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Clinical and genetic determinants of diabetes and complications

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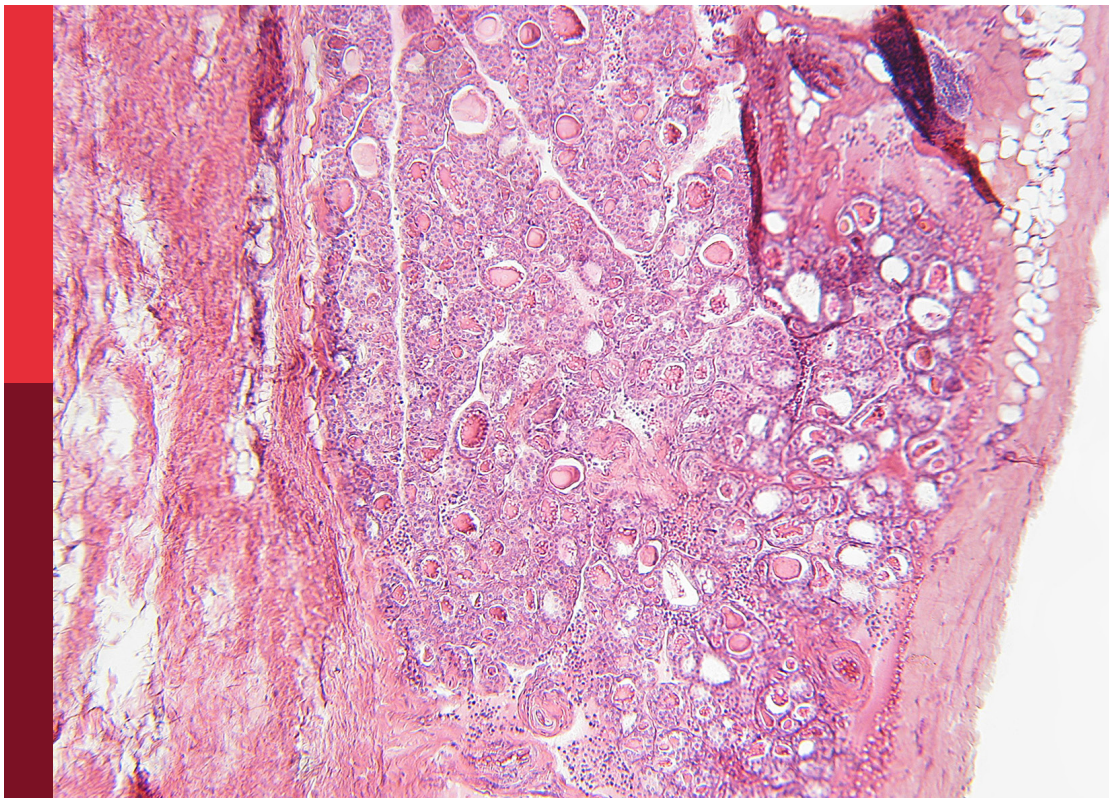

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Clinical and genetic determinants of diabetes and complications

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Editorial: Clinical and genetic determinants of diabetes and complications

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Editorial on the Research Topic
Clinical and genetic determinants of diabetes and complications

Diabetes Mellitus (DM) continues to be a significant cause of death worldwide, imposing a substantial burden on global public health. According to the data from International Diabetes Federation, the number of DM patients is expected to increase by 50% by 2030 compared to the 366 million cases reported in 2011. DM gives rise to various complications, resulting in organ damage, such as the heart and kidneys, ultimately leading to a diminished quality of life and an increased rate of premature mortality. For instance, individuals with diabetes have a twofold higher risk of cardiovascular mortality. The development of DM involves multiple factors, and several clinical risk factors, including overweight or obesity, have been suggested. However, the impact of several other potential factors on DM's pathogenesis remains inconclusive. At the genetic level, having a family history of DM elevates the risk of developing the condition, and more than 500 genetic loci have been identified as being associated with DM. Early efforts to find genes associated with diabetes complications relied on family linkage analyses, candidate gene studies susceptible to false positives, and underpowered genome-wide association studies (GWAS) constrained by sample size. Detecting individuals who are very vulnerable to the disease may help with disease prevention. Nevertheless, the genetic determinants of DM complications are not yet well comprehended.

This Research Topic encompasses a collection of 30 studies that explore various aspects of diabetes and its complications. Specifically, it includes 15 studies examining the epidemiological characteristics and risk factors associated with diabetes and its complications. Furthermore, five studies analyze potential biochemical markers relevant to the pathogenesis of diabetes and diabetic complications, and seven studies evaluate genetic information for predicting diabetes and its complications, and three studies that assess treatment options.

The incidence and death rates associated with diabetic complications differ based on the population and the underlying factors contributing to the disease. For instance, a cross-sectional study conducted by Bundó et al. revealed a lower prevalence of diabetic foot disease in Catalonia (Spain) compared to previous similar studies. Meanwhile, in a systematic review and meta-analysis by Akhtar et al., the prevalence of foot ulcers in diabetic patients in Pakistan was investigated, indicating a relatively high prevalence of diabetic foot ulcers in the country. Alizadeh et al. conducted a cohort study involving 1329 participants aged 20 to 70 years with prediabetes, finding that the risk of progressing to diabetes was elevated in individuals with combined impaired fasting glycemia (IFG)/impaired glucose tolerance (IGT) compared to IFG alone. The results of a study by Liu et al. suggested that the OTUD3 gene variant rs78466831 is associated with type 2 DM (T2DM) and may serve as a risk factor for diabetic retinopathy. In another Chinese follow-up study, Shi et al. revealed that frailty is common among older adults with diabetes and is correlated with an elevated risk of adverse health outcomes.

Abnormalities in glucose and lipid metabolism play a crucial role in the progression of diabetic complications. Xiao et al. discovered that bile acids independently contribute to adverse renal outcomes in patients with diabetic kidney disease (DKD). Song et al. observed higher levels of remnant cholesterol in T2DM patients with the peripheral arterial disease (PAD), which were independently associated with the severity of PAD. In their study, Guan et al. compared the circulating adiponectin levels in Japanese women with varying levels of physical activity. They found that adiponectin primarily correlated with regional adiposity and high-density lipoprotein cholesterol (HDL-C). Li et al. summarized in their review that obesity can induce oxidative stress, which can contribute to insulin resistance, inflammation, and disorders in lipid metabolism, ultimately impacting cognitive dysfunction in individuals with diabetes. According to the findings of Lin et al., admission hyperglycemia in critically ill sepsis patients with diabetes was not found to be a contributing factor to the short-term prognosis.

With the increasing prevalence of diabetes, there is a proportional rise in the incidence of diabetic complications. Within this Research Topic, numerous papers explore the causal association between diabetes and its associated complications. Hao et al. provided evidence of a causal association between T2DM and systolic blood pressure. Guo et al. demonstrated a causal association between T2DM and coronary artery disease in East Asians but not atrial fibrillation. Previous research has identified a bidirectional link between nonalcoholic fatty liver disease (NAFLD) and T2DM. Yu et al. revealed the causal effect of NAFLD on the development of T2DM, emphasizing the need for further verification regarding the lack of a causal association between T2DM and NAFLD. Xu et al. indicated that lymphoid leukemia increases the risk of developing diabetes. Guo et al. suggested that T2DM is an independent risk factor for elevated risk of synovitis and tenosynovitis.

Biomarkers play a crucial role in the identification, diagnosis, prevention, and treatment monitoring of diseases. Since many complications of diabetes are difficult to detect, the discovery of biomarkers is essential for early detection and management. In a retrospective observational study, Song et al. identified that the combination model of the neutrophil/HDL-C ratio and the systemic inflammation response index was the most valuable in predicting PAD in individuals with T2DM. In another retrospective study, Li et al. suggested that the triglycerides/HDL-C ratio could be an effective marker for assessing the risk of NAFLD in patients newly diagnosed with T2DM. Mitra et al.'s review summarized the prospective potential of exosomal microRNAs in diagnosis and clinically prognosis of gestational DM (GDM) and its impact on pregnancy outcomes. Huo et al. conducted a cross-sectional study, revealing that increased levels of circulating glycoprotein non-metastatic melanoma protein B are associated with both DM and cataracts, thus serving as a potential biomarker for DM-associated cataracts. Ferraz et al. suggested that 41 miRNAs were differentially regulated between T1DM and control individuals. In particular, hsa-miR-26b-5p and hsa-miR-21-5p may influence nuclear and mitochondrial dysfunction, leading to dysregulation in type 1 DM.

The progression of T2DM varies significantly and can be influenced by genetic factors. Therefore, numerous studies have explored genetic information related to diabetic complications in this field. Wang et al. conducted a cross-sectional study involving 120 T2DM patients from Han and Tibetan ethnic groups, revealing subtle differences in clinical characteristics between various ethnic groups that may be associated with epigenetic modifications. Liu et al. reviewed the association between epigenetic changes and DKD, emphasizing that DNA methylation, histone modification, and changes in noncoding RNA expression profiles are deeply involved in DKD-related inflammation, oxidative stress, hemodynamics, and abnormal signaling pathways. Ramos-Levi et al. identified a core set of single nucleotide polymorphisms (SNPs) associated with diabetes and GDM, suggesting the usefulness of identifying these genetic variants for designing preventive strategies, even in nutritional interventions. In their research, Mansour et al. performed an Exome-Wide Association Study on Emirati individuals diagnosed with T2DM. Through their study, they identified specific genetic loci that are linked to various categories of T2DM-related complications within the Emirati population. Zhang et al. investigated the distribution pattern of the CYP2C9 gene in Chinese Han individuals and identified variants that may impact drug metabolic activities. Yu et al. reported one colocalized locus and 14 additional candidate loci shared between T2DM and periodontal disease (PD)/oral health. Zhang et al. revealed that the MUC5B SNP rs2943512 (A > C) or the up-regulation of MUC5B in bronchial epithelial cells might significantly promote interstitial lung disease in patients with T2DM.

In this Research Topic, there are also papers focused on treating diabetic complications, aiming to improve the management and control of their progression, considering their high rates of disability and fatality. In a randomized controlled trial conducted in China, Cai et al. demonstrated that a subcutaneous administration of polyethylene glycol loxenatide, along with regular treatment, led to a more significant weight reduction than metformin in overweight or obese patients with T2DM. Akiyama et al.'s review highlighted that SGLT2 inhibitors reduce blood glucose levels and decrease the likelihood of being admitted to the

hospital due to heart failure and worsening renal function in patients with T2DM. Lastly, in a mini-review, Renuka et al. discussed the use of stimuli-responsive nanocomposite scaffolds in addressing specific issues related to wound healing and angiogenesis in diabetic patients, demonstrating their potential to interact with wound microenvironment, release bioactive materials in a regulated manner, and act as dressings for diabetic wound healing.

The Research Topic underscores the significance of clinical and genetic factors in the progression of diabetes and its complications, which holds important implications for prevention and treatment strategies. These findings provide valuable insights for clinical practice.

Author contributions

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

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Conflict of interest

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

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Are the determinants of the progression to type 2 diabetes and regression to normoglycemia in the populations with pre-diabetes the same?

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Background: We aimed to determine the predictors of regression to normoglycemia and progression to diabetes among subjects with pre-diabetes in a single model concurrently.

Methods: The present study included 1329 participants aged 20 to 70 years with prediabetes from the population-based cohort of the Tehran Lipid and Glucose Study, with a 10-year follow-up. Glycemic status at follow-up was categorized as regression to normoglycemia: fasting plasma glucose [FPG] of <5.55 and 2h-plasma glucose [PG] of <7.77 mmol/L, and not taking antidiabetic medications. Glycemic status at follow-up was categorized as progression to diabetes: FPG ≥7 or 2h-PG of ≥11.1 mmol/L, or taking antidiabetic medications. Glycemic status determined whether the patients remained in prediabetes category (isolated impaired fasting glycaemia [iIFG] [(5.55≤FPG<7 and 2h-PG<7.77 mmol/L); isolated impared glucose tolarence [iIGT] (7.77 ≤ 2h-PG<11.1 and FGP<5.55 mmol/L)]. With prediabetes as a reference, multinomial logistic regression was utilized to identify the determinants of glycemic changes.

Results: Approximately 40% of participants returned to normoglycemia (n = 578), and similar percentage of participants progressed to diabetes (n = 518). Based on the multivariable multinomial model, regression to normoglycemia was associated with age (relative risk ratio [RRR] = 0.97; 95% CI, 0.95-0.99), female sex (RRR = 1.72; 95% CI, 1.18-2.50), high education level of ≥12 years (RRR = 2.10; 95% CI, 1.19-3.70), and combined IFG/impaired glucose tolerance

(IGT) versus IFG (RRR = 0.45; 95% CI, 0.29-0.70). The risk of progression to diabetes increased with body mass index (RRR = 1.10; 95% CI, 1.05-1.15), waist circumference (RRR = 0.97; 95% CI, 0.96-0.99), positive familial history of diabetes (RRR = 1.62; 95% CI, 1.07-2.45), and combined IFG/IGT versus IFG (RRR = 2.54; 95% CI, 1.71-3.77).

Conclusion: A small percentage of patients with prediabetes remain in this condition, but the majority go on to develop diabetes or regress to normoglycemia. Both directions had distinct predictors.

KEYWORDS

normoglycemia, pre-diabetes, type 2 diabetes, Cardiometabolic disorders, progression, regression

Introduction

Prediabetes is understood to be a critical metabolic stage in the onset of diabetes and its complications. In prediabetes, glucose levels are higher than normal but not yet at the threshold for diabetes. The number of people with prediabetes is rapidly rising in all countries around the world. In terms of disease burden, high fasting plasma glucose (FPG) ranked fifth in 2017. Globally, 352 million adults (7.3%) had prediabetes, and that number was projected to rise to 587 million (8.3%) by the year (1) 2045. Rates of progression to diabetes and regression to normoglycemia from prediabetes have been reported differently in previous studies. Every year, 5% to 10% of those with prediabetes may develop diabetes, while the same number may develop normoglycemia. According to the American Diabetes Association expert panel, 70% of people with prediabetes will eventually develop diabetes (2).

Regression ranged from 33% to 59% within 1 to 5 years' follow-up in 47 studies (3). Clinical studies have confirmed that lifestyle modification programs focusing on consuming a healthier diet and engaging in more physical activity can lower the risk of developing diabetes. Reversion from prediabetes to normoglycemia is associated with improving a range of cardiovascular risk factors (1).

In this study, our objective was to determine the predictors of the regression to normoglycemia and progression to diabetes among adults with pre-diabetes in a single model simultaneously using a population-based cohort study with ten years of follow-up.

Methods

Study design and population

The Tehran Lipid and Glucose Study (TLGS)—the first community-based large-scale, long-term cohort study in Iran—was designed (4) in 1998. The TLGS was initiated in 1999 to investigate noncommunicable disease (NCD) and its associated risk factors or determinants among a representative population of Tehran. The baseline measurement was conducted between February 1999 and August 2001. In this study, those who were 3 years old or older and residing in the District 13 of Tehran were considered the reference population. Currently, the project is in its seventh phase. A total of 15,005 people aged 3 and older were recruited during the baseline data collection phase of the project (1999-2001), and they were examined for NCD risk factors—a procedure that is repeated every 3 years following the standardized protocol. Data for this study were taken from the third examination cycle (n = 9998). We considered adults aged 20-70 years with the diagnosis of prediabetes as the study population. So, those with the diagnosis of diabetes, defined as FPG ≥7 or 2h-PG ≥11.1 mmol/L or taking anti-diabetic medications (n=943), and those with normoglycemia (FPG<5.55 and 2h-PG<7.77 mmol/L and not taking anti-diabetic medications) were excluded. Because of comorbidities in people over 70 years old, we excluded these participants as well (n=624). The flowchart in Figure 1 shows the description of the study population. Finally,1329 participants with prediabetes aged 20 to 70 years remained eligible and were observed for 10

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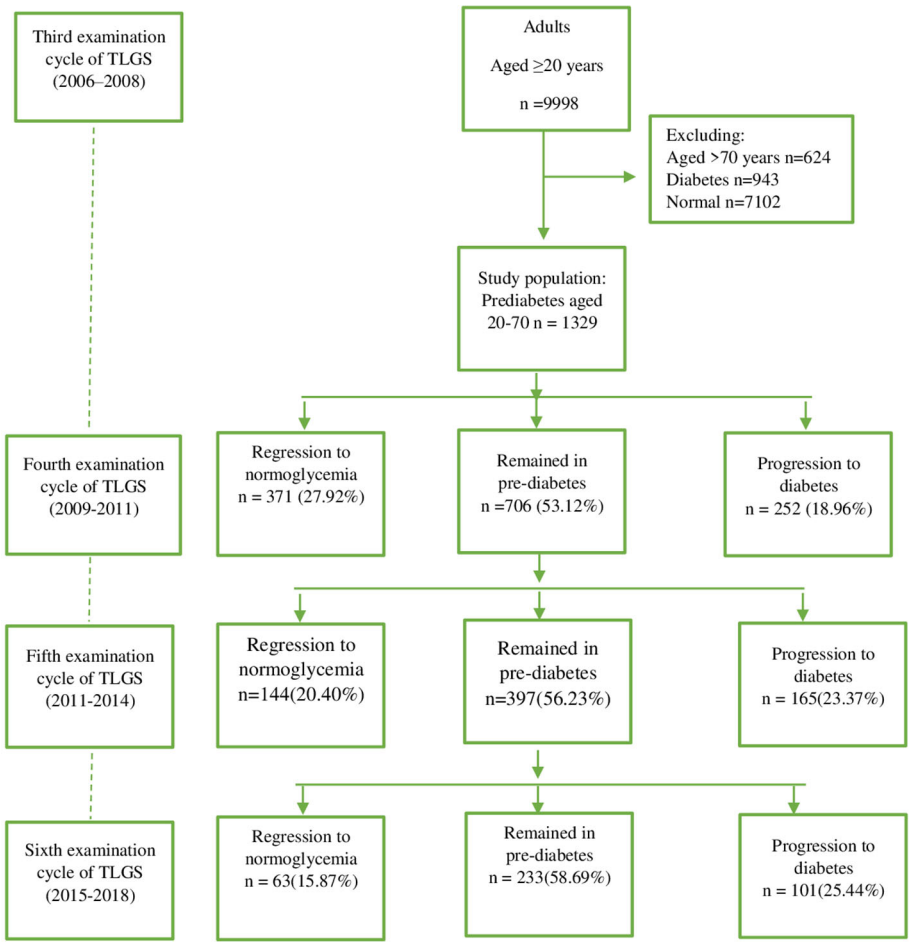

FIGURE 1
Flow diagram describing the study population.

years in the current analysis. The study was reviewed and approved by the ethics committee of Shahid Beheshti University of Medical Sciences (Ethics approval reference number: IR.SBMU.ENDOCRINE.REC.1400.113).

Measurements

Each interview was conducted through a structured questionnaire to collect demographic data, education level, smoking status, medication use, family history of diabetes, history of cardiovascular disease (CVD), or family history of CVD. The mercury column sphygmomanometer was used to measure the systolic and diastolic blood pressure (SBP, DBP), and the mean of 2 consecutive measurements on the same arm after at least 5 minutes of seated rest in a chair was calculated. The standard measurement techniques were used to determine the body weight, waist sizes, and height. A venous sample was taken between 7:00 AM and 9:00 AM after 12 to 14 hours of fasting for laboratory testing, and all samples were analyzed in the TLGS research laboratory on the day of blood sampling. The details and protocols of the TLGS clinical measurements were published elsewhere (5).

Definition of variables

At the baseline or each examination, participants were classified as (1) having diabetes (fasting plasma glucose [FPG] ≥7 or 2h-PG ≥11.1 mmol/L, or taking antidiabetic medications), (2) normoglycemia (FPG <5.55 and 2h-PG<7.77 mmol/L and not taking antidiabetic medications), and (3) as prediabetes (isolated impared fasting glucose [IFG] [5.55≤FPG<7 and 2h-PG<7.77 mmol/L]; isolated impared glucose tolerance [IGT] [7.77 ≤ 2h-PG<11.1 and FGP<5.55 mmol/L] and combined IFG/IGT [5.55≤FPG<7 and 7.77 ≤ 2h-

PG<11.1 mmol/L]). If any participant's first-degree relatives had type 2 diabetes, it was regarded as having a positive family history of the disease. Body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters. Smoking status was categorized as follows: current, former, and nonsmokers. Furthermore, participants were divided into 3 groups based on their length of education: 0 to 5, 6 to 12, and >12 years. The outcome of our study was evaluated by whether patients developed diabetes or normoglycemia for the first time or maintained prediabetes during our follow-up. The event date was taken into account as the point at which the person first experienced normoglycemia or diabetes and last experienced prediabetes; for those without a normoglycemia or diabetes event, the most recent follow-up time was taken into account.

Statistical analysis

Baseline characteristics were summarized as mean and standard deviation for continuous variables and frequencies (%) for categorical variables. The predictive mean matching method and a 5-time imputation with 50 iterations were used to perform multiple imputations for missing data at the baseline (up to 12.4% for different variables) and follow-up (up to 29% in separate examinations). One-way analysis of variance and chi-square tests were used to compare continuous and categorical variables between the groups. Multinomial logistic regression was performed to calculate the relative risk ratio (RRR) and 95% CI for the considered risk factors. First, a univariable analysis of potential predictors were performed that included age, sex, BMI, SBP, DBP, use of antihypertensive drugs, use of antihyperlipidemic drugs, positive familial history of type 2 diabetes, waist circumference, glycemic status, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), personal history of CVD, familial history of CVD, smoking status, and education level. For the next step, variables presenting P <.2 were included in the multivariable model— all variables were significant in one or both outcomes. The interaction of the selected variables with age and sex was assessed using the likelihood ratio test (LR test). Since the LR test was not significant, the interaction terms were not entered into the multivariable model. We also checked the interaction between type of prediabetes (IFG/IGT) and other predictors and did not find a significant interaction. BMI had a strong correlation with waist circumference (r = 0.78), thus,a sensitivity analysis was performed. Separate models for weight and waist circumference were developed. (see Supplementary Tables). Continuous variables were centralized to ease the interpretation of intercept terms. An RRR> 1 suggests a higher risk for regression in the case of regression to normoglycemia, which is a favorable outcome. In the case of a progression to diabetes, an RRR >1 indicates a higher risk of progression, which is an unfavorable outcome. Statistical significance was set at P <.05. All analyses were performed using STATA Version 16 (Stata Corp).

Results

We assessed 1329 people with prediabetes between 2006 and 2018, and the median follow-up time was 10 years (interquartile range, 0.9 years).

Baseline clinical characteristics and laboratory data according to transition status during follow-up are presented in Table 1. The BMI, systolic blood pressure, diastolic blood pressure, waist circumference, FPG, 2h-PG, TC, TG, likelihood of using a drug to treat hyperlipidemia, positive family history of type 2 diabetes, and dyslipidemia were all higher in participants who had progressed to diabetes. However, those who regressed to normoglycemia had more favorable values. No significant differences across categories were observed regarding CVD history, familial history of CVD, and smoking status. Overall, approximately 40% of participants (n = 578) (men, 43.03; women, 43.86) returned to normoglycemia, and 40% of participants (n = 518) (men, 36.56; women, 40.89) progressed to diabetes (see Figure 1 and Table 2).

Table 3 shows a univariable multinomial logistic regression analysis with unadjusted RRRs of variables used for the multivariable model.

We observed an age-related reversion to normoglycemia in the multivariable model. The regression probability decreases by 3% per year (RRR = 0.97; 95% CI, 0.95-0.99). Similarly, baseline combined IFG/IGT had a notable negative effect, and these participants had a 55% lower regression probability (RRR = 0.45; 95% CI, 0.29-0.70) compared with the iIFG participants. Women were 72% more likely to regress (RRR = 1.72; 95% CI, 1.18- 2.50). Higher education level (≥12 years) was positively associated with regression to normoglycemia (RRR = 2.10; 95% CI, 1.19-3.70) (Table 4).

A higher BMI significantly increased the likelihood of developing diabetes from prediabetes. The progression probability increases by 10% for each BMI unit increase (RRR = 1.10; 95% CI, 1.05-1.15), whereas waist circumference had a negative effect on the progression to diabetes (0.97 [0.96-0.99]). The risk of progression was 62% higher for those with a positive family history of diabetes compared with those with a negative history (RRR = 1.62; 95% CI, 1.07-2.45). This predictor had a strong positive correlation with the development of diabetes, in contrast to regression to normal condition, which was inversely related to the combined IFG/IGT. Those with combined IFG/IGT were 2.5 times (95% CI, 1.71-3.77) more likely to develop diabetes than the participants with iIFG (Table 4).

As previously explained, due to the significant negative relationship observed between waist circumference and

TABLE 1 Baseline characteristics of participants according to transition status[a].

Variables	Total Participants (N = 1329)	Remained in Prediabetes (n = 233)	Regression to Normoglycemia (n = 578)	Progression to Diabetes (n = 518)	*P*
Age, years	50.01 ± 12.11	52.46 ± 10.69	47.27 ± 13.20	51.97 ± 10.77	<.001
Sex, female	741 (55.76)	113 (48.50)	325 (56.23)	303 (58.49)	.037
BMI, Kg/m^2	29.43 ± 4.88	28.96 ± 4.30	28.64 ± 4.70	30.53 ± 5.12	.003
SBP, mmHg	121.29 ± 18.41	122.70 ± 17.30	117.80 ± 17.76	124.56 ± 18.95	<.001
DBP, mmHg	77.32 ± 10.30	77.42 ± 10.36	75.69 ± 9.84	79.10 ± 10.49	<.001
Antihypertensive drugs	98 (7.37)	19 (8.15)	30 (5.19)	49 (9.46)	.023
Antihyperlipidemic drugs	79 (5.94)	19 (8.15)	24 (4.15)	36 (6.95)	.043
Familial History of T2DM	282 (21.22)	41 (17.60)	106 (18.34)	135 (26.06)	.003
Waist circumference, cm	97.15 ± 11.42	97.52 ± 10.66	94.97 ± 11.64	99.42 ± 11.05	<.001
FPG, mmol/L	5.62 ± .52	5.63 ± .47	5.44 ± .50	5.81 ± .50	<.001
2h-PG, mmol/L	7.61 ± 1.75	7.46 ± 1.74	7.17 ± 1.66	8.18 ± 1.70	<.001
TC, mmol/L	5.26 ± 1.05	5.26 ± .99	5.18 ± 1.01	5.36 ± 1.11	.0171
TG, mmol/L	2.13 ± 1.27	2.16 ± 1.08	1.99 ± 1.33	2.27 ± 1.26	.0008
HDL-C, mmol/L	1.04 ± .25	1.02 ± .23	1.06 ± .27	1.02 ± .23	.0147
Personal history of CVD	59 (4.44)	11 (4.72)	22 (3.81)	26 (5.02)	.606
Familial history of CVD	121 (9.10)	20 (8.58)	53 (9.17)	48 (9.27)	.953
Smoking					.484
Former	134 (10.08)	26 (11.16)	62 (10.73)	46 (8.88)	
Current	152 (11.44)	28 (12.02)	72 (12.46)	52 (10.04)	
Education					<.001
<6 years	496 (37.32)	103 (44.21)	182 (31.49)	211 (50.85)	
6-11 years	624 (46.95)	106 (45.49)	280 (48.44)	238 (38.75)	
≥12 years	209 (15.73)	24 (10.30)	116 (20.07)	69 (13.32)	

[a]Data are presented as mean ± SD or n (%). One-way analysis of variance and Chi-Square tests were used to compare continuous and categorical variables between the groups. BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure TC, total cholesterol; TG, triglyceride; T2DM, type 2 diabetes; 2h-PG, 2-hour plasma glucose.

progression to diabetes, the researchers decided to investigate the correlation between waist circumference and BMI. They observed a high correlation, and as a result, sensitivity analysis was done. The relationship in the model containing BMI remained significant (RRR = 1.06; 95% CI, 1.02-1.10)(see Supplementary Table 1). Significance disappeared in the model when waist circumference was included (RRR = 1; 95% CI, 0.98-1.01) (see Supplementary Table 2). There was no change in the significance of other variables (see Supplementary Tables 1, 2).

