one-way ANOVA followed by a *post-hoc* test (Tukey–Kramer). Two-way ANOVA was used to calculate statistical significance for NF-κB/p56 nuclear and cytoplasmic expression among various experimental groups. Statistical significance was considered at $p < 0.05$.

RESULTS

Effects of Dapagliflozin–Liraglutide Treatment on Blood Glucose and Serum Insulin Levels

Induction of experimental DM resulted in a 4.99-fold ($p < 0.0001$) increase in blood glucose level accompanied by a 65.3% reduction ($p < 0.0001$) in serum insulin as compared to normal control. Treatment with Dapa and Lira for 8 weeks resulted in 42.5% and 30.9% significant decrease ($p < 0.0001$) in blood glucose level and 1.69- and 1.55-fold ($p < 0.0001$, $p < 0.001$) increase in serum insulin level, respectively, as compared to the diabetic group. Combined treatment with Dapa and Lira significantly improved blood glucose level ($p < 0.05$, $p < 0.0001$, respectively) and produced non-significant elevation in serum insulin level as compared to single treatment groups (**Figures 1A, B**).

Effects of Dapagliflozin–Liraglutide Treatment on Biomarkers of Cardiac Injury

Serum levels of LDH and CK-MB were significantly increased by 2.47- and 4.31-fold ($p < 0.0001$ and $p < 0.0001$), respectively, in the DM group compared to the normal group. However, these levels were significantly reduced by 26.5% and 41.2% ($p < 0.0001$, $p < 0.001$), respectively, in the Dapa-treated group and by 18.7% and 43.2% ($p < 0.001$), respectively, in the Lira-treated group when compared to the diabetic group. Combined Dapa–Lira therapy produced more reduction in the levels of these cardiac markers by 31.6% and 47.7% ($p < 0.0001$, $p < 0.05$, respectively), as compared to the DM+Dapa group and by 38% and 45.9% ($p <$

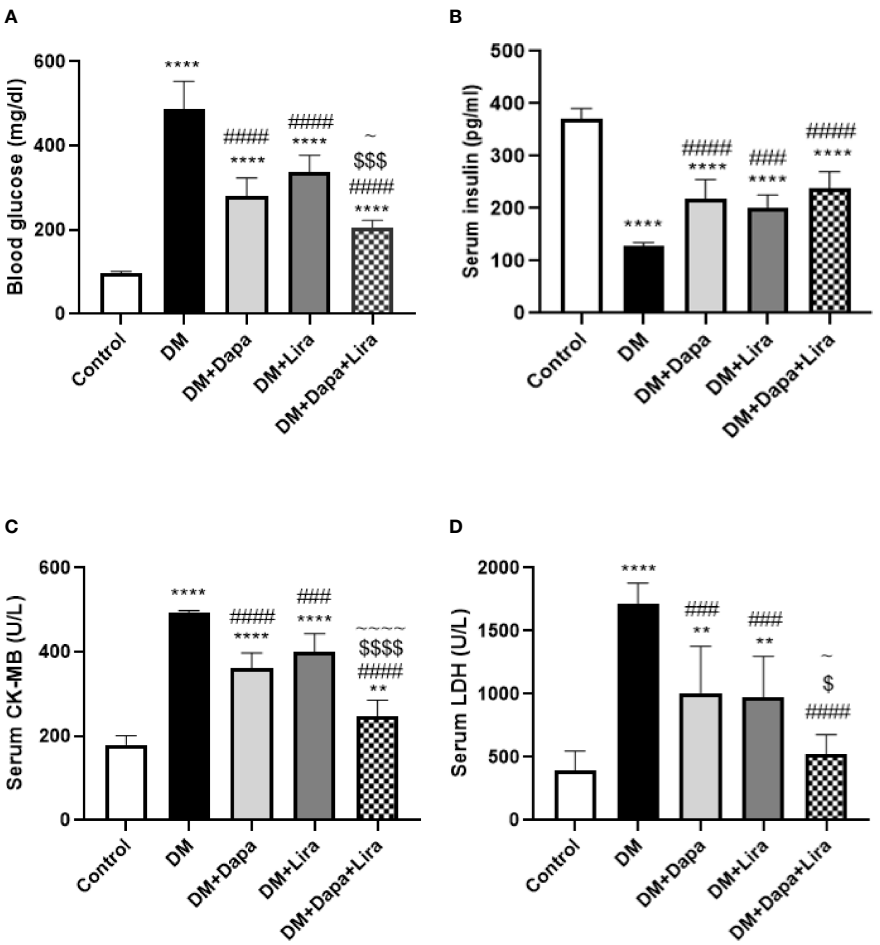

FIGURE 1 | Effect of dapagliflozin (Dapa) and liraglutide (Lira) or their combination on **(A)** blood glucose level, **(B)** serum creatine kinase-MB (CK-MB), and **(C)** serum lactate dehydrogenase (LDH). Data are represented as mean ± SE, n = 6. ** significance in comparison with control group at $p < 0.01$, **** at $p < 0.0001$, ### significance in comparison with diabetic group at $p < 0.001$, #### at $p < 0.0001$, ~ significance in comparison with DM+Dapa at $p < 0.05$, ~~~~ significance in comparison with DM+Dapa at $p < 0.0001$, $ significance in comparison with DM+Lira group at $p < 0.05$, $$$ at $p < 0.001$, $$$$ at $p < 0.0001$.

0.0001, $p < 0.05$, respectively) as compared to the DM+Lira group (**Figures 1C, D**).

Effects of Dapagliflozin–Liraglutide Treatment on Type 1 Diabetes Mellitus-Induced Cardiac Histological Changes

As shown in **Figure 2A**, H&E-stained heart sections from the control group demonstrated the normal histological structure of the cardiac muscle fibers. Sections from diabetic rat hearts showed disarray of the cardiac myocytes with myocardial fiber disorganization, myocardial fiber necrosis, and mild chronic inflammatory cells in the subpericardium, apoptotic myocyte with hypereosinophilic cytoplasm, pyknotic nuclei and increased intermyocyte, interstitial edema, and perivascular chronic inflammatory cells. The degree of injury appears to be moderate in the DM+Dapa-treated group and mild in the DM +Lira-treated group. The normal structure in cardiac sections from the DM+Dapa+Lira group was almost restored. Additionally, these results were further ascertained by morphometric analysis shown in **Table 1** and **Figure 2B**.

Effects of Dapagliflozin–Liraglutide Treatment on Oxidative Stress Markers in Cardiac Tissue

Cardiac tissue levels of MDA demonstrated a marked increase ($p < 0.0001$) in the DM group in comparison with the normal control group. Meanwhile, a significant decrease in the treated groups (DM+Dapa and DM+Lira groups) ($p < 0.0001$) was observed as compared to that of the untreated DM group. Additionally, the combination treatment produced a significant

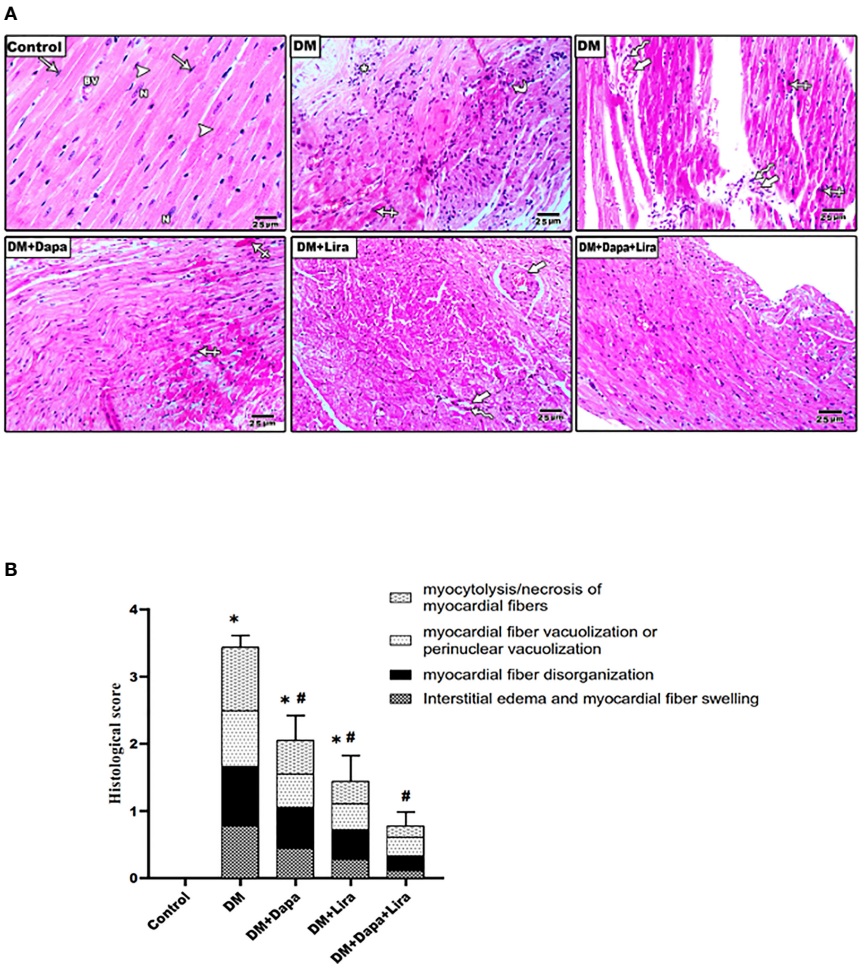

FIGURE 2 | **(A)** Representative photomicrographs of H&E-stained cardiac sections from different experimental groups. Normal control rats showed normal histological structure of the cardiac muscle diabetic group demonstrating disarray of the cardiac myocytes with myocardial fiber disorganization (curved arrow), myocardial fiber necrosis with chronic inflammatory cells in the subpericardium (astrix), apoptotic myocyte with hypereosinophilic cytoplasm, pyknotic nuclei (crossed arrow), increased intermyocyte (thick arrows), and perivascular chronic inflammatory cells (zigzag arrows) marked interstitial edema. Treatment groups (DM+Dapa) and (DM+Lira) showed less structural injury. Combination therapy group (DM+Dapa+Lira) showed almost restoration of normal cardiac structure. ×400 bar 25. **(B)** A graph showing histological score for cardiomyopathy. Data are expressed as mean ± SEM (n = 6). $^{*}p < 0.05$ versus control and $^{\#}p < 0.05$ versus diabetic group. Dapa, dapagliflozin; Lira, liraglutide.

TABLE 1 | Histopathological scores for myocardial necrosis among different experimental groups.

	Myocardial fiber swelling and interstitial edema	Disorganization of myocardial fiber with or without fibroblastic proliferation	Perinuclear vacuolization or myocardial fiber vacuolization	Myocardial fibers myocytolysis/necrosis	Total severity score
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
DM	0.78 ± 0.14*	0.89 ± 0.08*	0.83 ± 0.09*	0.94 ± 0.06*	3.44 ± 0.17*
DM+Dapa	0.44 ± 0.13*#	0.66 ± 0.11*#	0.50 ± 0.12*#	0.50 ± 0.13*#	2.06 ± 0.37*#
DM+Lira	0.28 ± 0.10*#	0.45 ± 0.12*#	0.39 ± 0.12*#	0.33 ± 0.11*#	1.44 ± 0.38*#
DM+Dapa +Lira	0.06 ± 0.03#	0.23 ± 0.10#	0.28 ± 0.11#	0.17 ± 0.09#	0.78 ± 0.21#

Tissue pathological changes within each slide were inspected and scored in three high-power fields based on severity and extent of myocardial injury. A semiquantitative scoring from 0 to 3 or more was used, with no damage = 0 and severe damage = 3 or more. Values represent the average score for each animal. Results are considered significantly different when p < 0.05. Data are expressed as mean ± SEM (n = 6).

DM, diabetes mellitus; Dapa, dapagliflozin; Lira, liraglutide.

**p < 0.05 versus control.*

#p < 0.05 versus diabetic group.

decrease in MDA as compared to the DM+Lira group ($p < 0.05$). In contrast, the level of the antioxidant GSH and activity of antioxidant enzyme CAT were significantly decreased in cardiac tissues of the DM group ($p < 0.0001$) in comparison with the normal control group. However, a significant increase in the levels of these antioxidant moieties was observed in the treated groups (DM+Dapa and DM+Lira groups) when compared to the untreated DM group ($p < 0.0001$). Also, the combination therapy markedly increased tissue GSH ($p < 0.0001$) as compared to the DM+Lira group as well as tissue CAT ($p < 0.05$) compared to the DM+Dapa and DM+Lira groups (**Figures 3A–C**).

Effect of Dapagliflozin–Liraglutide Treatment on mRNA Expression of TNF-α and Caspase-3 in Cardiac Tissue

TNF-α mRNA levels were significantly increased in the cardiac tissue of the DM group ($p < 0.0001$) compared to normal control. On the other hand, the pro-inflammatory cytokine mRNA levels were markedly decreased in the treated groups (DM+Dapa and DM+Lira groups, $p < 0.0001$) compared to the DM group. Additionally, the combination treatment resulted in a significant decrease in TNF-α mRNA level when compared with single treatment, $p < 0.0001$ (**Figure 4A**).

Regarding caspase-3, the mRNA levels of the apoptotic enzyme were significantly elevated in cardiac tissues of the DM group, $p < 0.0001$, in comparison with those of the normal control group. However, a marked decrease in its level was observed in groups treated with Dapa or Lira, $p < 0.0001$, as compared to the untreated DM group. Further, the combination therapy resulted in a more significant reduction in caspase-3 mRNA level compared to single treatment groups, $p < 0.0001$ (**Figure 4B**).

Effect of Dapagliflozin–Liraglutide Treatment on Cardiac Tissue Inflammatory Cytokines IL-1β and IL-6

Cardiac tissue of the DM group demonstrated significantly increased levels of inflammatory cytokines IL-1β and IL-6 ($p < 0.0001$) as compared to normal control. However, the pro-inflammatory cytokine levels were significantly decreased in cardiac tissue of the DM+Dapa group ($p < 0.01$, $p < 0.0001$, respectively) and DM+Lira group ($p < 0.0001$) as compared to the untreated DM group. Also, the combination treatment group demonstrated a significant decrease in cardiac IL-1β levels ($p < 0.0001$) when compared with the DM+Dapa group and in cardiac IL-6 levels ($p < 0.0001$) when compared with treatment groups (DM+Dapa and DM+Lira groups) (**Figure 5**).

Effect of Dapagliflozin–Liraglutide Treatment on Immunostaining of NF-κB/p56, TNF-α, and Cleaved Caspase-3 in Cardiac Tissue

Results from IHC further reinforce RT-PCR findings. Indeed, immunostaining of cardiac tissues from different experimental groups demonstrated increased immunostaining of NF-κB/p56 in cardiac tissue of the diabetic group ($p < 0.0001$) when compared to normal control. The immunostaining was markedly decreased in

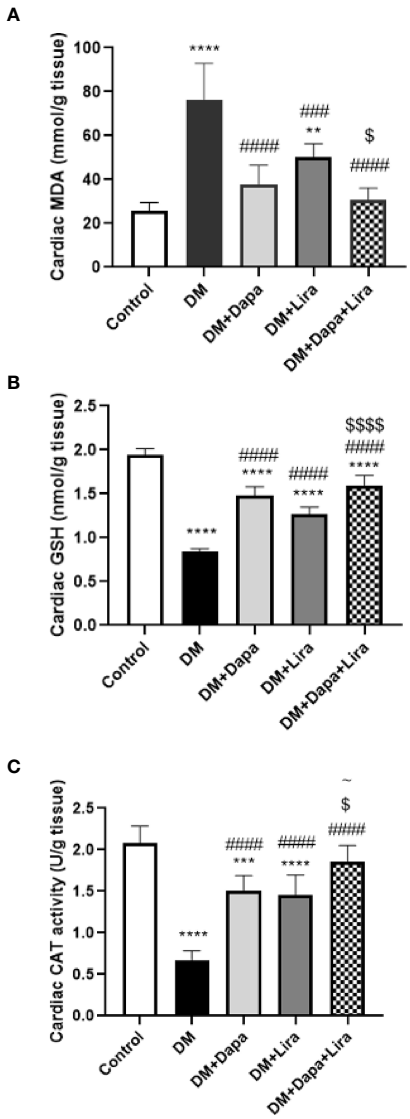

FIGURE 3 | Effect of dapagliflozin (Dapa) and liraglutide (Lira) or their combination on oxidative stress biomarkers in rat heart tissues: **(A)** tissue malondialdehyde (MDA), **(B)** tissue reduced glutathione (GSH), and **(C)** tissue catalase activity (CAT). Data are represented as mean ± SE, n = 6. ** significance in comparison with control group at $p < 0.01$, *** at $p < 0.001$, **** at $p < 0.0001$, ### significance in comparison with diabetic group at $p < 0.001$, #### at $p < 0.0001$, ~ significance in comparison with DM+Dapa at $p < 0.05$, $ significance in comparison with DM+Lira group at $p < 0.05$, $$$$ at $p < 0.0001$.