TABLE 2 Cumulative incidence of regression to normoglycemia and progression to diabetes by sex during a 10-year follow-up among people with prediabetes[a].

	Total	Male	Female
Regression to normoglycemia	578 (43.49)	253 (43.03)	325 (43.86)
Remained in prediabetes	233 (17.53)	120 (20.41)	113 (15.25)
Progression to diabetes	518 (38.98)	215 (36.56)	303 (40.89)
Total	1329	588	741

[a]Data are presented as n (%).

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TABLE 3 Prognostic factors associated with regression and progression in prediabetes over a 10-year follow-up in a uni-variable analysis[a].

	Regression to Normoglycemia	Progression to Diabetes
Variables		
Age, years	0.96 (0.95-0.97)	1 (0.99-1.01)
Sex, female	1.76 (1.60-1.94)	1.67 (1.51-1.84)
BMI, Kg/m^2	1.09 (1.06-1.12)	1.12 (1.10-1.15)
SBP, mmHg	1.007 (1.005-1.008)	1.006 (1.005-1.007)
DBP, mmHg	1.011 (1.009-1.013)	1.010 (1.008-1.012)
Antihypertensive drugs	1.57 (.88-2.80)	2.57 (1.51-4.38)
Antihyperlipidemic drugs	1.26 (.69-2.30)	1.89 (1.08-3.30)
Familial history of T2DM	2.58 (1.80-3.70)	3.29 (2.32-4.67)
Waist circumference, cm	1.009 (1.007-1.010)	1.008 (1.006-1.009)
Glycemic status		
iIFG	Reference	
iIGT	3.40 (2.65-4.56)	1.71 (1.26-2.31)
Combined IFG/IGT	1.10 (.77-1.59)	4.07 (3.03-5.46)
TC, mmol/L	1.17 (1.14-1.21)	1.16 (1.12-1.19)
TG, mmol/L	1.38 (1.29-1.48)	1.39 (1.30-1.49)
HDL-C, mmol/L	2.39 (2.07-2.76)	2.09 (1.80-2.42)
Personal history of CVD	2 (.96-4.12)	2.36 (1.16-4.78)
Familial history of CVD	2.64 (1.58-4.43)	2.39 (1.42-4.04)
Smoking		
Nonsmoker	Reference	
Ex-smoker	2.38 (1.50-3.76)	1.76 (1.09-2.86)
Smoker	2.57 (1.66-3.97)	1.85 (1.17- 2.94)
Education		
<6 years	Reference	
6-12 years	2.64 (2.11-3.30)	2.24 (1.78-2.82)
≥12 years	4.83 (3.11-7.50)	2.87 (1.80-4.57)

[a]Data are presented as RRR (95% CI). A total of 1329 participants remained in prediabetes as reference group. BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; iIFG, isolated impared fasting glucose; iIGT, isolated impared glucose tolerance; SBP, systolic blood pressure TC, total cholesterol; T2DM, type 2 diabetes; TG, triglyceride; RR, relative risk ratio.

Discussion

The aim of the study was to simultaneously identify the determinants of regression to normoglycemia and progression to diabetes in individuals with prediabetes. In this population-based cohort study with a 10-year follow-up, we observed similar conversion rates of approximately 40% for progression and regression from prediabetes in participants, but with different predictors. A study on middle-aged participants with prediabetes showed that during a 10-year follow-up, the rates of regression to normoglycemia and progression to diabetes were about 23% and 30%, respectively (6). Another study among the middle-aged Swedish population with prediabetes reported a rate of regression of bout 36% during 8 to10 years (7). The KORA S4/F4 study of those aged 55 to 74 years in Germany found a reversion rate of 16.3% over 7 years of follow-up using an oral glucose tolerance test as the diagnostic criterion (8). The conversion rate varies based on the population characteristics, length of follow-up, and the definition used to define normoglycemia, prediabetes, and diabetes.

Our results showed that the factors that lead to regression from prediabetes to normoglycemia are not the same as factors that predict progression to diabetes. Age, sex, education level, and combined IFG/IGT predicted the regression. BMI, familial history of type 2 diabetes, and combined IFG/IGT are determinants of diabetes progression. The combined IFG/IGT were inversely associated with regression to normoglycemia and directly associated with the development of diabetes.

This analysis showed that younger age—independent of other factors—was related to a higher probability of regression to normoglycemia, which is in line with previous studies (1, 9). Aging is an inevitable risk factor for insulin resistance (10, 11). To reestablish the normal state, identification and intervention at younger ages may be considered. In this cohort, women had a higher probability of regression to normoglycemia. This finding may reflect a higher use of health care services and health awareness among women. A previous study (12) found an association between female sex and regression to normoglycemia, whereas other studies did not (13, 14). However, a study reported that women had a

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TABLE 4 Prognostic factors associated with regression and progression in prediabetes over a 10-years follow-up in a multi-variable analysis[a].

	Regression to Normoglycemia	Progression to Diabetes
Variables		
Age, years	0.97 (0.95-0.99)	1 (.98-1.02)
Sex, female	0.72 (1.18- 2.50)	1.11 (.76-1.62)
BMI, Kg/m^2	0.99 (0.94-1.04)	1.10 (1.05-1.15)
SBP, mmHg	0.99 (.98-1.01)	1 (.99-1.01)
DBP, mmHg	0.99 (.97-1.01)	1.01 (0.99-1.03)
Antihypertensive drugs	0.95 (.49-1.82)	0.95 (0.52-1.76)
Antihyperlipidemic drugs	0.55 (0.28-1.08)	0.76 (0.40-1.42)
Familial History of T2DM	0.84 (0.55-1.29)	1.62 (1.07-2.45)
Waist circumference, cm	0.99 (0.98-1.01)	0.97 (0.96-0.99)
Glycemic status		
iIFG	Reference	
iIGT	1.43 (0.99-2.06)	1.08 (0.72-1.60)
Combined IFG/IGT	0.45 (0.29-0.70)	2.54 (1.71- 3.77)
TC, mmol/L	0.97 (0.81-1.15)	1.04 (0.87-1.24)
TG, mmol/L	0.98 (.84-1.14)	1.03 (0.89-1.20)
HDL-C, mmol/L	1.92 (.93-3.97)	.96 (0.45-2.04)
Personal history of CVD	1.31 (.60-2.88)	0.96 (0.44-2.08)
Familial history of CVD	1.02 (0.58-1.79)	.94 (0.53-1.66)
Smoking		
Non-smoker	Reference	
Ex-smoker	1.45 (0.84-2.50)	.94 (0.53-1.65)
Smoker	1.41 (0.83-2.38)	.96 (0.55-1.65)
Education		
<6 years	Reference	
6-12 years	1.19 (.81-1.76)	1.21 (.82-1.80)
≥12 years	2.10 (1.19-3.70)	1.72 (.95-3.09)

[a]Data are presented as RRR (95% CI). A total of 1329 participants remained in prediabetes as reference group. BMI, body mass index; CVD, cardiovascular disease; DBP, diastolic blood pressure; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; iIFG, isolated impared fasting glucose; iIGT, isolated impared glucose tolerance; RRR, relative risk ratio; SBP, systolic blood pressure TC, total cholesterol; T2DM, type 2 diabetes; TG, triglyceride.

higher insulin secretion index (15). It is well documented that diabetes complications and burden vary between the sexes (16). However, there is not much proof of this problem in the stage of prediabetes. Previous studies have reported that women with prediabetes have a higher burden of cardiovascular disease as a complication of diabetes than their men counterparts (17). All of the aforementioned information suggests that among people with prediabetes, gender-related factors may need to be taken into account before diabetes actually develops. Age and sex are nonmodifiable factors that were associated with regression to normal glucose levels; however, they can be valuable for screening and intervention programs. This study found no correlation between conversions and blood pressure or dyslipidemia (low HDL-C and high cholesterol levels). Measures of lipid metabolism in relation to glycemic status have only been investigated in a small number of previous studies, and the results have been inconsistent (6, 18). In their study on 1610 participants with prediabetes, Ahmadi et al (19) showed that rising the trend of HDL-C was an independent risk factor for conversion to diabetes 9 years before the incidence of diabetes. In our study, although there were some associations between lipid measures and both regression and progression, this association disappeared after adjustment with other possible predictors. Based on these results, it appears that additional research may be required to examine how lipid components, which are frequently utilized in clinical laboratories as metabolites, contribute to the onset of diabetes. Lipid-lowering medication was associated with an increased risk of progression to diabetes in univariable analysis (RRR = 1.89; 95% CI, 1.08-3.30) but decreased the regression to diabetes by about 2-fold with a borderline significance (RRR = 0.55; 95% CI, 0.28-1.08). This outcome is expected given that statins make up the majority of antihyperlipidemic medications and that they raise the risk of dysglycemia. Our findings regarding BMI and waist circumference mostly agree with those of previous studies on the progression toward diabetes. In line with our findings, previous studies from India and South Africa did not detect any association between waist circumference and progression to diabetes (20, 21). However, weight gain, particularly visceral fat accumulation, could

increase impaired insulin signaling, leading to insulin resistance and increasing the risk of progression from prediabetes to diabetes (22). In our study, this was shown with BMI.

In addition, our findings about the parental history of diabetes are consistent with findings from previous studies. Although the increased risk of progression to diabetes among those with a family history of diabetes shows some genetic effects, it may also indicate that individuals with a family history of diabetes are more likely to have their glucose level tested (23), and that a family history of diabetes probably affects an individual's knowledge of having diabetes (24).

Diabetes risk is increased by both IFG and IGT, and it is increased by the two together more than by either one alone. This is consistent with the concept that any rise in glucose is not benign and reflects an endocrine pancreatic defect. The annual incidence of diabetes in people with IFG or IGT varied from 5% to 10%. Compared with normoglycemic people, the meta-analyzed relative risk and 95% CI for diabetes was 5.52 (3.13-7.91) in people with iIGT, 7.54 (4.63-10.45) in people with iIFG, and 12.13 (4.27-20) in people with both IFG and IGT (25). With the iIFG group chosen as the reference group in this study, we demonstrated that iIFG and iIGT had no differences in the progression to diabetes, but iIGT had a higher likelihood of regressing to normoglycemia with a borderline significance (P = .054). Insulin resistance in subjects with IFG is due to increased hepatic insulin resistance while in subjects with IGT it is related to the increased insulin resistance in skeletal muscles. However, there is a strong association between increased insulin resistance in liver and skeletal muscles. In both kinds of pre-diabetes, insulin resistance combined with β-cell dysfunction would be responsible for the increased risk of type 2 diabetes. ***(Abdul-Ghani MA, DeFronzo RA. Pathophysiology of prediabetes. Current diabetes reports. 2009 Jun;9(3):193-9.)***

Regression to normoglycemia was more likely to occur in participants with higher education levels. Education level is associated with income, occupation, and physical activity. Education also improves the willingness to seek health information and encourages healthy lifestyle behaviors. The inverse association between education level and diabetes and obesity has been supported by previous studies (26, 27).

In terms of possible clinical and public health significance of our findings, since identifying high-risk populations is considered a critical issue in diabetes prevention and intervention programs, pre-diabetes is an appropriate state in which high-risk individuals could be identified and followed for appropriate interventions. Therefore, identifying high-risk pre-diabetes people who progress to diabetes can help us carry out effective interventions to prevent diabetes, and even better control of these risk factors can increase the regression to normoglycemia.

Strengths and limitations

The population-based longitudinal study design, multiple measurements from both clinical and paraclinical sources, including tests and questionnaires, repeated blood sampling, extensive follow-up, and use of an analysis that takes into account all outcomes simultaneously are the main strengths of our study. The loss of follow-up is a limitation in our study, as it is in any cohort study, which we tried to resolve *via* imputation. Another limitation is the definition of diabetes and normoglycemia, which was determined by a single blood glucose measurement; however, this is common in epidemiological studies. The diagnosis of diabetes in clinical practice is based on at least two measurements of hyperglycemia and using one measurement in epidemiological studies makes the results unreliable. In our study the fluctuation of glucose level could be on both sides, i.e toward diabetes or normoglycemia, so although it may decrease the reliability of the results, a measurement bias is not plausible. Furthermore, although there is a growing body of evidence describing how the types of prediabetes are physiologically different, because of the low sample size we could not separate the analysis based on the type of prediabetes. Nevertheless, the likelihood ratio test did not show a significant interaction between the type of prediabetes and different predictors.

Conclusion

The magnitude of regression to normal glucose levels was the same as progression toward diabetes. We did not aim to investigate the reasons why people with prediabetes progressed to diabetes and regressed to normoglycemia, however, we did demonstrate that different factors can predict these related outcomes. Factors associated with regression to normal glucose levels were age, sex, and education level, and factors associated with progression to diabetes were BMI and familial history of type 2 diabetes. The combined IFG/IGT had a notable significant relationship with both, which indicates the major determinant role for prediabetes transitions.

In diabetes preventive and intervention programs, identifying high-risk people is thought to be a challenging task. It seems that prediabetes is a state in which high-risk populations should be identified, and essential interventions should be done. Identification of high-risk prediabetes individuals who go on to develop diabetes is crucial for effective diabetes prevention. Prediabetic individuals may progress to normoglycemia if these risk factors are better managed.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Shahid Beheshti University of Medical Sciences (Ethics approval reference number: IR.SBMU.ENDOCRINE.REC.1400.113). The patients/participants provided their written informed consent to participate in this study.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: FH, FA, and DK. Data collection: FH, FA, and DK. Analysis and interpretation of results: ZA, KK, and DK. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1041808/full#supplementary-material

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Lower bile acids as an independent risk factor for renal outcomes in patients with type 2 diabetes mellitus and biopsy-proven diabetic kidney disease

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Aims: Abnormalities of glucolipid metabolism are critical mechanisms involved in the progression of diabetic kidney disease (DKD). Bile acids have an essential role in regulating glucolipid metabolism. This study investigated the clinicopathological characteristics of DKD patients with different bile acid levels and explored the relationship between bile acids and renal outcomes of DKD patients.

Methods: We retrospectively reviewed and evaluated the histopathological features and clinical features of our cohort of 184 patients with type 2 diabetes mellitus and biopsy-proven DKD. Patients were divided into the lower bile acids group (≤2.8 mmol/L) and higher bile acids group (>2.8 mmol/L) based on the cutoff value of bile acids obtained using the time-dependent receiver-operating characteristic curve. Renal outcomes were defined as end-stage renal disease (ESRD). The influence of bile acids on renal outcomes and correlations between bile acids and clinicopathological indicators were evaluated.

Results: Bile acids were positively correlated with age (r = 0.152; P = 0.040) and serum albumin (r = 0.148; P = 0.045) and negatively correlated with total cholesterol (r = -0.151; P = 0.041) and glomerular class (r = -0.164; P =0.027). During follow-up, 64 of 184 patients (34.78%) experienced progression to ESRD. Lower levels of proteinuria, serum albumin, and bile acids were independently associated with an increased risk of ESRD (hazard ratio, R=5.319; 95% confidence interval, 1.208–23.425).

Conclusions: Bile acids are an independent risk factor for adverse renal outcomes of DKD patients. The serum level of bile acids should be maintained at more than 2.8 mmol/L in DKD patients. Bile acid analogs or

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their downstream signaling pathway agonists may offer a promising strategy for treating DKD.

KEYWORDS

bile acids, diabetic kidney disease, glucolipid metabolism, end-stage renal disease, renal outcomes, risk factors

Introduction

Data collected from 142 countries comprising 97.3% of the worldwide population showed that the global prevalence of diabetes among patients with end-stage renal disease (ESRD) increased from 19.0% in 2000 to 29.7% in 2015, and that the proportion of patients with ESRD attributable to diabetes increased from 22.1% to 31.3% (1). Diabetic kidney disease (DKD) is a significant microvascular complication that has become the leading cause of chronic kidney disease and ESRD, resulting in large health and economic burdens worldwide (2–4).

The management of risk factors, such as hyperglycemia, hypertension, dyslipidemia, and the use of renin-angiotensin-aldosterone system blockers, has helped to delay the progression of DKD. Recently, new therapeutic agents, including sodium-glucose transporter 2 inhibitors, endothelin antagonists, glucagon-like peptide-1 receptor agonists, and mineralocorticoid receptor antagonists, have provided additional treatment options for patients with DKD (5). Although more treatment options are available, a significant number of patients still experience progression to ESRD. Therefore, it is urgent to actively explore the pathogenesis of DKD to find more effective intervention targets.

Abnormalities of glycolipid metabolism are crucial in the development and progression of DKD. Bile acids are the main components of bile (approximately 50% of the organic bile composition) and are mainly synthesized by the liver; furthermore, they have been confirmed to regulate glycolipid metabolism (6, 7). The improvement of glycolipid metabolism has been proven to be renoprotective; therefore, bile acids may indirectly exert renoprotective effects by improving glycolipid metabolism. Additionally, many studies have shown that bile acid signaling molecules exert metabolic effects by interacting with nuclear receptors (farnesoid X receptor [FXR], pregnane X receptor, vitamin D receptor, G-protein-coupled receptors [TGR5]), and cellular signal transduction pathways (e.g., c-Jun N-terminal kinase and extracellular signal-regulated kinase) (8). This suggests that bile acids and their analogs may exert direct physiological effects by activating receptors in other organs. Some studies confirmed that bile acid derivatives or analogs can directly act on the bile acid receptors (TGR5/FXR) of the kidney to protect the kidney (9–13). Whether improving glucose and lipid metabolism or modulating energy metabolism or directly activating renal bile acid receptors, bile acids are closely related to the prognosis of DKD patients; therefore, bile acid analogs are likely to become a new treatment for DKD.

No study has confirmed whether bile acids are associated with renal outcomes of patients with DKD. Therefore, during this retrospective cohort study, we explored whether bile acid levels could predict the renal prognosis of Chinese patients with type 2 diabetes mellitus (T2DM) and biopsy-proven DKD.

Materials and methods

Study design and patients

This was a retrospective cohort study including T2DM patients with biopsy-confirmed DKD at the West China Hospital of Sichuan University from April 2009 to December 2021. The diagnosis and classification of T2DM were based on the criteria of the American Diabetes Association (14). DKD was diagnosed according to the standards of the Renal Pathology Society in 2010. The inclusion criteria were age 18 years or older, diagnosis of T2DM, and diagnosis of DKD proven by renal biopsy. The exclusion criteria were malignant tumors, coexistence with other glomerular diseases, hepatobiliary disease (active hepatitis, cirrhosis, hepatobiliary stones), estimated glomerular filtration rate (eGFR) <15 mL/min/1.73 m^2 or dialysis, and incomplete data (Figure 1). This study was approved by the ethics committee of West China Hospital of Sichuan University. The study protocol complied with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients.

Clinical and pathologic characteristics

Clinical and pathologic characteristics were collected from the electronic medical records at the time of renal biopsy. Subsequent follow-up evaluations of these patients were performed two to

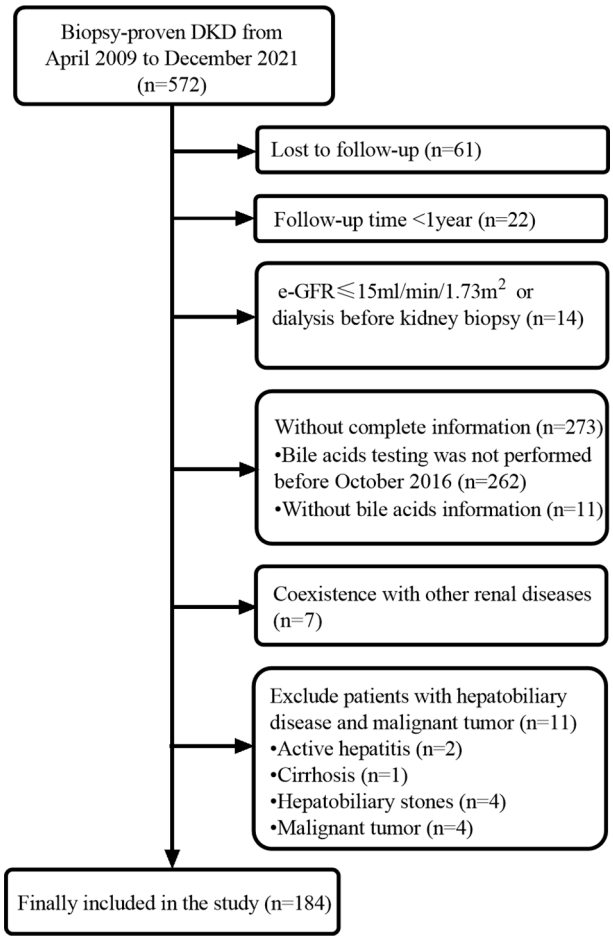

FIGURE 1
Flowchart of included patients in this study.

four times per year depending on the patient's condition. The renal outcomes were defined by ESRD, which was considered the requirement for renal replacement therapy (kidney transplantation and/or hemodialysis and/or peritoneal dialysis), and/or eGFR <15 mL/min/1.73 m^2. The eGFR was calculated using the creatinine-based Chronic Kidney Disease Epidemiology Collaboration equation. Bile acid tests were performed using an enzymatic cycling assay. All biopsy specimens were routinely examined by light immunofluorescence. The histological lesions were evaluated according to the criteria of the Renal Pathology Society (15).

Statistical analysis

All statistical tests were analyzed using SPSS version 26.0 (SPSS Inc., Chicago, IL, USA). The normally distributed continuous variables were expressed as the mean ± standard deviation or median and interquartile range. Categorical data were presented as the number and percentage. The time-dependent receiver-operating characteristic curve (ROC) was used to evaluate the prognostic accuracy of bile acids, and the cutoff value was calculated using R4.03 (R Foundation for Statistical Computing, Vienna, Austria). When comparing two groups, we used the t test, Mann–Whitney U test, and chi-square test, as appropriate. Correlations between bile acids and clinical and pathological findings were calculated using correlation analysis. Pearson's correlation was used for normally distributed numerical variables, and Spearman's correlation was used for other variables. The renal survival curves were assessed using the Kaplan–Meier method and compared using the log-rank test. Cox proportional hazard models were performed to analyze the influence of bile acids on renal outcomes. A two-sided $P < 0.05$ was considered statistically significant.

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Results

Baseline characteristics

This study cohort comprised a total of 184 individuals with biopsy-proven DKD (Figure 1). Clinical data are provided in Table 1. The median bile acid level was 2.80 mmol/L (1.60–4.85 mmol/L) for all patients. The median age was 51.0 years (44.0 –56.0 years), and 74.5% of patients were male. The median duration of diabetes was 108.00 months (60.00–144.00 months). Complications of diabetic retinopathy were observed in 53.06% of patients. Comorbidities of hypertension were observed in 85.33% of patients. The mean proteinuria and eGFR levels were 5.16 ± 4.27 g/d and 63.21 ± 26.59 mL/min/1.73 m^2, respectively. The patients had more severe proteinuria and lower eGFR. Furthermore, 78.0% of the patients used renin-angiotensin system inhibitors (RASI). A restricted cubic spline was used to calculate the cutoff value of bile acids (Figure 2). Then, patients were divided into the lower bile acids group (≤2.8 mmol/L) and the higher bile acids group (>2.8 mmol/L) according to the cutoff value. Compared with the lower bile acids group, the higher bile acids group had lower total cholesterol levels, lower low-density lipoprotein cholesterol levels, older ages, higher serum albumin levels, and higher eGFR levels (Table 1). There were no significant differences in the pathologic changes and use of RASI (Table 2).

Clinical and pathological features associated with bile acids

The bile acid level was positively correlated with age (r = 0.152; P = 0.040) and serum albumin (r = 0.148; P = 0.045) and negatively correlated with total cholesterol (r = -0.151; P = 0.041) and glomerular class (r = -0.164; P =0.027) (Figure 3, Supplementary Table 1).

Risk of progression to ESRD

During the median follow-up of 19.02 months (8.65-32.39 months), 64 of 184 (34.78%) patients experienced progression to ESRD. Compared with patients with lower bile acid levels, those with higher bile acid levels were likely to have a lower incidence of ESRD (Table 1). A Kaplan-Meier analysis indicated that patients with lower bile acid levels at baseline were at significantly higher risk for progression to ESRD. The time-dependent ROC was used to evaluate the prognostic accuracy of bile acid levels of patients with DKD and showed that the predictive ability of bile acids for ESRD was relatively stable over time (Figure 4, Supplementary Figure 1). The Cox regression analysis evaluated the association between baseline clinicopathological variables and the renal prognosis. Univariate analyses revealed that bile acids, diabetic retinopathy (DR), body

TABLE 1 Baseline clinical features of 184 DKD patients.