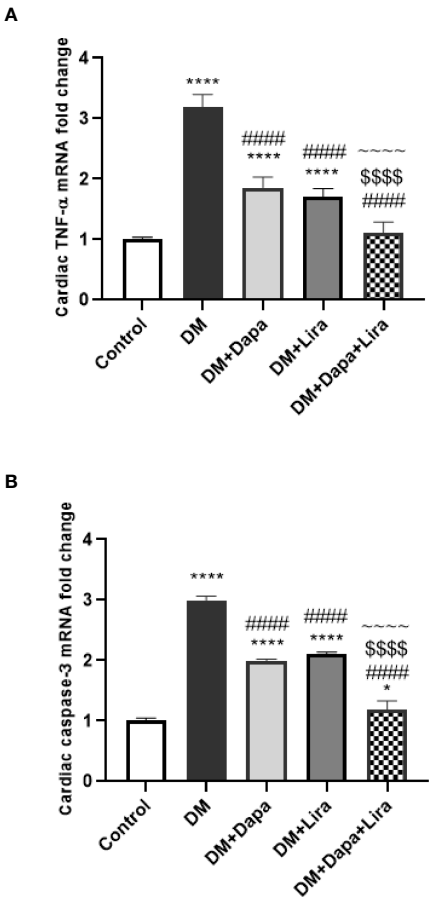

FIGURE 4 | Effect of dapagliflozin (Dapa) and liraglutide (Lira) or their combination on mRNA expression of **(A)** tissue TNF-α and **(B)** tissue caspase-3. Data are represented as mean ± SE, n = 5. **** significance in comparison with control group at $p < 0.0001$, #### significance in comparison with diabetic group at $p < 0.0001$, ~~~~ significance in comparison with DM+Dapa at $p < 0.0001$, $$$$ significance in comparison with DM+Lira group at $p < 0.0001$.

treatment groups (DM+Dapa and DM+Lira groups, $p < 0.0001$) compared to the diabetic group. Additionally, the combination treatment resulted in a significant decrease in immunostaining of NF-κB/p56 in cardiac tissue compared with the DM+Dapa group ($p < 0.0001$) and DM+Lira group ($p < 0.001$) (**Figures 6A, B**). Further, the IRS score was used to compare the nuclear and cytoplasmic expression of NF-κB/p56 among different experimental groups. As shown in **Figure 6C**, the nuclear expression of NF-κB/p56 in the diabetic group was significantly different as compared to normal control ($p < 0.0001$). Treatment with Lira resulted in a significant reduction in NF-κB/p56 nuclear expression compared to the diabetic group ($p < 0.001$). Further, combination treatment resulted in a significant reduction in nuclear expression of NF-κB/p56 as compared to the DM+Dapa-treated group ($p < 0.0001$).

Immunostaining of TNF-α and cleaved caspase-3 was significantly increased in cardiac tissue of the diabetic group ($p < 0.0001$) compared to normal control. However, immunostaining of TNF-α and cleaved caspase-3 was significantly decreased in cardiac tissue of treatment groups (DM+Dapa and DM+Lira groups and combination treatment groups, $p < 0.0001$) compared to the diabetic group (**Figures 7**, **8**).

DISCUSSION

Despite using insulin as the mainstay for T1DM treatment, non-insulin adjunct therapy is a new trend. The purpose of this

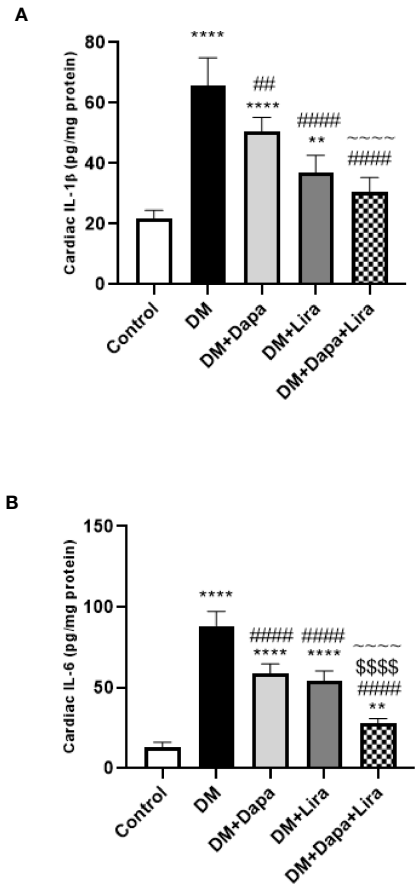

FIGURE 5 | Effect of dapagliflozin (Dapa) and liraglutide (Lira) or their combination on protein levels of **(A)** tissue IL-1β and **(B)** tissue IL-6. Data are represented as mean ± SE, n = 5. ** significance in comparison with control group at $p < 0.01$, **** at $p < 0.0001$, ## significance in comparison with diabetic group at $p < 0.01$, #### at $p < 0.0001$, ~~~~ significance in comparison with DM+Dapa at $p < 0.0001$, $$$$ significance in comparison with DM+Lira group at $p < 0.0001$.

therapy is to slow down autoimmune processes, to regulate glucagon secretion, and to protect pancreatic β cells. Thereby, the use of this therapy has improved glycemic control, provided nephroprotection, and protected vascular endothelium in both clinical and experimental studies The present study demonstrated the protective efficacy of a selective SGLT2 inhibitor Dapa and a long-acting GLP-1 receptor agonist Lira and their combination therapy in DM-accompanied organ injury (24).

Oxidative stress plays a crucial role in the pathogenesis of DM and its induced complications. Disruption of the normal homeostasis of free radicals has been implicated in the impairment of pancreatic β-cell function (25). Further, oxidation of glucose and non-enzymatic glycation of proteins trigger free radical formation, which in turn causes damage to macromolecules, cellular machinery, and antioxidant enzymes (26). Indeed, several *in vivo* and *in vitro* experimental studies reported that diabetes-accompanied metabolic abnormalities cause mitochondrial superoxide overproduction. This event is considered a central and major mediator of diabetes tissue damage *via* activation of key pathogenic pathways involved in the pathogenesis of diabetic organ complications (27). Besides, oxidative stress inflammation is a common feature of DM. Both effectors reportedly share the same stimulus, reactive oxygen species (ROS). Of note, oxidative stress triggers inflammatory cascades, which in turn promote ROS production, creating a vicious cycle that increases the complexity of diabetes-associated multi-organ complications (28). Furthermore, DM-related oxidative stress and inflammation can trigger cellular apoptosis leading to cellular damage and organ failure (29). In agreement, we previously reported signs of oxidative stress, inflammation, and apoptosis in diabetic tissue using the same experimental model (30–33). Currently, our data were consistent as evident by an increased tissue biomarker of oxidative stress, MDA, and reduced cellular antioxidant defense, GSH and CAT. This was accompanied by increased tissue pro-inflammatory cytokine expression TNF-α as well as upregulated expression of the apoptotic enzyme, caspase-3.

In addition to its role as a crucial modulator of the inflammation process *via* regulation of the expression of hundreds of genes involved in cellular inflammatory events, NF-κB is also a redox-sensitive nuclear factor that subsequently modulates a large number

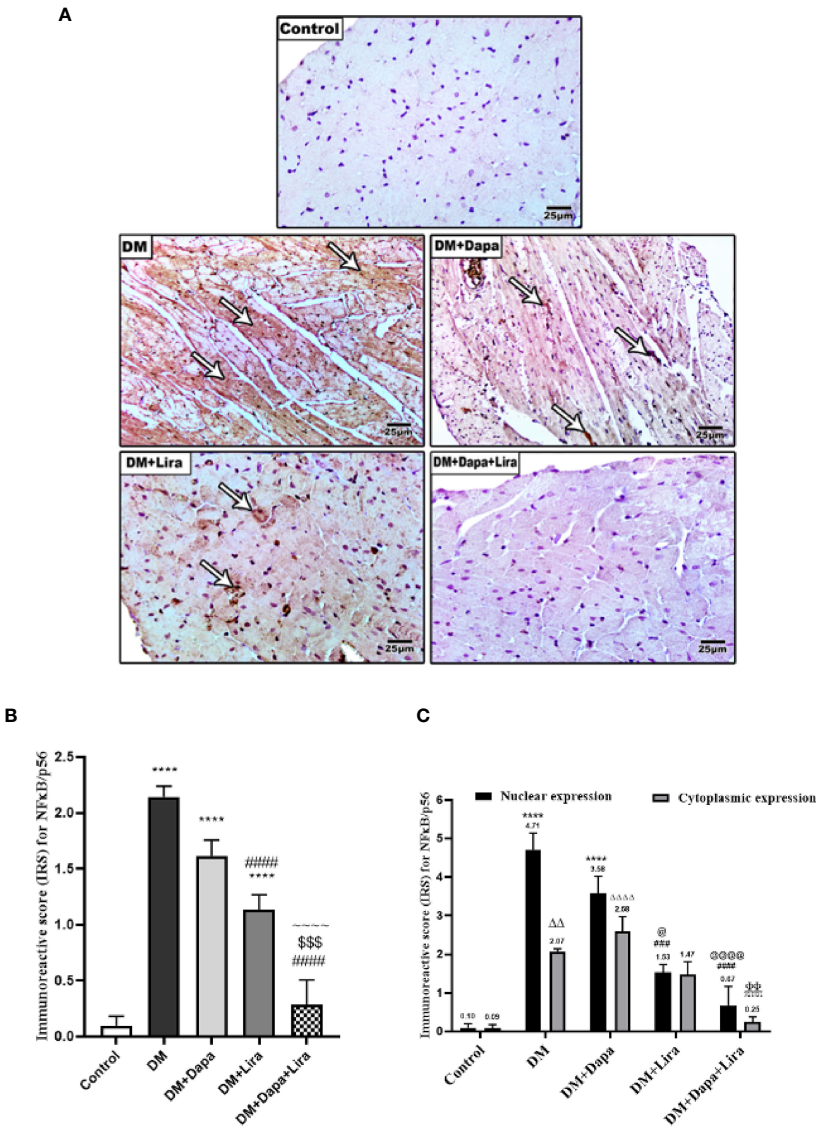

FIGURE 6 | **(A)** Representative photomicrographs of NF-κB/p56 immuno-stained cardiac sections from different experimental groups, ×200 bar 50. **(B)** A graph showing immunoreactive score for NF-κB/p56 cellular expression. Data are represented as mean ± SE, n = 6. **** significance in comparison with control group at $p < 0.0001$, #### significance in comparison with diabetic group at $p < 0.0001$, ~~~~ significance in comparison with DM+Dapa at $p < 0.0001$, $$$ significance in comparison with DM+Lira group at $p < 0.001$. **(C)** A graph showing immunoreactive score for NF-κB/p56 differentially in the nucleus and cytoplasm. Data are expressed as mean ± SEM ($n = 6$). $^{****}p < 0.0001$ versus control, $^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p < 0.001$ versus diabetic group and $p < 0.0001$, $^{@}p < 0.05$ versus DM+Dapa for nuclear expression, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta\Delta}p < 0.0001$ versus control, $^{\Phi\Phi}p < 0.01$ versus diabetic group, $^{\pi\pi\pi}p < 0.001$ versus DM+Dapa for cytoplasmic expression. Dapa, dapagliflozin; Lira, liraglutide.

of processes to maintain tissue homeostasis. Thereby, NF-κB possesses a strategic position at the crossroad between inflammation and oxidative stress, emphasizing its role as a potential target for the management of DM-accompanied organ injury (34). Herein, our data showed upregulated expression of NF-κB along with increased NF-κB/p56 immunostaining in rat diabetic cardiac tissues.

Accumulating evidence demonstrated therapeutic and protective efficacies of Dapa and Lira in T2DM-associated tissue injury. Chen et al. reported that Dapa administration protected against DM-induced oxidative stress in lens (35). Wei et al. demonstrated that Dapa improved pancreatic β-cell function in db/db mice (36). Tang et al. reported antioxidant and anti-inflammatory effects of Dapa with subsequent inhibition of glomerulosclerosis and liver fibrosis in db/db mice (37). In T2DM patients, Dapa administration resulted in beneficial outcomes pertaining to the microvascular sequelae including improvement of the renal resistive index, arterial stiffness, and systemic endothelial function (38, 39). With respect to Lira, it has been found to slow down memory function decline (40), improve

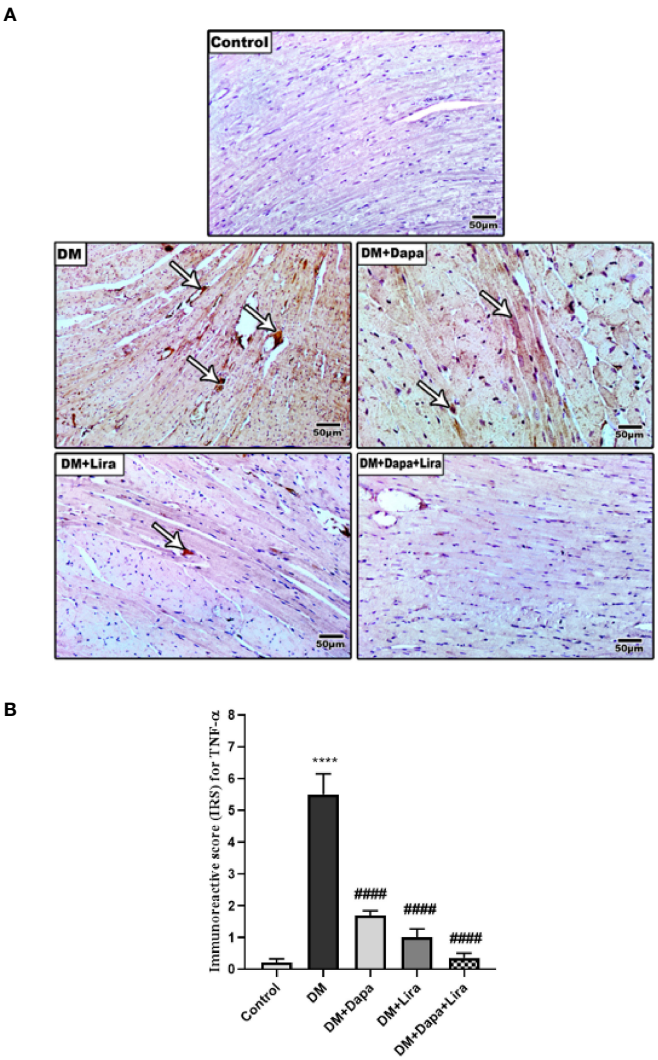

FIGURE 7 | **(A)** Representative photomicrographs of TNF-α immuno-stained cardiac sections from different experimental groups, ×200 bar 50. **(B)** A graph showing immunoreactive score for TNF-α cellular expression. Data are represented as mean ± SE, n = 6. **** significance in comparison with control group at $p < 0.0001$, #### significance in comparison with diabetic group at $p < 0.0001$. Dapa, dapagliflozin; Lira, liraglutide.

cardiovascular and kidney outcomes, and reduce mortality in T2DM patients (41). Moreover, Lira treatment ameliorated the severity of T2DM complicated with non-alcoholic fatty liver disease (42). In experimental models of T2DM, Lira treatment protected against cognitive deficits (43) and exerted a renoprotective effect (44) *via* autophagy activation and endoplasmic reticulum stress attenuation (45). Moreover, Lira administration has modulated the gut microbiome and ameliorated fatty liver in db/db mice (46). Interestingly, a recent study using Dapa–Lira combined therapy showed beneficial metabolic and neuroprotective effects in diet-induced diabetic mice (17). Additionally, a clinical trial performed by Petrie et al. demonstrated that Dapa administration reduced cardiovascular morbidity and mortality in patients with heart failure independent of DM (47). Qin et al. reported protective effects of Dapa against the development of ventricular arrhythmia in pulmonary artery hypertension rats. The mechanism involves modulation of TLR4/NF-κB signaling pathway (48). Similarly, Lira has been reported to protect against myocardial pyroptosis in diabetic rats *via* activation of Sirt1/AMPK signaling pathways (49).