Variables	All (n=184)	Lower bile acids (n=93)≤2.8mmol/L	Higher bile acids (n=91)>2.8mmol/L	p-value
Age (years)	51.00 (44.00–56.00)	50.00 (43.00–53.00)	54.5 (46.5–59.5)	0.002
Gender (male, %)	137 (74.5)	69 (74.2)	68 (74.7)	0.934
DR [n (%)]	52 (53.1)	27 (54. 0)	25 (52.1)	0.849
Duration of diabetes (Months)	108.00 (60.00–144.00)	96.00 (60.00–156.00)	108.00 (60.00–138.00)	0.936
BMI (kg/m^2)	24.74 (22.23–26.89)	24.38 (21.51–26.53)	25.01 (22.41–27.97)	0.413
Hypertension [n (%)]	157 (85.3)	82 (88.2)	75 (82.4)	0.270
Initial proteinuria (g/day)	5.16 ± 4.27	5.45 ± 4.23	4.81 ± 4.34	0.379
e-GFR ($ml/min/1.73m^2$)	63.21 ± 26.59	58.94 ± 25.30	67.46 ± 27.31	0.030
Serum creatinine (mg/dL)	133.42 ± 107.91	148.65 ± 141.96	118.02 ± 51.92	0.055
Serum albumin (g/L)	35.35 ± 7.38	33.08 ± 6.87	37.67 ± 7.20	<0.001
Hemoglobin (g/L)	120.54 ± 22.53	117.99 ± 22.51	123.15 ± 22.38	0.120
HbA1c (%)	7.89 ± 1.99	7.76 ± 2.25	8.02 ± 1.67	0.411
FBS (mmol/L)	7.15 (5.70–10.09)	7.03 (5.71–13.88)	7.71 (5.63–9.80)	0.816
Triglyceride (mmol/L)	2.12 ± 1.22	2.08 ± 1.14	2.25 ± 1.64	0.410
Total cholesterol (mmol/L)	5.08 ± 1.64	5.56 ± 1.54	4.59 ± 1.60	<0.001
LDL-c (mmol/L)	2.92 ± 1.34	3.37 ± 1.34	2.47 ± 1.18	<0.001
HDL-c (mmol/L)	1.36 ± 0.67	1.43 ± 0.73	1.28 ± 0.60	0.128
RASI [n (%)]	142 (78.0)	71 (78.0)	71 (78.0)	1.000
Progressed to ESRD (%)	64 (34.8)	40 (43.0)	24 (26.4)	0.018

DR, diabetic retinopathy; e-GFR, estimated glomerular filtration rate; FBS, fasting blood sugar; LDL, low density lipoprotein; HDL, high density lipoprotein; RASI, renin-angiotensin system inhibitor; ESRD, end-stage renal disease;

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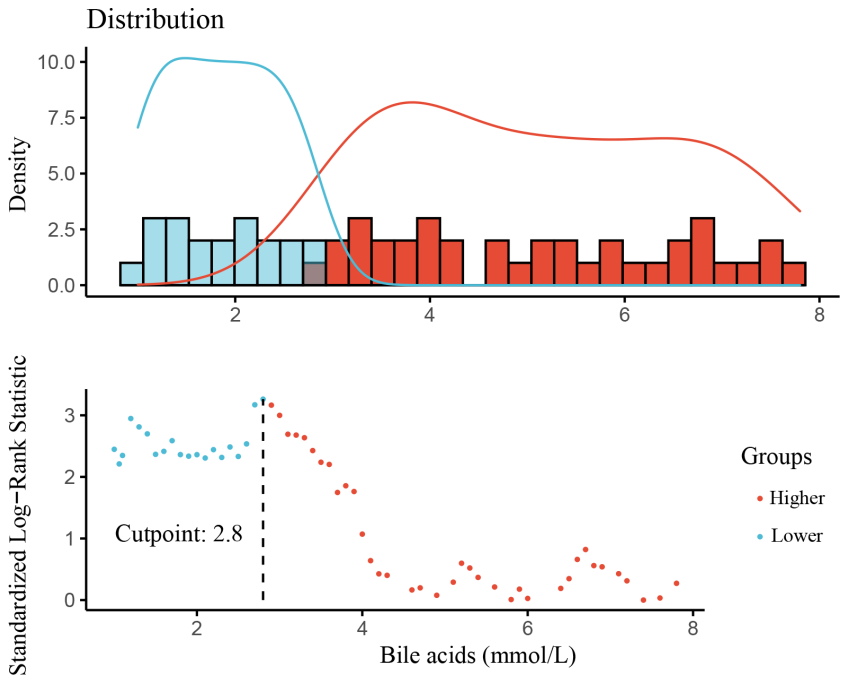

FIGURE 2
The optimal cut-point value of variables by restricted cubic spline.

TABLE 2 Baseline pathologic features of 184 DKD patients.

Variables	All (n=184)	Lower bile acids (n=93)≤2.8mmol/L	Higher bile acids (n=91)>2.8mmol/L	p-value
Glomerular class [n (%)]				0.130
I	10 (5.4)	5 (5.4)	5 (5.5)	
IIa	38 (20.7)	14 (15.1)	24 (26.4)	
IIb	34 (18.5)	16 (17.2)	18 (19.8)	
III	74 (40.2)	39 (41.9)	35 (38.5)	
IV	28 (15.2)	19 (20.4)	9 (9.9)	
IFTA [n (%)]				0.118
0	2 (1.1)	1 (1.1)	1 (1.1)	
1	78 (42.4)	35 (37.6)	40 (47.3)	
2	74 (40.2)	36 (38.7)	38 (41.8)	
3	30 (16.3)	21 (22.6)	9 (9.9)	
Interstitial inflammation [n (%)]				0.118
0	4 (3.1)	3 (5.0)	1 (1.5)	
1	89 (70.1)	38 (60.0)	53 (79.1)	
2	32 (25.2)	20 (33.3)	12 (17.9)	
3	2 (1.6)	1 (1.7)	1 (1.5)	
Arteriolar hyalinosis [n (%)]				0.353
0	8 (5.6)	3 (4.4)	5 (6. 6)	
1	75 (52.0)	32 (47.1)	43 (56.6)	
2	61 (42.4)	33 (48.5)	28 (36.8)	

IFTA, interstitial fibrosis and tubular atrophy.

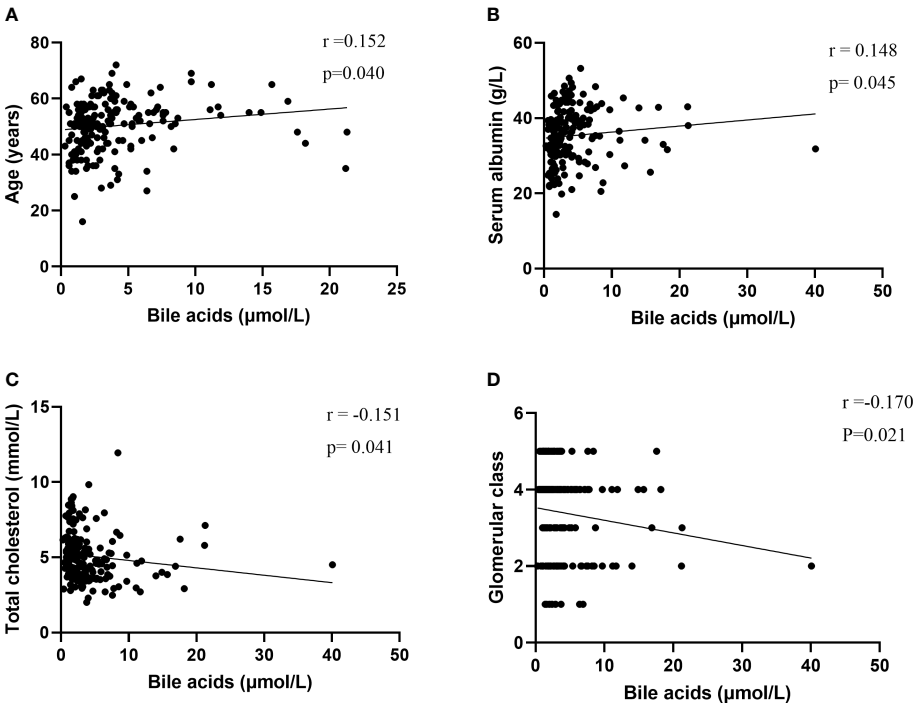

FIGURE 3
Correlations of bile acids with **(A)** Age, **(B)** serum albumin, **(C)** total cholesterol, **(D)** Glomerular class.

mass index (BMI), eGFR, hemoglobin, serum albumin, initial proteinuria, glomerular class, interstitial fibrosis, and tubular atrophy, and the use of RASI were risk factors for progression to ESRD ($P < 0.05$) (Supplementary Table 2). Lower bile acid levels remained independently associated with a higher risk of progression to ESRD with DKD after adjusting for baseline age, sex, BMI, DR, hypertension, DM duration, eGFR, initial proteinuria, hemoglobin, serum albumin, glomerular class, interstitial fibrosis and tubular atrophy, and RASI use (in model 3). The hazard ratio for the lower bile acids group was

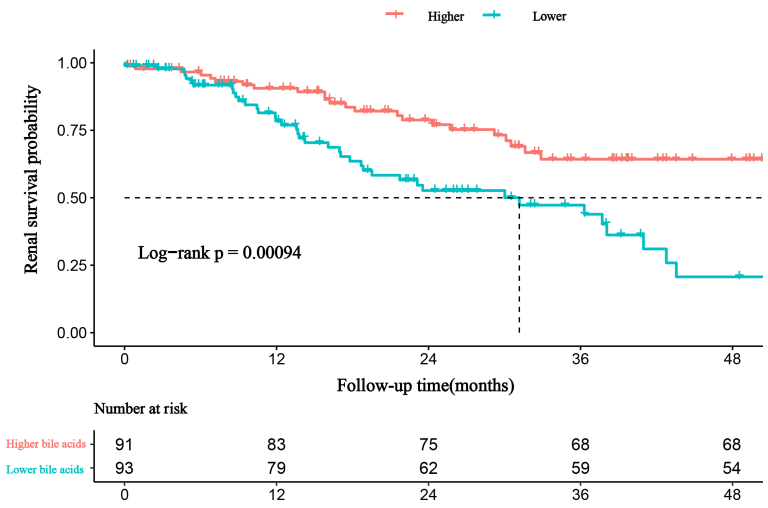

FIGURE 4
The prediction of bile acids for ESRD in DKD patients.

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5.319 (95% confidence interval, 1.208–23.425; P = 0.027) (Table 3). Additionally, initial proteinuria and serum albumin levels were independent risk factors for renal outcomes of patients with DKD.

Discussion

To the best of our knowledge, this is the first cohort study to relate bile acids to renal outcomes of patients with DKD. We explored the associations among bile acids, clinicopathological features, and renal outcomes of 184 patients with T2DM and biopsy-proven DKD. The results indicated that bile acids are an independent predictor of DKD progression to ESRD in T2DM patients in addition to traditional factors, including proteinuria and serum albumin levels, that serum bile acid, as a noninvasive marker, was associated with adverse renal outcomes, and that bile acid analogs and their targeting downstream signaling pathway might be promising therapeutic agents for the treatment of DKD.

Bile acids are synthesized intrahepatically from cholesterol and are the major organic component of bile. Eating foods that are high in protein can lead to increased bile acid secretion. Vagus nerve excitation can also lead to increased bile acid secretion. Humoral factors such as gastrin, pancreatin, cholecystokinin, and bile salts, can cause increased bile acid secretion. Pathological factors such as hepatobiliary disease can also lead to increased bile acid secretion. Additionally, studies have suggested that metformin (16, 17) and metabolic surgery (18, 19) increase bile acid levels.

There have been no reports of the relationship between bile acids and the renal prognosis of patients with DKD. We found that the risk of ESRD decreased with increasing bile acid levels. We obtained the cutoff value using the restricted cubic spline. Patients with DKD and bile acid levels less than 2.8 mmol/L have a poor renal prognosis. Additionally, the cutoff value is a reference value for discriminating those at clinically higher risk for ESRD. However, we believe that there is an upper limit to the bile acid level that is beneficial to renal outcomes. Exceeding the upper limit, however, may mean that more bile acid receptors will be activated and more side effects may occur, thus leading to more harm than good. The time-dependent ROC was used to evaluate the prognostic accuracy of bile acid levels of patients with DKD and showed that the predictive ability of bile acids for ESRD was relatively stable over time. The survival analysis performed during our study confirmed that patients with higher bile acid levels have a better renal prognosis. Furthermore, the risk of ESRD was 5.319-times higher for patients with lower bile acid levels compared to those with higher bile acid levels, suggesting the importance of bile acids to patient outcomes. Further exploration of the mechanisms of their protective effects is necessary.

TABLE 3 Associations between bile acid levels and renal outcomes.

	Hazard Ratio (95% Confidence Interval)		
	Lower bile acids (≤2.8mmol/L)	Higher bile acids (>2.8mmol/L)	p-value
Unadjusted	2.311 (1.386-3.852)	1	0.001
Model 1[a]	6.006 (1.512-23.857)	1	0.011
Model 2[b]	6.338 (1.555-25.834)	1	0.10
Model 3[c]	5.319 (1.208-23.425)	1	0.027

Model 1[a] adjusted for baseline age, gender, BMI, hypertension (yes or no), DR (yes or no), DM duration, e-GFR, and proteinuria, Hemoglobin, Serum albumin. **Model 2**[b] adjusted for covariates in model 1 plus renal pathological findings (the glomerular class, IFTA). **Model 3**[c] adjusted for covariates in model 2 plus RASI use. CI, confidence interval; DR, diabetic retinopathy; e-GFR, estimated glomerular filtration rate; IFTA, interstitial fibrosis and tubular atrophy; RASI, renin-angiotensin system inhibitor.

A better renal prognosis for DKD patients with higher bile acid levels might be achieved by improvements in glucose metabolism disorders. Increasing studies have shown that bile acids are involved in glycometabolism. Wang et al. (20) demonstrated that bile acids can regulate postprandial glucose metabolism levels, suggesting a direct role of bile acids in the regulation of blood glucose. Sang et al. (21) demonstrated an increased risk of dysglycemia for Chinese community-dwelling individuals who underwent cholecystectomy, indirectly suggesting that bile acids have an important role in maintaining blood glucose. Many studies have shown that regulating blood glucose can delay DKD progression. The United Kingdom Prospective Diabetes Study was a landmark randomized, multicenter trial of glycemic therapies for 5102 patients with newly diagnosed T2DM that was conducted for 20 years (1977-1997) at 23 clinical sites in the United Kingdom and conclusively showed that intensive control can reduce the risk of microvascular complications, including progression to DKD (22–24). The ACCORD (25), ADVANCE (26), and VADT (27) studies also confirmed the same conclusion for patients with T2DM. Furthermore, a meta-study evaluated seven trials involving 28,065 adults who were monitored for 2 to 15 years and showed that compared with conventional control, intensive glucose control reduced the risks of microalbuminuria and macroalbuminuria (28). However, our study showed no correlation between bile acid levels and HbA1c and fasting blood sugar levels of patients with DKD. This may have occurred because most patients had been treated with glucose-lowering therapy. In our study, glucose-lowering therapy, including the use of insulin and oral hypoglycemic agents, was used for 89.9% of the patients. Therefore, we think that the renal protective effects of bile acids may be attributed to improved glucose metabolism.

The better renal prognosis for DKD patients with higher bile acid levels might be achieved by their improved glycolipid metabolism disorders. We initially recognized that the primary

role of bile acids is to promote the digestion and absorption of lipid nutrients, thus serving as amphipathic biological detergents for lipid metabolism (6). Bile acids are the end products of cholesterol catabolism and have an important role in maintaining cholesterol homeostasis and preventing the build-up of toxic metabolites and the accumulation of cholesterol (7). However, hyperlipemia is a traditionally recognized risk factor for cardiovascular disease for patients with T2DM and DKD (29). Several studies have shown that high triglyceride levels and/or low high-density lipoprotein cholesterol levels are independent risk factors for DKD in patients with the recommended target values of blood glucose and blood pressure for type 1 diabetes mellitus and T2DM (30). Muntner et al. (31) investigated the relationship between plasma lipids and kidney disease indicated by an increase of ≥0.4 mg/dL in the serum creatinine level of the large cohort of the Atherosclerosis Risk in Communities study that included patients with T2DM. The United Kingdom Prospective Diabetes Study investigating baseline clinical risk factors associated with the later development of kidney dysfunction in more than 4000 participants, all with T2DM, identified that higher triglyceride and low-density lipoprotein cholesterol levels significantly and independently predicted incident renal impairment (32). The Early Treatment Diabetic Retinopathy Study revealed that increased serum triglyceride and total cholesterol levels were independently associated with kidney outcomes (33). The role of bile acids in regulating lipid metabolism was also confirmed during our study. We found that there was a negative correlation between bile acids and total cholesterol with DKD. Therefore, we consider that the renoprotective effect of bile acids may be attributable to the improved lipid metabolism of patients with DKD.

Bile acids may improve renal outcomes of patients with DKD by directly activating renal FXR or TGR5. In human and animal models, tubular cells and glomerular cells of the kidney highly express FXR, and FXR is downregulated in diabetic kidney disease (9). Wang et al. (10) demonstrated accelerated renal injury in diabetic FXR knockout mice. In contrast, treatment with the FXR agonist INT-747 improved renal injury by decreasing proteinuria, glomerulosclerosis, and tubulointerstitial fibrosis and modulating renal lipid metabolism. Similarly, Jiang et al. (11) reported that FXR modulates renal lipid metabolism, fibrosis, and DKD. Many studies have suggested that FXR activation inhibits inflammation in DKD (12). Moreover, FXR activation improves diabetic tubular function and tubular toxicity (34–36). TGR5 was identified as a membrane receptor for bile acids which is highly expressed in tubules, podocytes, and mesangial cells in the kidney (37, 38). It has been confirmed that the TGR5 agonist INT-777 induced mitochondrial biogenesis, decreased oxidative stress, increased fatty acid beta oxidation, and decreased renal lipid accumulation (39). We found that the bile acid level was negatively correlated with the severity of the glomerular injury, suggesting that bile acids may activate receptors and downstream signaling pathways in glomerular cells. Therefore, the direct relationship between bile acids and kidney injury must be explored.

Metformin can increase bile acid levels and the glucose-lowering effect, which may benefit the kidneys. Possible mechanisms for metformin-induced suppression of active bile acid reabsorption in the ileum are inhibition of the apical sodium-dependent bile acid transporter and modulation of the transcriptional activity of FXR via an AMPK-mediated mechanism in enterocytes (16). However, metformin is contraindicated for many individuals with impaired kidney function because of concerns of lactic acidosis (40). Nevertheless, many studies have suggested that metformin may have renoprotective effects on DKD. A recent retrospective study confirmed that metformin for advanced chronic kidney disease patients decreased the risk of all-cause mortality and incident ESRD. Additionally, metformin did not increase the risk of lactic acidosis. However, because of the remaining bias even after propensity score matching, further randomized, controlled experiments with large samples are necessary to change real-world practice (41). Therefore, metformin may exert renoprotective effects through bile acids in DKD, but the specific mechanism requires further investigation. Unfortunately, our data lacked information regarding metformin treatment, and it was impossible to analyze the relationship between metformin and bile acids during our study.

Bariatric and metabolic surgeries, including Roux-en-Y gastric bypass and vertical sleeve gastrectomy, are known to increase bile acid secretion and alter bile acid composition, particularly after Roux-en-Y gastric bypass (18, 19). The mechanisms underlying the benefits of bariatric and metabolic surgeries likely involve the bile acids signaling pathway mediated mainly by nuclear FXR and the membrane TGR5, the interaction of bile acids and gut microbiota, and exosomes (18, 19). Bariatric and metabolic surgeries have been shown to improve hyperglycemia, insulin sensitivity, and hyperlipidemia (19). These renoprotective effects may be closely related to the bile acid and glycolipid metabolic benefits associated with bariatric and metabolic surgeries. However, the effects on important endpoints of kidneys, such as ESRD and eGFR changes, must be further confirmed by randomized controlled experiments with large samples. Furthermore, the mechanism of action in DKD requires more research for further elucidation.

Higher levels of bile acids with better renal outcomes may be attributed to the indirect effects of bile acids that result in improved glycolipid metabolism and the direct effects of activating bile acids receptors to protect the kidney.

We also found a negative correlation between bile acid levels and age; this may have occurred because the synthesis and secretion of bile acids are different in individuals of different ages. We found a positive correlation between bile acid and serum albumin levels; however, more studies exploring the possible mechanism are necessary.

This study had some limitations. First, this was a retrospective study; therefore, some selection bias was inevitable. Second, the patients had biopsy-proven DKD, and

the sample size was insufficient. Finally, we did not control all therapeutic interventions (such as glucagon-like peptide-1 and sodium-glucose transporter 2), which could have been confounders of the results.

In conclusion, our study describes a novel marker for predicting the renal outcomes of DKD and indicates that the serum level of bile acids should be maintained at more than 2.8 mmol/L in patients with DKD. Our study also predicted that bile acid analogs and their targeting downstream signaling pathway might be promising therapeutic agents for the treatment of DKD.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

Conception and design of the study: XX, JZ, SJ, and FL. Acquisition and analysis of data: XX, SJ, JZ, YTZ, JY, YW, YCZ, and QY. Drafting the manuscript or figures: XX, JZ, SJ, and FL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1026995/full#supplementary-material

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Genetic variants for prediction of gestational diabetes mellitus and modulation of susceptibility by a nutritional intervention based on a Mediterranean diet

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Hypothesis: Gestational diabetes mellitus (GDM) entails a complex underlying pathogenesis, with a specific genetic background and the effect of environmental factors. This study examines the link between a set of single nucleotide polymorphisms (SNPs) associated with diabetes and the development of GDM in pregnant women with different ethnicities, and evaluates its potential modulation with a clinical intervention based on a Mediterranean diet.

Methods: 2418 women from our hospital-based cohort of pregnant women screened for GDM from January 2015 to November 2017 (the San Carlos Cohort, randomized controlled trial for the prevention of GDM ISRCTN84389045 and real-world study ISRCTN13389832) were assessed for evaluation. Diagnosis of GDM was made according to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria. Genotyping was performed by IPLEX MassARRAY PCR using the Agena platform (Agena Bioscience, SanDiego, CA). 110 SNPs were selected for analysis based on selected literature references. Statistical analyses regarding

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patients' characteristics were performed in SPSS (Chicago, IL, USA) version 24.0. Genetic association tests were performed using PLINK v.1.9 and 2.0 software. Bioinformatics analysis, with mapping of SNPs was performed using STRING, version 11.5.

Results: Quality controls retrieved a total 98 SNPs and 1573 samples, 272 (17.3%) with GDM and 1301 (82.7%) without GDM. 1104 (70.2%) were Caucasian (CAU) and 469 (29.8%) Hispanic (HIS). 415 (26.4%) were from the control group (CG), 418 (26.6%) from the nutritional intervention group (IG) and 740 (47.0%) from the real-world group (RW). 40 SNPs (40.8%) presented some kind of significant association with GDM in at least one of the genetic tests considered. The nutritional intervention presented a significant association with GDM, regardless of the variant considered. In CAU, variants rs4402960, rs7651090, IGF2BP2; rs1387153, rs10830963, MTNR1B; rs17676067, GLP2R; rs1371614, DPYSL5; rs5215, KCNJ1; and rs2293941, PDX1 were significantly associated with an increased risk of GDM, whilst rs780094, GCKR; rs7607980, COBLL1; rs3746750, SLC17A9; rs6048205, FOXA2; rs7041847, rs7034200, rs10814916, GLIS3; rs3783347, WARS; and rs1805087, MTR, were significantly associated with a decreased risk of GDM, In HIS, variants significantly associated with increased risk of GDM were rs9368222, CDKAL1; rs2302593, GIPR; rs10885122, ADRA2A; rs1387153, MTNR1B; rs737288, BACE2; rs1371614, DPYSL5; and rs2293941, PDX1, whilst rs340874, PROX1; rs2943634, IRS1; rs7041847, GLIS3; rs780094, GCKR; rs563694, G6PC2; and rs11605924, CRY2 were significantly associated with decreased risk for GDM.

Conclusions: We identify a core set of SNPs in their association with diabetes and GDM in a large cohort of patients from two main ethnicities from a single center. Identification of these genetic variants, even in the setting of a nutritional intervention, deems useful to design preventive and therapeutic strategies.

KEYWORDS

genetic risk variants, genetic polymorphisms, gestational diabetes mellitus, single nucleotide polymorphisms, SNPs, Mediterranean diet, nutritional intervention

Introduction

Gestational diabetes mellitus (GDM), defined as diabetes newly diagnosed in the second or third trimester of pregnancy, and was not clearly overt diabetes prior to gestation (1), is a frequent gestational metabolic complication that has become a major public health issue. Its prevalence has significantly increased in parallel with increasing rates of obesity, older age at pregnancy, and the implementation of the International Association of the Diabetes and Pregnancy Study Groups criteria (IADPSG criteria) (2). GDM is associated with adverse maternal and neonatal outcomes and an increased risk for the future development of type 2 diabetes both in the mother and the offspring (1, 2), so strategies for early detection and prevention, and interventions to control maternal glucose levels have become a priority.

The complex underlying pathogenesis of GDM includes a specific genetic background and the effect of environmental factors. Although there is still much to be known regarding the underlying mechanisms responsible for the development of GDM, several modifiable and non-modifiable factors have been acknowledged; for instance, increased adiposity, lifestyle, ethnicity, increased maternal age, polycystic ovary syndrome or a family history for type 2 diabetes. Regarding the genetic background, several genetic polymorphisms have been identified as potentially associated with an increased risk of developing GDM, most of them overlapping with those associated with the risk of type 2 diabetes. However, there is still controversy on the

true impact of genetic polymorphisms on the risk of these metabolic alterations, and whether this increased risk could be modulated by clinical interventions such as diet. In previous studies (3, 4) we found that an early nutritional intervention with a supplemented Mediterranean diet (MedDiet) reduces the incidence of GDM and, consequently, our hospital recommended the adoption of this nutritional intervention to all pregnant women.

The objective of this study is to examine the link between a set of single nucleotide polymorphisms (SNPs) associated with diabetes and GDM, according to different bibliographical references, and the development of GDM in pregnant women of different ethnicities, in the setting of a clinical intervention based on the MedDiet.

Methods

Study population

The study population originates from our hospital-based cohort of pregnant women screened for GDM from January 2015 to November 2017 (the San Carlos Cohort, randomized controlled trial (RCT) for the prevention of GDM registered December 4, 2013 at ISRCTN84389045 (DOI 10.1186/ISRCTN84389045) and real-world study, registered October 11th, 2016 at ISRCTN13389832 (DOI 10.1186/ISRCTN13389832) (3, 4) with approval by the Clinical Trials Committee of the Hospital Clínico San Carlos (July 17, 2013, CI 13/296-E and October 1st, 2016, CI16/442-E, respectively), and compliance with the Declaration of Helsinki). The central location of our hospital and its relatively large reference healthcare population of around 445,000 implied that our study sample could adequately represent the population living in our country.

Figure 1 shows the CONSORT 2010 flowchart of our study population. From January 2015 to November 2017, a total of 2418 women who attended their first gestational visit (at 8 ± 2 gestational weeks (GW), in which the first ultrasound is performed and analytical screening for chromosomal alterations is carried out), with fasting plasma glucose (FPG) < 92 mg/dL, were assessed for the clinical trial. Inclusion criteria were ≥18 years old, singleton gestation, and willingness to participate in the study. Exclusion criteria comprised gestational age at entry >14 weeks, pre-gestational diabetes, diseases affecting carbohydrate metabolism, intolerance to nuts or extra-virgin olive oil (EVOO), and medical conditions or pharmacological therapy that could compromise the effect of the intervention and/or the follow-up program. All patients included signed a written informed consent.

A sample of 1000 women was selected and randomly divided into two groups of the same size, control group (CG) and intervention group (IG), according to two nutritional intervention models. The same basic MedDiet and daily exercise habits were recommended for both groups. Participants allocated to IG received lifestyle guidance from dieticians one week after inclusion in a unique 1-hour group session. The key IG recommendation was a daily consumption of at least 40 mL of EVOO and a handful (25-30g) of pistachios. To ensure the consumption of the minimum amount recommended, women were provided with 20 L of EVOO and 4 Kg of roasted pistachios. Women in the CG were advised by midwives to restrict consumption of dietary fat, including EVOO and nuts. These recommendations are provided in local antenatal clinics as part of the available guidelines in pregnancy standard care (5). The first women was included on January 2nd, 2015 and the last one was included on December 27th, 2015. The follow up until delivery on July 2016. The study was completed by 874 women (440/434, CG/IG). This group is the initial sub-cohort of this paper.

The aforementioned RCT concluded that an early nutritional intervention with a supplemented MedDiet reduces the incidence of GDM (3). Based on these results, our hospital recommended the adoption of this nutritional intervention (i.e., MedDiet enriched with EVOO and nuts), without providing these specific products, to all pregnant women, from the beginning of gestation, in real word (4). Thus, from November 2016 onwards, every pregnant woman who attended the first gestational visit were invited to participate in our study based on the implementation of the RCT results in clinical practice. The last women included on November 30, 2017 was follow up until delivery on July 2018. In accordance with the inclusion and exclusion criteria indicated above, a new sub-cohort (real-world group, RW) was defined, with 768 samples that are included in this study.

Ethnicity of participants includes mainly Caucasian and Hispanic, as well as some minority ethnicities (Chinese, African and others). Given the characteristics of this study, samples corresponding to these minority ethnic groups were excluded. Therefore, samples from 1586 pregnant women were available and were used for this study. The characteristics of patients included in the study are displayed in Table 1.

Patient data collection

Data regarding clinical, demographic and anthropometric characteristics was collected from medical records and follow-up visits. Specifically, we collected information on maternal age, ethnicity, gestational week at the time of the oral glucose tolerance test (OGTT), body mass index, family history of type 2 diabetes, past medical history of GDM, past obstetric history and parity, gestational weight gain, associated comorbidities, and the newborn's birthweight.

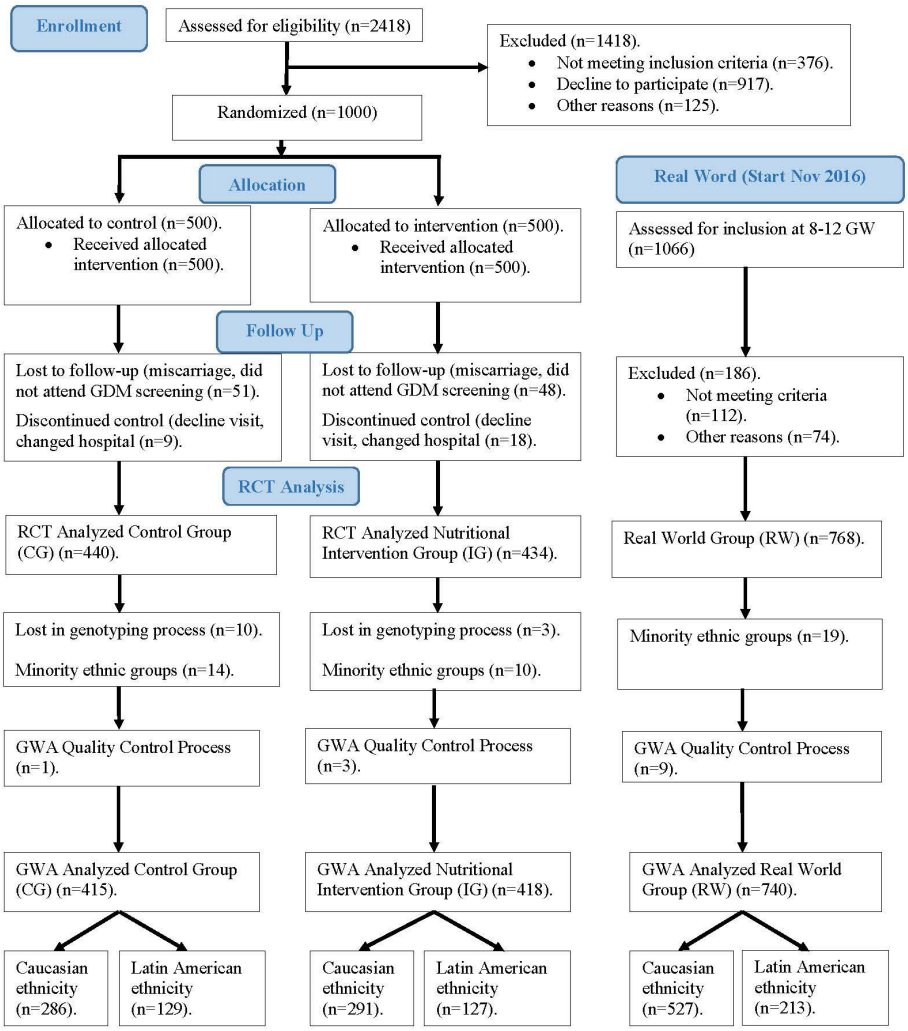

FIGURE 1
Flow diagram of women included in our study.

Diagnosis of gestational diabetes mellitus

A 2-hour OGTT with 75-g glucose was performed at 24-28 weeks of gestation. FPG levels were determined by the glucose oxidase method in fresh plasma samples. The International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria were used for the diagnosis of GDM (2).

Genotype analysis

Genomic DNA was extracted from EDTA-stabilized blood samples taken during the OGTT using the Maxwell RSC instrument (Promega, Dubendorf, Switzerland).

Genotyping was performed by IPLEX MassARRAY PCR using the Agena platform (Agena Bioscience, SanDiego, CA). IPLEX MassARRAY PCR and extension primers were designed from sequences containing each target SNP and 150 upstream and downstream bases with AssayDesign Suite (http://agenabio.com/assay-design-suite-20-software) using the default settings. Single base extension reactions were performed on the PCR reactions with the iPLEX Gold Kit (AgenaBioscience) and 0.8µl of the custom UEP pool. The kit contains mass modified terminator nucleotides that increase the mass difference between extended UEPs, allowing for greater accuracy in genotyping. The mass difference with unmodified terminator nucleotides ranges from 9 to 40 kDa, depending on the two nucleotides compared. With the mass-modified terminator nucleotides the mass difference increases to 16–80 kDa. The single base extension reactions were cycled with a nested PCR protocol that used five cycles of annealing and extension nested with a denaturation step in a cycle that was repeated 40 times for a total of 200 annealing and extension

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TABLE 1 Main characteristics of patients included in the study.

		Gestational diabetes mellitus	
		NO N (%)	YES N (%)
Ethnicity	Caucasian	915 (70.3)	189 (69.5)
	Hispanic	386 (29.7)	83 (30.5)
	Total	1301 (100)	272 (100)
Intervention nutritional group	Control (CG)	319 (24.5)	96 (35.3)
	Intervention (IG)	349 (26.8)	69 (25.4)
	Real Word (RW)	633 (48.7)	107 (39.3)
	Total	1301 (100)	272 (100)
Age (years)		33 ± 5	34 ± 5
Prior body weight (kg)		59.4 ± 9.72	62.82 ± 10.99
Prior BMI		22.47± 3.43	23.99 ± 4.01
Parity	1	567 (43.6)	117 (43.0)
	2	394 (30.3)	86 (31.6)
	3	203 (15.6)	41 (15.1)
	≥ 4	129 (9.9)	28 (10.3)
	NA	8 (0.6)	0 (0)
	Total	1301 (100)	272 (100)
Obstetric history	None	804 (61.8)	162 (59.6)
	Abortion	422 (32.4)	85 (31.2)
	GDM	28 (2.2)	10 (3.7)
	HT	14 (1.1)	1 (0.4)
	Other	33 (2.5)	14 (5.1)
	Total	1301 (100)	272 (100)

Data are presented as number and percentage for categorical values and mean ± standard deviation for quantitative values

steps. The goal was to extend nearly all of the UEPs. Following single base extension, the reactions were diluted with 16μl of water and deionized with 6 ng of resin. After deionizing for 20 min the reactions were dispensed onto SpectroChipArrays with a Nanodispenser (Agena Bioscience). The speed of dispensation was optimized to deliver an average of 20 nl of each reaction to a matrix pad on the SpectroChip. An Agena Bioscience Compact MassArray Spectrometer was used to perform MALDI-TOF mass spectrometry according to the iPLEX Gold Application Guide. The Typer 4 software package (Agena Bioscience) was used to analyze the resulting spectra and the composition of the target bases was determined from the mass of each extended oligo. These panels were designed in collaboration with PATIA and Genotyping was performed at the Agena platform located at the Epigenetics and Genotyping laboratory, Central Unit for Research in Medicine (UCIM), Faculty of Medicine, University of Valencia, Valencia, Spain.

Selection of SNPs

The 110 single-nucleotide polymorphisms were based on literature references (6–12). Specifically, SNPs were prioritized according to the results of large meta-analysis of genome-wide association studies (GWAS) performed in European and other populations, and with the presumption that their effects can be extrapolated and generalized, and that large sample sizes allow solid estimations of the true size effect. Allele frequencies were considered to maximize the SNPs' predictive power (effect size x allele frequency). In addition, significant SNPs identified in smaller association studies were also included. As a result, the selected SNPs for analysis fulfilled the following criteria: odds ratio (OR) >1.2, Rare Allele Frequency (RAF) >0.20 and Association Statistical Significance of p <1 × 10-5 (Supplementary Table 1).

GWA quality control

Quality control steps removed participants with a high missing genotype rate (MIND >5%, 13 samples), removed SNPs with a high missing genotype data (GENO > 5%, 1 variant), removed SNPs due to Hardy-Weinberg exact test (HWE, $p < 1 \times 10^{-6}$, 7 variants), and removed SNPs due to allele low frequency threshold (MAF < 5%, 4 variants). As a result, our data warehouse included 1573 women

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and 98 SNPs, with a total genotyping rate of 0.996544 (Supplementary Table 1).

Statistical analysis

Statistical analyses regarding patients' characteristics were performed in SPSS (Chicago, IL, USA) version 24.0. Data are presented as mean ± standard deviation or median and interquartile range according to the normality of their distribution. $\chi 2$ test was used to compare qualitative characteristics and quantitative characteristics were assessed with Student's t-test. A two-sided p-value <0.05 was considered statistically significant.

The association between each SNP and GDM risk was evaluated by genetic binary logistic regression models. All genetic association tests were performed using PLINK v.1.9 and 2.0 software (13). Specifically, we used the following models and tests: ADDITIVE model – test ADD; DOMINANT model – test DOM; RECESSIVE model – test REC and HETHOM model -test HOM and HET.

In all the logistic regression models, a variable was added to represent the nutritional intervention group [GROUP]. We defined this variable with values 1, 2 and 3 corresponding respectively to the CG, IF and RW groups of Figure 1. The reference group for the logistic regression model was the CG group.

The analysis was carried out by stratifying the sample by ethnicity, according to the two categories present in the data: Caucasian (CAU) and Hispanic (HIS). The allele indicated in the previous literature was taken as the reference allele (REF). In the logistic regression models, the minor allele (A1) was always taken as the base category, meaning that it can be a risk allele when OR > 1 or a protective allele when OR < 1. For each test of a model, the corresponding p-value was obtained using the PLINK software. As false discovery rate control (FDR), we started with the set of p-values and then we calculated the q-values (i.e. minimum FDR incurred when calling a test significant) and *lfdr*-values (*local false discovery rate*, i.e. the empirical Bayesian posterior probability that the null hypothesis is true, conditional on the observed p-value) using the *qvalue* package (version 2.24.0) of R software (version 4.1.2) (14), with smoother method option and adjustment of lambda parameter in the interval 0.01-0.95 with increment of 0.01 (14). As association significance criteria we used the following thresholds: p-value ≤ 0.05, q-value ≤ 0.05, *lfdr*-value ≤ 0.1.

Bioinformatics analysis

We mapped each SNP to its nearest corresponding protein-coding gene and then we performed gene ontology (GO) enrichment analysis and protein–protein interaction (PPI) analysis for the set of SNPs that reached significance in any of the criteria indicated above. The analysis was performed using STRING, version 11.5 (15).

Results

Patient data and SNP data

Quality controls retrieved a total 98 SNPs and 1573 samples, 272 (17.3%) with GDM and 1301 (82.7%) without GDM. 1104 (70.2%) were Caucasian (CAU) and 469 (29.8%) Hispanic (HIS). 415 (26.4%) were from the control group (CG), 418 (26.6%) from the nutritional intervention group (IG) and 740 (47.0%) from the real-world group (RW). Women's main demographic and anthropometric characteristics are represented in Table 1. Table 2a CAU and 2b HIS show the main characteristics of the variants for the Caucasian and Hispanic ethnicities, respectively.

Supplementary Tables 2 CAU-2HIS show, respectively, for each ethnicity, logistic regression analysis performed for the 98 SNPs and 1573 samples. Tables 3a CAU and 3b HIS extract, respectively, the main relevant findings for the two ethnic strata considered; specifically, these tables show the SNPs for which a discovery (p-value ≤0.05, or q-value ≤ 0.05, or *lfdr* ≤ 0.1) was obtained in at least one of the SNP genetic tests performed.

General findings and effect of the nutritional intervention

Of a total of 110 variants included in the study, 98 (89.1%) passed the quality control. Of these, 40 (40.8%) presented some kind of significant association with GDM in at least one of the genetic tests considered, that is, the corresponding threshold was reached in some assessment criteria, with the following distribution by ethnicity: 13 (32.5%) only in the Caucasian ethnic stratum, 19 (47.5%) only in the Hispanic ethnic stratum and 8 (20.0%) in both ethnic strata (Table 3a CAU, 3b HISP). The nutritional intervention presented a significant association with GDM, regardless of the variant considered; we obtained an OR < 1 for GROUP variable in favor of MedDiet, with all the significance criteria satisfied in practically all the tests of each model (Supplementary Tables 1CAU and 1HIS).

Caucasian ethnicity findings

Table 3a CAU summarizes the most relevant findings for Caucasian pregnant women. The genetic variants significantly associated with increased risk of GDM were rs4402960, rs7651090, IGF2BP2; rs1387153, rs10830963, rs10830962, MTNR1B; rs17676067, GLP2R, rs1371614, DPYSL5; rs5215, KCNJ11; and rs2293941, PDX1. Variants significantly associated with decreased risk of GDM were rs780094, GCKR; rs7607980, COBLL1; rs3746750, SLC17A9; rs6048205, FOXA2; rs7041847, rs7034200, rs10814916, GLIS3; rs3783347, WARS; and rs1805087, MTR.

TABLE 2 CAU Characteristics of variants. CAUCASIAN.

CHROM	LOCUS	POS	ID	REF	ALT	A1	A1_CT	ALLELE_CT	A1_CASE_CT	A1_CTRL_CT	CASE_ALLELE_CT	CTRL_ALLELE_CT	CASE_NON_A1_CT	CASE_HET_A1_CT	CASE_HOM_A1_CT	CTRL_NON_A1_CT	CTRL_HET_A1_CT	CTRL_HOM_A1_CT	A1_FREQ	A1_CASE_FREQ	A1_CTRL_FREQ	OBS_CT
1	MTHFR	11794419	rs1801131	T	G	G	630	2172	103	527	374	1798	95	81	11	437	397	65	0.290	0.275	0.293	1086
1	MTHFR	11796321	rs1801133	G	A	A	847	2204	157	690	376	1828	63	93	32	339	460	115	0.384	0.418	0.377	1102
1	PROX1	213985913	rs340874	T	C	T	1065	2206	185	880	376	1830	51	89	48	236	478	201	0.483	0.492	0.481	1103
1	LYPLAL1	219527177	rs2785980	T	C	C	717	2202	107	610	376	1826	96	77	15	396	424	93	0.326	0.285	0.334	1101
1	MTR	236885200	rs1805087	A	G	G	397	2204	54	343	378	1826	137	50	2	600	283	30	0.180	0.143	0.188	1102
2	DPYSL5	26930006	rs13716l4	C	T	T	572	2204	111	461	378	1826	87	93	9	521	323	69	0.260	0.294	0.252	1102
2	GCKR	27518370	rs780094	T	C	T	1043	2208	153	890	378	1830	69	87	33	251	438	226	0.472	0.405	0.486	1104
2	MAP3K19	134998059	rs1530559	A	G	A	765	2202	139	626	374	1828	73	89	25	391	420	103	0.347	0.372	0.342	1101
2	RBMS1	160460949	rs6742799	A	C	C	389	2200	62	327	374	1826	130	52	5	618	263	32	0.177	0.166	0.179	1100
2	FIGN	163641436	rs21119289	C	G	C	284	2204	44	240	378	1826	149	36	4	686	214	13	0.129	0.116	0.131	1102
2	COBLL1	164694691	rs7607980	T	C	C	318	2204	41	277	378	1826	151	35	3	655	239	19	0.144	0.108	0.152	1102
2	G6PC2	168906638	rs560887	T	C	T	581	2208	88	493	378	1830	114	62	13	491	355	69	0.263	0.233	0.269	1104
2	G6PC2	168917561	rs563694	C	A	C	679	2194	110	569	378	1816	94	80	15	419	409	80	0.309	0.291	0.313	1097
2	IRS1	226203364	rs2943634	A	C	A	673	2176	112	561	374	1802	88	86	13	424	393	84	0.309	0.299	0.311	1088
2	IRS1	226795828	rs1801278	C	T	T	188	2206	39	149	376	1830	151	35	2	770	141	4	0.085	0.104	0.081	1103
3	PPARG	12348985	rs17036328	T	C	C	205	2208	31	174	378	1830	161	25	3	752	152	11	0.093	0.082	0.095	1104
3	PPARG	12351626	rs1801282	C	G	G	194	2208	29	165	378	1830	163	23	3	759	147	9	0.088	0.077	0.090	1104
3	UBE2E2	23413299	rs1496653	A	G	G	394	2208	62	332	378	1830	135	46	8	611	276	28	0.178	0.164	0.181	1104
3	AMT	49417897	rs11715915	C	T	T	713	2188	128	585	376	1812	83	82	23	422	383	101	0.326	0.340	0.323	1094
3	ADCY5	123346931	rs11708067	A	G	G	359	2192	70	289	376	1816	124	58	6	636	255	17	0.164	0.186	0.159	1096
3	SLC2A2	170999732	rs11920090	T	A	A	352	2204	57	295	376	1828	136	47	5	644	245	25	0.160	0.152	0.161	1102
3	IGF2BP2	185793899	rs4402960	G	T	T	695	2208	148	547	378	1830	68	94	27	441	401	73	0.315	0.392	0.299	1104
3	IGF2BP2	185795604	rs7651090	A	G	G	696	2208	147	549	378	1830	67	97	25	436	409	70	0.315	0.389	0.300	1104
3	ADIPOQ	186853103	rs2241766	T	G	G	400	2206	70	330	378	1828	126	56	7	608	282	24	0.181	0.185	0.181	1103
4	WFS1	6288259	rs4458523	T	G	T	825	2194	140	685	372	1822	73	86	27	361	415	135	0.376	0.376	0.376	1097
4	FAM13A	88820118	rs3822072	G	A	A	1065	2196	189	876	374	1822	43	99	45	230	486	195	0.485	0.505	0.481	1098
4	TET2	105160479	rs9884482	T	C	C	870	2196	142	728	374	1822	75	82	30	319	456	136	0.396	0.380	0.400	1098
4	PDGFC	156798972	rs4691380	C	T	T	827	2204	145	682	376	1828	69	93	26	353	440	121	0.375	0.386	0.373	1102
5	IRX1	4355595	rs17727202	T	C	C	165	2208	27	138	378	1830	162	27	0	779	134	2	0.075	0.071	0.075	1104
5	ANKRD55	56510924	rs459193	A	G	A	663	2208	105	558	378	1830	100	73	16	451	370	94	0.300	0.278	0.305	1104
5	ZBED3	77130042	rs7708285	G	A	G	658	2208	128	530	378	1830	84	82	23	460	380	75	0.298	0.339	0.290	1104
5	PCSK1	96207022	rs13179048	C	A	A	623	2208	99	524	378	1830	102	75	12	454	398	63	0.282	0.262	0.286	1104
5	PCSK1	96295001	rs17085593	C	G	G	637	2204	103	534	376	1828	99	75	14	443	408	63	0.289	0.274	0.292	1102
5	PCSK1	96393194	rs6235	C	G	G	565	2200	88	477	374	1826	107	72	8	481	387	45	0.257	0.235	0.261	1100
6	RRB1	7212967	rs17762454	C	T	T	615	2188	95	520	372	1816	102	73	11	462	372	74	0.281	0.255	0.286	1094
6	RREB1	7231610	rs9379084	G	A	A	332	2208	66	266	378	1830	130	52	7	668	228	19	0.150	0.175	0.145	1104
6	CDKAL1	20679478	rs7756992	A	G	G	549	2192	86	463	374	1818	108	72	7	503	349	57	0.250	0.230	0.255	1096
6	CDKAL1	20686765	rs9368222	C	A	A	522	2206	80	442	378	1828	115	68	6	523	340	51	0.237	0.212	0.242	1103
6	RSPO3	127131790	rs2745353	C	T	T	1075	2206	177	898	378	1828	52	97	40	240	450	224	0.487	0.468	0.491	1103
7	DGKB	15024684	rs2191349	G	T	G	987	2202	175	812	376	1826	56	89	43	286	442	185	0.448	0.465	0.445	1101
7	GCK	44189469	rs1799884	C	T	T	424	2208	77	347	378	1830	119	63	7	599	285	31	0.192	0.204	0.190	1104
7	GCK	44196069	rs4607517	G	A	A	409	2184	74	335	378	1806	120	64	5	597	277	29	0.187	0.196	0.185	1092
7	GRB10	50690548	rs933360	C	T	C	530	2196	91	439	374	1822	108	67	12	523	337	51	0.241	0.243	0.241	1098
7	GRB10	50723882	rs6943153	T	C	T	602	2184	102	500	372	1812	98	74	14	469	374	63	0.276	0.274	0.276	1092
7	HIP1	75546898	rs1167800	A	G	G	974	2208	156	818	378	1830	62	98	29	281	450	184	0.441	0.413	0.447	1104
8	PPP1R3B	9326086	rs4841132	A	G	A	135	2208	22	113	378	1830	167	22	0	809	99	7	0.061	0.058	0.062	1104
8	PPP1R3B	9330085	rs7004769	A	G	A	407	2204	63	344	378	1826	131	53	5	609	264	40	0.185	0.167	0.188	1102
8	ANK1	41651740	rs12549902	G	A	G	1025	2198	175	850	378	1820	54	95	40	238	494	178	0.466	0.463	0.467	1099
8	SLC30A8	117172544	rs13266634	C	T	T	581	2208	98	483	378	1830	104	72	13	485	377	53	0.263	0.259	0.264	1104
8	SLC30A8	117172786	rs3802177	G	A	A	567	2208	95	472	378	1830	106	71	12	490	378	47	0.257	0.251	0.258	1104
8	SLC30A8	117173494	rs11558471	A	G	G	601	2206	99	502	378	1828	103	73	13	466	394	54	0.272	0.262	0.275	1103
9	GLIS3	4287466	rs7041847	A	G	G	1034	2202	167	867	376	1826	63	83	42	245	469	199	0.470	0.444	0.475	1101

(Continued)

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TABLE 2 Continued

CHROM	LOCUS	POS	ID	REF	ALT	A1	A1_CT	ALLELE_CT	A1_CASE_CT	A1_CTRL_CT	CASE_ALLELE_CT	CTRL_ALLELE_CT	CASE_NON_A1_CT	CASE_HET_A1_CT	CASE_HOM_A1_CT	CTRL_NON_A1_CT	CTRL_HET_A1_CT	CTRL_HOM_A1_CT	A1_FREQ	A1_CASE_FREQ	A1_CTRL_FREQ	OBS_CT
9	GLIS3	4289050	rs7034200	C	A	C	1092	2206	180	912	378	1828	60	78	51	225	466	223	0.495	0.476	0.499	1103
9	GLIS3	4293150	rs10814916	A	C	A	1043	2194	171	872	378	1816	63	81	45	234	476	198	0.475	0.452	0.480	1097
9	CDKN2B	22134095	rs10811661	T	C	C	423	2188	73	350	374	1814	122	57	8	592	280	35	0.193	0.195	0.193	1094
9	SARDH	133734024	rs573904	C	T	T	626	2206	120	506	378	1828	85	88	16	479	364	71	0.284	0.317	0.277	1103
10	CDC123	12265895	rs11257655	C	T	T	503	2208	93	410	378	1830	107	71	11	545	330	40	0.228	0.246	0.224	1104
10	CDC123	12286011	rs12779790	A	G	G	433	2208	78	355	378	1830	120	60	9	587	301	27	0.196	0.206	0.194	1104
10	CUBN	17114152	rs1801222	A	G	A	607	2198	114	493	376	1822	90	82	16	488	353	70	0.276	0.303	0.271	1099
10	HKDC1	69223185	rs4746822	C	T	C	968	2204	161	807	378	1826	60	97	32	276	467	170	0.439	0.426	0.442	1102
10	HHEX	92722319	rs7923866	C	T	T	783	2206	132	651	378	1828	75	96	18	374	429	111	0.355	0.349	0.356	1103
10	ADRA2A	111282335	rs10885122	T	G	T	292	2206	48	244	376	1830	144	40	4	687	212	16	0.132	0.128	0.133	1103
10	TCF7L2	112994312	rs34872471	T	C	C	759	2208	140	619	378	1830	72	94	23	394	423	98	0.344	0.370	0.338	1104
10	TCF7L2	112996282	rs4506565	A	T	T	819	2204	147	672	378	1826	68	95	26	356	442	115	0.372	0.389	0.368	1102
10	TCF7L2	112998590	rs7903146	C	T	T	774	2206	144	630	378	1828	73	88	28	387	424	103	0.351	0.381	0.345	1103
11	DUSP8	1675619	rs2334499	C	T	T	967	2180	172	795	376	1804	50	104	34	279	451	172	0.444	0.457	0.441	1090
11	KCNJ11	17387083	rs5215	C	T	C	772	2200	146	626	376	1824	66	98	24	396	406	110	0.351	0.388	0.343	1100
11	CRY2	45851540	rs11605924	A	C	C	1096	2208	178	918	378	1830	52	96	41	238	436	241	0.496	0.471	0.502	1104
11	MADD	47314769	rs7944584	A	T	T	711	2204	110	601	378	1826	92	84	13	412	401	100	0.323	0.291	0.329	1102
11	OR4S1	48311808	rs1483121	G	A	A	340	2204	53	287	378	1826	139	47	3	640	259	14	0.154	0.140	0.157	1102
11	FADS1	61804006	rs174550	T	C	C	675	2196	118	557	378	1818	88	84	17	432	397	80	0.307	0.312	0.306	1098
11	ARAP1	72721940	rs11603334	G	A	A	283	2208	43	240	378	1830	149	37	3	691	208	16	0.128	0.114	0.131	1104
11	MTNR1B	92940662	rs1387153	C	T	T	646	2206	136	510	378	1828	75	92	22	470	378	66	0.293	0.360	0.279	1103
11	MTNR1B	92965261	rs10830962	C	G	G	935	2196	180	755	374	1822	47	100	40	310	447	154	0.426	0.481	0.414	1098
11	MTNR1B	92975544	rs10830963	C	G	G	607	2204	132	475	378	1826	78	90	21	504	343	66	0.275	0.349	0.260	1102
12	GLS2	56471554	rs2657879	A	G	G	473	2206	83	390	378	1828	113	69	7	568	302	44	0.214	0.220	0.213	1103
12	IGF1	102481791	rs35767	A	G	A	346	2202	54	292	378	1824	139	46	4	650	232	30	0.157	0.143	0.160	1101
12	HNF1A	121022883	rs7957197	T	A	A	464	2200	71	393	376	1824	125	55	8	560	311	41	0.211	0.189	0.215	1100
12	P2RX2	132465032	rs10747083	G	A	G	769	2206	130	639	378	1828	87	74	28	373	443	98	0.349	0.344	0.350	1103
13	PDX1	27917061	rs2293941	G	A	A	534	2204	103	431	378	1826	97	81	11	538	319	56	0.242	0.272	0.236	1102
13	KL	32980164	rs576674	G	A	G	504	2192	83	421	376	1816	112	69	7	524	347	37	0.230	0.221	0.232	1096
14	WARS	100372924	rs3783347	G	T	T	383	2208	53	330	378	1830	141	43	5	609	282	24	0.173	0.140	0.180	1104
15	C2CD4A	62090956	rs4502156	T	C	C	1011	2202	174	837	378	1824	57	90	42	264	459	189	0.459	0.460	0.459	1101
15	C2CD4B	62141763	rs11071657	A	G	G	875	2208	156	719	378	1830	61	100	28	328	455	132	0.396	0.413	0.393	1104
16	FTO	53767042	rs1421085	T	C	C	914	2204	149	765	376	1828	65	97	26	303	457	154	0.415	0.396	0.418	1102
16	FTO	53782363	rs8050136	C	A	A	896	2194	154	742	376	1818	66	90	32	317	442	150	0.408	0.410	0.408	1097
16	CTRB2	75211105	rs9921586	G	T	T	281	2208	47	234	378	1830	143	45	1	693	210	12	0.127	0.124	0.128	1104
17	GLP2R	9888058	rs17676067	T	C	C	598	2206	120	478	376	1830	90	76	22	498	356	61	0.271	0.319	0.261	1103
17	HNF1B	37738049	rs4430796	A	G	A	1009	2206	169	840	378	1828	63	83	43	269	450	195	0.457	0.447	0.460	1103
19	CILP2	19547663	rs16996148	G	T	T	171	2208	26	145	378	1830	163	26	0	774	137	4	0.077	0.069	0.079	1104
19	PEPD	33408159	rs731839	G	A	G	762	2196	136	626	376	1820	75	90	23	383	428	99	0.347	0.362	0.344	1098
19	GIPR	45693376	rs2302593	C	G	G	1082	2198	188	894	378	1820	43	104	42	235	456	219	0.492	0.497	0.491	1099
20	FOXA2	22578963	rs6048205	A	G	G	110	2208	10	100	378	1830	179	10	0	819	92	4	0.050	0.026	0.055	1104
20	TOP1	41115265	rs6072275	G	A	A	336	2206	54	282	378	1828	138	48	3	654	238	22	0.152	0.143	0.154	1103
20	ZHX3	41203988	rs17265513	T	C	C	406	2204	68	338	378	1826	127	56	6	609	270	34	0.184	0.180	0.185	1102
20	SLC17A9	62967547	rs3746750	A	G	A	759	2200	111	648	376	1824	94	77	17	362	452	98	0.345	0.295	0.355	1100
21	BACE2	41209710	rs737288	G	T	T	773	2188	130	643	374	1814	74	96	17	373	425	109	0.353	0.348	0.354	1094
21	BACE2	41211811	rs6517656	G	A	A	458	2208	78	380	378	1830	118	64	7	573	304	38	0.207	0.206	0.208	1104

Main characteristics of the variants for the Caucasian (CAU) ethnicity.