Given the aforementioned evidence and due to their multiple beneficial effects, Dapa and Lira seem to be a very attractive treatment option in T1DM. Indeed, using Dapa in combination with insulin and Lira resulted in a marked improvement in blood glucose and weight loss in T1DM patients (13). Moreover, Dapa treatment demonstrated an anti-atherogenic effect in T1DM mice (50). Interestingly, administration of Dapa or Lira to T1DM mice enhanced β-cell proliferation and decreased β-cell apoptosis (51). Similarly, our data revealed that administration of Dapa and/or Lira to diabetic animals attenuated accompanying

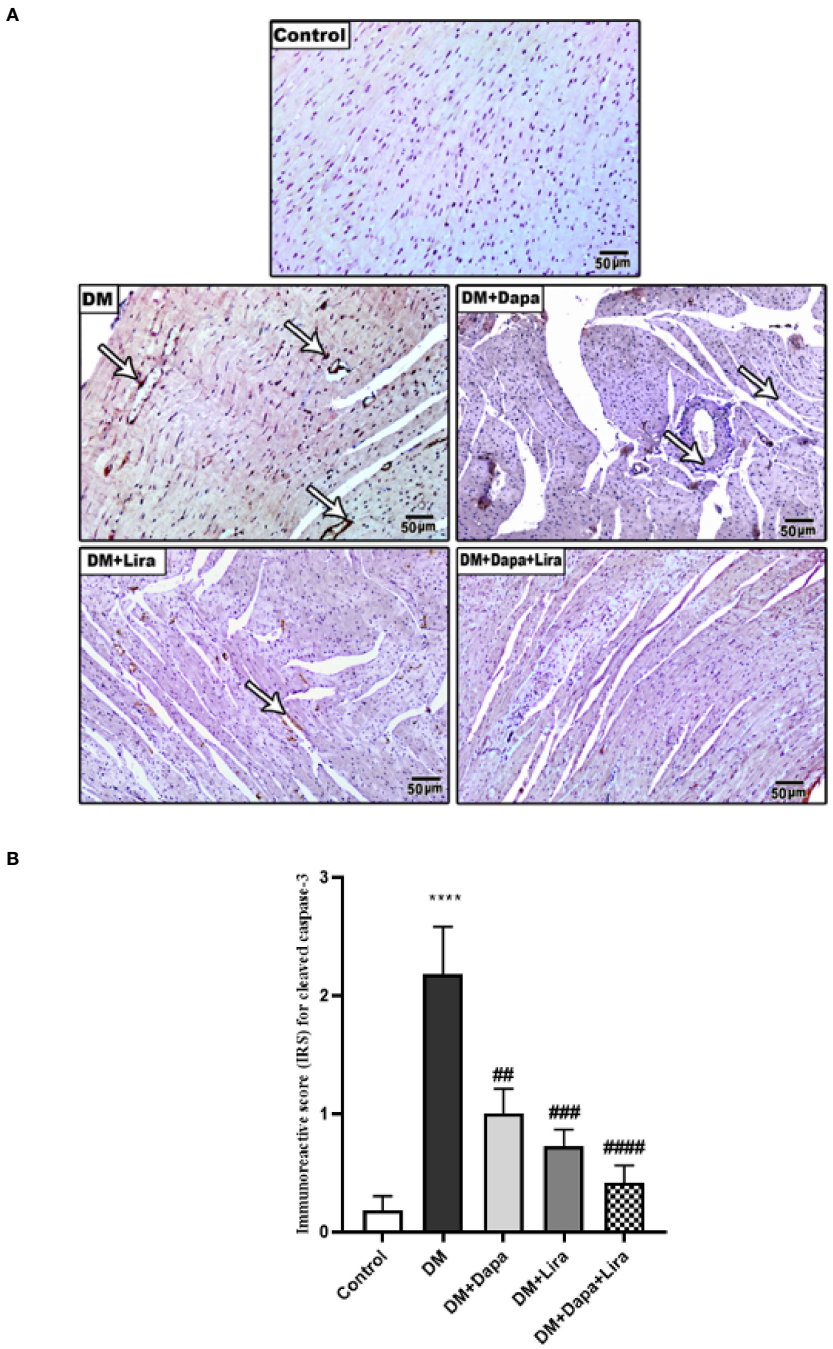

FIGURE 8 | **(A)** Representative photomicrographs of cleaved caspase-3 immuno-stained cardiac sections from different experimental groups. ×200 bar 50. **(B)** A graph showing immunoreactive score for cleaved caspase-3 cellular expression. **** Significance in comparison with control group at $p < 0.0001$, #### significance in comparison with diabetic group at $p < 0.0001$, ### significance in comparison with diabetic group at $p < 0.001$, ## significance in comparison with diabetic group at $p < 0.01$

cardiac tissue injury *via* modulating key elements of oxidative stress, inflammation, and apoptosis. Combination treatment was more effective as compared to sole treatment.

In conclusion, the present study demonstrated beneficial protective effects of Dapa and/or Lira administration against T1DM-related cardiac injury. The mechanisms underlying these effects are attenuating oxidative stress, downregulated inflammation, and apoptosis. Both non-insulin pharmacological drugs could be used as adjunct therapy given their beneficial protective effects on body organs during the diabetic course (**Figure 9**).

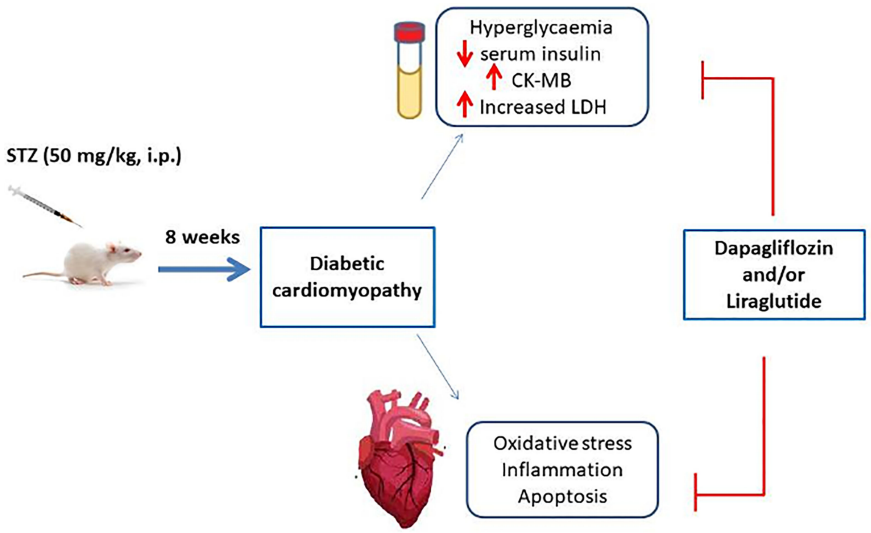

FIGURE 9 | Graphical abstract.

DATA AVAILABILITY STATEMENT

Data are available upon request from the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Research Ethics Committee, Faculty of Medicine, Mansoura University, Egypt.

AUTHOR CONTRIBUTIONS

Conceptualization, ME-Sha, ME-A, M-She; Funding acquisition, HE, M-She; Investigation, ME-Sha, ME-A, ME, HE, DE, ME-She, SA and NE; Methodology; ME-Sha, ME-A, ME, HE, DE, ME-She; Resources, ME-Sha, ME-A, ME, HE, DE, ME-She, SA and NE; Software, ME-Sha, ME-A, ME, HE, DDE, ME-She, SA and NE; Visualization, HE, ME-She, SA; Writing – original draft, ME-Sha, MA-A, DDE, ME-She, NE; Writing – review & editing, ME-Sha, ME-A, DE, ME-Sha, NE.

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Impact of Insulin Resistance on Cardiometabolic Risk Factors and an Anthropometry-Based Predictive Nomogram for Insulin Resistance Among Adolescents in China

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Objective: We aimed to investigate the impact of insulin resistance (IR), as determined by the homeostasis model assessment of insulin resistance (HOMA-IR), on cardiometabolic risk factors (CMRFs), and develop an anthropometry-based predictive nomogram for IR among adolescents in China.

Design: Data were acquired from a cross-sectional study with a stratified cluster sampling method, conducted among adolescents in Northeast China.

Participants: A total of 882 adolescents (aged 12–16 years, 468 boys) were included.

Measurements: All participants underwent anthropometric and biochemical examinations. The thresholds of IR included the 90th percentile of the HOMA-IR for adolescents with a normal body mass index (BMI) and fasting plasma glucose (FPG) level within each sex group (Cutoff A), and the 75th percentile for all participants of the same sex (Cutoff B).

Results: The HOMA-IR was associated with CMRFs. IR, as defined by both cutoffs A and B, was significantly associated with most CMRFs, except decreased HDL-C levels. Excellent concordance ($\kappa = 0.825$) was found between these two criteria in diagnosing IR. However, IR using cutoff A, was more closely associated with cardiometabolic risk. The incidence of IR, as defined by cutoff A, was 18.93% and increased from 10.99% to 43.87% based on the different BMI categories. Further, an anthropometry-based predictive model for IR, incorporating sex, age, waist-to-hip ratio, weight and BMI, was developed and presented as a nomogram.

Conclusions: IR among adolescents is strongly related to cardiometabolic risk. We developed an anthropometry-based predictive nomogram for IR among adolescents, which may facilitate health counselling and self-risk assessments.

Keywords: adolescent, anthropometry, cardiometabolic risk factors, China, homeostasis model assessment, insulin resistance, nomogram

INTRODUCTION

Insulin resistance (IR) is often associated with the cluster of metabolically related cardiovascular risk factors [cardiometabolic risk factors (CMRFs)] that lead to morbidity and mortality worldwide (1–3). Owing to the global epidemic of obesity among children and adolescents, IR is also present in early life and contributes to metabolic disorders in childhood that tend to persist into adulthood (1). It is difficult to directly measure the incidence of IR due to the practical, ethical and economic issues involved with using the hyperinsulinemic-euglycemic clamp, which is considered the gold standard for measuring insulin sensitivity (4). The homeostasis model assessment of IR (HOMA-IR) has been proposed as a robust surrogate method for classifying IR in epidemiological studies (5, 6), and has been found to be more reliable than the fasting plasma glucose-to-insulin ratio and quantitative insulin sensitivity check index for quantifying the degree of IR in children with obesity (7). Additionally, puberty has also been associated with marked IR, and is known as "physiological IR" (1, 8). Among adults, the universally accepted HOMA-IR cutoff value for IR is 2.5 (5). However, among adolescents, a specific cutoff criterion for defining IR is still urgently needed to avoid under- or over-diagnosis. Here, using the data from a population-based study among adolescents in China, we aimed to classify IR in adolescents based on specific criteria, prevalence, and the extent of influences on CMRFs.

Owing to the use of anthropometric methods being convenient, noninvasive, and inexpensive, the importance of using these for the clinical diagnosis of obesity-related health risks is emphasized (9, 10). Overweight and obesity are frequently associated with IR (1, 2, 11). Weight, body mass index (BMI), waist circumference (WC), waist-to-hip ratio (WHR) and waist-to-height ratio (WHtR) are commonly used anthropometric indices for assessing global or local obesity in individuals. When identifying adolescents at risk of IR, anthropometric methods may be effective if the diagnostic values are appropriately specified in a statistical model. The nomogram is a simple and personalized visualization tool, that has been extensively used in the diagnosis of diseases. Here, we further aimed to develop an anthropometry-based predictive nomogram for early detection of IR among adolescents in China.

MATERIALS AND METHODS

Study Design and Data Collection

Data were acquired from a cross-sectional study that used stratified cluster sampling to include a total of 933 adolescents from the urban district of Liaoyang, a moderately sized city in northeast China. A total of 936 participants without known metabolic syndrome (MetS) were recruited, and 3 of which with HbA1c of ≥ 6.5% or FPG of ≥ 7.0 mmol/L were excluded because of the possibility of a diagnosis of diabetes. Ethical permission from the hospital's Ethics Committee and written informed consent from the students and their parents were obtained prior to commencing the original study.

The following data were analyzed for the current study: age, sex, weight, height, WC, hip circumference (HC), systolic blood pressure (SBP), diastolic blood pressure (DBP), hemoglobin A1c (HbA1c), fasting plasma glucose (FPG), fasting plasma insulin (FINS), serum uric acid (SUA), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and a family history of type 2 diabetes mellitus (T2DM, defined as having a first- or second-degree relative with T2DM). A total of 882 participants (aged 12–16 years, 468 boys), without missing data on the variables described above, were included in the current analysis.

Physical examinations were conducted by trained physicians. Anthropometric indices were measured in a standing position while lightly clothed and without shoes. Weight was measured to the nearest 0.1 kg and height was measured to the nearest 0.1 cm. WC and HC were measured at the narrowest point between the iliac crest and the lowest rib during minimal respiration and at the largest girth, respectively. SBP and DBP were measured in the sitting position after a 10-minute rest, and calculated as the mean value of two repeated measurements. BMI was calculated as weight divided by height squared (kg/m^2). WHR and WHtR were calculated as the quotient of the circumference of the waist to that of the hips and WC divided by height, respectively.

Blood samples were collected after an overnight fast (≥10 h). FPG, TG, TC, HDL-C and SUA were measured using an Olympus AU system (Olympus 400, Olympus Optical Company, Japan) in the laboratory at Liaoyang Diabetes Hospital. We further estimated LDL-C using Friedwald's formula (12). HbA1c levels were measured using high performance liquid chromatography with a D-10 Hemoglobin Testing System (Bio-Rad Laboratories, Inc., Hercules, CA) in the central laboratory at Shengjing Hospital of China Medical University. HbA1c was standardized to the Diabetes Complications and Control Trial method. FINS was determined by radioimmunoassay (China Institute of Atomic Energy, Beijing, China). HOMA-IR was calculated as FINS (μU/mL) × FPG (mmol/L)/22.5 (5).

Definitions

Obesity was defined as a BMI ≥95th percentile, and overweight was defined as a BMI ≥85th percentile and <95th percentile for children and adolescents of the same age and sex in China (13). Although IR is affected by pubertal status (8), Tanner stage data were not available for use in our study. Boys aged 12-18 years and girls aged 11-18 years are usually regarded as adolescents in China, because pubertal spurt is between ages 12 and 14 in boys and 11 and 13 in girls (14–18); thus, the participants (aged 12–16 years) in our study were all considered to be adolescents. Currently, there is no universally accepted method for defining the optimal threshold for IR among adolescents. Therefore, we adopted the 90th percentile of the HOMA-IR for adolescents with a normal BMI and FPG level within each sex group (Cutoff A) (7, 19), and the 75th percentile for all participants of the same sex (Cutoff B), as the thresholds of IR (20, 21). IR was identified when the HOMA-IR was above the following cutoff points: A: 5.64 (boy), 5.73 (girl) and B: 5.24 (boy), 5.20 (girl). According to the unified definition of pediatric metabolic syndrome (MetS) by the International

Diabetes Federation (IDF) (22), MetS was identified in adolescents with abdominal obesity (defined as a WC ≥90th percentile in children and adolescents of the same age and sex in China) (23) and at least two of the following four criteria: TG levels of ≥1.70 mmol/L; HDL-C levels of <1.03 mmol/L for individuals aged 10–15 years and boys aged ≥16 years, or < 1.29 mmol/L for girls aged ≥16 years; an SBP of ≥130 mmHg and/or a DBP of ≥85 mmHg; and a FPG level of ≥5.6 mmol/L or T2DM.

Abdominal obesity, hypertension, increased FPG levels, dyslipidemia and hyperuricemia were assessed as CMRFs in this study. We used the definition of MetS (IDF) as a reference, which includes abdominal obesity, hypertension, increased FPG levels, increased TG levels and decreased HDL-C levels. Since neither the IDF nor the recommendations in China included threshold values for TC and LDL-C in children; we adopted the threshold values of a TC level of ≥5.2 mmol/L and a LDL-C level of ≥3.4 mmol/L as proposed by the American Academy of Pediatrics (24). Dyslipidemia was defined as the presence of at least one of the following: hypercholesterolemia, hypertriglyceridemia, hypo-HDL-C, and hyper-LDL-C. Due to the lack of universally accepted clinical diagnostic criteria for hyperuricemia in adolescents, we defined hyperuricemia as a SUA level of ≥357 μmol/L, which is consistent with similar previous studies (25). Clustering of CMRFs was defined as the presence of two or more CMRFs (26).

Statistical Analysis

The Shapiro–Wilk and Levene tests were used to assess the normal distribution and homogeneity of each variable, respectively. Quantitative variables are expressed as the mean ± standard deviation or median (interquartile range) depending on the normality of their distributions, whereas qualitative variables are presented as counts and percentages. Quantitative data were analyzed using the Student's t-test or Mann–Whitney U-test, and qualitative data were analyzed using the Chi-square test. Significance tests for differences in proportions were conducted using the two-tailed two-sample test approach. A partial correlation analysis was used to determine the relationship between HOMA-IR and CMRFs following adjustments for age and sex. We performed binary logistic regression analysis to determine the influence of IR on CMRFs. The kappa coefficient (κ) was calculated to determine the degree of agreement between the different definitions of IR. The prevalence of IR was calculated while considering BMI stratification. A least absolute shrinkage and selection operator (LASSO) regression analysis was used to select the most predictive anthropometric indices for IR (27, 28). Subsequently, a logistic regression analysis was performed to establish a prediction model by introducing the variables based on the LASSO regression, and the nomogram was constructed to achieve individual predictions. To validate the classification abilities of the nomogram, a bootstrapping calibration approach was used and randomly repeated 1000 times with replacement. Decision curve analysis and clinical impact curves were used to determine clinical usefulness. Data processing and statistical analyses were performed using R software (version 4.0.3; R project for Statistical Computing) and *P*-values <0.05 were considered statistically significant.

RESULTS

Patient Characteristics

Among the 882 adolescents (468 boys), the median age was 14 (12–15) years, and 69 had MetS. The demographic data, physical examination results, and biochemical characteristics of the study participants according to gender are presented in **Table 1**. Boys had higher values for weight, BMI, WC, WHR, WHtR, SBP, FPG, SUA and HbA1c. Girls had higher values for TC, DBP and insulin levels, while there were no significant differences in values of HOMA-IR or other indices evaluated. Adolescent males were more likely to have abdominal obesity and hyperglycemia, contributing to a higher incidence of MetS.

Association Between Insulin Resistance and Cardiometabolic Risk Factors

The HOMA-IR was more significantly correlated with WC, FPG, TG, and SUA ($r > 2$, $P < 0.001$), compared with other CMRFs evaluated (**Figure 1**). We analyzed the influence of IR, as defined by the different HOMA-IR thresholds (Cutoffs A and B), on CMRFs using a logistic regression analysis (**Table 2**). Whether adjusted for age and sex or not, IR as defined by both cutoffs A and B, was significantly associated with most CMRFs, except decreased HDL-C levels. More specifically, IR, as defined by cutoff B, was not correlated with hypercholesterolemia, and was associated with dyslipidemia only after adjustments for sex and age. Among participants with IR, the cardiometabolic risk increased by 3.132- and 2.662-fold according to cutoffs A and B, respectively. Excellent concordance ($\kappa = 0.825$, $P < 0.001$) was found between these two criteria in diagnosing IR among adolescents in the Northeast of China (**Table 3**). Owing to the higher odds ratios for CMRFs, IR as defined by cutoff A was chosen for subsequent analyses.