CHROM, Chromosome code; LOCUS, Locus/Gene; POS, Base-pair coordinate [GRCh38]; ID, Variant ID; REF, Reference allele; ALT, Alternate allele; A1, Counted allele in logistic regression; A1_CT, Total A1 allele count; ALLELE_CT, Allele observation count; A1_CASE_CT, A1 count in cases; A1_CTRL_CT, A1 count in controls; CASE_ALLELE_CT, Case allele observation count; CTRL_ALLELE_CT, Control allele observation count; CASE_NON_A1_CT, Case genotypes with 0 copies of A1; CASE_HET_A1_CT, Case genotypes with 1 copy of A1; CASE_HOM_A1_CT, Case genotypes with 2 copies of A1; CTRL_NON_A1_CT, Control genotypes with 0 copies of A1; CTRL_HET_A1_CT, Control genotypes with 1 copy of A1; CTRL_HOM_A1_CT, Control genotypes with 2 copies of A1; A1_FREQ, A1 allele frequency; A1_CASE_FREQ, A1 allele frequency in cases; A1_CTRL_FREQ, A1 allele frequency in controls; OBS_CT, Number of samples in the regression.

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TABLE 2 HIS Characteristics of variants. HISPANIC.

CHROM	LOCUS	POS	ID	REF	ALT	A1	A1_CT	ALLELE_CT	A1_CASE_CT	A1_CTRL_CT	CASE_ALLELE_CT	CTRL_ALLELE_CT	CASE_NON_A1_CT	CASE_HET_A1_CT	CASE_HOM_A1_CT	CTRL_NON_A1_CT	CTRL_HET_A1_CT	CTRL_HOM_A1_CT	A1_FREQ	A1_CASE_FREQ	A1_CTRL_FREQ	OBS_CT
1	MTHFR	11794419	rs1801131	T	G	G	137	930	25	112	166	764	59	23	1	279	94	9	0.147	0.151	0.147	465
1	MTHFR	11796321	rs1801133	G	A	A	373	936	65	308	166	770	30	41	12	139	184	62	0.399	0.392	0.400	468
1	PROX1	213985913	rs340874	T	C	C	330	936	50	280	166	770	44	28	11	155	180	50	0.353	0.301	0.364	468
1	LYPLAL1-AS1	219527177	rs2785980	T	C	T	406	936	78	328	166	770	29	30	24	145	152	88	0.434	0.470	0.426	468
1	MTR	236885200	rs1805087	A	G	G	195	938	33	162	166	772	54	25	4	240	130	16	0.208	0.199	0.210	469
2	DPYSL5	26930006	rs1371614	C	T	T	396	934	80	316	164	770	21	42	19	145	164	76	0.424	0.488	0.410	467
2	GCKR	27518370	rs780094	T	C	T	308	930	48	260	164	766	44	28	10	164	178	41	0.331	0.293	0.339	465
2	MAP3K19	134998059	rs1530559	A	G	A	308	934	49	259	164	770	41	33	8	174	163	48	0.330	0.299	0.336	467
2	RBMS1	160460949	rs6742799	A	C	C	130	928	22	108	166	762	62	20	1	279	96	6	0.140	0.133	0.142	464
2	FIGN	163641436	rs2119289	C	G	C	99	938	22	77	166	772	61	22	0	311	73	2	0.106	0.133	0.100	469
2	COBLL1	164694691	rs7607980	T	C	C	65	938	12	53	166	772	71	12	0	335	49	2	0.069	0.072	0.069	469
2	G6PC2	168906638	rs560887	T	C	T	94	938	12	82	166	772	72	10	1	307	76	3	0.100	0.072	0.106	469
2	G6PC2	168917561	rs563694	C	A	C	119	938	15	104	166	772	70	11	2	286	96	4	0.127	0.090	0.135	469
2	IRS1	226203364	rs2943634	A	C	A	190	932	25	165	164	768	61	17	4	247	109	28	0.204	0.152	0.215	466
2	IRS1	226795828	rs1801278	C	T	T	60	938	8	52	166	772	75	8	0	338	44	4	0.064	0.048	0.067	469
3	PPARG	12348985	rs17036328	T	C	C	146	938	21	125	166	772	64	17	2	273	101	12	0.156	0.127	0.162	469
3	PPARG	12351626	rs1801282	C	G	G	123	938	17	106	166	772	67	15	1	291	84	11	0.131	0.102	0.137	469
3	UBE2E2	23413299	rs1496653	A	G	G	107	938	13	94	166	772	71	11	1	299	80	7	0.114	0.078	0.122	469
3	AMT	49417897	rs11715915	C	T	T	140	938	30	110	166	772	58	20	5	289	84	13	0.149	0.181	0.142	469
3	ADCY5	123346931	rs11708067	A	G	G	335	936	54	281	166	770	39	34	10	154	181	50	0.358	0.325	0.365	468
3	SLC2A2	170999732	rs11920090	T	A	A	129	938	19	110	166	772	64	19	0	288	86	12	0.138	0.114	0.142	469
3	IGF2BP2	185793899	rs4402960	G	T	T	237	938	51	186	166	772	41	33	9	222	142	22	0.253	0.307	0.241	469
3	IGF2BP2	185795604	rs7651090	A	G	G	232	934	50	182	166	768	41	34	8	221	144	19	0.248	0.301	0.237	467
3	ADIPOQ	186853103	rs2241766	T	G	G	168	938	32	136	166	772	54	26	3	259	118	9	0.179	0.193	0.176	469
4	WFS1	6288259	rs4458523	T	G	T	292	926	46	246	162	764	38	40	3	174	170	38	0.315	0.284	0.322	463
4	FAM13A	88820118	rs3822072	G	A	A	401	934	72	329	166	768	26	42	15	121	197	66	0.429	0.434	0.428	467
4	TET2	105160479	rs9884482	T	C	C	398	934	62	336	166	768	33	38	12	123	186	75	0.426	0.373	0.438	467
4	PDGFC	156798972	rs4691380	C	T	T	325	932	64	261	166	766	34	34	15	171	163	49	0.349	0.386	0.341	466
5	IRX1	4355595	rs17727202	T	C	C	40	938	5	35	166	772	78	5	0	351	35	0	0.043	0.030	0.045	469
5	ANKRD55	56510924	rs459193	A	G	A	219	934	47	172	166	768	46	27	10	235	126	23	0.234	0.283	0.224	467
5	ZBED3	77130042	rs7708285	G	A	G	338	938	62	276	166	772	30	44	9	164	168	54	0.360	0.373	0.358	469
5	PCSK1	96207022	rs13179048	C	A	A	173	936	25	148	166	770	59	23	1	253	116	16	0.185	0.151	0.192	468
5	PCSK1	96295001	rs17085593	C	G	G	182	938	27	155	166	772	57	25	1	248	121	17	0.194	0.163	0.201	469
5	PCSK1	96393194	rs6235	C	G	G	182	938	27	155	166	772	56	27	0	251	115	20	0.194	0.163	0.201	469
6	RRB1	7212967	rs17762454	C	T	T	360	936	70	290	166	770	29	38	16	148	184	53	0.385	0.422	0.377	468
6	RREB1	7231610	rs9379084	G	A	A	51	938	7	44	166	772	76	7	0	344	40	2	0.054	0.042	0.057	469
6	CDKAL1	20679478	rs7756992	A	G	G	288	934	57	231	166	768	36	37	10	190	157	37	0.308	0.343	0.301	467
6	CDKAL1	20686765	rs9368222	C	A	A	212	938	48	164	166	772	40	38	5	241	126	19	0.226	0.289	0.212	469
6	RSPO3	127131790	rs2745353	C	T	C	376	938	60	316	166	772	35	36	12	125	206	55	0.401	0.361	0.409	469
7	DGKB	15024684	rs2191349	G	T	T	384	936	78	306	166	770	20	48	15	132	200	53	0.410	0.470	0.397	468
7	GCK	44189469	rs1799884	C	T	T	180	936	38	142	166	770	51	26	6	258	112	15	0.192	0.229	0.184	468
7	GCK	44196069	rs4607517	G	A	A	168	928	32	136	162	766	54	22	5	261	108	14	0.181	0.198	0.178	464
7	GRB10	50690548	rs933360	C	T	C	341	936	72	269	166	770	29	36	18	166	169	50	0.364	0.434	0.349	468
7	GRB10	50723882	rs6943153	T	C	C	460	932	76	384	166	766	24	42	17	94	194	95	0.494	0.458	0.501	466
7	HIP1	75546898	rs1167800	A	G	G	285	938	50	235	166	772	42	32	9	186	165	35	0.304	0.301	0.304	469
8	PPP1R3B	9326086	rs4841132	A	G	A	226	936	39	187	164	772	49	27	6	219	147	20	0.241	0.238	0.242	468
8	PPP1R3B	9330085	rs7004769	A	G	A	367	938	63	304	166	772	30	43	10	135	198	53	0.391	0.380	0.394	469
8	ANK1	41651740	rs12549902	G	A	G	387	932	72	315	164	768	27	38	17	124	205	55	0.415	0.439	0.410	466
8	SLC30A8	117172544	rs13266634	C	T	T	235	936	43	192	166	770	48	27	8	219	140	26	0.251	0.259	0.249	468
8	SLC30A8	117172786	rs3802177	G	A	A	232	938	41	191	166	772	49	27	7	222	137	27	0.247	0.247	0.247	469
8	SLC30A8	117173494	rs11558471	A	G	G	244	938	44	200	166	772	47	28	8	216	140	30	0.260	0.265	0.259	469
9	GLIS3	4287466	rs7041847	A	G	G	394	936	57	337	166	770	37	35	11	121	191	73	0.421	0.343	0.438	468

(Continued)

10.3389/fendo.2022.1036088

TABLE 2 Continued

CHROM	LOCUS	POS	ID	REF	ALT	A1	A1_CT	ALLELE_CT	A1_CASE_CT	A1_CTRL_CT	CASE_ALLELE_CT	CTRL_ALLELE_CT	CASE_NON_A1_CT	CASE_HET_A1_CT	CASE_HOM_A1_CT	CTRL_NON_A1_CT	CTRL_HET_A1_CT	CTRL_HOM_A1_CT	A1_FREQ	A1_CASE_FREQ	A1_CTRL_FREQ	OBS_CT
9	GLIS3	4289050	rs7034200	C	A	C	461	936	71	390	166	770	27	41	15	93	194	98	0.493	0.428	0.506	468
9	GLIS3	4293150	rs10814916	A	C	A	433	932	66	367	166	766	28	44	11	105	189	89	0.465	0.398	0.479	466
9	CDKN2B	22134095	rs10811661	T	C	C	119	934	24	95	166	768	60	22	1	295	83	6	0.127	0.145	0.124	467
9	SARDH	133734024	rs573904	C	T	T	201	934	36	165	166	768	51	28	4	234	135	15	0.215	0.217	0.215	467
10	CDC123	12265895	rs11257655	C	T	T	245	938	46	199	166	772	44	32	7	213	147	26	0.261	0.277	0.258	469
10	CDC123	12286011	rs12779790	A	G	G	135	936	23	112	166	770	61	21	1	282	94	9	0.144	0.139	0.145	468
10	CUBN	17114152	rs1801222	A	G	A	245	936	39	206	166	770	51	25	7	200	164	21	0.262	0.235	0.268	468
10	HKDC1	69223185	rs4746822	C	T	T	457	936	84	373	166	770	22	38	23	105	187	93	0.488	0.506	0.484	468
10	HHEX	92722319	rs7923866	C	T	C	466	936	90	376	166	770	17	42	24	104	186	95	0.498	0.542	0.488	468
10	ADRA2A	111282335	rs10885122	T	G	T	172	938	37	135	166	772	54	21	8	262	113	11	0.183	0.223	0.175	469
10	TCF7L2	112994312	rs34872471	T	C	C	192	938	29	163	166	772	58	21	4	240	129	17	0.205	0.175	0.211	469
10	TCF7L2	112996282	rs4506565	A	T	T	215	936	38	177	166	770	52	24	7	228	137	20	0.230	0.229	0.230	468
10	TCF7L2	112998590	rs7903146	C	T	T	190	936	30	160	166	770	57	22	4	240	130	15	0.203	0.181	0.208	468
11	DUSP8	1675619	rs2334499	C	T	T	425	934	85	340	166	768	23	35	25	123	182	79	0.455	0.512	0.443	467
11	KCNJ11	17387083	rs5215	C	T	C	294	932	55	239	166	766	36	39	8	185	157	41	0.315	0.331	0.312	466
11	CRY2	45851540	rs11605924	A	C	C	468	938	72	396	166	772	23	48	12	95	186	105	0.499	0.434	0.513	469
11	MADD	47314769	rs7944584	A	T	T	138	938	24	114	166	772	60	22	1	283	92	11	0.147	0.145	0.148	469
11	OR4S1	48311808	rs1483121	G	A	A	57	938	14	43	166	772	70	12	1	344	41	1	0.061	0.084	0.056	469
11	FADS1	61804006	rs174550	T	C	T	359	928	62	297	164	764	37	28	17	160	147	75	0.387	0.378	0.389	464
11	ARAP1	72721940	rs11603334	G	A	A	71	936	8	63	166	770	76	6	1	326	55	4	0.076	0.048	0.082	468
11	MTNR1B	92940662	rs1387153	C	T	T	163	936	38	125	166	770	50	28	5	270	105	10	0.174	0.229	0.162	468
11	MTNR1B	92965261	rs10830962	C	G	G	309	938	62	247	166	772	34	36	13	182	161	43	0.329	0.373	0.320	469
11	MTNR1B	92975544	rs10830963	C	G	G	124	938	27	97	166	772	56	27	0	294	87	5	0.132	0.163	0.126	469
12	GLS2	56471554	rs2657879	A	G	G	234	938	34	200	166	772	53	26	4	212	148	26	0.249	0.205	0.259	469
12	IGF1	102481791	rs35767	A	G	A	239	936	50	189	166	770	39	38	6	219	143	23	0.255	0.301	0.245	468
12	HNF1A	121022883	rs7957197	T	A	A	106	938	18	88	166	772	66	16	1	310	64	12	0.113	0.108	0.114	469
12	P2RX2	132465032	rs10747083	G	A	G	243	938	37	206	166	772	52	25	6	201	164	21	0.259	0.223	0.267	469
13	PDX1	27917061	rs2293941	G	A	A	277	938	53	224	166	772	35	43	5	206	136	44	0.295	0.319	0.290	469
13	KL	32980164	rs576674	G	A	G	344	934	60	284	164	770	31	42	9	161	164	60	0.368	0.366	0.369	467
14	WARS	100372924	rs3783347	G	T	T	96	938	14	82	166	772	69	14	0	308	74	4	0.102	0.084	0.106	469
15	C2CD4A	62090956	rs4502156	T	C	T	289	936	50	239	166	770	43	30	10	184	163	38	0.309	0.301	0.310	468
15	C2CD4B	62141763	rs11071657	A	G	A	393	936	65	328	166	770	34	33	16	138	166	81	0.420	0.392	0.426	468
16	FTO	53767042	rs1421085	T	C	C	151	938	22	129	166	772	61	22	0	267	109	10	0.161	0.133	0.167	469
16	FTO	53782363	rs8050136	C	A	A	195	938	31	164	166	772	54	27	2	240	128	18	0.208	0.187	0.212	469
16	CTRB2	75211105	rs9921586	G	T	T	118	938	19	99	166	772	65	17	1	298	77	11	0.126	0.114	0.128	469
17	GLP2R	9888058	rs17676067	T	C	C	109	936	23	86	166	770	62	19	2	301	82	2	0.116	0.139	0.112	468
17	HNF1B	37738049	rs4430796	A	G	G	322	938	66	256	166	772	32	36	15	181	154	51	0.343	0.398	0.332	469
19	CILP2	19547663	rs16996148	G	T	T	57	938	13	44	166	772	71	11	1	343	42	1	0.061	0.078	0.057	469
19	PEPD	33408159	rs731839	G	A	G	416	934	78	338	164	770	22	42	18	118	196	71	0.445	0.476	0.439	467
19	GIPR	45693376	rs2302593	C	G	C	387	934	83	304	166	768	21	41	21	146	172	66	0.414	0.500	0.396	467
20	FOXA2	22578963	rs6048205	A	G	G	50	938	12	38	166	772	72	10	1	352	30	4	0.053	0.072	0.049	469
20	TOP1	41115265	rs6072275	G	A	A	118	938	20	98	166	772	65	16	2	293	88	5	0.126	0.120	0.127	469
20	ZHX3	41203988	rs17265513	T	C	C	72	938	15	57	166	772	69	13	1	330	55	1	0.077	0.090	0.074	469
20	SLC17A9	62967547	rs3746750	A	G	A	314	934	63	251	164	770	30	41	11	167	185	33	0.336	0.384	0.326	467
21	BACE2	41209710	rs737288	G	T	T	194	934	39	155	164	770	52	21	9	246	123	16	0.208	0.238	0.201	467
21	BACE2	41211811	rs6517656	G	A	A	167	936	36	131	166	770	55	20	8	270	99	16	0.178	0.217	0.170	468

Main characteristics of the variants for the Hispanic (HIS) ethnicity.

CHROM, Chromosome code; LOCUS, Locus/Gene; POS, Base-pair coordinate [GRCh38]; ID, Variant ID; REF, Reference allele; ALT, Alternate allele; A1, Counted allele in logistic regression; A1_CT, Total A1 allele count; ALLELE_CT, Allele observation count; A1_CASE_CT, A1 count in cases; A1_CTRL_CT, A1 count in controls; CASE_ALLELE_CT, Case allele observation count; CTRL_ALLELE_CT, Control allele observation count; CASE_NON_A1_CT, Case genotypes with 0 copies of A1; CASE_HET_A1_CT, Case genotypes with 1 copy of A1; CASE_HOM_A1_CT, Case genotypes with 2 copies of A1; CTRL_NON_A1_CT, Control genotypes with 0 copies of A1; CTRL_HET_A1_CT, Control genotypes with 1 copy of A1; CTRL_HOM_A1_CT, Control genotypes with 2 copies of A1; A1_FREQ, A1 allele frequency; A1_CASE_FREQ, A1 allele frequency in cases; A1_CTRL_FREQ, A1 allele frequency in controls; OBS_CT, Number of samples in the regression.

TABLE 3 CAU (SNP + GROUP) MODELS. SIGNIFICANT SNPs. CAUCASIAN.

									ADDITIVE				DOMINANT				RECESSIVE				HETHOM							
									ADD				DOM				REC				HOM				HET			
CHROM	LOCUS	POS	ID	REF	ALT	A1	A1_FREQ	OBS_CT	OR_CI95	pvalue	qvalue	lfdr	OR_CI95	pvalue	qvalue	lfdr	OR_CI95	pvalue	qvalue	lfdr	OR_CI95	pvalue	qvalue	lfdr	OR_CI95	pvalue	qvalue	lfdr
1	LYPLAL1	219527177	rs2785980	T	C	C	0.326	1101	0.79 (0.62-1.01)	0.062	0.037	0.224	0.74 (0.54-1.02)	0.064	0.036	0.231	0.74 (0.42-1.31)	0.308	0.219	0.997	0.65 (0.36-1.18)	0.161	0.196	0.865	0.76 (0.55-1.06)	0.110	0.141	0.714
1	MTR	236885200	rs1805087	A	G	G	0.180	1102	0.73 (0.53-1.00)	0.050	0.030	0.169	0.75 (0.53-1.06)	0.098	0.054	0.383	0.31 (0.08-1.22)	0.095	0.081	0.512	0.29 (0.07-1.24)	0.096	0.125	0.645	0.79 (0.56-1.13)	0.205	0.235	0.926
2	DPYSL5	26930006	rs1371614	C	T	T	0.260	1102	1.21 (0.95-1.54)	0.132	0.076	0.488	1.53 (1.12-2.10)	0.008	0.006	0.020	0.59 (0.29-1.20)	0.145	0.118	0.744	0.75 (0.36-1.57)	0.448	0.385	0.981	1.70 (1.23-2.35)	0.001	0.015	0.014
2	GCKR	27518370	rs780094	T	C	T	0.472	1104	0.74 (0.59-0.92)	0.007	0.006	0.019	0.67 (0.48-0.93)	0.016	0.010	0.042	0.66 (0.44-0.99)	0.042	0.037	0.196	0.54 (0.35-0.86)	0.009	0.015	0.051	0.73 (0.51-1.04)	0.080	0.107	0.549
2	COBLL1	164694691	rs7607980	T	C	C	0.144	1102	0.67 (0.47-0.95)	0.023	0.014	0.066	0.63 (0.43-0.92)	0.016	0.010	0.041	0.73 (0.24-2.28)	0.594	0.344	1.000	0.66 (0.20-2.15)	0.489	0.406	0.981	0.62 (0.42-0.93)	0.020	0.029	0.117
3	IGF2BP2	185793899	rs4402960	G	T	T	0.315	1104	1.54 (1.21-1.95)	0.000	0.006	0.004	1.66 (1.20-2.30)	0.002	0.006	0.008	1.89 (1.18-3.04)	0.008	0.008	0.033	2.37 (1.42-3.95)	0.001	0.015	0.012	1.53 (1.09-2.15)	0.015	0.022	0.085
3	IGF2BP2	185795604	rs7651090	A	G	G	0.315	1104	1.52 (1.20-1.94)	0.001	0.006	0.004	1.67 (1.20-2.31)	0.002	0.006	0.008	1.79 (1.10-2.92)	0.020	0.018	0.078	2.27 (1.34-3.85)	0.002	0.015	0.019	1.56 (1.11-2.19)	0.011	0.016	0.062
5	ZBED3	77130042	rs7708285	G	A	G	0.298	1104	1.24 (0.98-1.57)	0.078	0.046	0.292	1.24 (0.90-1.70)	0.180	0.097	0.623	1.52 (0.92-2.50)	0.099	0.084	0.536	1.63 (0.97-2.76)	0.066	0.090	0.460	1.16 (0.83-1.62)	0.376	0.361	0.979
9	GLIS3	4287466	rs7041847	A	G	G	0.470	1101	0.87 (0.69-1.09)	0.228	0.119	0.663	0.71 (0.51-1.00)	0.048	0.028	0.156	1.02 (0.70-1.49)	0.924	0.444	1.000	0.80 (0.52-1.23)	0.311	0.316	0.973	0.67 (0.47-0.97)	0.033	0.047	0.210
9	GLIS3	4289050	rs7034200	C	A	C	0.495	1103	0.90 (0.72-1.13)	0.363	0.169	0.769	0.68 (0.49-0.96)	0.030	0.018	0.086	1.14 (0.80-1.62)	0.485	0.300	1.000	0.84 (0.55-1.27)	0.401	0.368	0.980	0.61 (0.42-0.89)	0.010	0.016	0.059
9	GLIS3	4293150	rs10814916	A	C	A	0.475	1097	0.87 (0.70-1.10)	0.244	0.126	0.681	0.67 (0.48-0.94)	0.021	0.013	0.055	1.10 (0.76-1.59)	0.621	0.346	1.000	0.81 (0.53-1.24)	0.335	0.331	0.976	0.61 (0.43-0.89)	0.009	0.015	0.053
9	SARDH	133734024	rs573904	C	T	T	0.284	1103	1.20 (0.94-1.53)	0.140	0.081	0.512	1.32 (0.96-1.81)	0.083	0.047	0.318	1.08 (0.62-1.90)	0.786	0.392	1.000	1.24 (0.69-2.24)	0.475	0.400	0.981	1.34 (0.96-1.86)	0.084	0.111	0.574
11	KCNJ11	17387083	rs5215	C	T	C	0.351	1100	1.24 (0.98-1.56)	0.071	0.042	0.262	1.45 (1.04-2.01)	0.027	0.016	0.073	1.09 (0.68-1.75)	0.722	0.372	1.000	1.35 (0.81-2.26)	0.251	0.282	0.956	1.48 (1.05-2.08)	0.026	0.038	0.158
11	MTNR1B	92940662	rs1387153	C	T	T	0.293	1103	1.49 (1.17-1.89)	0.001	0.006	0.006	1.63 (1.18-2.24)	0.003	0.006	0.009	1.71 (1.02-2.85)	0.040	0.036	0.185	2.12 (1.23-3.65)	0.007	0.015	0.041	1.54 (1.10-2.16)	0.011	0.017	0.065
11	MTNR1B	92965261	rs10830962	C	G	G	0.426	1098	1.31 (1.05-1.64)	0.019	0.012	0.053	1.55 (1.08-2.21)	0.017	0.010	0.042	1.31 (0.88-1.93)	0.182	0.144	0.854	1.69 (1.06-2.69)	0.028	0.040	0.172	1.50 (1.03-2.18)	0.036	0.050	0.232
11	MTNR1B	92975544	rs10830963	C	G	G	0.275	1102	1.51 (1.19-1.91)	0.001	0.006	0.005	1.73 (1.26-2.37)	0.001	0.006	0.004	1.60 (0.96-2.67)	0.072	0.063	0.378	2.04 (1.18-3.51)	0.010	0.016	0.060	1.67 (1.20-2.33)	0.003	0.015	0.021
13	PDX1	27917061	rs2293941	G	A	A	0.242	1102	1.20 (0.93-1.54)	0.154	0.086	0.545	1.36 (0.99-1.87)	0.055	0.031	0.187	0.90 (0.46-1.75)	0.750	0.382	1.000	1.04 (0.52-2.05)	0.920	0.542	0.982	1.42 (1.03-1.97)	0.035	0.049	0.222
14	WARS	100372924	rs3783347	G	T	T	0.173	1104	0.73 (0.53-1.01)	0.057	0.034	0.200	0.68 (0.47-0.97)	0.032	0.018	0.091	0.99 (0.37-2.63)	0.981	0.455	1.000	0.88 (0.33-2.36)	0.802	0.507	0.982	0.66 (0.45-0.95)	0.027	0.040	0.168
17	GLP2R	9888058	rs17676067	T	C	C	0.271	1103	1.30 (1.02-1.65)	0.035	0.022	0.110	1.27 (0.92-1.74)	0.140	0.077	0.533	1.80 (1.07-3.01)	0.027	0.024	0.111	1.92 (1.12-3.29)	0.018	0.027	0.107	1.16 (0.83-1.62)	0.396	0.368	0.980
20	FOXA2	22578963	rs6048205	A	G	G	0.050	1104	0.47 (0.24-0.90)	0.023	0.014	0.065	0.47 (0.24-0.91)	0.026	0.016	0.072	0.50 (0.02-13.38)	0.682	0.362	1.000	0.48 (0.02-12.67)	0.658	0.456	0.982	0.51 (0.26-0.99)	0.045	0.063	0.301
20	SLC17A9	62967547	rs3746750	A	G	A	0.345	1100	0.73 (0.57-0.94)	0.015	0.009	0.039	0.65 (0.47-0.89)	0.008	0.006	0.019	0.78 (0.45-1.34)	0.369	0.255	1.000	0.63 (0.36-1.11)	0.109	0.140	0.706	0.65 (0.47-0.91)	0.012	0.019	0.071

Table that summarizes the most relevant results of the analysis of SNPs + Group models in Caucasian (CAU) ethnicity. ADD, Additive model; DOM, dominant model; REC, recessive model; HETHOM, heterozygous-homozygous model; CHROM, Chromosome code; LOCUS, Locus/Gene; POS, Base-pair coordinate [GRCh38]; ID, Variant ID; REF, Reference allele; ALT, Alternate allele; A1, Counted allele in logistic regression; A1_FREQ, minor allele frequency; OBS_CT, Number of samples in the regression; OR_CI95, odds ratio with 95% confidence interval.