The Incidence of Insulin Resistance Based on Body Mass Index Categories

A total of 167 adolescents were classified as having IR based on cutoff A (90th percentile of the HOMA-IR for adolescents with a normal BMI and FPG level within each sex group), with a prevalence of 18.93%. The prevalence of IR varied among different BMI categories (**Figure 2**), and was highest in adolescents with obesity (43.87%) and lowest in adolescents with a BMI of <85th percentile (10.99%). There were no significant differences in the prevalence of IR between boys and girls either in total or in each subgroup alone.

Development of an Anthropometry-Based Predictive Nomogram for Insulin Resistance

Overweight and obesity are often associated with IR. Weight, BMI, WC, WHR and WHtR are common anthropometric indices used to assess global or local obesity in individuals. Based on the LASSO regression analysis, WHR, weight, and BMI had nonzero coefficients in addition to sex and age, with the greatest predictive power for IR (**Figure 3A**). A model incorporating sex, age, WHR, weight, and BMI was developed based on logistic

TABLE 1 | Characteristics of the study participants.

	Total (n = 882)	Male (n = 468)	Female (n = 414)
Age (years)	14 (12–15)	14 (12–15)	14 (12.25–15)
Ethnic (Han)	786 (89.12%)	420 (89.74%)	366 (88.41%)
WC (cm)	74.5 (69.5,83.0)	77.25 (70.50–87.50)	73.50 (68.50–78.50)***
HC (cm)	93.5 (88.5,99.5)	93.5 (88.5–100.5)	93.5 (88.5–98.5)
WHR	0.81 (0.77,0.85)	0.83 (0.79–0.87)	0.79 (0.75–0.83)***
WHtR	0.46 (0.43–0.50)	0.47 (0.43–0.52)	0.46 (0.43–0.49)*
Weight (kg)	55.5 (48.5–64.5)	59.5 (50.5–72.5)	51.5 (46.5–59.5)***
BMI (kg/m^2)	20.63 (18.55,23.83)	21.23 (18.89–24.95)	19.99 (18.25–22.91)***
SBP (mmHg)	117 (109–126)	121(111–130)	115 (107–122)***
DBP (mmHg)	72.87 ± 10.78	71.76 ± 11.03	74.12 ± 10.35**
Family history of T2DM	487 (55.22%)	255 (54.49%)	232 (56.04%)
FPG (mmol/L)	4.7 (4.4–5.0)	4.8 (4.5–5.1)	4.7 (4.4–5.0)**
TC (mmol/L)	4.46 (3.92–5.04)	4.35 (3.84–4.92)	4.60 (4.03–5.13)***
HDL-C (mmol/L)	1.06 (0.87–1.27)	1.06 (0.85–1.26)	1.06 (0.90–1.29)
TG (mmol/L)	0.94 (0.67–1.28)	0.90 (0.65–1.27)	0.96 (0.70–1.30)
LDL-C (mmol/L)	3.30 (3.25–3.48)	3.30 (3.25–3.47)	3.31 (3.25–3.49)
SUA (μmol/l)	301.50 (248.25,367.00)	347.50 (297.75–412.25)	260.00 (225.25–300.75)***
HbA1c (%)	5.4 (5.3–5.6)	5.5 (5.3–5.6)	5.4 (5.2–5.6)**
FINS (μU/mL)	18 (13–24)	17.00 (12.00–24.13)	19.00 (14.05–24.00)*
HOMA-IR	3.77 (2.72–5.22)	3.71 (2.84–5.23)	3.86 (2.48–5.18)
MetS	69 (7.82%)	53 (11.32%)	16 (3.86%)***
Abdominal obesity	184 (20.86%)	111 (23.72%)	71 (17.63%)*
Hypertension	228 (25.85%)	148 (31.62%)	80 (19.32%)***
Increased FPG	56 (6.35%)	36 (7.69%)	20 (4.83%)
Increased TG	86 (9.75%)	54 (11.54%)	32 (7.73%)
Decreased HDL	432 (48.98%)	230 (49.14%)	202 (48.79%)

Quantitative data are expressed as the mean ± SD or median (25th–75th percentile), and qualitative data as the number (percent).
****P < 0.001, **P < 0.01; *P < 0.05 compared with males.*
BMI, body mass index; DBP, diastolic blood pressure; FINS, fasting plasma insulin; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HC, hip circumference; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; MetS, metabolic syndrome; SBP, systolic blood pressure; SUA, serum uric acid; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglycerides; WC, waist circumference; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio.

regression analyses and presented as a nomogram (**Figure 3B**). The probability of IR was accurately predicted using the calibration curve (**Figure 3C**). The clinical impact and decision curves (**Figures 3D, E**) revealed that our model demonstrated a positive net benefit without increasing the number of false positives.

A dynamic nomogram using a random participant was established to predict the risk of IR (**Figure 3F**). The participant's characteristics were as follows: 12-year-old boy, WHR = 1.08, weight = 81.5 kg and BMI = 32.65 kg/m^2. The total points were 213 and the probability of IR was 67.1%. Therefore, this tool provides an individualized estimate of the risk of IR and should be useful to patients and healthcare providers in the long-term management of adolescents with cardiometabolic risk. We also developed a dynamic nomogram using online software (https://doctordu.shinyapps.io/InsulinResistanceRiskNomogram/).

DISCUSSION

In China, the prevalence of overweight and obesity among children and adolescents has dramatically increased over the decades and in 2019, was 11.1% and 7.9%, respectively (29). With the earlier onset of obesity, it is also likely that the prevalence of IR has increased among children and adolescents, which may explain the concomitant earlier onset of pediatric metabolic disorders (2, 3, 30). Although IR has been extensively studied among adults (31, 32), there remains a lack of population-based studies on classifying IR in adolescents using specific criteria, prevalence, or the extent of influences on CMRFs.

Owing to the influence of pubertal development, it is imperative that the reference ranges used for diagnosis of IR in adolescents are appropriate for the relevant population. Currently, there are no universally agreed upon reference ranges that can be used to diagnose IR in adolescents. We summarized the main cut-off values of HOMA-IR for defining insulin resistance in children and adolescents in recent literatures (**Table 4**). Researchers in Turkey calculated the cut-off values for HOMA-IR by receiver operating characteristic (ROC) analysis, upon the IR criterion of a total blood insulin level exceeding 300 μU/mL during a standard oral glucose tolerance test (7, 38, 39). However, this method is not applicable for use in population-wide screening, owing to the relatively high cost and complexity. It is also inappropriate to apply their cutoff values directly in other races. Consequently, cutoff values for the diagnosis of IR have been defined based on the HOMA-IR distribution of a healthy reference population or the study population itself (19, 21, 33–37). The cutoff values of the HOMA-IR used for defining IR in adolescents varies widely according to ethnicity, age, gender, and screening methods, and ranges from 2.4 to 5.22 (19, 34, 36, 39). Due to the subjectivity and limitation in population studies of adding the Tanner criteria in identifying IR, there is a proposal to use cutoff values for HOMA-IR according to gender and pubertal staging (39). Here, we defined IR using the threshold of the 90th percentile of the HOMA-IR for adolescents with a normal BMI and FPG level within each sex

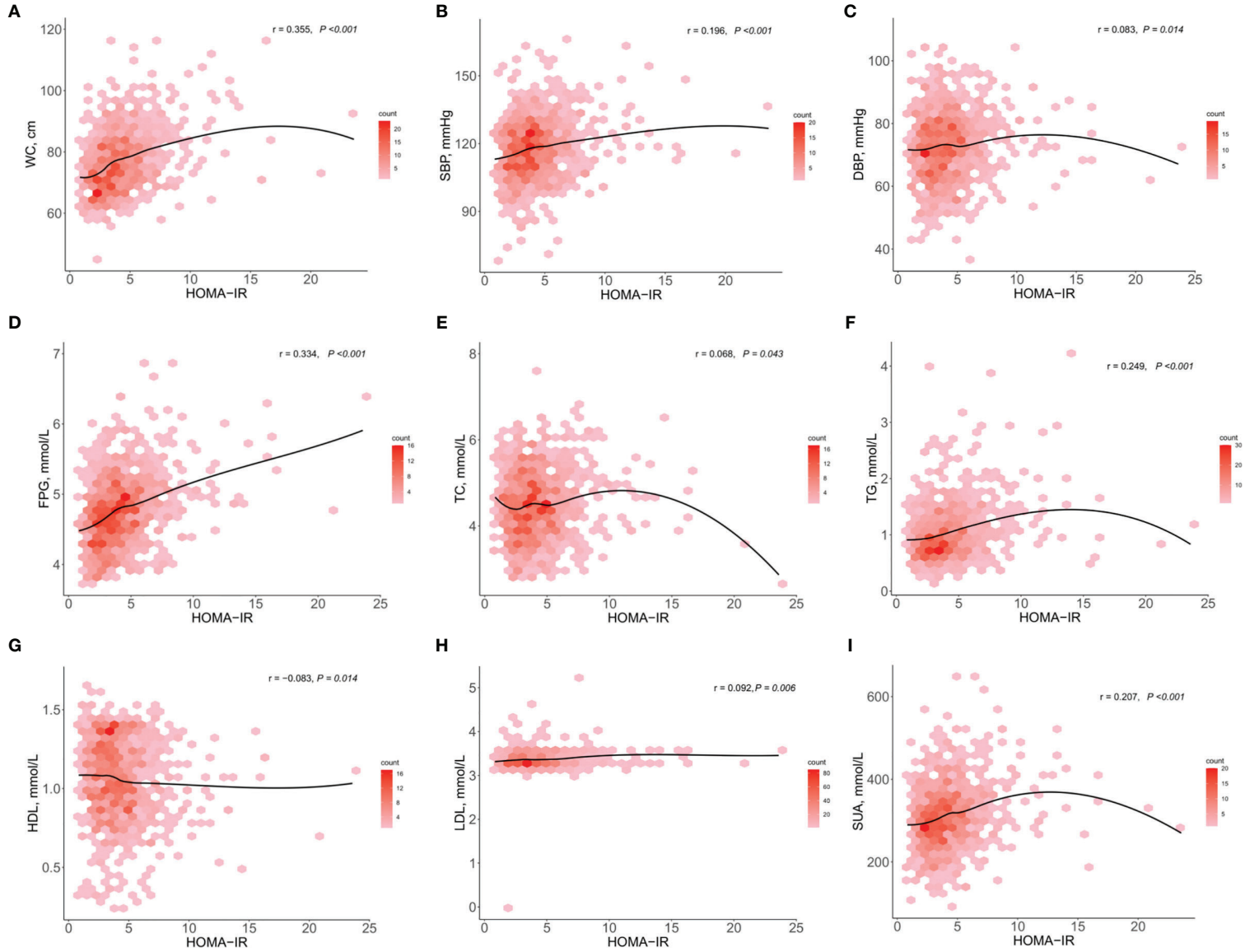


FIGURE 1 | Scatterplots of the homeostasis model assessment of insulin resistance against cardiometabolic risk factors (n = 882). Partial correlation coefficient of the HOMA-IR with WC, SBP, DBP, FPG, TC, TG, HDL, LDL, and SUA **(A–I)**, adjusted for age and sex. Darker red denotes higher density. The black line is a smoothed condition mean, denoting the mean value of the cardiometabolic risk factor at a given HOMA-IR. DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; SUA, serum uric acid; TC, total cholesterol; TG, triglycerides; WC, waist circumference.

TABLE 2 | The influence of insulin resistance on cardiometabolic risk factors according to different definitions of insulin resistance.

Items		Cutoff A	*P*	Cutoff B	*P*
Abdominal obesity	Crude OR (95% CI)	3.081 (2.131–4.454)	<0.001	2.972 (2.104–4.199)	<0.001
	Adjusted OR (95% CI)	3.027 (2.090–4.386)	<0.001	2.973 (2.099–4.210)	<0.001
Hypertension	Crude OR (95% CI)	1.888 (1.318–2.705)	0.001	1.624 (1.164–2.267)	0.004
	Adjusted OR (95% CI)	1.964 (1.359–2.838)	<0.001	1.724 (1.225–2.425)	0.002
Increased FPG	Crude OR (95% CI)	3.885 (2.226–6.780)	<0.001	3.571 (2.063–6.180)	<0.001
	Adjusted OR (95% CI)	3.730 (2.108–6.600)	<0.001	3.468 (1.979–6.077)	<0.001
Dyslipidemia	Crude OR (95% CI)	1.538 (1.011–2.339)	0.044	1.380 (0.956–1.994)	0.086
	Adjusted OR (95% CI)	1.697 (1.108–2.600)	0.015	1.495 (1.028-2.174)	0.035
Increased TC	Crude OR (95% CI)	1.676 (1.133–2.478)	0.01	1.322 (0.914–1.913)	0.138
	Adjusted OR (95% CI)	1.748 (1.177–2.596)	0.006	1.345 (0.927–1.951)	0.119
Hypertriglyceridemia	Crude OR (95% CI)	2.735 (1.697–4.409)	<0.001	2.395 (1.513–3.790)	<0.001
	Adjusted OR (95% CI)	2.624 (1.601–4.300)	<0.001	2.311 (1.439–3.713)	0.001
Increased LDL-C	Crude OR (95% CI)	1.536 (1.091–2.163)	0.014	1.534 (1.124-2.095)	0.007
	Adjusted OR (95% CI)	1.478 (1.044–2.092)	0.028	1.467 (1.070-2.013)	0.017
Decreased HDL-C	Crude OR (95% CI)	1.067 (0.762–1.495)	0.705	1.031 (0.760–1.398)	0.846
	Adjusted OR (95% CI)	1.245 (0.871–1.780)	0.229	1.186 (0.857–1.640)	0.304
Hyperuricemia	Crude OR (95% CI)	2.193 (1.544–3.116)	<0.001	1.694 (1.223–2.346)	0.002
	Adjusted OR (95% CI)	2.465 (1.647–3.691)	<0.001	1.974 (1.361–2.863)	<0.001
Clustering of cardiometabolic risk factors	Crude OR (95% CI)	2.861 (2.010–4.073)	<0.001	2.349 (1.719–3.210)	<0.001
	Adjusted OR (95% CI)	3.132 (2.160–4.540)	<0.001	2.662 (1.913–3.705)	<0.001

Crude OR is the OR before adjusting for sex and age; adjusted OR is the OR after adjusting for sex and age. Clustering of cardiometabolic risk factors: defined as ≥2 cardiometabolic risk factors.

CI, confidence interval; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; OR, odds ratio; TC, total cholesterol.

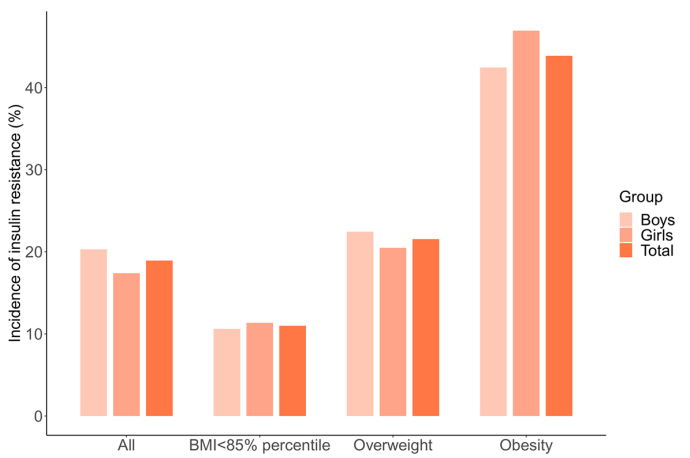
TABLE 3 | Concordance between the different homeostasis model assessment of insulin resistance cutoff values used in diagnosing insulin resistance.

	IR diagnosed by cutoff B				κ (95% CI)	*P*
		-	+	Total		
IR diagnosed by cutoff A	-	662	53	715	0.825 (0.780, 0.870)	<0.001
	+	0	167	167		
	Total	662	220	882		

+ with IR; – without IR.

IR, insulin resistance; CI, confidence interval.

group (5.64 for boys and 5.73 for girls), and the 75th percentile for adolescents of the same sex (5.24 for boys and 5.20 for girls), namely cutoffs A and B. A previous study evaluated the frequencies of dyslipidemia and hyperglycemia in children with IR assessed by cutoff points of HOMA-IR; the frequencies of increased TC, LDL-C and TG, decreased HDL-C were all above 50% (35). Another study demonstrated that in a sample of Korean children and adolescents, metabolic syndrome increased 18.4-fold and each MetS component (including central obesity, hypertriglyceridemia, hypertension, low HDL-C, and hyperglycemia) increased 2.12–7.42-fold among participants



	All	BMI<85% percentile	Overweight	Obesity
Total	18.93	10.99	21.55	43.87
Boys	20.30	10.61	22.45	42.45
Girls	17.39	11.35	20.48	46.94

FIGURE 2 | The incidence of insulin resistance based on body mass index categories.

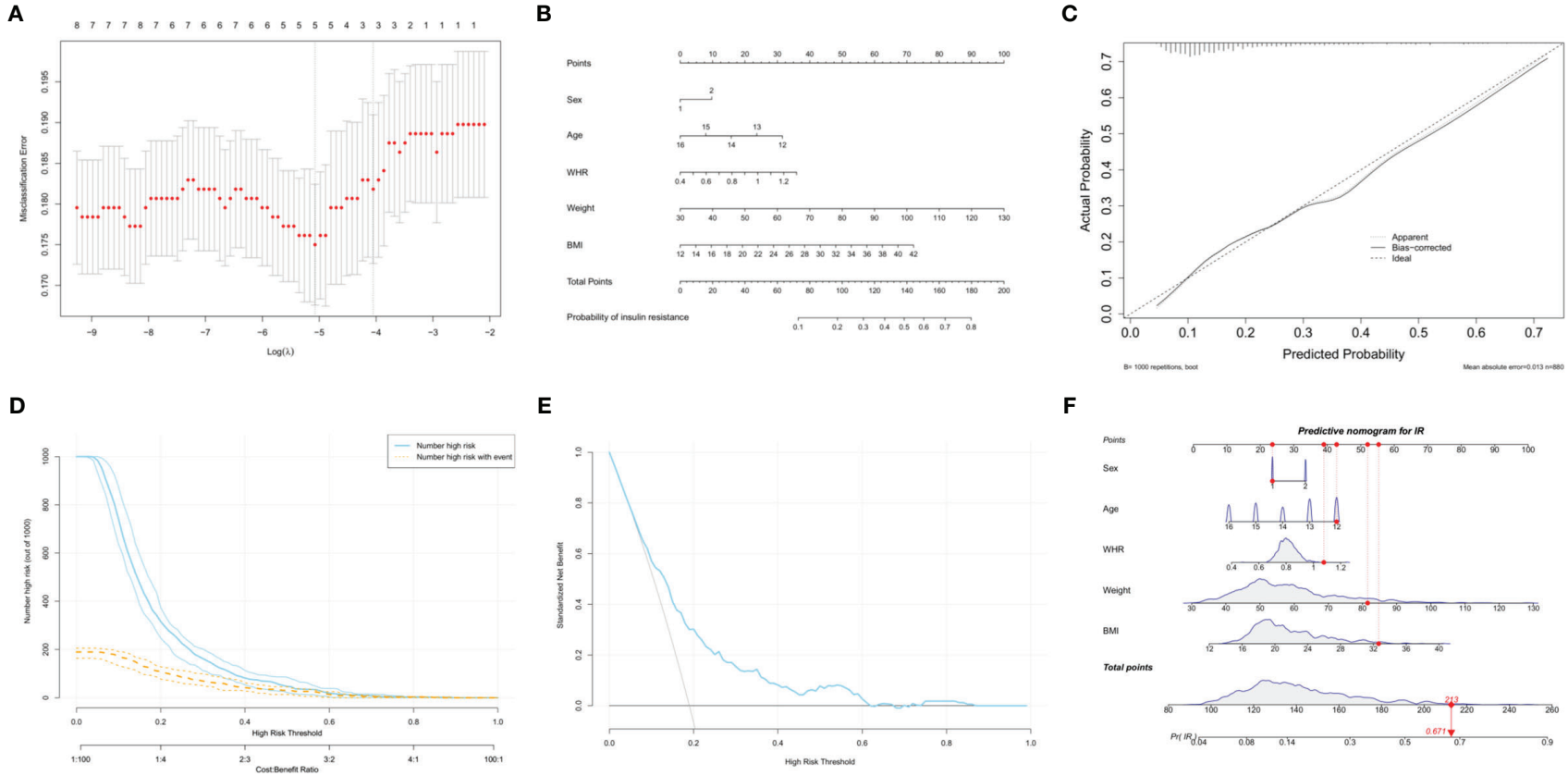


FIGURE 3 | Development of an anthropometry-based nomogram for predicting insulin resistance among adolescents in China. **(A)** The LASSO coefficient profiles of eight variables. The coefficient profile plot was produced against the log(lambda) sequence. Five features with nonzero coefficients were selected by optimal lambda (0.006259), including sex, age, waist-to-hip ratio (WHR), weight and body mass index (BMI). **(B)** An anthropometry-based nomogram for the prediction of insulin resistance (IR) among adolescents in China, that includes sex, age, WHR, weight and BMI. **(C)** The calibration analysis. The x- and y-axes represents the nomogram-predicted probability of IR and the actual rate of IR, respectively. The solid line represents the performance of the nomogram. A more favorable performance is indicated by a closer fit to the ideal calibration line (the diagonal dotted line). **(D)** The clinical impact curve for evaluating clinical use. **(E)** The decision curve for evaluating clinical use. **(F)** Dynamic nomogram. An adolescent was randomly selected from the population, and the risk of IR was predicted using the five anthropometric indices of the nomogram.

TABLE 4 | Main cut-off values of HOMA-IR for defining IR in children and adolescents in recent literatures.

Country	Sample size	Prevalence of IR	Population	Cut-off values	Criteria	Refs
America	1802	52.1% in obese adolescents	Adolescents without diabetes aged 12-19 years	4.39	2 SD above the average	(33)
Argentina	226		Healthy children and adolescents aged 1 to 18 years old	Prepubertal 1.9 (≤ 7.5years 1.4, > 7.5years 2.0), pubertal 2.5 (girls 2.6, boys 2.4)	97th percentile	(34)
Brazil	383	56.10%	Children and adolescents aged 7 to 17.9 years	Boys (7-8.9 years 1.76, 9-10.9 years 1.97, 11-12.9 years 2.65, 13-14.9 years 3.21, 15-17.9 years 2.39), girls (7-8.9 years 1.39, 9-10.9 years 2.62, 11-12.9 years 3.02, 13-14.9 years 3.46, 15-17.9 years 2.89)	2 SD above the average	(35)
Chile	1192		Children and adolescents with normal BMI and fasting blood fasting blood glucose aged 10-15 years	Tanner I and II (boys 3.2, girls 4.1), Tanner III and IV (boys 4.2, girls 5.0)	90th percentile	(19)
China	1037	44.3% in obese participants	Children and adolescents with normal weight status and without any components of metabolic syndrome aged 6 to 18 years	Total 3.0, prepubertal 2.6, pubertal 3.2 6-9 years 2.1, 10-18 years 3.2 Male 3.1, Female 2.9	95th percentile	(36)
China	831		Children and adolescents aged 7 to 18 years	Boys (prepubertal 2.94, pubertal 4.43, postpubertal 4.66), girls (prepubertal 2.62, pubertal 4.56, postpubertal 3.95)	75th percentile	(21)
Korean	2116	4.7%, 25.6%, and 47.1% in normal-weight, over-weight, and obese participants	Children and adolescents with normal BMI and fasting blood fasting blood glucose aged 10 to 20 years	Boys (10-11 years 3.7, 11-12 years 4.4, 12-13 years 4.52, 13-14 years 4.58, 14-15 years 5.01, 15-16 years 4.5, 16-17 years 4.08), girls (10-11 years 4.1, 11-12 years 4.87, 12-13 years 5.65, 13-14 years 5.45, 14-15 years 4.15, 15-16 years 4.09, 16-17 years 4.5)	95th percentile	(37)
Turkey	57	43.86%	Pubertal obese children and adolescents (mean age: 12.04 ± 2.90 years; mean BMI: 29.57 ± 5.53)	3.16	ROC curve	(7)
Turkey	148	37.16%	Obese children and adolescents (mean age: 10.86 ± 3.08 years, mean BMI: 27.7 ± 4.2)	2.7	ROC curve	(38)
Turkey	208	Prepubertal (boys 37%, girls 27.8%), pubertal (boys 61.7%, girls 66.7%)	Obese children and adolescents aged 5 to 18 years	Prepubertal (boys 2.67, girls 2.22), pubertal (boys 5.22, girls 3.82)	ROC curve	(39)

BMI, body mass index; IR, insulin resistance; ROC, receiver operating characteristic curve; SD, standard deviation.

with IR (40). However, they used different cutoff values of HOMA-IR for the diagnosis of IR. Our study focused on more CMRFs and the clustering of CMRFs, and was not limited to only MetS components (36, 41). The partial correlation analysis revealed a significant association between the HOMA-IR and CMRFs, and HOMA-IR was more significantly correlated with WC, FPG, TG, and SUA compared with other CMRFs evaluated. The logistic regression, showed that IR was significantly associated with most CMRFs, except decreased HDL-C levels. Although, the direction of causality among IR and CMRFs could not be determined owing to the cross-sectional nature of this study, adolescents with IR have a 3.132- and 2.662-fold increased risk of clustering of CMRFs based on cutoffs A and B, respectively. Therefore, the early identification of IR is critical for early intervention and prevention of cardiometabolic disorders. The kappa coefficient (κ) suggested excellent agreement between cutoffs A and B for determining IR. However, based on cutoff B, IR was not correlated with hypercholesterolemia, and was associated with dyslipidemia only after adjustments for sex and age. Moreover, IR, as defined by cutoff A, was more closely associated with the clustering of CMRFs than IR as defined by cutoff B. Overall, the results suggested that cutoff A is the optimal indicator for clinical application. Using cutoff A, we further assessed the incidence of IR, which was 18.93% in this study, and increased from 10.99% to 43.87% based on the different BMI categories. This finding was similar to that of previous population-based studies (36, 37). Several studies have reported widely varying prevalence rates of IR among adolescents with obesity in different countries, such as the United States (52.1%) (33) and Brazil (29.1%) (42). Similar to other studies, there were no obvious differences based on sex in the frequency of IR (35, 40, 41).

Although several studies have been conducted to identify the anthropometric indices for IR among adolescents (42–44), few studies have integrated multiple anthropometric indices in a prediction model or scoring system to predict the individual risk of IR among adolescents. Nomograms, which allow for the seamless incorporation of risk prediction into clinical decision making, are widely used as personalized risk-prediction tools. According to the LASSO analysis, age, gender, BMI, weight, and WHR are important predictors of IR in adolescents. Our study quantified the above factors in a statistical prediction model, which we converted to a clinically usable nomogram that potentially illustrates the extent to which individual risk indicators contribute to the risk of IR. Its classifying power and clinical use were supported by the calibration, clinical impact and decision curves. As depicted in our example, the probability of a 12-year-old boy with a WHR of 1.08, weight of 81.5 kg, and BMI of 32.65 kg/m^2, having IR is 67.1%. This was the first study to develop an anthropometry-based nomogram for predicting individualized probabilities of IR among adolescents in China.

This study has some limitations. The Tanner stage data, which may be necessary for accurately classifying adolescence, were not included. Owing to the limited sample size, we were unable to stratify the analyses by age and pubertal stage (early, mid, and late puberty). Further research focused on the association of IR with other CMRFs (such as nonalcoholic fatty liver disease) among adolescents are awaited to make the results more valuable for reference. Our study included a single-center cohort, which was not representative of the entire adolescent population in China. Further evaluation in large-scaled, multicenter-study populations is imperative to generalize these findings.

In conclusions, IR, as defined by the HOMA-IR, was significantly associated with an increased cardiometabolic risk. For defining IR among adolescents in China, the optimal thresholds, using the HOMA-IR, were above 5.64 for boys and 5.73 for girls. The incidence of IR increases with an increased BMI, and is highest among adolescents with obesity. Additionally, we developed an anthropometry-based nomogram for predicting IR among adolescents, that incorporated sex, age, WHR, weight and BMI, and which may facilitate health counselling, self-risk assessments and early detection of IR.

DATA AVAILABILITY STATEMENT

The processed data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Liaoyang Diabetes Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RD and YW conceptualized the study, carried out the initial analyses, and drafted the initial manuscript. PL and LL designed the data collection instruments, collected data, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Burden of Type 2 Diabetes and Associated Cardiometabolic Traits and Their Heritability Estimates in Endogamous Ethnic Groups of India: Findings From the INDIGENIUS Consortium

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To assess the burden of type 2 diabetes (T2D) and its genetic profile in endogamous populations of India given the paucity of data, we aimed to determine the prevalence of T2D and estimate its heritability using family-based cohorts from three distinct Endogamous Ethnic Groups (EEGs) representing Northern (Rajasthan [Agarwals: AG]) and Southern (Tamil Nadu [Chettiars: CH] and Andhra Pradesh [Reddys: RE]) states of India. For comparison, family-based data collected previously from another North Indian Punjabi Sikh (SI) EEG was used. In addition, we examined various T2D-related cardiometabolic traits and determined their heritabilities. These studies were conducted

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as part of the Indian Diabetes Genetic Studies in collaboration with US (INDIGENIUS) Consortium. The pedigree, demographic, phenotypic, covariate data and samples were collected from the CH, AG, and RE EEGs. The status of T2D was defined by ADA guidelines (fasting glucose ≥ 126 mg/dl or HbA1c ≥ 6.5% and/or use of diabetes medication/history). The prevalence of T2D in CH (N = 517, families = 21, mean age = 47y, mean BMI = 27), AG (N = 530, Families = 25, mean age = 43y, mean BMI = 27), and RE (N = 500, Families = 22, mean age = 46y, mean BMI = 27) was found to be 33%, 37%, and 36%, respectively, Also, the study participants from these EEGs were found to be at increased cardiometabolic risk (e.g., obesity and prediabetes). Similar characteristics for the SI EEG (N = 1,260, Families = 324, Age = 51y, BMI = 27, T2D = 75%) were obtained previously. We used the variance components approach to carry out genetic analyses after adjusting for covariate effects. The heritability (h^2) estimates of T2D in the CH, RE, SI, and AG were found to be 30%, 46%, 54%, and 82% respectively, and statistically significant ($P \leq 0.05$). Other T2D related traits (e.g., BMI, lipids, blood pressure) in AG, CH, and RE EEGs exhibited strong additive genetic influences (h^2 range: 17% [triglycerides/AG and hs-CRP/RE] - 86% [glucose/non-T2D/AG]). Our findings highlight the high burden of T2D in Indian EEGs with significant and differential additive genetic influences on T2D and related traits.

Keywords: type 2 diabetes, cardiometabolic traits, Indian population, epidemiology, genetic epidemiology, family study, heritability

INTRODUCTION

Type 2 diabetes (T2D) is a complex blood glucose-homeostasis disorder characterized by both insulin resistance and pancreatic β-cell dysfunction (1). The compound burden of an increasing global T2D epidemic together with its comorbid conditions such as obesity, hypertension, and cardiovascular disease (CVD) has become a major global public health problem, particularly in countries such as China, India, and the United States (US) (2–5). Indeed, Asia including the Indian subcontinent has become the epicenter of the escalating diabetes epidemic; currently, India has the second highest number of people affected with diabetes worldwide next to China (4–6). According to the International Diabetes Federation (IDF), an estimated 77 million people (20-79 years) have diabetes in India in 2019, which is projected to be 101 million people in 2030 and ~134 million people in 2045, respectively (4). Given its unique population genetic background and cultural history, the contemporary Indian population is composed of numerous sub-populations (e.g., tribal vs. caste [from now onwards referred at as Endogamous Ethnic Groups/EEGs] groups) with remarkable cultural, linguistic, regional, and genetic diversity (7–9).

Numerous epidemiological studies, local and national, have shown that the occurrence of T2D exhibits remarkable variation by geography (rural vs. urban and Northern vs. Southern regions of India) and socio-economic status (3, 5, 10–18). For example, the national Indian Council of Medical Research (ICMR)-INdia DIABetes (INDIAB) population-based study involving 15 states of India estimated the prevalence of T2D to be 7.3%; and, it varied by state/region, ranging from 4.3% in Bihar to 10.0% in Punjab and was higher in urban areas than in rural areas (3). Uniquely, Indian populations (and other South Asian [SA] populations), compared with other populations, are at increased risk for the development of T2D at younger ages and at lower body mass index (BMI) levels (18–22). It is shown that Indians have increased levels of insulin resistance and a stronger genetic predisposition to T2D (23–27).

Although India represents nearly one fifth of the global population, there have only been a few genome-wide association studies (GWASs) of T2D involving populations in India including our own study of the Punjabi Sikh population or immigrant populations of Indian ancestry, which localized a few T2D susceptibility loci (28–32). In addition, there is a paucity of data on EEG specific family-based genetic epidemiological studies (26, 33–37). In general, Indian populations are ideal to conduct genetic epidemiologic investigations of complex diseases such as T2D and obesity, given their high levels of endogamy, large family structures, historic admixture patterns mirroring a North-South gradient (Ancestral North Indians vs. Ancestral South Indians), and a high degree of genetic differentiation among them reflecting the importance of local biocultural backgrounds (38–42).

Therefore, the purpose of the current study is to compare the epidemiological (e.g., phenotype differences) and genetic epidemiological (e.g., genetic and environmental influences) profiles among four EEGs based on pedigree-based data sets, two representing north Indian states of Punjab (data already available) and Rajasthan and other two representing the south Indian states of Tamil Nadu and Andhra Pradesh. It should be noted that, in addition to the determination of overall genetic

and environmental influences on a given phenotype based on pedigree information, compared to population-based studies, pedigree-based studies provide several advantages to the identification of rare variation, the main advantage being that rarer variants (with larger effect sizes) will be present at a much higher frequency than in the general population (43, 44). Our follow-up studies will assess the extent to which the common and rare variants to be found through targeted sequencing of selected SA-specific T2D risk loci including our own GWAS T2D signal found in the Sikh population are transportable to other three EEGs to be examined in this study. Thus, here we report the findings of T2D burden in the families of the selected EEGs and genetic and environmental influences on T2D and its related cardiometabolic phenotypes.