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TABLE 3 HIS (SNP + GROUP) MODELS. SIGNIFICANT SNPs. HISPANIC.

									ADDITIVE				DOMINANT				RECESSIVE				HETHOM							
									ADD				DOM				REC				HOM				HET			
CHROM	LOCUS	POS	ID	REF	ALT	A1	A1_FREQ	OBS_CT	OR_CI95	pvalue	qvalue	lfdr	OR_CI95	pvalue	qvalue	lfdr	OR_CI95	pvalue	qvalue	lfdr	OR_CI95	pvalue	qvalue	lfdr	OR_CI95	pvalue	qvalue	lfdr
1	PROX1	213985913	rs340874	T	C	C	0.353	468	0.78 (0.54-1.12)	0.177	0.139	0.594	0.62 (0.38-1.00)	0.049	0.023	0.079	1.06 (0.52-2.15)	0.869	0.219	0.643	0.81 (0.39-1.70)	0.585	0.371	0.858	0.56 (0.33-0.95)	0.032	0.044	0.120
2	DPYSL5	26930006	rs1371614	C	T	T	0.424	467	1.36 (0.98-1.88)	0.069	0.064	0.229	1.77 (1.03-3.03)	0.039	0.019	0.060	1.28 (0.72-2.27)	0.407	0.129	0.521	1.79 (0.90-3.55)	0.096	0.108	0.368	1.76 (0.99-3.12)	0.054	0.069	0.202
2	GCKR	27518370	rs780094	T	C	T	0.331	465	0.80 (0.55-1.16)	0.241	0.172	0.745	0.63 (0.39-1.02)	0.063	0.029	0.107	1.20 (0.57-2.53)	0.627	0.175	0.605	0.93 (0.43-2.01)	0.852	0.443	0.858	0.57 (0.34-0.96)	0.033	0.045	0.125
2	G6PC2	168917561	rs5636694	C	A	C	0.127	469	0.64 (0.36-1.14)	0.132	0.111	0.460	0.54 (0.29-1.00)	0.051	0.024	0.083	2.25 (0.55-9.11)	0.258	0.093	0.417	1.96 (0.35-11.06)	0.447	0.325	0.849	0.48 (0.24-0.95)	0.034	0.046	0.126
2	IRS1	226203364	rs2943634	A	C	A	0.204	466	0.65 (0.42-1.01)	0.053	0.051	0.168	0.57 (0.33-0.98)	0.041	0.020	0.064	0.61 (0.21-1.80)	0.371	0.119	0.501	0.52 (0.18-1.56)	0.247	0.223	0.730	0.58 (0.32-1.04)	0.069	0.082	0.260
3	UBE2E2	23413299	rs1496653	A	G	G	0.114	469	0.60 (0.33-1.09)	0.095	0.084	0.330	0.56 (0.29-1.05)	0.073	0.033	0.130	0.60 (0.12-2.92)	0.526	0.158	0.573	0.54 (0.07-4.50)	0.569	0.371	0.858	0.56 (0.28-1.11)	0.097	0.108	0.371
3	IGF2BP2	185793899	rs4402960	G	T	T	0.253	469	1.39 (0.96-2.01)	0.085	0.077	0.291	1.36 (0.85-2.18)	0.197	0.076	0.379	2.07 (0.95-4.51)	0.067	0.030	0.114	2.26 (0.96-5.29)	0.061	0.076	0.230	1.23 (0.74-2.04)	0.428	0.314	0.845
3	IGF2BP2	185795604	rs7651090	A	G	G	0.248	467	1.38 (0.94-2.02)	0.096	0.084	0.333	1.35 (0.84-2.18)	0.215	0.081	0.403	2.08 (0.87-4.97)	0.099	0.043	0.180	2.28 (0.93-5.58)	0.073	0.086	0.277	1.23 (0.75-2.04)	0.412	0.311	0.841
4	WFS1	6288259	rs4458523	T	G	T	0.315	463	0.80 (0.54-1.17)	0.250	0.176	0.764	0.92 (0.57-1.49)	0.730	0.198	0.632	0.31 (0.09-1.05)	0.060	0.027	0.101	0.32 (0.09-1.11)	0.073	0.086	0.278	1.06 (0.65-1.74)	0.820	0.438	0.858
5	ANKRD55	56510924	rs459193	A	G	A	0.234	467	1.35 (0.94-1.94)	0.108	0.094	0.378	1.29 (0.80-2.09)	0.304	0.108	0.485	2.18 (0.99-4.80)	0.054	0.025	0.089	2.26 (1.00-5.11)	0.050	0.064	0.185	1.11 (0.66-1.88)	0.695	0.405	0.858
5	PCSK1	96393194	rs6235	C	G	G	0.194	469	0.74 (0.48-1.16)	0.196	0.149	0.641	0.85 (0.51-1.42)	0.539	0.162	0.595	0.10 (0.01-1.79)	0.118	0.049	0.218	0.10 (0.01-1.80)	0.118	0.125	0.447	1.01 (0.61-1.69)	0.965	0.468	0.858
6	CDKAL1	20686765	rs9368222	C	A	A	0.226	469	1.50 (1.03-2.20)	0.036	0.035	0.107	1.81 (1.13-2.90)	0.014	0.011	0.022	1.17 (0.46-2.99)	0.746	0.199	0.627	1.50 (0.55-4.15)	0.430	0.314	0.846	1.86 (1.13-3.05)	0.015	0.032	0.063
6	RSPO3	127131790	rs2745353	C	T	C	0.401	469	0.80 (0.55-1.15)	0.223	0.164	0.705	0.65 (0.40-1.05)	0.081	0.036	0.149	1.00 (0.52-1.92)	0.996	0.245	0.666	0.76 (0.37-1.57)	0.465	0.331	0.852	0.62 (0.37-1.05)	0.073	0.086	0.279
7	DGKB	15024684	rs2191349	G	T	T	0.410	468	1.42 (0.99-2.03)	0.058	0.054	0.184	1.71 (0.99-2.96)	0.056	0.026	0.092	1.39 (0.74-2.62)	0.308	0.106	0.459	1.93 (0.91-4.07)	0.085	0.097	0.324	1.65 (0.93-2.92)	0.085	0.097	0.324
7	GRB10	50690548	rs933360	C	T	C	0.364	468	1.38 (0.99-1.93)	0.061	0.056	0.197	1.38 (0.84-2.27)	0.201	0.077	0.386	1.81 (0.99-3.31)	0.056	0.026	0.092	1.99 (1.02-3.90)	0.045	0.059	0.167	1.20 (0.70-2.05)	0.504	0.349	0.857
9	GLIS3	4287466	rs7041847	A	G	G	0.421	468	0.70 (0.49-1.00)	0.051	0.049	0.159	0.59 (0.36-0.96)	0.034	0.017	0.052	0.72 (0.36-1.44)	0.356	0.117	0.491	0.55 (0.26-1.15)	0.112	0.121	0.425	0.61 (0.36-1.02)	0.058	0.074	0.220
9	GLIS3	4293150	rs10814916	A	C	A	0.465	466	0.74 (0.52-1.04)	0.085	0.077	0.290	0.76 (0.46-1.26)	0.291	0.105	0.476	0.54 (0.27-1.05)	0.070	0.031	0.121	0.50 (0.24-1.04)	0.064	0.078	0.244	0.88 (0.52-1.50)	0.633	0.387	0.858
10	CUBN	17114152	rs1801222	A	G	A	0.262	468	0.83 (0.55-1.24)	0.366	0.233	1.000	0.68 (0.41-1.10)	0.116	0.050	0.231	1.57 (0.64-3.84)	0.326	0.112	0.472	1.28 (0.51-3.21)	0.594	0.374	0.858	0.60 (0.35-1.01)	0.054	0.069	0.201
10	ADRA2A	111282335	rs10885122	T	G	T	0.183	469	1.31 (0.87-1.96)	0.195	0.149	0.639	1.09 (0.67-1.79)	0.718	0.197	0.631	3.69 (1.52-8.95)	0.004	0.010	0.011	3.54 (1.38-9.07)	0.009	0.032	0.045	0.86 (0.50-1.50)	0.605	0.375	0.858
11	DUSP8	1675619	rs2334499	C	T	T	0.455	467	1.33 (0.96-1.85)	0.090	0.080	0.309	1.28 (0.75-2.17)	0.370	0.124	0.526	1.71 (1.00-2.91)	0.050	0.024	0.082	1.77 (0.93-3.36)	0.080	0.093	0.305	1.06 (0.60-1.90)	0.836	0.443	0.858
11	CRY2	45851540	rs11605924	A	C	C	0.499	469	0.72 (0.51-1.01)	0.057	0.054	0.182	0.84 (0.50-1.41)	0.505	0.158	0.584	0.45 (0.24-0.84)	0.012	0.010	0.022	0.46 (0.22-0.98)	0.044	0.059	0.164	1.05 (0.60-1.84)	0.863	0.443	0.858
11	MTNR1B	92940662	rs1387153	C	T	T	0.174	468	1.61 (1.06-2.43)	0.025	0.025	0.074	1.65 (1.00-2.71)	0.049	0.023	0.078	2.53 (0.84-7.68)	0.101	0.043	0.183	2.91 (0.94-8.97)	0.063	0.078	0.238	1.53 (0.91-2.58)	0.109	0.120	0.415
12	P2RX2	132465032	rs10747083	G	A	G	0.259	469	0.80 (0.53-1.21)	0.298	0.198	0.868	0.67 (0.41-1.10)	0.113	0.049	0.226	1.40 (0.54-3.62)	0.482	0.147	0.556	1.16 (0.44-3.04)	0.762	0.423	0.858	0.61 (0.36-1.03)	0.065	0.078	0.245
13	PDX1	27917061	rs2293941	G	A	A	0.295	469	1.09 (0.76-1.54)	0.647	0.364	1.000	1.50 (0.92-2.43)	0.103	0.045	0.201	0.46 (0.17-1.20)	0.110	0.047	0.203	0.61 (0.22-1.65)	0.325	0.264	0.802	1.79 (1.09-2.96)	0.022	0.033	0.086
19	GIPR	45693376	rs2302593	C	G	C	0.414	467	1.48 (1.06-2.07)	0.020	0.021	0.061	1.79 (1.04-3.07)	0.034	0.017	0.051	1.62 (0.92-2.85)	0.096	0.042	0.173	2.18 (1.11-4.28)	0.024	0.035	0.091	1.64 (0.92-2.91)	0.091	0.102	0.346
21	BACE2	41209710	rs737288	G	T	T	0.208	467	1.21 (0.82-1.79)	0.329	0.212	0.934	1.04 (0.63-1.72)	0.869	0.227	0.634	2.56 (1.07-6.08)	0.034	0.016	0.054	2.43 (1.01-5.86)	0.049	0.064	0.181	0.84 (0.48-1.46)	0.540	0.361	0.858
21	BACE2	41211811	rs6517656	G	A	A	0.178	468	1.24 (0.84-1.84)	0.277	0.190	0.824	1.15 (0.69-1.92)	0.584	0.171	0.608	2.13 (0.87-5.23)	0.100	0.043	0.182	2.12 (0.85-5.27)	0.107	0.118	0.408	0.98 (0.56-1.73)	0.951	0.466	0.858

Table that summarizes the most relevant results of the analysis of SNPs + Group models in Hispanic (HIS) ethnicity. ADD, Additive model; DOM, dominant model; REC, recessive model; HETHOM, heterozygous-homozygous model; CHROM, Chromosome code; LOCUS, Locus/Gene; POS, Base-pair coordinate [GRCh38]; ID, Variant ID; REF, Reference allele; ALT, Alternate allele; A1, Counted allele in logistic regression; A1_FREQ, minor allele frequency; OBS_CT, Number of samples in the regression; OR_CI95, odds ratio with 95% confidence interval.

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Hispanic ethnicity findings

Table 3b HIS summarizes the most relevant findings for Hispanic pregnant women. The genetic variants significantly associated with increased risk of GDM were rs9368222, CDKAL1; rs2302593, GIPR; rs10885122, ADRA2A; rs1387153, MTNR1B; rs737288, BACE2; rs1371614, DPYSL5; and rs2293941, PDX1. Variants significantly associated with decreased risk for GDM were rs340874, PROX1; rs2943634, IRS1; rs7041847, GLIS3; rs780094, GCKR; rs563694, G6PC2; and rs11605924, CRY2.

OR and *p* and *q*-values can be seen in the tables.

Additional findings

There are some variants for which some indication of association with GDM was obtained, but the results were not conclusive. Specifically, for CAU we can point to variants rs2785980 (LYPLAL1), rs7708285 (ZBED3) and rs573904 (SARDH), while for HIS we can point to variants rs1496653 (UBE2E2), rs4402960 (IGF2BP2), rs7651090 (IGF2BP2), rs4458523 (WFS1), rs459193 (ANKRD55), rs6235 (PCSK1), rs2745353 (RSPO3), rs2191349 (DGKB), rs933360 (GRB10), rs10814916 (GLIS3), rs1801222 (CUBN), rs2334499 (DUSP8), rs10747083 (P2RX2) and rs6517656 (BACE2) (Table 3a CAU and Table 3b HISP).

Bioinformatics analysis results

The 40 variants that presented some type of association with GDM were mapped to the closest gene/locus, resulting in a total of 34 encoding proteins that were used as STRING input data (Supplementary Table 3). Basic settings of analysis were: full STRING network, edges indicate both functional and physical protein associations, evidence as meaning of network edges, all active interaction sources, medium confidence (0.400) as minimum required interaction score. The complete results provided by the software can be found in Supplementary Table 4. The aspects that were considered most relevant to the objective of the work were selected by inspection so that Supplementary Table 5. Table 4 displayed the bioinformatic analysis of relevant results, and the graph in Figure 2 were obtained.

Discussion

In this study, we have evaluated the association of 98 susceptibility genetic variants with the diagnosis of GDM in a large population of pregnant women from two ethnic groups, from a single center, living in Spain, in the setting of an ongoing nutritional intervention program. To our knowledge, this is the first time that a large relevant set of SNPs has been analyzed in such a large sample of GDM patients, and with a close follow-up regarding their diet and lifestyle.

We have observed that the nutritional intervention presented a significant association with GDM, regardless of the variant considered, OR < 1 ($p < 0.05$, $q < 0.05$, $lfdr < 0.1$), in practically all models for both ethnicities [Supplementary Table 2 CAU-2HIS], confirming the protective effect of the MedDiet for GDM, as previously reported (3, 4, 16, 17) and, at the same time, confirming the significance of the observed SNPs. The variable of the logistic regression model that represents the nutritional group [GROUP] provided relevant information to assess the association of the genetic variants with GDM. The analysis showed that the SNP-GDM association tests identified as significant, when adjusted by the GROUP variable, had a lower FDR, that is, the discoveries have a low proportion of false significant identified associations, evaluated by *q*-values, and a low local false discovery rate, evaluated by *lfdr*-values. Furthermore, *q*-values indicate that it is possible to qualify as discovery a null hypothesis with a *p*-value greater than the usual threshold of 0.05, increasing the set of variants that deserve further investigation, without significantly increasing the false discovery rate.

Although case-control-based GWAS usually refer to the additive model, it is currently recommended to also consider other genetic models (18) for a better understanding of the variant-disease relationship. Our study includes four genetic models that provide joint information on this relationship, aiding in the understanding of genetic analysis and providing further strengths to our findings. We can point out that, with some minor exceptions, when a significant association is observed for a given SNP in several models, the corresponding OR verify $OR_{ADD} < OR_{DOM} < OR_{REC} < OR_{HOM}$, when minor allele is a risk allele or $OR_{ADD} > OR_{DOM} > OR_{REC} > OR_{HOM}$ when minor allele is protective (Table 3a CAU-3b HIS).

Logistic regression results are consistent with information collected on STRING databases relative to PPI, both known and predicted, or associations identified by co-expression, protein homology, or text mining. The most significant variants in genetic tests are located in locus/genes encoding proteins annotated in the knowledge database as associated with biological processes related to diabetes and GDM (Table 4). Most of the nodes in Figure 2 have the name of a locus/gene that are well referenced in the literature because several SNPs with a significant association with diabetes and GDM are located nearby. Specifically, the nodes located in the central core of the graph, MNTR1B (rs1387153, rs10830962, rs10830963), IGF2BP2 (rs4402960, rs7651090), KCNJ11 (rs5215), GCKR (rs780094), CDKAL1 (rs9368222), IRS1 (rs2943634), ADRA2A (rs10885122), CRY2(rs11605924), DKGB (rs2191349), G6PC2 (rs563694), GLIS3 (rs7041847, rs7034200, rs10814916), GIPR (rs2302593), WFS1 (rs4458523), ZBED3 (rs7708285), PROX1 (rs340874), FOXA2 (rs6048205), PDX1 (rs2293941), PCSK1 (rs6235), have been referred in various GWAS as associated to diabetes (6, 19–25), GDM (26–34) or both (35, 36).

TABLE 4 Bioinformatic analysis relevant results.

QueryIndex	QueryItem	StringId	Disease	Diabetes Mellitus	Gestational Diabetes	Regulation of Biological Quality	Regulation of cell Communication	Glucose Homeostasis	Regulation of Insulin Secretion	Cobalamin
1	ADRA2A	9606.ENSP00000280155	√			√	√	√	√	
2	ANKRD55	9606.ENSP00000342295	√							
3	BACE2	9606.ENSP00000332979				√		√		
4	CDKAL1	9606.ENSP00000274695	√	√	√					
5	COBLL1	9606.ENSP00000341360								
6	CRY2	9606.ENSP00000478187	√			√	√	√		
7	CUBN	9606.ENSP00000367064	√			√				√
8	DGKB	9606.ENSP00000385780				√	√			
9	DPYSL5	9606.ENSP00000288699	√							
10	DUSP8	9606.ENSP00000380530					√			
11	FOXA2	9606.ENSP00000400341				√	√		√	
12	G6PC2	9606.ENSP00000364512				√	√	√	√	
13	GCKR	9606.ENSP00000264717	√			√		√		
14	GIPR	9606.ENSP00000467494				√	√		√	
15	GLIS3	9606.ENSP00000371398	√	√						
16	GLP2R	9606.ENSP00000262441	√							
17	GRB10	9606.ENSP00000381793	√			√	√			
18	IGF2BP2	9606.ENSP00000371634	√	√	√	√				
19	IRS1	9606.ENSP00000304895	√	√	√	√	√	√	√	
20	KCNJ11	9606.ENSP00000345708	√	√	√	√	√	√	√	
21	LYPLAL1	9606.ENSP00000355895								
22	MTNR1B	9606.ENSP00000257068	√	√	√	√	√	√	√	
23	MTR	9606.ENSP00000355536	√							√
24	P2RX2	9606.ENSP00000343339	√			√	√			
25	PCSK1	9606.ENSP00000308024	√			√				
26	PDX1	9606.ENSP00000370421	√	√		√	√	√	√	
27	PROX1	9606.ENSP00000355925	√							
28	RSPO3	9606.ENSP00000349131					√			
29	SARDH	9606.ENSP00000360938								
30	SLC17A9	9606.ENSP00000359376	√							
31	UBE2E2	9606.ENSP00000379931								
32	WARS	9606.ENSP00000347495	√							
33	WFS1	9606.ENSP00000226760	√	√		√	√	√		
34	ZBED3	9606.ENSP00000255198				√	√			

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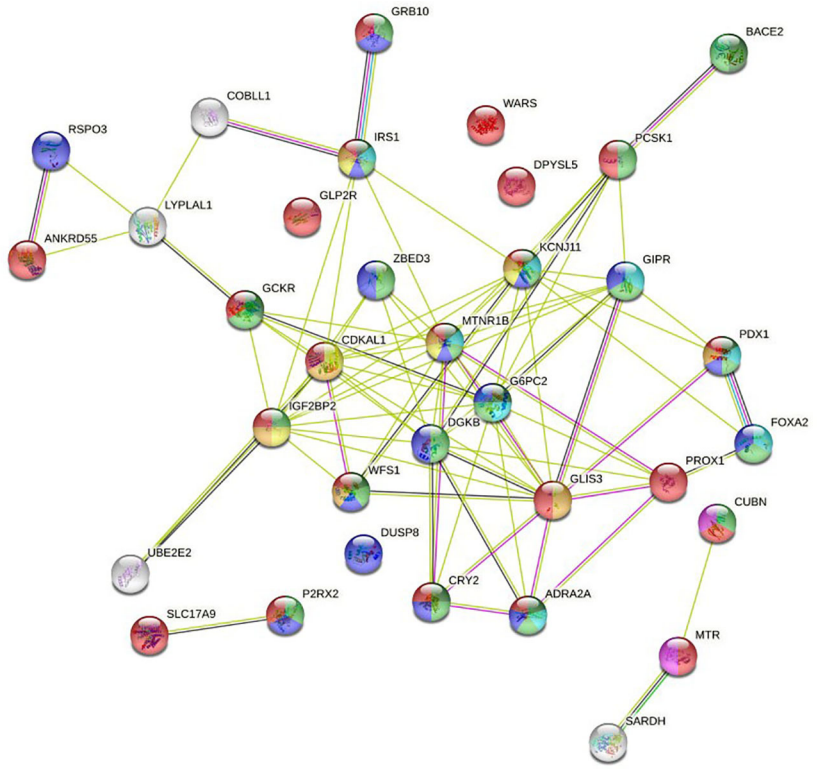

FIGURE 2
Full STRING network of both functional and physical protein associations. The edges indicate both functional and physical protein associations.

We can observe a subnetwork made up of the RSPO3 (rs2745353), ANKRD55 (rs459193), LYPLAL1 (rs2785980) and COBLL1 (RS7607980) nodes. Although this is not annotated in STRING gene ontology, the revised literature reports that all of them are related to fasting insulin and show a significant association with diabetes and GDM (6, 19, 21–23, 35, 36).

In addition to the central core, where the nodes with the highest intensity of interaction are located, the network has three terminal nodes, four isolated nodes, and two isolated subnetworks, one made up of two nodes and the other made up of three nodes.

BACE2 (rs737288, rs6517656) node has been associated with GDM in some studies (8, 37), but not in others (28, 38). It is related to higher fasting C-peptide levels. As can be seen in the graph, it has a close interaction with PCSK1. In our work, the association for the Hispanic ethnic stratum is significant. GRB10 (rs933360) node has strong interaction with the IRS1 node, an insulin receptor substrate 1 that may mediate the control of various cellular processes by insulin. It is associated with diabetes in some studies (32–34), and with both diabetes and GDM in other (35, 36). We have found an association with GDM in the Hispanic ethnic stratum. UBE2E2 (rs1496653) node is an ubiquitin-conjugating enzyme associated with diabetes in some reports (20, 21, 25), and with GDM in other studies (27, 35, 36). In our work, it shows interaction with IGF2BP2, but it barely reaches significance in the Hispanic ethnicity.

DPYSL5 (rs1371614) has been associated with diabetes (6, 23, 24) and GDM (35, 36). It is a dihydropyrimidinase-related protein that has been linked with fasting glucose. In our study, we found an association in some models for both ethnic groups. WARS (rs3783347) is a shear stress-responsive gene that has been associated with diabetes (19, 22–24). In our study, it is significant in some models for Caucasian ethnicity. DUSP8 (rs2334499), dual specificity protein phosphatase 8, has phosphatase activity with synthetic phosphatase substrates and negatively regulates mitogen-activated protein kinase activity. Some studies (20, 21) report association with diabetes, while others (27, 36) do so with GDM. Our work shows association in a model for Hispanics. GLP2R (rs17676067) is a receptor for glucagon-like peptide 2, which has been reported as associated with diabetes (21). Our work shows association in the ADD, REC and HOM models for Caucasian ethnicity.

SLC17A9 (rs3746750), Solute Carrier Family 17 Member 9, is a protein coding gene related with transporter activity and

involved in vesicular storage and exocytosis of ATP. It has been related to purinergic signaling and diabetes (39, 40). In our work, it shows a significant association in the ADD, DOM and HET models for Caucasian ethnicity. In the graph, we can see a strong association of SLC17A9 with P2RX2 (rs10747083), purinoceptor 2, ion channel gated by extracellular ATP involved in a variety of cellular responses. It is included in some studies as associated with diabetes (19, 22, 23) and GDM (36). In our study, it hardly reaches significance in the DOM model of the Hispanic ethnicity.