MATERIALS AND METHODS

Indian Diabetes Genetic Studies in Collaboration With US (INDIGENIUS) Consortium

As part of the joint Indian Council of Medical Research (ICMR) and National Institute of Diabetes and Digestive and Kidney Diseases/National Institutes of Health (NIDDK/NIH), US, Collaborative Research Partnership (CRP), we developed a new Indo-US bilateral CRP on genetics of diabetes research. The research activities of this study were initiated after obtaining the project-specific (i.e., Indian and US Institutions) Institutional Review Board (IRB) approvals in accordance with both the Government of India and the US regulations for the protection of human subjects as well as institution-specific collaborative research policies. Prior to the conduct of the study, a workshop/symposium involving the Indian and US investigators was conducted at Sri Ramachandra Institute for Higher Education and Research (SRIHER), Chennai to present and discuss the country-specific protocols, standardization of research procedures and conduct, and the overall collaborative structure to establish the INDIGENIUS Consortium.

Study Design, Populations (i.e., EEGs), and Recruitment

The study design was developed jointly by the Indian and US investigators. As part of the ICMR project, three independent Family Diabetes Research Centers (FDRCs), the Tamil Nadu Family Diabetes Study (TNFDS), Chennai, Tamil Nadu (South India); the Jaipur Family Diabetes Study (JFDS), Jaipur, Rajasthan (Northwest India); and the Nellore Family Diabetes Study (NFDS), Nellore, Andhra Pradesh (South India) were established. The FDRC at SRIHER, Chennai has served as the Data Coordinating Center (DCC). Each of the FDRCs recruitment goals were 500 individuals from ~20 large families; and the families were ascertained on probands that were previously identified as having T2D based on medical records or information from existing case registries available at the FDRCs. The study design and methodological tools used are summarized in **Figure 1**.

The planned sample size of 500 individuals per EGG corresponds to the general target heritability of 0.20. After the actual recruitment, we found that the following heritability estimates per EEG/pedigree structures could be detected at 80% power: TNFDS = 0.210, NFDS = 0.215, and JFDS = 0.163. Our sampling strategy was to recruit study participants in random order without any attempt to recruit multiplex families preferentially. Thus, a family from each EEG was ascertained through a single proband with T2D. The T2D probands in this study constitute a community-based case series of T2D, representing a community-based sample of pedigrees. Once a family was identified for recruitment, all 1st, 2nd, and 3rd degree relatives (T2Ds and non-T2Ds), aged from 18 years or above (i.e., adults), living in a household and its surroundings were invited to participate in a given FDRC study. In addition, every effort was made to recruit family members away from homes as much as possible. Children aged 17 years and below were not recruited for this study. All of the field activities including family member recruitment; collection of demographic, phenotypic and covariate data; and, collection of blood specimens were performed under the direct supervision of the primary investigators of a given FDRC, who were assisted by the research assistants and/or the clinical staff.

The JFDS recruited families from the Agarwal EEG, one of the largest business communities in India found throughout northern India including the state of Rajasthan (45). The NFDS recruited families from the Reddy EEG, one of the dominant farming communities composed of wealthy landowners, businessmen, and people in other professions including government jobs mainly inhabited in the state of Andhra Pradesh (46). The TNFDS recruited families from the Chettiar EEG, a sub-group of the Tamil population originating from Chettinad in Tamil Nadu (47). Chettinadu literally means Chettiars' state. It is a community of traders and financiers for many centuries. The EEG identity was self-declared by study participants with information supported by parental and grandparental native backgrounds. The data from a Sikh Khatri EEG from the state of Punjab were already collected and used in this study for the purpose of comparisons. Through NIH support, over 4,700 individuals were recruited as part of the Asian Indian Diabetic Heart Study/Sikh Diabetes Study [AIDHS/SDS], and the details of these studies were reported previously (34, 36, 48). Briefly, the Sikh Khatri EEG family study (Phase I of the AIDHS/SDS) was comprised of 1,260 individuals distributed across 340 families (mostly nuclear in nature) for whom demographic, phenotypic/covariate data, and blood samples were already available (30, 34, 36, 49, 50). The Agarwal and Sikh EEGs are speakers of Indo-Europeans languages, and the Reddy and Chettiar EEGs are speakers of Dravidian languages. The geographic locations of the study sites are depicted in **Figure 2**.

Phenotypic and Covariate Data Collection

The pedigree, demographic, phenotypic, environmental/covariate data and blood/urine samples were obtained through family/household visits (TNFDS - Chidambaram/Karaikudi and vicinities and JFDS - Jaipur) and clinic visits (NFDS - Nellore) by

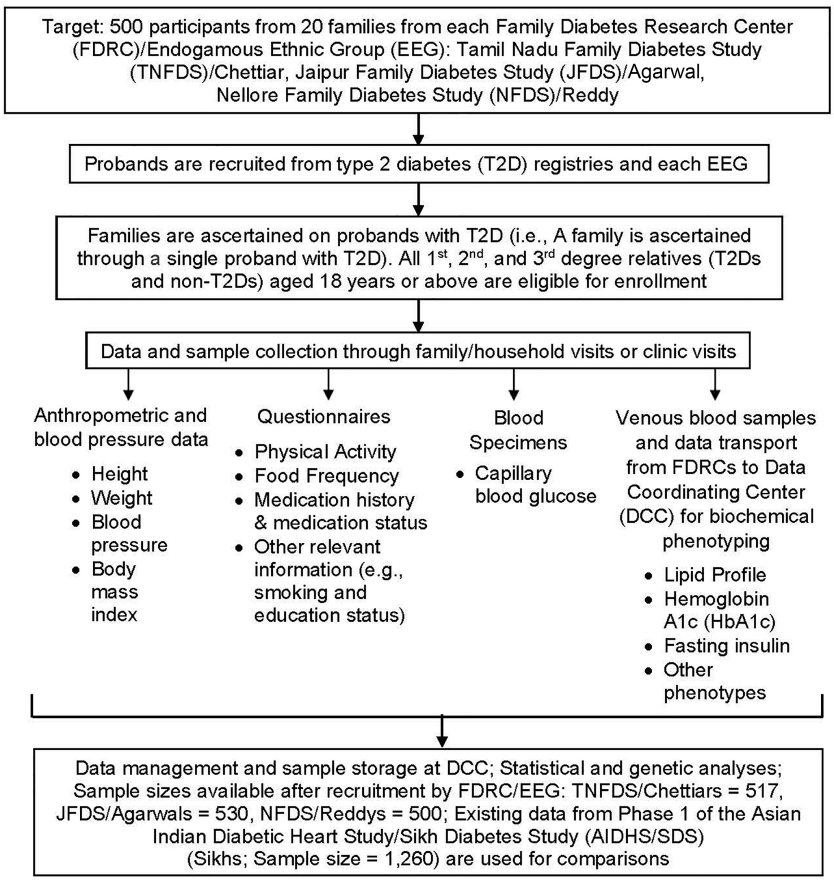

FIGURE 1 | Flow chart depicting study design and methodological tools including recruitment, data collection, and analyses.

the trained research/clinical staff members of each of the three FDRCs during the years 2016-2018. In regard to the TNFDS, family members were recruited from the towns of Chidambaram and Karaikudi (straight line distance between the two towns is approximately 110 miles) and their surrounding areas. Anthropometric data including weight, height, waist and hip circumferences (i.e., average of three values collected for a given trait) were collected using standardized procedures (51). Body mass index (BMI) was measured as weight (kg) divided by height (m^2). Systolic and diastolic blood pressure and heart rate (i.e., average of three values collected for a given trait) were measured using Omron HEM -8712 blood pressure monitor. Fasting (at least 8-hour overnight fast) and post prandial capillary blood glucose levels were measured using Accu-Chek instant S glucometer at the study sites. The serum and EDTA blood samples and urine samples collected from all the participants from study sites were transported on dry ice within 24 hrs to the DCC at SRIHER for processing and biochemical analysis in an accredited laboratory and storage of biospecimens for use in the future. All assays were performed using standardized procedures, and all data were screened through standard QC measures prior to data analyses. Fasting plasma total cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol were measured based on enzymatic photometric method using AU680 Clinical chemistry analyzer (Beckman Coulter Inc, Indianapolis, US). Hemoglobin A1c (HbA1c) levels were measured based on the principle of high performance liquid chromatography method using automated D-10 hemoglobin testing system (Bio-Rad Laboratories Inc., Hercules, CA, US). Fasting serum insulin was quantified based on chemiluminescent immunoassay using DxH 800 hematology analyzer (Beckman Coulter Inc., Indianapolis, US). Serum and urine creatinine levels were determined by Jaffe's method using AU5800 Clinical chemistry analyzer (Beckman Coulter Inc., Indianapolis, US). The high-sensitivity C-reactive Protein (hs-CRP) was measured using the AU5800 Beckman coulter system.

T2D was defined by fasting capillary blood glucose ≥ 126 mg/dl and/or HbA1c ≥ 6.5% (52). Participants who did not meet these criteria but who reported that they were under treatment with either oral antidiabetic agents or insulin and who gave a history of diabetes were also considered to have T2D. In addition, all non-T2D study participants were examined for the presence of prediabetes using the following criteria: fasting capillary blood glucose = 100-125 mg/dl and/or HbA1c = 5.7% - 6.4% (52). From the fasting glucose and insulin concentrations, we estimated insulin resistance using the homeostasis model assessment of insulin resistance (HOMA-IR) (53). Following the

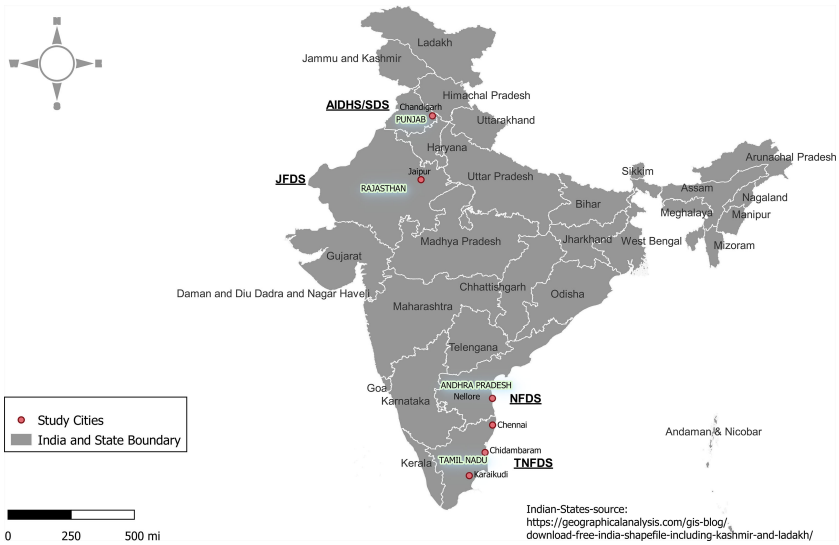

FIGURE 2 | The geographic locations of the study sites in the states of Andhra Pradesh, Punjab, Rajasthan, and Tamil Nadu in India.

World Health Organization (WHO) Asia Pacific Guidelines (54) and the Phase I of the ICMR-INDIAB study on obesity (55), generalized obesity (GO) was defined as a BMI ≥ 25 kg/m^2 for both genders and abdominal obesity (AO) as a waist circumference ≥ 90 cm for men and ≥ 80 cm for women with or without GO. A questionnaire was used to collect information on demographic and environmental factors and covariate data including household information, family history of diabetes, medical history of diabetes and related health conditions, medication status, smoking status, alcohol consumption, socioeconomic status, educational status, psychological or behavioral attributes, dietary intake, and physical activity based on standardized questionnaires (56–58).

Statistical/Genetic Analysis

Different statistical techniques were used to analyze the data including descriptive statistics. Group differences between the three EEGs were examined using ANOVA (Continuous traits) or Chi-square test (Discrete traits); superscript letters a, b, and c were used to refer to homogenous groups identified by Bonferroni's *post hoc* contrast; and, similarities were denoted by sharing the same letter. The hierarchical multiple logistic regression analysis was used to assess association between T2D and correlated factors (e.g., GO, Education, EEG) through odds ratio (OR) statistic. For this analysis, the combined sample of the three EEGs with blocks (i.e., Block 1 = sex, age/groups, and EEG; Block 2 = obesity types (e.g., GO); and Block 3 = Socioeconomic status, Education status, Smoking status, and Alcohol consumption status) was used. All variables were analyzed as dummy variables. Block 1 variables were held constant, and Blocks 2 and 3 were analyzed using the backward elimination procedure (P-values for entry and retention were 0.05 and 0.10, respectively). All analyses were carried out using IBM® SPSS.

The heritabilities of T2D and its related traits were determined using a variance components (VC) approach as implemented in the program SOLAR. To address the issue of non-normality, all quantitative traits were transformed using inverse normal transformation. In a simple model, variances or covariances between relatives as a function of the genetic relationships were specified, and the proportion of phenotypic variance that is attributed to (additive) genetic effects (i.e., heritability: h^2) was estimated from the components of variance (59, 60). For such a model, the covariance matrix for a family (Ω) is given by: $\boldsymbol{\Omega} = 2\Phi\sigma_g^2 + I\sigma_e^2$, where Φ is the kinship matrix, σ_g^2 is the genetic variance due to additive genetic effects, I is the identity matrix, and σ_e^2 is the variance due to individual-specific environmental effects. A likelihood ratio test was used to test whether the heritability of a given trait is significant ($P \leq 0.05$). Covariates (i.e., age, sex, age x sex, age^2, age^2 x sex, or BMI [T2D analysis only] were included in all analyses if found to be significant ($P \leq 0.10$). This method was extended to the dichotomous traits such as T2D, using a threshold or liability model (61). Given our family ascertainment scheme, all genetic analyses were performed by correcting for the ascertainment, as described previously (62). All genetic analyses were performed using the computer program SOLAR (63).

RESULTS

Given the goal of enrolling 1,500 individuals from the three studies, we actually recruited 1,547 individuals from the three study sites: TNFDS = 517 (518 individuals were recruited, but one individual with type 1 diabetes was excluded from all analyses) from 21 families; JFDS = 530 from 25 families; and NFDS = 500 from 22 families (**Table 1**). The CH, AG, RE, and SI

abbreviations are used from here forth to refer to the EEGs of Chettiars (TNFDS), Agarwals (JFDS), Reddys (NFDS), and Sikhs (AIDHS/SDS; for the purpose of comparison based on available data), respectively. The average family size in the combined sample of the three EEGS (i.e., CH, AG, and RE) is ~23, which ranged from 12-41. The characteristics of the study participants by EEG/FDRC for the demographic and selected T2D and its related glycemic and other cardiometabolic traits for this study are shown in **Table 1**, including prediabetes, generalized obesity (GO), abdominal obesity (AO), BMI, waist circumference (WC), fasting capillary blood glucose (FG), HOMA-IR, fasting insulin (FI), HbA1c, total cholesterol (TCHOL), triglycerides (TG), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), systolic (SBP) and diastolic (DBP) blood pressure, and high-sensitivity CRP (hs-CRP).

The characteristics of the participants of TNFDS (CH), JFDS (AG), and NFDS (RE) are reported in **Table 1**. A number of variables exhibited significant differences ($P \leq 0.05$) between the EEGs, while the differences regarding traits such as T2D prevalence, age of T2D onset, BMI, and HbA1c were found to be non-significant. As can be seen, above 50% of the study participants from CH and RE EEGs were females, while 41% of the AG sample were females. Based on average ages, the AG sample was relatively younger (43 y) compared to CH (47 y) and RE (46 y), respectively. The prevalence of T2D was found to be high, ranging from 33% (CH) to 37% (AG), while the average

TABLE 1 | Characteristics of the participants of TNFDS, JFDS, and NFDS.

EEG (FDRC, N)[#] / Variable[@]	All FDRCs (N = 1,547)	Chettiar (CH)TNFDS (N = 517)	Agarwal (AG)JFDS (N = 530)	Reddy (RE)NFDS (N = 500)	P-value^
Number of families	68	21	25	22	–
Average family size	23	25	21	23	–
Family size range	12-41	14-41	12-35	12-39	–
Age (years)	45.48 (16.37)	47.18[a] (16.38)	43.41[b] (16.44)	45.92[a,b] (16.09)^	<0.001
Age of T2D onset (years)	48.57 (12.25)	50.41 (11.55)	47.35 (11.59)	48.16 (13.40)	*0.051*
BMI (kg/m^2)	26.70 (5.19)	26.81 (5.29)	26.83 (4.70)	26.45 (5.55)	*0.420*
Waist circumference [WC] (cm)	95.4 (13.23)	97.98[a] (13.69)	93.36[b] (11.96)	94.89[b] (13.60)	<0.001
Fasting blood glucose [FG] (mg/dL)[$]	119.3 (42.72)	125.5[a] (51.29)	118.5[b] (29.76)	113.6[b] (43.85)	<0.001
HOMA-IR[$]	3.41 (7.42)	2.89[b] (2.69)	4.44[a] (8.10)	2.84[b] (9.58)	<0.001
Fasting Insulin [FI] (IU/ml)[$]	10.97 (17.47)	9.22[b] (6.75)	14.29[a] (22.18)	9.27[b] (18.96)	<0.001
HbA1c (%)[$]	6.40 (1.73)	6.34 (1.81)	6.52 (1.64)	6.32 (1.75)	*0.110*
Total cholesterol [TCHOL] (mg/dL)	161.1 (46.52)	180.5[a] (40.92)	145.0[c] (48.44)	158.2[b] (42.56)	<0.001
Triglycerides [TG] (mg/dL)	125.9 (80.41)	136.0[a] (86.65)	118.2[b] (70.13)	123.6[b] (82.95)	0.001
LDL-Cholesterol [LDL-C] (mg/dL)	100.8 (35.53)	116.2[a] (33.45)	86.88[c] (34.89)	99.77[b] (31.78)	<0.001
HDL-Cholesterol [HDL-C] (mg/dL)	43.98 (13.94)	47.13[a] (12.26)	40.55[c] (15.97)	44.36[b] (12.38)	<0.001
Systolic blood pressure [SBP] (mmHg)	128.0 (18.79)	132.6[a] (18.66)	125.5[b] (18.43)	125.9[b] (18.46)	<0.001
Diastolic blood pressure [DBP] (mmHg)	81.98 (11.41)	82.80[a] (11.28)	82.42[a] (10.74)	80.67[b] (12.13)	0.007
High-sensitivity CRP [hs-CRP] (mg/L)	0.44 (0.79)	0.51 (1.02)[a]	0.35 (0.47)[b]	0.45 (0.79)[a]	<0.001
Sex (F/M, %F)	761/786 (49)	267/250[a] (52)	217/313[b] (41)	277/223[a] (55)	<0.001
T2D (n,%)	543 (35)	169 (33)	196 (37)	178 (36)	*0.333*
Prediabetes (n,%)	606 (60)	228[a] (66)	217[a] (65)	161[b] (50)	<0.001
Generalized obesity (GO; n/%)	957 (62)	311[a,b] (60)	353[b] (67)	293[a] (59)	*0.190*
Abdominal obesity (AO; n/%)	1257 (81)	445[a] (86)	406[b] (77)	406[a,b] (81)	<0.001
Education Status (n, %)					
Uneducated	102 (6.59)	5[a] (0.96)	2[a] (0.37)	95[b] (19)	<0.001
School	675 (43.6)	268[a] (51.8)	133[b] (25.0)	274[a] (54.8)	
Graduate	576 (37.2)	180[a] (34.8)	298[b] (56.2)	98[c] (19.6)	
Postgraduate	194 (12.5)	64[a] (12.3)	97[b] (18.3)	33[c] (6.6)	
Socioeconomic Status (n, %)					
Lower	29 (1.87)	3[a] (0.58)	0[a] (0)	26[b] (5.2)	<0.001
Middle	1314 (84.9)	491[a] (94.9)	383[b] (72.2)	440[c] (88)	
Upper	204 (13.1)	23[a] (4.44)	147[b] (27.7)	34[a] (6.8)	
Smoking Status (n, %)					
Never	1382 (89.3)	495[a] (95.7)	433[b] (81.7)	454[c] (90.8)	<0.001
Former	51 (3.3)	7[a] (1.4)	22[b] (4.2)	22[b] (4.4)	
Current	114 (7.4)	15[a] (2.9)	75[b] (14.2)	24[a] (4.8)	
Alcohol Consumption Status (n, %)					
Never	1393 (91.3)	493[a] (95.4)	436[b] (85.7)	464[a] (92.8)	<0.001
Former	30 (2)	5[a] (1)	10[a] (2)	30[a] (3)	
Current	103 (6.7)	19[a] (3.7)	63[b] (12.4)	21[a] (4.2)	

[#]EEG, Endogamous ethnic group; FDRC, Family Diabetes Research Center; N, Sample size; [@]BMI, Body Mass Index; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; HbA1c, Hemoglobin A1c; High-sensitivity CRP (hs-CRP), High-sensitivity C-Reactive Protein. Continuous variables are expressed as mean (standard deviation) and Discrete variables are shown as counts and percentages (n, %), Group differences are tested using Analysis of variance (Continuous traits) or Chi-square test (Discrete traits) - superscript letters a, b, and c refer to homogenous groups identified by Bonferroni's post hoc contrast and similarities are denoted by sharing the same letter. Please see text for trait definitions; ^Includes one participant aged 15 years; ^Non-significant differences (i.e., P > 0.05) are shown in italics, and P-values are not corrected for non-independence; [$]Glycemic traits information including individuals with T2D (on medication) is provided for the purpose of comparison; however, their information related to non-T2D individuals is provided in ***Table 2****.*

BMI among the three EEGs was found to be more or less similar (~27). The prevalence rates of obesity and prediabetes were also found to be high among the three EEGs. The differences in GO (range: 59%-67%) between EEGs were not significant, while AO exhibited significant differences between EEGs ranging from 77% (AG) to 86% (CH). The prevalence rates of prediabetes differed significantly among EEGs (range: 50% [RE]-66% [CH]). Although the three EEGs differed from each other regarding a number of T2D-related traits, certain EEG pairs exhibited non-significant differences or similarities in regard to certain traits (**Table 1**). For example, CH and RE contrasted with AG in regard to FI, HOMA-IR, and hs-CRP, while CH contrasted with AG and RE regarding WC, FG, TG, and SBP.

As reported in **Table 1**, significant differences were found regarding education, socioeconomic, smoking, and alcohol consumption statuses among the three EEGs. For example, more than 56% of AGs had graduate level education compared to 35% in CH and 20% in RE, respectively, while 19% of RE sample were uneducated versus less than 1% uneducated individuals from CH and AG EEGs. A majority of the families from the three EEGs were reported to belong middle socioeconomic status (range: 72% [AG] - 95% [CH]), and about 28% of AGs were found to belong to upper socioeconomic status compared to 4% (CH) and 7% (RE), respectively. The smoking and alcohol consumption behaviors (males only) were largely absent in the three EEGs. However, there were more smokers (former/current) in AG (4.0%/14.2%) and RE (4.4%/4.8%) compared to CH (1.4%/2.9%) and more alcohol drinkers (former/current) in AG (2%/12%) compared to RE (3%/4%) and CH (1%-4%), respectively.

The results from hierarchical logistic regression analysis of the combined sample of the three EEGs including the significant predictors of T2D are shown in **Table 2**. As can be seen, the occurrence of T2D was more in males and T2D's risk is increased by age category in a stepwise fashion in reference to the age group 24 years and below. For example, based on odds ratio (OR), individuals in age group 35-44 years are approximately 7 times more likely to be T2D, while those in age group 65 and above years are approximately 42 times more likely to have T2D. In reference to CH, AG EEG is almost 2 times more likely to have T2D. AO is a strong correlate of T2D; individuals with AO are 2 times more likely to have T2D. Of the demographic and habitual behavioral traits considered, former smokers are more than 2 times likely to be affected with T2D.

The characteristics of selected traits by T2D status and EEG and the findings of group differences between EEGs are reported in **Table 3**. For the purpose of comparison, the SI data are included. In general, the trait differences between T2D and non-T2D individuals within a given EEG were found to be as expected. For example, FG means of T2D individuals ranged from 147.2 mg/dl (AG) to 188.6 mg/dl (SI), and CH and SI FG profiles differed from both AG and RE, respectively. In non-T2D individuals, it ranged from 93.74 mg/dl (RE) to 102.2 mg/dl (CH), and CH and AG as homogenous groups differed from homogenous groups of RE and SI. In regard to mean TG values, it ranged from 144.3 mg/dl (AG) to 187.9 mg/dl (SI) in individuals with T2D; although SI shares similarity with CH, it differs from AG and RE in mean TG profiles, respectively. In non-T2D individuals, mean TG values ranged from 102.8 mg/dl (AG) to 158.9 mg/dl (SI); SI exhibits its distinction from the other three groups; and RE aligns with both CH and AG groups, although CH and AG fail to be homogenous groups. The mean FI values in non-T2D individuals ranged from 7.39 (IU/ml) (RE) to 12.24 (IU/ml) (SI); the AG and SI groups are found to be homogenous in their mean FI profiles and they differed from both CH and RE, respectively.

Since T2D and its related traits are complex phenotypes that are influenced by genetic and environmental factors, we determined heritability (i.e., h^2 = the proportion of phenotypic variation in a given trait attributable to additive genetic influences) estimates for selected traits using family data from CH, AG, and RE, respectively. For the purpose of comparison, the already available heritability estimates for T2D and a few T2D related traits using SI family data are also reported. The number of families and related information from each EEG are provided in **Tables 1** (CH, AG, and RE) and 3 (SI). The types and numbers of relative pairs among study participants by EEG are reported in **Table 4**. The total number of relative pairs generated from each of the four EEGs family data sets are as follows: CH = 2,899, RE = 2,477, AG = 5,456, and SI = 2,393.

The heritability estimates obtained from CH, AG, and RE family data sets after accounting for ascertainment correction (excluding traits from non-T2D individuals only) are reported in **Table 5**. All quantitative traits were transformed using inverse normal transformation for genetic analysis and T2D was analyzed as a dichotomous trait (i.e., liability model) using the VC approach. All traits were adjusted for age and sex terms as stated earlier if found to be significant. T2D was analyzed with and without BMI as a covariate in addition to adjustment for age and sex terms. All h^2 estimates for the selected traits by EEG reported in **Table 5** were found to be significant ($P \leq 0.05$), excluding SBP and DBP in the RE sample. The h^2s for T2D by EEG are as follows: CH = 0.30 (30%), AG = 0.82 (82%), and RE = 0.46 (46%). For the purpose of comparison, it was found to be 54% in SI (**Table 5**; h^2 estimates for FG, HDL-C, and LDL-C are also available as shown for the purpose of comparison). Additional adjustment for BMI in the T2D analyses yielded more or less similar h^2 estimates. For the remaining traits, significant h^2 estimates ranged from 25% (hs-CRP) to 81% (FI; non-T2D only) in CH, from 17% (TG) to 86% (FG; non-T2D only) in AG, and from 17% (hs-CRP) to 68% (HbA1c; non-T2D only) in RE, respectively. Overall, the T2D and its related traits in AG, CH, and RE EEGs exhibited strong genetic influences (h^2 range: 17% [TG/AG and hs-CRP/RE] - 86% [FG/non-T2D/AG]).

DISCUSSION

In this study, we aimed to assess the burden of T2D and its related traits using family data collected from three EEGs (i.e., Chettiar/CH, Agarwal/AG, and Reddy/RE) with the same study

TABLE 2 | Hierarchical logistic regression analysis with blocks of significant predictor variables of type 2 diabetes in the combined data sets of TNFDS, JFDS, and NFDS.

Block/Variables[#]	β (SE)	OR (95% CI)	P-value[@]
Block 1: Sex (Male)	0.30 (0.13)	1.35 (1.04, 1.75)	0.0260
Age groups			
24 and below	–	–	–
25-34	0.83 (0.45)	2.29 (0.95, 5.54)	0.0650
35-44	1.90 (0.41)	6.71 (2.98, 15.13)	< 0.0001
45-54	2.87 (0.41)	17.61 (7.94, 39.02)	< 0.0001
55-64	3.51 (0.41)	33.43 (14.96, 74.69)	< 0.0001
65 and above	3.73 (0.42)	41.59 (18.30, 94.55)	< 0.0001
EEG			
CH	–	–	
AG	0.47 (0.16)	1.61 (1.18, 2.18)	0.0020
RE	0.25 (0.15)	1.29 (0.96, 1.73)	0.0960
Block 2: AO	0.69 (0.19)	2.00 (1.38, 2.92)	0.0003
Block 3: Smoking status			
Never	–	–	–
Former	0.77 (0.35)	2.15 (1.08, 4.31)	0.0300
Current	0.38 (0.24)	1.46 (0.91, 2.33)	0.1140

[#]Hierarchical logistic regression model included three blocks: Block 1, Sex, age, and EEG; which were held constant; Block 2, GO and AO, which were adjusted for sex. In addition, combined obesity (i.e., GO + AO) was also included as a variable for model efficiency; Block 3, Socioeconomic status, Education status, Smoking status, and Alcohol consumption status. All variables were analyzed as dummy variables. Blocks 2 and 3 were analyzed using the backward elimination procedure and the significance thresholds considered for entry and retention were 0.05 and 0.10, respectively; [@]P-values are not corrected for non-independence.

TABLE 3 | Comparison of selected traits by T2D status and EEG.

EEG[#] / Variables[@]	CH	AG	RE	SI[$]
A. T2D Individuals				
Age (years)	57.19 (13.24)	55.94 (12.50)	54.23 (14.26)	53.8 (11.4)
Age of T2D onset (years)	50.41^{a} (11.55)	$47.35^{a,b}$ (11.59)	$48.16^{a,b}$ (13.40)	46.1^{b} (10.7)
BMI (kg/m^2)	27.77 (4.88)	27.76 (4.66)	27.10 (5.90)	27.5 (4.7)
WC (cm)	100.3^{a} (12.84)	97.93^{a} (11.28)	98.26^{a} (13.05)	94.4^{b} (10.9)
SBP (mmHg)	139.7^{b} (20.23)	133.6^{c} (19.56)	132.6^{c} (18.72)	149.82^{a} (23.81)
DBP (mmHg)	$84.48^{a,b}$ (12.34)	$85.17^{a,b}$ (11.42)	82.68^{b} (10.69)	86.37^{a} (11.66)
FG (mg/dl)	173.5^{a} (65.97)	147.2^{c} (31.22)	149.8^{b} (56.32)	188.6^{a} (72.1)
HbA1c (%)	$8.11^{a,b}$ (2.242)	$8.10^{a,b}$ (1.71)	7.90^{b} (2.046)	8.8^{a} (2.4)
TCHOL (mg/dl)	185.9^{a} (44.38)	141.3^{c} (48.98)	164.2^{b} (45.98)	181.2^{a} (45.7)
TG (mg/dl)	$169.2^{a,b}$ (108.8)	144.3^{b} (84.31)	146.0^{b} (101.9)	187.9^{a} (106.6)
LDL-C (mg/dl)	116.6^{a} (37.85)	82.31^{c} (35.42)	103.1^{b} (34.57)	$104.2^{a,b}$ (37.6)
HDL-C (mg/dl)	47.02^{a} (12.62)	38.73^{b} (15.78)	44.56^{a} (14.90)	39.5^{b} (12.6)
B. Non-T2D Individuals				
Age (years)	42.31^{b} (15.55)	36.06^{c} (13.83)	41.33^{b} (15.19)	46.2^{a} (14.7)
BMI ((kg/m^2)	26.34 (5.422)	26.28 (4.65)	26.08 (5.32)	27.2 (4.7)
WC (cm)	96.82^{a} (13.95)	90.66^{b} (11.54)	93.02^{b} (13.54)	91.7^{b} (11.7)
SBP (mmHg)	129.1^{b} (16.82)	120.7^{c} (15.95)	122.2^{c} (17.27)	137.71^{a} (23.30)
DBP (mmHg)	$81.99^{a,b}$ (10.65)	$80.80^{a,b}$ (9.991)	79.56^{b} (12.74)	83.26^{a} (12.15)
FG (mg/dl)	102.2^{a} (11.75)	101.7^{a} (8.25)	93.74^{b} (11.15)	96.2^{b} (10.7)
HbA1c (%)	$5.49^{b,c}$ (0.42)	5.59^{b} (0.430)	5.44^{c} (0.524)	6.43^{a} (1.7)
FI (IU/ml)	9.31^{b} (7.21)	11.53^{a} (11.53)	7.39^{c} (5.91)	12.24^{a} (10.41)
HOMA-IR	2.37^{b} (1.93)	2.93^{a} (3.03)	1.72^{c} (1.46)	$2.4^{a,b}$ (2.1)
TCHOL (mg/dl)	177.9^{a} (38.93)	147.1^{b} (48.07)	154.9^{b} (40.24)	175.7^{a} (43.3)
TG (mg/dl)	120.0^{b} (68.04)	102.8^{c} (54.86)	$111.2^{b,c}$ (67.31)	158.9^{a} (78.9)
LDL-C (mg/dl)	116.0^{a} (31.14)	89.56^{c} (34.34)	97.91^{b} (30.02)	100.1^{b} (33.8)
HDL-C (mg/dl)	47.18^{a} (12.09)	41.61^{c} (16.01)	44.25^{b} (10.75)	41.0^{c} (10.9)

[#]EEG, Endogamous ethnic group; [$]SI/AIDHS/SDS, Sikh EEG representing the Asian Indian Diabetic Heart Study/Sikh Diabetes Study (data were previously collected and used here for the purpose of comparison; please see text for references); N, 1,260; Families, 324 (families were ascertained on multiple siblings with T2D), Average family size, 6.7; Family size range, 2-59; Females (%), 38.5%; T2D (%), 74.7; [@]T2D, Type 2 diabetes; non-T2D, nondiabetics; BMI, Body Mass Index; WC, Waist circumference; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; FG, Fasting blood glucose; HbA1c, Hemoglobin A1c; FI, Fasting insulin; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; TCHOL, Total cholesterol; TG, Triglycerides; LDL-C, LDL-cholesterol; HDL-C, HDL cholesterol. All variables are expressed as mean (standard deviation) and group differences are tested using Analysis of variance - superscript letters a, b, and c refer to homogenous groups identified by Bonferroni's post hoc contrast (P < 0.001) and similarities are denoted by sharing the same letter. P-values are not corrected for non-independence. Please see text for trait definitions. Values of FI and HOMA-IR for individuals with T2D are not shown in the table given the potential impact of medication on their concentrations. However, HbA1c and FG values for diabetics are shown for depicting the glycemic status and profile comparisons across the EEGs, respectively. Please note that the variables reported in the table were not adjusted for any medication influences.