The CUBN (rs18001222), MTR (rs1805087), and SARDH (rs573904) proteins define a subnetwork in the graph that play a role in one-carbon metabolism with functions in many cellular processes. Also, genetic variants in the transport and metabolism of folate modify glycemic control and risk of GDM, and the effect of folic acid on homocysteine levels is modulated by CUBN (rs1801222) (41). CUBN, cubilin, is a cotransporter which plays a role in lipoprotein, vitamin and iron metabolism; serves as transporter in several absorptive epithelia, including embryonic yolk sac. In a study by Böger et al. (42) it is described as "*a gene locus for albuminuria*", an idea that is reiterated in subsequent works (43). It has also been associated with type 2 diabetes in an elderly population (44). In our work, it is in the limits of significance in the DOM and HET model in the Hispanic ethnicity. MTR, 5 -methyltetrahydrofolate–homocysteine methyltransferase, catalyzes the transfer of a methyl group from methyl- cobalamin to homocysteine; belongs to the vitamin-B12 dependent methionine synthase family, and has been associated with various biological processes related to pregnancy (45). In our work, it has been significant in the ADD model for Caucasian ethnicity.

It should be noted that some studies are partially in disagreement with the most widely accepted results, that is, they report no association with diabetes or GDM in some of the variants mentioned above. In this regard, the following works can be consulted (9, 38, 46–49):. As an example, in our study some SNPs included in the initial list of variants and clearly identified in the literature, such as TCFL2, KCNQ1, HNFA1A, SCL30A8, have not reached a level of significance in any association model with GDM. This could be related to the complex genetic and epigenetic architecture, with both similarities and differences between diabetes and GDM, which deserves further investigation.

The idea of considering the evaluation of the impact of diet and lifestyle on the significance of SNPs in their association with GDM is currently attracting the interest of investigators (50). In this regard, we remark that our study has been performed with a meticulous evaluation of lifestyle habits, showing the protective effect of a healthy MedDiet, and that significant SNPs remained as such, after performing a rigorous genetic and statistical bioinformatic analysis.

Conclusion

Identifying the potential susceptibility genetic variants that could be associated with developing GDM and their modulation due to a nutritional intervention deems useful to design preventive and therapeutic strategies, especially in the setting of the increasing prevalence of GDM. In this study, we have examined a set of 98 SNPs in a large cohort of patients from two main ethnicities from a single center, and in the setting of an ongoing clearly beneficial nutritional intervention. The study confirms previous works that promote the therapeutic recommendation of Mediterranean Diet to all pregnant women to prevent GDM. In addition, we have confirmed a core set of SNPs reported in the literature as associated with diabetes and GDM. However, our statistical models, that include the nutritional intervention as an additional variable, highlight and reinforce the significance of the association effects, reducing the FDR levels. This means that a safer tool is available to control the risk of GDM based on the genomic profile of the individual. Therefore, genotypic analysis of women of child-bearing age and recommending a MedDiet, will assist the prompt identification and management of GDM.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Clinical Trials Committee of the Hospital Clínico San Carlos. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization and design: AR-L, AB, AC-P, NT, ADu, MH, MR, LM, MZ, PM, ADi, LV, VM, JV. Data curation, and analysis and interpretation of data: AR-L, AB, AC-P, NT, ADu, CF, IJ, LV, VM, IM, JV. Funding acquisition: AC-P, NT. Investigation: AC-P, MT, PM, ADi, AB, MA, LS, LM, MZ, MR, MT. Methodology: AC-P, NT, ADu, CF, IJ, MH, MT, IM, PM, MA, LS, LM, MZ, AB, LV, VM, JV. MR. Software: AR-L. Supervision, Validation and Visualization: AC-P, AR-L, AB, NT, MR. Writing – original draft: AC-P, AR-L, AB, NT. Writing – review & editing: AR-L, AC-P, AB, NT, ADu, MR, MA, LS, LM, MZ. All authors have seen and agree with the content of the full last version of manuscript.

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Conflict of interest

LM, MZ, LS, MA are employees of Patia Europe.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1036088/full#supplementary-material

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Prevalence and risk factors of diabetic foot disease among the people with type 2 diabetes using real-world practice data from Catalonia during 2018

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Background: Our study aimed to assess the prevalence of diabetic foot disease (DFD) and its associated risk factors among subjects attending primary care centers in Catalonia (Spain).

Methods: We undertook a cross-sectional analysis of data from the primary health care (SIDIAP) database. The presence of comorbidities and concomitant medication were analyzed for subjects with or without DFD. DFD prevalence was estimated from 1st January 2018 to 31st December 2018.

Results: During the 12-month observational period, out of 394,266 people with type 2 diabetes, we identified 3,277 (0.83%) active episodes of DFD in the database. The majority of these episodes were foot ulcers (82%). The mean age of patients with DFD was 70.3 (± 12.5) years and 55% were male. In the multivariable descriptive models, male gender, diabetes duration, hypertension, macrovascular, microvascular complications, and insulin and antiplatelet agents were strongly associated with DFD. A previous history of

DFD was the stronger risk factor for DFD occurrence in subjects with T2DM (OR: 13.19, 95%CI: 11.81; 14.72).

Conclusions: In this real-world primary care practice database, we found a lower prevalence of DFD compared to similar previous studies. Risk factors such as male sex, duration of diabetes, diabetes complications and previous history of DFD were associated with the presence of DFD.

KEYWORDS

Catalonia, diabetic foot disease, primary healthcare, prevalence, SIDIAP

Introduction

Diabetic foot disease (DFD) and its complications herald the high morbidity and mortality among patients with type 2 diabetes mellitus (T2DM). Globally, it is estimated that the subjects with DFD have a similar life expectancy compared to some frequent cancer types such as colon and breast (1). Actually, DFD is the leading cause of hospitalization among T2DM subjects (2). In Catalonia (Spain), people with T2DM and DFD are three times at higher risk for hospital admission, and even five times more for admissions to socio-sanitary facilities (day care facilities and residences) than the rest the population (3). This entails a higher health cost and a decreased quality of life of these subjects.

There is a 25% risk probability of developing a foot ulcer among people with diabetes during the disease course (4). DFD will evolve towards healing, amputation, or even death depending on the severity, underlying comorbidities, and care received. The prevalence of DFD varies among countries and even within different regions in the same country (5, 6). This variability could be due to differences in the type of population studied, the definition of foot ulcers, and the methodology used to identify the cases and the setting where the study was performed (primary vs secondary care) (7).

Catalonia is situated in the northeast of Spain with a population of 7.5 million whose capital is the city of Barcelona. The primary care electronic medical records started in 2006 and currently the health system is entirely electronic. Due to this process, large amounts of routinely collected electronic health data are available through different population databases. Measuring the real burden of DFD could help us to better quantify the impact of this highly complex and costly diabetes complication on life expectancy and morbidity among persons with T2DM in our primary health care settings. Moreover, it could help us to identify factors associated with this condition more efficiently. So far, to the best of our knowledge, there are no real-world data (RWD) studies on DFD in our primary health care settings. Our study aimed to estimate the prevalence of the DFD and its associated risk factors in subjects who attended the centers of the largest public healthcare provider in Catalonia in 2018 (northeast region of Spain).

Materials and methods

Study population

At the "cut-off" date (31st December 2018), we included all live adult subjects (age > 18 years) in the database with a diagnosis of T2DM defined as the presence of diagnostic codes (International Statistical Classification of Diseases and Related Health Problems 10th Revision-ICD-10): E11 and E14. Subjects with other types of diabetes, such as type 1, secondary, gestational or other types of diabetes (ICD-10: E10, E12, O24 or E13) were excluded from the analysis.

Study intervention and data source

We performed a cross-sectional study using the primary health care population SIDIAP database from 1st January 2018 until 31st December 2018. The SIDIAP (Sistema d'Informació per al desenvolupament de la Investigació en Atenció Primària) database includes the routinely collected healthcare data from users attending the primary healthcare centers from Institut Català de la Salut (ICS) (8). The cross-sectional analysis was chosen as well validated method in epidemiology to collect and analyze the data from many different individuals from our primary health care database at a single point in time and to investigate the association between a putative risk factors and a health outcome (9, 10). ICS is the major local public healthcare provider, covering 80% (5,564,292 users) of the Catalonian population. The SIDIAP database is a well-validated primary health database in diabetes research in Spain (11).

Study variables and comparison

We defined a DFD episode as the presence of one or a combination of different diagnostic codes and sub-codes for lower-extremity ulcers (ICD-10: L97, E11.621), osteomyelitis (ICD-10: M86), gangrene (ICD-10: I96, E11.52), lower-extremity amputation (ICD-10: Z89), or surgical detachment procedures-0Y6) or Charcot neuroarthropathy (M14.6, E11.61) at the cut-off date. All those diagnostic codes and procedures referring to amputations below the ankle were defined as minor amputations and included amputations of one or more toes and trans-metatarsal amputations. Those amputations above and through the foot or ankle were defined as major amputations (12, 13). The diagnostic codes related to low-extremity amputations but without specific locations were considered non-specific amputations. During the study period, we also analyzed the prevalence of other comorbidities such as hypertension and hyperlipidaemia identified by ICD-10 diagnostic codes and/or pharmacologic treatment, macrovascular (coronary heart diseases, cerebral vascular accident and heart failure) and microvascular complications (diabetic retinopathy, diabetic neuropathy, and chronic kidney disease, the latter defined as a combination of CKD-EPI glomerular filtration rate <60 ml/min/1,73m^2 and/or an albumin/creatinine ratio >30mg). We also analyzed other clinical variables, such as diabetes duration, body mass index (BMI), and systolic and diastolic blood pressure. Variables related to lipid, renal profile, glycosylated hemoglobin (HbA1c), and pharmacologic treatments were also extracted from the database and analyzed.

Two groups of subjects were created, i.e. groups with and without an episode of DFD that occurred during 2018. We compared the groups for different clinical characteristics at "cut-off" date.

Statistical analysis

We described all the variables during the study period. The mean values and standard deviation for continuous variables were estimated, while we calculated the number and frequencies for categorical variables.

The prevalence of DFD was calculated as the proportion of subjects with DFD divided by the total number of alive people with T2DM in the database. In the case of multiple episodes of DFD in different moments, we counted the episodes only once per person and the episode closest to the cut-off date to prevent possible overestimation of the DFD prevalence in the database. We calculated the prevalence of active episodes of DFD during 2018 (a 12-month period from the cut-off date). We created the variable "previous history of DFD" with this approach. As a history of DFD, we considered all previous episodes that occurred before 1st January 2018 the period to estimated DFD prevalence, i.e. the 2018-year period).

To evaluate the association between different factors and DFD, we performed multivariable logistic models to describe the association between the different clinically important variables and the presence of DFD during the study period. Furthermore, additional models were performed to evaluate the association between antidiabetic drugs and presence of or history of previous DFD (before 2018). All the analyzes were done with R statistical software version 3.5.1.

Results

Between 1st January 2018 and 31st December 2018, a total of 394,376 live subjects were identified in the database. Of these subjects, 110 were excluded due to the double codification of other types of diabetes. Thus, we finally included 394,266 subjects meeting the study eligibility criteria. Figure 1 shows the study flowchart.

Characteristics of subjects with and without DFD

Table 1 shows the clinical characteristics of the study subjects. The mean age was 70.3 (± 12.5) years, with a male predominance (55%). DFD episodes were more frequent among people aged 75 or older. There were more current smokers and "at-risk" alcohol users in the group with DFD than in the non-DFD group.

We observed a worse comorbidity profile among people with DFD. These subjects had longer diabetes duration (3.7 years longer) than those without DFD. Microvascular and macrovascular complications were more prevalent among participants with DFD. We observed minimum differences in BMI and blood pressure between groups, and slightly poorer glycemic control among subjects with DFD. The lipid profile was poorer among subjects without DFD, while we observed lower glomerular filtration rates among those with DFD.

Regarding antidiabetic treatment, lifestyle and dietary measures, non-insulin antidiabetic drugs (NIAD) as a single therapy and dual therapy were more frequent among subjects without DFD. Accordingly, insulin alone or in combination was more frequently used as a treatment option among the subjects with DFD. We also observed a higher prevalence of other concomitant drug treatments among subjects with DFD, especially antiplatelet agents. The results of antidiabetic and other concomitant treatments are summarized in Table 2.

FIGURE 1
Study flowchart DFD: diabetic foot disease; n: number.

DFD prevalence

During the last 12 months from the "cut-off" date (31/12/2018), we identified 3,277 (0.83%) active episodes of DFD, of which 82% were due to active foot ulcers. During this period, 28.8% of subjects underwent lower-limb amputations, while 7.9% of subjects had foot gangrene. The prevalence of DFD is summarized in Table 3.

Factors related to the DFD

Supplement Table 1 and Figure 2 show different comorbidity models. In all the multivariable descriptive models, male sex, diabetes duration, at-risk alcohol use and higher BMI were independent risk factors for DFD. Concerning the comorbidities, the presence of hypertension, and macrovascular and microvascular complications were positively associated with DFD. As expected, peripheral artery disease and diabetic neuropathy were associated with increased risk for DFD in the fully itemized model. These associations were even stronger in models merging conditions under macrovascular and microvascular categories. The presence of hyperlipidaemia was negatively associated with DFD.

In the additional models that included antidiabetic treatment, insulin use was associated with DFD episodes (Supplement Table 2 and Figure 3A). In contrast, treatment with NIAD or lifestyle and dietary measures were negatively associated with DFD. A previous history of DFD was strongly associated (OR: 13.19, 95%CI: 11.82; 14.72) with the DFD events in this additional model (Supplement Table 3 and Figure 3B).

Discussion

Our real-world evidence study from the SIDIAP primary care database in Catalonia in the 12-month 2018 period found that the prevalence of diabetic foot disease among live T2DM subjects of 0.83%. Few studies have described the prevalence of DFD among subjects with diabetes mellitus. The meta-analyzes and systematic reviews done by Lazzarini et al. (5) and Zhang et al. (6) reported a prevalence of DFD of 4.7% (95% CI: 0.2–11.9%) and 6.3% (95% CI: 5.4-7.3%), respectively. Both studies described a significant variability in the prevalence from one

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TABLE 1 Clinical characteristics of the study subjects.

Variable	Patients without DFD (N=390989)	Patients with DFD (N=3277)	Total (N=394266)
Age, mean, years	70.3 (12.5)	74.0 (12.0)	70.3 (12.5)
Age ≥75 years, n (%)	146757(37.5)	1628 (49.7)	148385 (37.6)
Sex (male), n (%)	214618 (54.9)	2090 (63.8)	216708 (55.0)
Current Smoker, n (%)	55553 (14.4)	507 (15.6)	56060 (14.4)
"Low risk" alcohol use, n (%)	90533 (34.9)	649 (28.4)	91182 (34.8)
"At risk" alcohol use, n (%)	3294 (1.27)	41 (1.79)	3335 (1.27)
Diabetes duration, mean (SD), years,	10.2 (6.55)	13.4 (7.35)	10.2 (6.57)
Body mass index, mean (SD),kg/m^2	30.0 (5.20)	30.1 (6.11)	30.0 (5.21)
SBP, mean (SD), mmHg	133 (13.6)	132 (16.7)	133 (13.7)
DBP, mean (SD), mmHg	75.1 (9.73)	71.8 (10.5)	75.1 (9.74)
Comorbidities, n (%)			
Hypertension,	305581 (78.2)	3018 (92.1)	308599 (78.3)
Hyperlipidaemia,	272111 (69.6)	2408 (73.5)	274519 (69.6)
Ischemic heart disease	51252 (13.1)	771 (23.5)	52023 (13.2)
Heart failure	28127 (7.19)	743 (22.7)	28870 (7.32)
Cerebrovascular disease	38027 (9.73)	639 (19.5)	38666 (9.81)
Peripheral arterial disease	28577 (7.31)	1575 (48.1)	30152 (7.65)
Macrovascular complications	94340 (24.1)	2039 (62.2)	96379 (24.4)
Diabetic neuropathy	24199 (6.19)	883 (26.9)	25082 (6.36)
Diabetic retinopathy	39490 (10.1)	1186 (36.2)	40676 (10.3)
Chronic kidney disease	122122 (31.2)	1956 (59.7)	124078 (31.5)
Microvascular complications	64061 (16.4)	1666 (50.8)	65727 (16.7)
Laboratory parameters			
HbA1c, mean, (SD), %	7.09 (1.29)	7.35 (1.54)	7.09 (1.29)
HbA1c ≥ 8%, n (%)	56595 (18.84)	681 (27.7)	57276 (18.86)
Total cholesterol (mg/dL), mean (SD)	182 (40.4)	164 (43.6)	182 (40.4)
HDL cholesterol (mg/dL), mean (SD)	48.7 (12.7)	45.3 (13.3)	48.7 (12.7)
LDL cholesterol (mg/dL), mean (SD)	103 (33.3)	91.1 (34.9)	103 (33.3)
Triglycerides (mg/dL), mean (SD)	159 (104)	152 (97.9)	159 (104)
Estimated glomerular filtration rate, mL/min/1.73m^2, mean (SD)	73.5 (18.4)	62.5 (23.5)	73.4 (18.5)

DFD, diabetic foot disease; SD, standard deviation; HbA1c, glycosylate haemoglobin; SBP, systolic blood pressure; DPB, diastolic blood pressure.

continent to another, and among the different regions where the studies were carried out. Their great limitation was the heterogeneity among the data, even within the same country. In Zhang's study, the prevalence in Europe was 5.1%. Analyzing the included studies, great methodological variability was observed, most with a small number of patients included; further, more than 66% of the studies were old, published before 2010 (6).

A large amount of routinely collected health care data in recent years allowed the performance of real clinical practice studies. Several studies have been published to determine the prevalence of DFD using different registry systems (databases). These studies bring us closer to the reality of the health care area studied. In Spain, Alonso et al. (14), in a study of diabetes-related complications in the Basque Country, found a prevalence of foot ulcers of 1.93%, very similar to the figure found in Israel (15) (1.2%) and in Taiwan (2%) (16). In Saudi Arabia, the overall prevalence of DFD was 3.3%, while the prevalence of foot ulcers, gangrene, and amputations were 2.05%, 0.19%, and 1.06%, respectively (17). These prevalences are higher compared to those observed in our study.

In the current analysis, during 2018, a prevalence of 0.68% (2,687) of new episodes of diabetic ulcer were recorded. This percentage was lower if we compare this with the prevalence observed in a retrospective registry-based study (2.05%) from 65,534 Saudi diabetic patients during the 2000 and 2012 regardless of the type of diabetes (17). In a recent cross-sectional study developed in the southern area of the metropolitan region of Barcelona, the point prevalence of foot ulcers during a 2-month period in 2013 was 0.16% (18). That

TABLE 2 Antidiabetic and other concomitant treatment.

	Patients without DFD (N=390989)	Patients with DFD (N=3277)	Total (N=394266)
Antidiabetic treatment *, n (%)			
Diet and lifestyle only	90301 (23.1)	547 (16.7)	90848 (23.0)
NIAD monotherapy	133538 (34.2)	653 (19.9)	134191 (34.0)
Dual NIAD therapy	65360 (16.7)	399 (12.2)	65759 (16.7)
Triple NIAD therapy	26592 (6.80)	146 (4.46)	26738 (6.78)
Insulin alone	19612 (5.02)	599 (18.3)	20211 (5.13)
Insulin in combination	55586 (14.2)	933 (28.5)	56519 (14.3)
Other concomitant drugs, n (%)**			
Anticoagulants	24015 (6.14)	441 (13.5)	24456 (6.20)
Antiplatelet agents	113289 (29.0)	1733 (52.9)	115022 (29.2)
Antihypertensive	275630 (70.5)	2726 (83.2)	278356 (70.6)
Lipid-lowering	206959 (52.9)	1881 (57.4)	208840 (53.0)
Antibiotics	62926 (16.1)	1415 (43.2)	64341 (16.3)

DFD: diabetic foot disease; NIAD: non-insulin antidiabetic drugs.
*In the last three months.
**In the last 12 months.

study was not specifically designed to assess the prevalence of DFD, and included the recorded diagnostic codes of different types of ulcers (including venous ulcers), without including other forms of DFD, like those of our study (amputations, osteomyelitis, Charcot disease). Additionally, that study did not characterize subjects with diabetes. Furthermore, our study is more representative of the Catalonian population. Therefore, our findings are hardly comparable to those of this recent study (18).

In our study population, there were 943 (0.24%) new episodes of amputations. According to a systematic review by Narres et al. (19), the incidence of lower-limb amputations in the diabetic population ranged from 78 to 704 per 100,000 people with diabetes/year. Also, high variation exists for these procedures, from one country to another and even within the same country. In Spain, the incidence of amputations also shows significant variation from one region to another, and in the case of major lower-limb amputations, Catalonia is in an intermediate situation among the different health care regions (20). The rate of amputations in Catalonia in 2016 among the diabetic population aged between 45 and 74 years was 27.4 per 10,000 people with diabetes (3). The results provided in our study are lower, suggesting a decrease in the number of episodes, as was the case for other countries (19); however, this finding will need to be confirmed in further studies. Regarding Charcot foot disease, we could only identify 39 newly diagnosed patients (0.01%) in 2018. There are few published studies for comparison. In a retrospective hospital-based study, Fabric et al. (21) found an annual incidence of 0.3%.

Comparing diabetic patients with and without DFD in our study, in the DFD group, there were more men, they were older, with a longer diabetes duration, with a higher percentage of

TABLE 3 DFD prevalence and DFD related variables.

	Total (N=394266)	Patients with DFD*(N=3277)
DFD, n (%)	3,277 (0.83)	3,277(100)
Foot ulcers, n (%)	2687 (0.682)	2687 (82.0)
Osteomyelitis, n (%)	220 (0.06)	220 (6.71)
Gangrene, n (%)	261 (0.07)	261 (7.96)
Charcot foot, n (%)	39 (0.01)	39 (1.19)
Amputations, n (%)	943 (0.24)	943 (28.8)
Major amputations, n (%)	168 (0.04)	168 (5.13)
Minor amputations, n (%)	393 (0.1)	393 (12.0)
Non-specific amputations, n (%)	596 (0.2)	596 (18.2)
Previous history of DFD	10852 (2.75)	3105 (94.8)

*Active episodes of DFD during 2018; DFD: diabetic foot disease.

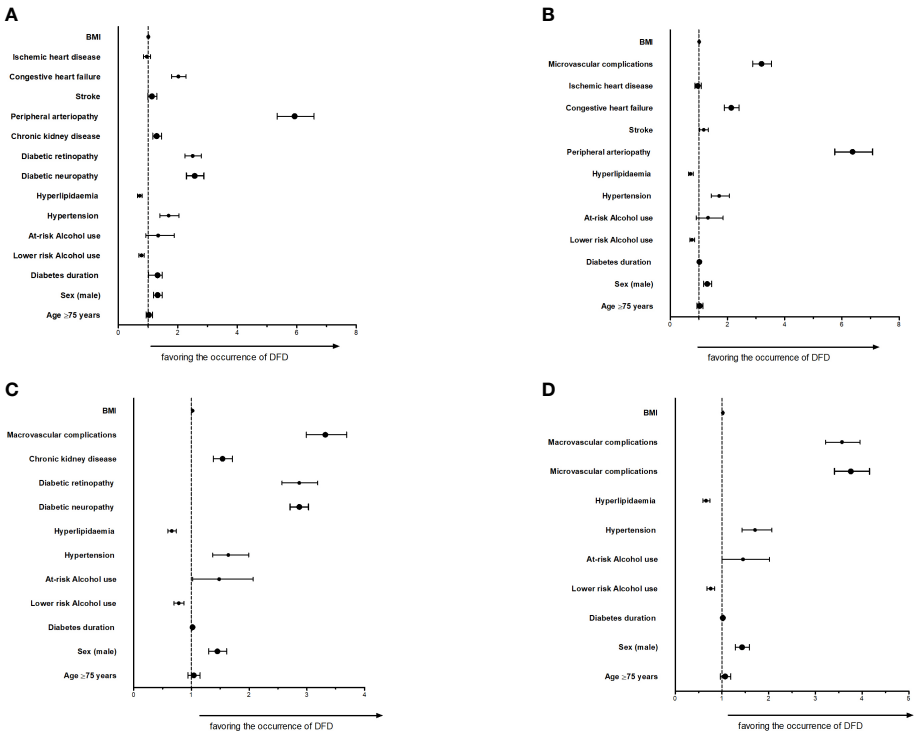

FIGURE 2
DFD and different comorbidities models **(A)** Fully itemized model; **(B)** Microvascular complications merged model; **(C)** Macrovascular complications merged model; **(D)** Microvascular and Macrovascular merged model BMI: body mass index; DFD: diabetic foot disease.

smokers and patients with hypertension, and a higher proportion of micro- and macrovascular complications. These findings are in line with other similar studies. It is known that the risk of ulcers and amputation increases with age (6, 14, 22), duration of diabetes (6, 23), poor metabolic control (15, 23), and smoking (6), and are more prevalent in men (6, 23), but is yet to be explained (6). In our analysis, only 7.31% of the patients without DFD had a recorded diagnosis of peripheral artery disease compared to 48.1% of those diagnosed with DFD. These results are similar to previous studies, and its presence dramatically worsens the prognosis of these patients (24). It is surprising that only 6.19% of patients without DFD and 26.9% of those who had an episode of DFD had a recorded diagnosis of peripheral neuropathy. This percentage is much lower than those previously reported by other authors (25). This is most probably due to the already-described underreporting of this complication in primary care electronic health care records (26) and it is in line with previously published similar studies with the same database (27, 28).

Concerning the risk factors in the multivariable descriptive models, we observed strong associations of macrovascular and microvascular complications in patients with DFD. These chronic complications are related with DFD as a consequence of a general vascular failure (2, 23, 29). A previous history of a DFD increases the risk 13-fold of a new DFD episode, which is in line with what has repeatedly described in multiple studies.

Our study has some limitations. Firstly, as in all studies based on routinely collected healthcare data, the underreporting or missing data is quite frequent and is a clear limitation. Also, to prevent possible overestimation of the prevalence, only one episode was recorded for each person with T2DM, the closest to the cut-off date. The multivariable models are descriptive and do not predict the occurrence of DFD in 2018. On the other hand, as strength, the large sample size provides valuable information and gives us an idea of the magnitude of the problem in our country and primary health care facilities.