TABLE 4 | Types and numbers of relative pairs among study participants by EEG.

Type of Relative Pair	Number of Pairs			
	CH	**RE**	**AG**	**SI**
Parent-Offspring	269	318	369	480
Siblings	234	180	407	833
Grandparent-Grandchild	46	90	111	17
Avuncular	343	329	1,021	337
Grand Avuncular	71	97	397	19
1st Cousins	365	369	1,115	347
1st Cousins, 1 rem	381	473	1,247	186
2nd Cousins	375	314	510	49
2nd Cousins, 1 rem	354	150	141	29
3rd Cousins	159	38	–	6
3rd Cousins, 1rem	41	4	–	–
Others	261	115	138	90
Total	**2,899**	**2,477**	**5,456**	**2,393**

design and methodological tools, representing three different languages and geographical locations in India, as part of the Indo-US (ICMR/NIH) joint collaborative research projects related to diabetes. In addition to these newly collected data, for the purpose of comparison, we used already available data from our other family study representing a linguistically and geographically distinct population of Khatri Sikhs (SI). Aside from depicting the prevalence and familial aggregation (i.e., clustering of diseases or traits within families due to genetic and/or environmental similarities) profiles of T2D and associated cardiometabolic traits, we determined the extent to which variation in a given trait is due to additive genetic influences using family data. These family studies represent our INDIGENIUS consortium studies (**Figure 2**).

The CH, AG, and RE EEGs bear substantial T2D and its related clinical burdens (**Table 1**). The T2D prevalence rates were high given its familial aggregation, which ranged from 33% (CH) to 37% (AG) (Overall prevalence = 35%), and the differences between them were found to be not statistically different despite the fact that they represented distinct linguistic and geographical affiliations. The proportion of newly diagnosed T2D in the total T2D sample by EEG is as follows: CH = 28%, RE = 41%, and AG = 18%. However, the three EEGs are mostly representative of the middle socioeconomic status and urban (AG)/semiurban (CH and RE) communities. Recently, using data from a family-based study of the Sindhi endogamous population, the prevalence of T2D without and with adjustment for the ascertainment criteria was found to be ~30% and 35%, respectively, which are very similar to the prevalence rates observed in our study (37). Given attention to the differences in the T2D diagnostic criteria used by different studies, these prevalence rates are much higher than those reported for population-based studies in India such as the ICMR-INDIAB study because of the family-based nature of our

TABLE 5 | Heritability estimates for the selected traits by EEG.

EEG / Variable	CH			AG			RE		
	$N^{\#}$	$h^2 \pm SE$	*P*-value	$N^{\#}$	$h^2 \pm SE$	*P*-value	$N^{\#}$	$h^2 \pm SE$	P-value^
T2D	517	0.30 ± 0.20	0.0466	530	0.82 ± 0.25	0.0003	500	0.46 ± 0.17	0.0028
T2D_adjBMI[@]	517	0.29 ± 0.17	0.0455	530	0.83 ± 0.25	0.0002	500	0.48 ± 0.18	0.0024
BMI	517	0.53 ± 0.09	1.4×10^{-10}	530	0.47 ± 0.09	3.1×10^{-10}	500	0.44 ± 0.09	2.0×10^{-07}
WC	517	0.51 ± 0.09	1.3×10^{-09}	530	0.46 ± 0.10	1.9×10^{-09}	500	0.61 ± 0.10	1.6×10^{-09}
HDL-C	517	0.65 ± 0.09	2.9×10^{-16}	530	0.71 ± 0.08	6.1×10^{-28}	500	0.49 ± 0.09	4.0×10^{-10}
LDL-C	517	0.36 ± 0.09	3.0×10^{-07}	530	0.36 ± 0.08	2.3×10^{-10}	500	0.49 ± 0.08	1.1×10^{-10}
TG	517	0.37 ± 0.08	7.9×10^{-09}	530	0.17 ± 0.10	0.0217	500	0.27 ± 0.11	0.0026
TCHOL	517	0.39 ± 0.08	1.4×10^{-08}	530	0.36 ± 0.08	1.6×10^{-09}	500	0.50 ± 0.09	6.2×10^{-11}
SBP	517	0.31 ± 0.09	0.0001	530	0.39 ± 0.09	9.5×10^{-09}	500	0.11 ± 0.10	*0.1289*
DBP	517	0.26 ± 0.10	0.0007	530	0.38 ± 0.08	4.6×10^{-11}	500	0.13 ± 0.09	*0.0578*
hs-CRP	517	0.25 ± 0.09	4.9×10^{-04}	530	0.33 ± 0.09	1.0×10^{-06}	500	0.17 ± 0.09	0.0156
FG (non-T2D)[$]	348	0.43 ± 0.14	0.0004	334	0.86 ± 0.11	1.1×10^{-15}	322	0.66 ± 0.11	2.3×10^{-09}
FI (non-T2D)[$]	348	0.81 ± 0.11	2.0×10^{-11}	334	0.32 ± 0.13	0.0020	322	0.43 ± 0.15	0.0011
HOMA-IR (non-T2D)[$]	348	0.74 ± 0.11	1.5×10^{-09}	334	0.33 ± 0.13	0.0023	322	0.37 ± 0.15	0.0051
HbA1c (non-T2D)[$]	348	0.39 ± 0.14	0.0016	330	0.64 ± 0.12	2.4×10^{-09}	322	0.68 ± 0.13	1.0×10^{-07}

[#]All quantitative traits were transformed using inverse normal transformation and adjusted for age and sex terms (i.e., age, sex, age x sex, age^2, age^2 x sex) if found to be significant for genetic analyses; [@]T2D (discrete trait) was analyzed with and without BMI as a covariate, in addition to age and sex terms as covariates; [$]Genetic analysis of glycemic traits were based on data from non-T2D individuals only; ^Heritability estimates that were not significant (i.e., P > 0.05) are shown in italics. For the purpose of comparison, h^2 estimates for selected traits from SI are as follows: T2D = 54%, FG = 54%, HDL-C = 87% and LDL-C = 44%.

studies and their ascertainment strategy. Hence, comparisons with other studies should be made with caution. Several studies have shown increased prevalence of T2D in urban areas compared to rural areas in India (3, 15, 64–68). For example, given that the CH and RE were recruited from the states of Tamil Nadu and Andhra Pradesh, the estimated prevalence rates of T2D in the urban (vs. rural) areas of the same states by the ICMR-INDIAB national population-based cross-sectional study were 13.7% (vs. 7.8%) and 12.6% (vs. 6.3%), respectively (3, 15). In a survey of 11 cities including Jaipur, the location of AG population in our study, the prevalence of T2D was found to be 15.7% (67) in middle class participants, which is comparable to other prevalence rates reported in the urban areas of Tamil Nadu (15.5%) and Andhra Pradesh (15.1%) states, respectively (66, 68). In another study involving an Urban population from Tamil Nadu, the occurrence of T2D increased along with ascending social class (Low = 12.0%, Middle = 18.4%, and High = 21.7%) (17). Also, our findings are compared to the age-adjusted prevalence rates of T2D reported for the US ethnic groups as part of the Mediators of Atherosclerosis in South Asians Living in America (MASALA) study and the Multi-Ethnic Study of Atherosclerosis (MESA) as follows: South Asians (23%), European Americans (6%), African Americans (18%), Latinos/Hispanics (17%), and Chinese Americans (13%) (69). In consideration of the above discussion, the prevalence of T2D found in the current study mirrors the substantial burden of T2D and its aggregation among Indian families.

To assess the high risk groups for T2D development, we estimated the prevalence of prediabetes using the ADA criteria. Its prevalence estimates ranged from 50% (RE) to 66% (CH) (Overall prevalence = 60%), and exhibited significant differences. Given attention to the issues such as the diagnostic criteria of prediabetes used, the choice of test, and the population being examined, the prevalence estimates across populations including those from India have been shown to vary greatly (70–75). According to the ICMR-INDIAB national data based on information from 15 states, the overall prevalence of prediabetes was estimated to be 10.3%, and its occurrence (i.e. urban vs. rural) in the States of Tamil Nadu, Andhra Pradesh, and Rajasthan was reported to be 9.8% vs. 7.1%, 11.1% vs. 9.6%, and 17.1% vs. 14.7%, respectively (3, 15, 76). However, the fasting glucose cutoffs used (i.e., ADA vs. WHO) to define impaired fasting glucose in the ICMR-INDIAB study national data resulted in a remarkable difference in the prevalence rates of both impaired fasting glucose and prediabetes: 20.8% vs. 6.5% and 24.7% vs. 10.3%, respectively (3). The familial aggregation profiles of prediabetes observed in our study are worrisome given the alarming rise in incidence rates of T2D and prediabetes based on longitudinal data in Indian populations as well as those from the MASALA Study (77–79).

The prevalence rates of generalized (GO: range = 59% [RE] – 67% [AG]), overall prevalence = 62%) and abdominal (AO: range = 77% [AG] – 86% [CH]), overall prevalence = 81%) obesity profiles observed in this study are disturbing taken together with the burden of T2D and prediabetes borne by the families within each EEG. Similar observations (i.e., prevalence of general obesity and central obesity was > 70%) were made in the Sindhi family study of T2D mentioned previously (37). As in the case of T2D, based on the national data (i.e., ICMR-INDIAB Study – Phase I), the occurrence of these obesity traits was high in urban areas compared to those from rural areas (55). For example, the prevalence rates of GO and AO in urban vs. rural areas in Tamil Nadu state were 35.7%, vs. 20.0%, and 37.4% vs. 22.1%, respectively. In another study from Chennai (urban), Tamil Nadu, the age standardized prevalence of GO (i.e., BMI ≥ 23 kg/m^2) and AO were reported to be 45.9% and 46.6%, respectively (80). In a study from the state of Andhra Pradesh, the prevalence rates of GO and AO were 56.0% and 71.2%, respectively (81). Likewise, high prevalence rates of GO and AO were found in a New Delhi urban population, which were 50.1% and 68.9%, respectively (82). As revealed by the hierarchical logistic regression analysis of the combined sample of the three EEGs, in addition to sex (male), age (groups), EEG, and past smoking status, AO was determined to be a significant predictor of T2D (**Table 2**). It is known that AO is one of the major risk factors for T2D as well as cardiovascular disease in Asian Indians (83, 84). In addition to the above T2D, prediabetes, and obesity profiles, we examined differences between the EEGs regarding 13 quantitative traits related T2D, and only two of the 13 examined traits (i.e., BMI and HbA1c) failed to exhibit significant differences between the EEGs (**Table 1**). Based on selected traits and inclusion of data form the SI EEG for the purpose of comparison, the trait differences examined between T2D and non-T2D individuals within a given EEG were found to be as expected (**Table 3**). In general, the EEGs of CH and SI appear to have increased burden of lipid and blood pressure related conditions; however, based on information from non-T2D individuals, the EEGS of AG and SI appear to have distinct hyperinsulinemia/insulin resistance profiles.

Following the observed differential epidemiological profiles of T2D and related traits, we determined the extent to which these phenotypes are influenced by additive genetic influences using family data. Given that an estimate of heritability is population-specific, T2D and its related quantitative traits in the EEGs exhibited strong additive genetic influences. The heritabilities of T2D were found to be 30%, 46%, 54%, and 82% in CH, RE, SI, and AG EEGs, respectively, and statistically significant. The T2D heritability in the Sindhi family study was estimated to be 35% (37). The heritability estimates for the remaining T2D-related traits across the EEGs were significant, excluding SBP and DBP in the RE sample, which ranged from 25% (hs-CRP) to 81% (FI; non-T2D only) in CH, from 17% (TG) to 86% (FG; non-T2D only) in AG, and from 17% (hs-CRP) to 68% (HbA1c; non-T2D only) in RE, respectively. The available family based studies examined the occurrence of T2D among relatives to reflect shared genetic predisposition and the heritability profiles of T2D related traits in various Indian populations or Asian Indians, which differed in their study designs and analytical tools (26, 27, 35, 51, 85–89). For example, the heritabilities for selected traits for the purpose of discussion including fasting glucose, HbA1c, HDL-C, triglycerides, and systolic blood pressure were reported to be 24%, 36%, 39%, 22%, and 33% in

the data obtained from multiplex families from Chennai, Tamil Nadu (26), and they were 37%, 60%, 53%, 40%, and 29% for the same traits in a subsample of the Asian Indian families from UK, respectively (35). For BMI, the heriatbilties were 44%, 31% and 25% in the above stated Chennai sample, UK sample, and a sample of selected EEGs including the Reddy EEG from Andhra Pradesh (51), respectively. In the Sindhi family study of T2D, heritability of anthropometric phenotypes ranged from 27% to 73%, while its range was 0% to 39% for T2D-related phenotypes (37). Following the above discussion, our genetic analyses of T2D and related traits revealed significant, substantial, and differential additive genetic influences on T2D and its related traits in the study samples. These findings set the scene for future studies to identify risk loci for the various cardiometabolic traits examined in this study using genome-wide association scans.

There are a few limitations of our study. We used capillary blood glucose estimates in our family- and community-based study for the purpose of comparison with our AIDHS/SDS study and the same procedure was used by some other population-based studies in India (3, 90). It has been shown that it is a feasible alternative to define T2D in epidemiological/population-based studies (3, 90–92). Moreover, T2D was defined in this study using information from HbA1c and/or use of medical records and diabetes medication/history in addition to fasting capillary blood glucose measures. Our methodological approach did not make an attempt to differentiate type 2 diabetes from type 1 diabetes or other types of diabetes based on any specific investigation, excepting exclusion of one individual with type 1 diabetes based on self-report/medical history as noted before.

In conclusion, our Indo-US exploratory/developmental collaborative study on T2D in Indian populations revealed high burden of T2D and its clinical correlates. In addition, these traits were found to be under substantial additive genetic influences in genetically and culturally diverse EEGs representing the northern and southern regions of India. Through comparisons with other populations, the Indian EEGs exhibited distinct T2D profiles underscoring the need for focused studies in near future with attention to genetic and sociocultural diversity of the Indian populations. Since the EEGs in this study are mostly representative of the middle socioeconomic status and urban/semiurban communities, there appears to be an immediate need to extend our approach to assess T2D burden in families of other diverse EEGs in India. Our efforts are reflective of feasibility of large-scale genetic studies of T2D through collaborations, both national and international. Given the worrisome T2D clinical profiles found in this study, it is imperative that aggressive public health awareness and preventive measures are implemented early on, and further suggesting the need for immediate plans for intervention studies. To be specific, individuals/families from the EEGs who participated in this study should be advised to start diabetes prevention measures early in life, either in adolescence or early youth, with greater focus on healthy diet, physical activity, and weight maintenance. There are numerous EEGs in India and similar studies could identify more EEGs where earlier intervention may be highly warranted.

These data also call for longitudinal assessments of the project participants to thoroughly understand the disease development and progression. In addition to our ongoing work on targeted sequencing of the GWAS-derived South Asian-specific T2D risk loci in the SI EEG sample and subsequent replication studies in the CH, AG, and RE EEGs, our immediate plans through potential future projects are 1) to generate omics data, especially whole genome sequencing data, from the family members of the diverse EEGs, current and new, representing various regions of India to understand the molecular basis of T2D in Indian populations; and 2) to conduct intervention studies (e.g., family-focused) that are culturally suited to Indian EEGs.

DATA AVAILABILITY STATEMENT

Given attention to the Institutional Ethics Committees policies and the INDIGENIUS Consortium data access policy, restrictions will apply to the availability of data used for this study publicly. However, data will be made available from the authors upon reasonable request, and data access requests should be submitted to the corresponding author.

ETHICS STATEMENT

The study protocols involving human participants were reviewed and approved by the Institutional Ethics Committees of the Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu (Tamil Nadu Family Diabetes Study/TNFDS), the Fortis Escort Hospital, Jaipur, Rajasthan (Jaipur Family Diabetes Study/JFDS), and the Narayana Medical College and Hospital, Nellore, Andhra Pradesh (Nellore Family Diabetes Study/NFDS), India. Written informed consent for participation was obtained following the approvals by the three Institutional Ethical Committees. Already available family data from Phase I of the Asian Indian Diabetic Heart Study/Sikh Diabetes (AIDHS/SDS) were used for this study for the purpose of comparison. The analysis of de-identified data was approved by the Institutional Review Board, University of Texas Rio Grande Valley, Edinburg, Texas, USA. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SP, RG, DK, TP, ST, KM, RV, DS, JB, and RD contributed to the study conception and study design. The clinical data and biospecimen sample collection, processing, and data management of TNFDS, JFDS, and NFDS were supervised by SP, CN, UR, RG, KM, DK, AP, and RD through direct contributions from VV, TK, DR, SS, SL, KS, MS, RR, PV, PR, NS, and JE. AIDHS/SDS data were collected and analyzed under the supervision of DS through direct interactions with CB, GW, SR, JS, and NM. VV, JL-A, RA, DR, MA, and RD performed the statistical analysis with contributions from SM, JB, SP, and DS

regarding the data analysis and interpretation of the data. VV, JL-A, RA, and DR wrote the initial draft of the manuscript, which was critically revised by RD, SM, RG, DK, DS, and SP. All authors contributed to the article and approved the submitted version.

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