In conclusion, our real-world primary care database study in Catalonia, Spain, shows a lower DFD prevalence than in other similar studies. In our study, type 2 diabetic subjects with DFD were older, with longer duration of diabetes, had more micro- and macro-vascular complications, and were more often treated with insulin and antiplatelet agents than those without DFD. Further, a previous history of DFD was the stronger risk factor for a new episode of DFD in subjects with T2DM. Moreover, interventions are needed in our primary health care settings in order to improve the DFD codification and detection. The strong

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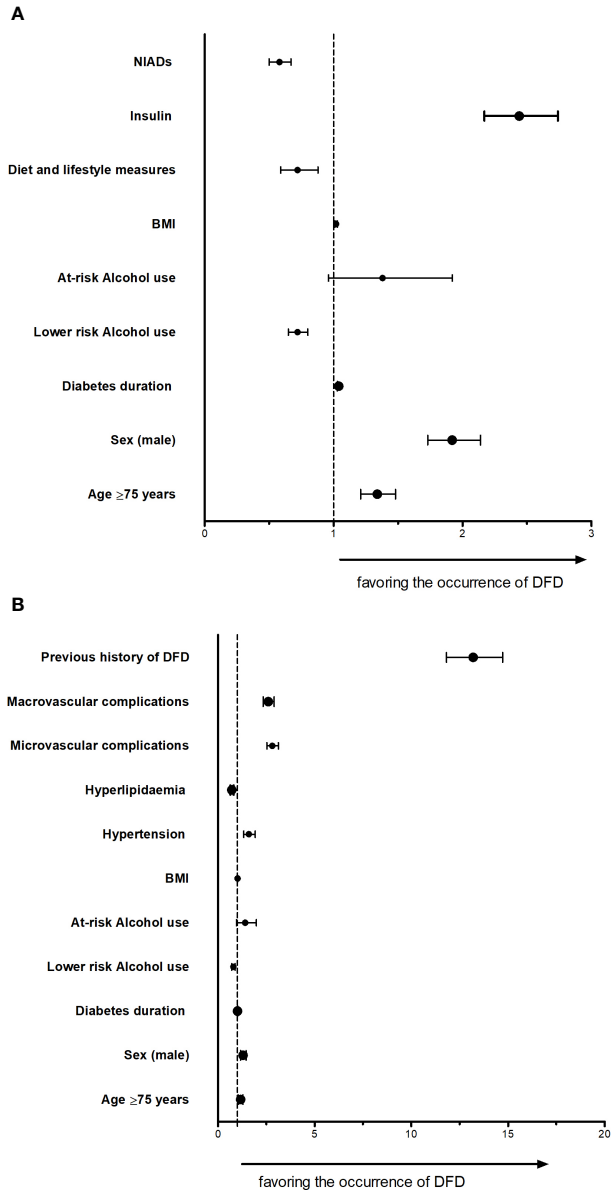

FIGURE 3
DFD models for association with antidiabetic drugs or previous history of DFD **(A)** DFD model adjusted by antidiabetic treatment **(B)** Fully adjusted model for DFD considering the previous history of DFD DFD: diabetic foot disease.

economic and social impact of DFD warrants future studies to evaluate the risk factors related to occurrence and prognosis, potentially increasing the knowledge of prevention and better treatment of this complex disease.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Restrictions apply to the availability of some or all data generated or analyzed during this study because they were used under license. Requests to access these datasets should be directed to Dr. Dídac Mauricio, didacmauricio@gmail.com.

Ethics statement

The studies involving human participants were reviewed and approved by Primary Health Care University Research Institute Jordi Gol (number 19/035-P). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization, MB. Methodology, MB, BV, DM, and JF-N. Software. Formal analysis, RP-T. Data curation, RP-T. Writing—original draft preparation, MB and BV. Writing—review and editing, MB, BV, DM, JF-N, JL, MM-C, EJ, and XC. Supervision, DM. Project administration, BV. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MM-C has received advisory and/or speaking fees from Astra-Zeneca, Bayer, Boehringer Ingelheim, GSK, Lilly, MSD, NOVARTIS, NovoNordisk, and Sanofi. He has received research grants to the institution from Astra-Zeneca, GSK, Lilly, MSD, Novartis, NovoNordisk, and Sanofi. He has received research grants from Institut Universitari d'Investigació en Atenció Primària Jordi Gol IDIAP Jordi Gol Barcelona, Spain, Instituto de Salud Carlos III Madrid, Spain, Generalitat de Catalunya. Peris 2016-2020. The Strategic Plan for Health Research and Innovation Barcelona, Spain.

JF-N has received advisory and/or speaking fees from Astra-Zeneca, Ascensia, Boehringer Ingelheim, GSK, Lilly, MSD, Novartis, NovoNordisk, and Sanofi. He has received research grants to the institution from Astra-Zeneca, GSK, Lilly, MSD, Novartis, NovoNordisk, Sanofi, and Boehringer. DM has received advisory and/or speaking fees from Almirall, Esteve, Ferrer, Janssen, Lilly, Menarini, MSD, NovoNordisk and Sanofi. MB and JL have received advisory and speaking fees from MSD. EJ has received educational, sponsorship and speaker fees from Astra Zeneca, Bayer, Lilly, Novonordisk and Sanofi. XC has received speaker's bureau and advisory board honoraria from AstraZeneca, Boehringer Ingelheim, Esteve, Lilly Diabetes, Novo Nordisk A/S, Roche, and Sanofi.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1024904/full#supplementary-material

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The prevalence of foot ulcers in diabetic patients in Pakistan: A systematic review and meta-analysis

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We aimed to determine the pooled prevalence of diabetic foot ulcers in Pakistan. MEDLINE (PubMed), Web of Science, Google scholars, and local databases were systematically searched for studies published up to August 10, 2022, on the prevalence of foot *ulcers* among diabetic patients in Pakistan. Random-effects meta-analysis was used to generate summary estimates. Subgroup analysis and meta-regression models were used to address the issue of high heterogeneity. Two authors independently identified eligible articles, collected data, and performed a risk of bias analysis. Twelve studies were included in the meta-analysis (14201, range 230–2199, diabetic patients), of which 7 were of "high" quality. The pooled prevalence of diabetic foot ulcers was 12.16% (95% CI: 5.91–20.23%). We found significant between-study heterogeneity ($I^2 = 99.3\%$; $p < 0.001$) but no statistical evidence of publication bias ($p = 0.8544$). Subgroup meta-analysis found significant differences in foot ulcer prevalence by publication year and by the duration of diabetes. An increasing trend was observed during the last two decades, with the prevalence of diabetic foot ulcers being the highest in the latest period from 2011 to 2022 (19.54%) than in the early 2000 s (4.55%). This study suggests that the prevalence of diabetic foot ulcers in Pakistan is relatively high, with significant variation between provinces. Further study is required to identify ways for early detection, prevention, and treatment in the population.

KEYWORDS

pooled prevalence, foot ulcer, diabetes, Pakistan, meta-analysis, systematic review

Introduction

A diabetic foot ulcer is a chronic consequence of diabetes characterized by lesions in the deep tissues. It causes neurological problems and peripheral vascular diseases in the lower extremities (1, 2). It poses a significant challenge for societies worldwide (3, 4). Foot ulceration and infection reduce patients' quality of life and significantly increase their risk of amputation, which is a tragic end for most people (4). It is an expensive disease to treat. Currently, 537 million adults are living with diabetes. This figure is forecast to

increase to over 783 million adults by 2045 (5). Throughout their lives, 25% of adults will develop foot ulcers (6). Diabetes-related foot and lower limb issues are severe and long-lasting. They affect 40–60 million people with diabetes around the world. Chronic foot ulcers and amputations among diabetic patients significantly reduce the quality of life and increase mortality risk (6). Diabetes foot is one of the most common, costly, and severe diabetic complications. Amputation is 10–20 times more common in people with diabetes than in non-diabetics. It is argued that a lower limb or part of a lower limb is amputated globally every 30 s due to diabetes (6). Particularly in low-income regions, diabetic foot ulcers can have a significant economic, social, and public health impact without an appropriate educational program and adequate and appropriate footwear (6). The prevalence of foot ulcers among diabetic patients is 6.3% around the world. The highest prevalence is in Belgium at 16.6%, and in Asia, it is 5.5%. The lowest prevalence of foot ulcers in Australia is 1.5% (1).

The prevalence of diabetes and associated complications in Pakistan is steadily rising (7–9). According to the International Diabetes Foundation, 33 million (26.7%) people are living with diabetes (10). Diabetic foot ulcers and infections place a significant financial and resource strain on healthcare systems by requiring hospital in-patients and outpatients to be handled by primary care and community care services. In terms of overall performance, Pakistan is ranked 154th out of 195 countries (11). Pakistan, as a developing country, struggles to sustain an effective healthcare system in the form of quality healthcare, healthcare education, and accessibility (12). With the limited number of diabetic foot ulcer management centers, Pakistan is ill-equipped to address the problem of diabetes and diabetic foot ulcer complications. According to published studies, the prevalence of diabetic foot ulcers in Pakistan ranges from 2.1 (13) to 50.9% (14). The rising prevalence of diabetic foot ulcers in Pakistan prompted this study to identify systematically, select, characterize, summarize, and estimate the pooled prevalence of diabetic foot ulcers in Pakistan till August 10, 2022.

Methods

Search strategy

The PRISMA Guidelines (15) were followed in this study. Similarly, to our previous studies (16–18), two of us (S.A. and F.H.) identified articles on the prevalence of diabetic foot ulcers in Pakistan published from inception to August 10, 2022. We thoroughly searched electronic databases such as Medline (PubMed), Web of Science, Google Scholar, and local databases. The following keywords were combined to explore the potential articles: "diabetic feet" OR "DFUs" OR "diabetic foot" OR "diabetic foot ulceration" OR "diabetic foot problem" OR "diabetic foot ulcer" AND "epidemiology" OR "prevalence" AND "Pakistan" OR "Pakistani" as well as variations thereof. We also looked through the reference lists of the selected studies for other potentially relevant studies. The PRISMA Guidelines Checklist is attached in the Supplementary File S1.

Inclusion and exclusion criteria

For this study, articles were included if they met the following criteria: (1) based on a population-based survey or hospital-based study published in English up to August 10, 2022; (2) participants must be Pakistan residents. The following articles were excluded if they were: (1) letters to the editor, reviews, case series, case studies, conference abstracts, qualitative studies, and intervention studies; (2) based on the Pakistani community living outside Pakistan; (3) did not report sufficient data; (4) were irrelevant to a diabetic foot ulcer, and (5) were based on duplicated information (data). Using a two-step procedure, the selection of articles was conducted. Two authors (S.A. and F.H.) separately examined the titles and abstracts of all identified articles. Second, the full texts of the pre-selected publications were independently evaluated based on the previously established inclusion criteria. When necessary, a third reviewer (A.A.) resolved conflicts.

Data extraction

A prepiloted data collection form was used by two independent investigators (S.A. and A.A.) to collect data on the following variables: author first, publication year, survey year, study design, the geographical location where the study was performed, the average age of diabetic patients, total sample size, the proportion of men, the number of participants with foot ulcers, sampling strategy, and setting (rural vs. urban). Discrepancies and uncertainties were explored and resolved through cross-checking of the data.

Study quality assessment

Two investigators (A.A. and F.H.) independently evaluated the risk of bias in the selected studies by adapting items from the JBI Critical Appraisal Checklist for Studies Reporting Prevalence Data (19). Disparities regarding methodological quality assessment scores were resolved by discussion and adjudication by a third author (SA). The studies were graded on a scale of 0 to 9. Using the score, we put each study into one of three categories: high risk (1–3), moderate risk (4–6), or low risk of bias (7–9).

Statistical analysis

The statistical software R (version 4.2.1) was used to conduct all analyses, and a *P* value of 0.05 was considered statistically significant. For the statistical pooling of the prevalence of foot ulcers among diabetic patients, random effects (Der Simonian-Laird) models were used (20, 21). The Cochrane Q-statistic was utilized to test for statistical heterogeneity, and I^2 was used to quantify it. Pooled results were presented with 95% confidence intervals (CIs) and a forest plot. Heterogeneity was defined as I^2 >50% (22, 23). Publication bias was initially analyzed visually using a funnel plot and later statistically with the Egger regression and Beggs tests (24, 25). Subgroup analysis was conducted to find potential sources of heterogeneity in the case of large heterogeneity.

Subgroup meta-analyses were performed according to different extracted variables (participant age, gender, geographical region, and time period). To further explore heterogeneity, meta-regression analyses were performed to determine the association between the prevalence of foot ulcers and study characteristics. The covariates in the meta-regression considered were: year of publication, setting (urban vs rural), sample size, year of investigation, mean age of diabetic patients, methodological quality, and gender (male vs. female). To examine the impact of individual studies on the pooled prevalence estimates, sensitivity analyses were carried out by

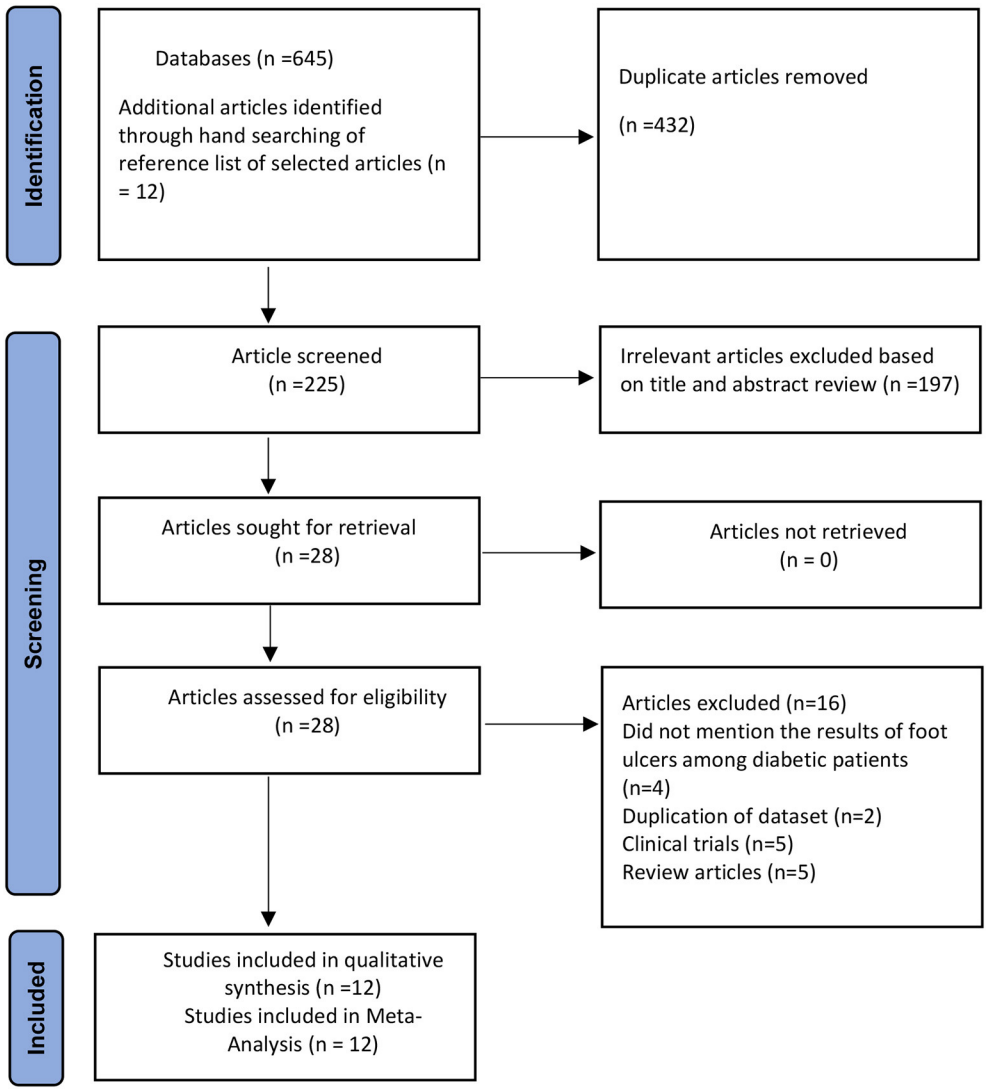

FIGURE 1
PRISMA flowchart of the prevalence of foot ulcer in diabetic patients in Pakistan (15).

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TABLE 1 General characteristics of studies selected in the systematic review ($n = 12$).

Rerferences	Sample size	Cases	Prevalence (%)	Study design	Setting	Province	Sex	Working year	% Male	Average age	Sampling	Risk of bias
Ahmed et al. (27)	500	20	4	Case-control	Urban	Sindh	Both	2004	32	55.2	Random Sampling	Low
Hashim et al. (13)	805	17	2.1	Cross-sectional	Urban	Punjab	Both	1999	47.2	49.26	NA	moderate
Khawaja et al. (28)	672	26	3.9	Cross-sectional	Urban	Sindh	Both	2000–2001	48.5	52.24	NA	moderate
Basit et al. (29)	2,199	229	10.4	Cross-sectional	Urban	Sindh	Both	1996–2001	48.5	51	NA	Low
Hussain et al. (30)	1,782	67	3.75	Case-control	Urban	Punjab	Both	2006–2008	73.13	55.7	NA	Low
Masood et al. (31)	318	22	6.9	Cross-sectional	Urban	Azad Kashmir	Both	2012	26.7	51.83	NA	Low
Khan et al. (32)	230	32	13.9	Cross-sectional	Urban	Pakistan	Both	2010–2011	40.86	53.82	Convenient	Low
Younis et al. (33)	1,940	136	7.02	Cross-sectional	Urban	Punjab	Both	2016–2017	37	51.24	NA	moderate
Khan et al. (34)	2,052	356	17.3	Cross-sectional	Urban	Punjab	Both	2017–2018	39.3	55	NA	moderate
Ejaz et al. (14)	1000	509	50.9	Cross-sectional	Urban	Punjab	Both	2017	66.6	48.26	Convenient	moderate
Akhtar et al. (35)	1,503	253	16.83	Cross-sectional	Both	Punjab	Both	2018–2019	33.53	51.58	Cluster Random	Low
Naseer et al. (36)	1,200	456	38	Cross-sectional	Both	Punjab	Both	NA	62.6	NA	NA	Low

excluding each study. The agreement between the investigators was evaluated by the Kappa statistic (26).

Result

Figure 1 displays the PRISMA selection and exclusion flowchart. A total of 657 studies were identified, including 645 via database searches and 12 from additional sources. After deduplication ($n = 432$), 197 studies were found ineligible after their titles and abstracts were thoroughly screened. The remaining 28 studies were subjected to a full-text evaluation to determine their eligibility; they were eliminated because they did not match the inclusion criteria. In the end, 12 papers were included in the analysis. The authors' inter-rater agreement for study inclusion was very good (Kappa = 0.83, $p = 0.001$).

Table 1 summarizes the key characteristics of the 12 studies included in this analysis. These articles included only Pakistani nationals, with sample sizes ranging from 230 (32) to 2199 (29), with a median of 1503 diabetic patients. Seven studies were conducted in Punjab province (13, 14, 30, 33–36), three studies were performed in Sindh (27–29), one was conducted in Azad Kashmir (31), and one study was conducted nationwide (32). Regarding the study design, a cross-sectional research design was utilized in 10 of the 12 studies; one study employed case-control, and the other used a prospective research design. Two studies were performed using convenient sampling procedures; one used simple random sampling techniques, one used cluster random cluster sampling; and the remaining four did not explicitly mention their sampling procedure. The reported foot ulcer prevalence rates in diabetic patients varied widely across provinces (Table 1). Ten studies were conducted on urban populations while two studies were conducted in both settings (urban and rural). The average participant age in the 11 studies providing this information was 52.29 years. The gender of the diabetic patients was provided in all papers. Regarding methodological quality bias, seven studies (27, 29–32, 35, 36) had a low risk of bias, five studies (13, 14, 28, 33, 34) had a moderate level, and none had a high risk of bias. The authors' agreement on the retrieved data was strong (Kappa score = 0.82, $p = 0.001$).

Quantitative synthesis

Pooled prevalence of diabetic foot ulcers

The pooled prevalence and subgroup meta-analysis for diabetic foot ulcers are summarized in Table 2. The prevalence of foot ulcers among diabetic patients was reported in 12 research articles (13, 14, 27–36) with a total of 14201 diabetic patients. The diabetic foot ulcer prevalence estimates in the included studies ranged from 2.11% (95% CI: 1.23–3.36%) to 50.90% (95% CI: 47.75–54.04%). The pooled prevalence of foot ulcers among diabetic patients was 12.16% (95% CI: 5.91–20.23%).

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TABLE 2 Summary estimates from meta-analyses of diabetic foot ulcers in Pakistan.

Variable	No. of articles	No. of participants	No. of cases	Prevalence, (95% CI)	I^2, %	95%, Prediction interval	*P*-Value			
							Q test	Egger test	Begg test	Subgroup difference
Foot Ulcer in Diabetic patients	12	1,4201	2,123	12.16 (5.91–20.23)	99.4	0.00–53.07	<0.001	0.911	0.6808	
By Sex								0.664	0.1857	0.3274
Male	6	3,755	459	12.04 (6.56–18.88)	96.2	0.00–41.71	<0.001			
Female	5	4,680	484	7.29 (1.92–15.69)	98.7	0.00–51.67	<0.001			
Time period								0.854	0.9379	0.0023
1999–2010	5	5,958	359	4.55 (2.35–7.42)	96.6	0.00–18.97	<0.001			
2011–2022	7	7,043	1,308	19.54 (9.54–32.03)	99.3	0.00–69.76	<0.001			
Ulcer duration										0.0491
≤10 years	3	4,440	291	6.16 (4.11–8.58)	87.6	0.00–54.37	<0.001			
>10 years	2	1,202	307	26.60 (6.36–54.30)	99		<0.001			
By location								0.205	0.7884	0.2335
Punjab	7	10,282	1,338	16.13 (5.57–30.79)	93.6	0.00–76.19	<0.001			
Sindh	3	3,371	275	5.86 (2.51–10.48)	96	0.00–93.90	<0.001			
Azad Kashmir	1	318	22	6.92 (4.36–9.99)						

The 95% prediction intervals were 0.0–52.07% (Figure 2). The I^2 value (99.4%, $P < 0.0001$) indicated high between-study heterogeneity across the findings of different studies. The funnel plot (Figure 3), Begg's rank test ($z = 0.41$; $p = 0.6808$) and Egger's test ($t = -0.11$; $p = 0.9110$) suggested no publication bias in the meta-analysis. The sensitivity analysis showed that the pooled prevalence of diabetic foot ulcers varied from 9.67% (95% CI: 5.23–15.28%) to 13.44% (95% CI: 6.68–22.06%) by excluding each study individually. The analysis found that no single study substantially affected the pooled prevalence of foot ulcers in diabetic patients.

To analyze the substantial sources of statistical heterogeneity, subgroup meta-analyses were conducted using age group, gender, geographical location, and time period. The subgroup meta-analysis based on geographical location showed that the prevalence of foot ulcers in diabetic patients was highest in studies conducted in Punjab province [16.13% (95% CI: 5.57–30.79%); $n = 7$], followed by Azad Kashmir and 6.92% (95% CI: 4.36–9.99; $n = 1$), and was lowest in Sindh (5.86% (95% CI: 2.51–10.48%; $n = 3$). When stratified by publication year, the pooled prevalence for diabetic foot ulcers estimates were 4.55% (95% CI: 2.37–7.42%; $n = 5$) from 1999 to 2010 and 19.54% (95% CI: 79.54–32.03%; $n = 7$) during 2011–2022. The highest prevalence of diabetic foot ulcers has been detected in recent years. When stratified by gender, the pooled prevalence of foot ulcers in male diabetic patients (12.04%; 95% CI: 3.48–18.88%; $n = 6$) was higher than in female diabetic patients (7.29%; 95% CI: 1.92–15.69%; $n = 5$).

The meta-regression analysis (Table 3) revealed that the prevalence of foot ulcers among diabetic patients significantly increased with the publication year ($\beta = 0.0179$; 95% CI: 0.0075–0.0282; $p = 0.0007$; $R^2 = 49.07\%$), as well as the year of investigation ($\beta = 0.0144$; 95% CI: 0.0021–0.0267; $p = 0.0222$; $R^2 = 29.93$). The findings also showed that neither the percentage of men in the sample, the sample size, nor the methodological quality of the studies was significantly associated with the prevalence of foot ulcers in diabetic patients.

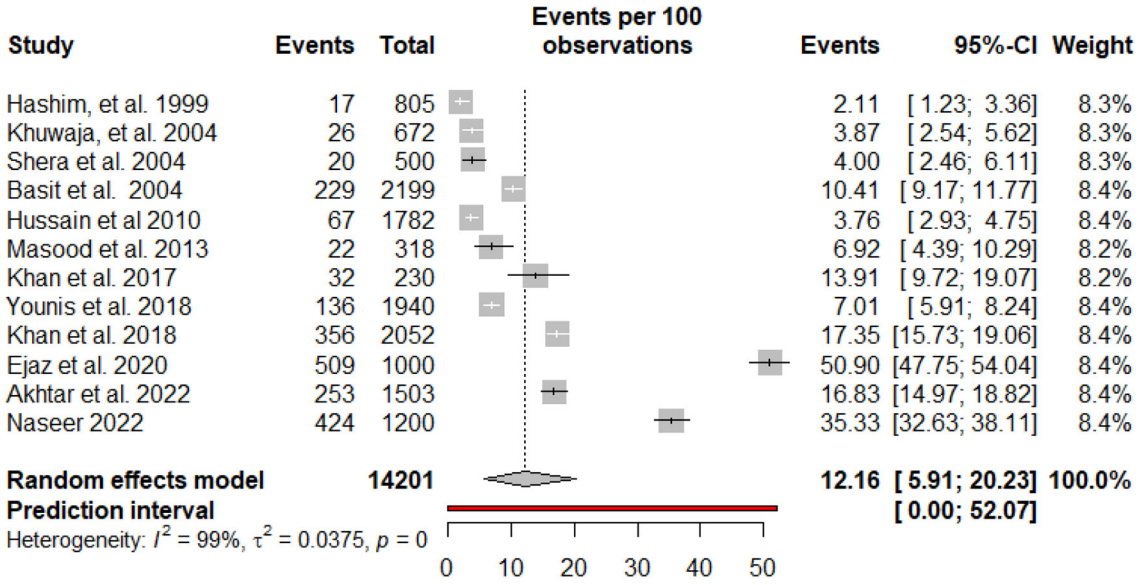

FIGURE 2
Forest plot of the prevalence of foot ulcers among diabetic patients in Pakistan.

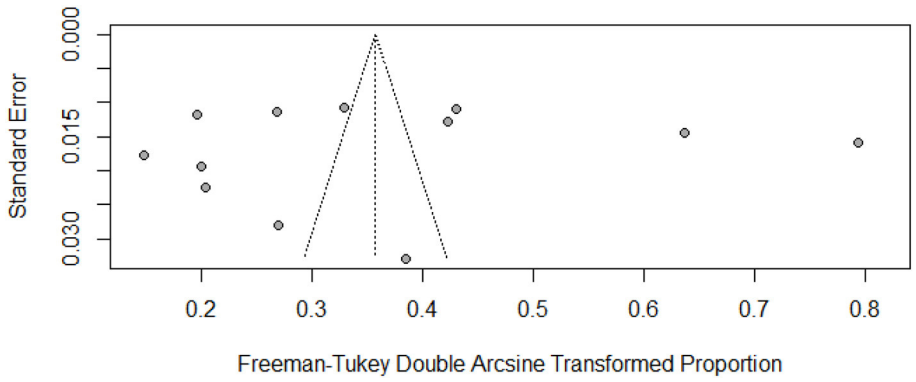

FIGURE 3
Funnel plot of the prevalence of foot ulcers among diabetic patients in Pakistan.

Discussion

Over the last few decades, diabetes and its associated consequences have become more widespread. Diabetes-related hospitalizations are disproportionately impacted by foot ulcers, which account for half of the hospitalizations (37). The development of a diabetic foot ulcer is a significant predictive indication of mortality risk. Over half of patients who acquire a foot ulcer will die within 5 years, primarily from cardiovascular disease and diabetes complications (38). We did the first systematic review and meta-analysis to determine the pooled prevalence estimate of diabetic foot ulcers in Pakistan from January 1999 to August 2022. This study combined information from 12 distinct data sets involving 14201 diabetic patients from varied geographical regions of Pakistan. This study's findings will contribute to developing public health policies to reduce the prevalence of diabetic foot ulcers in Pakistan. The pooled rate of diabetic foot ulcers was 12.16% (95% CI: 5.91–20.23%). Wide variability is observed in the prevalence estimate across the studies, ranging from 2.1 to 50.9%. Significant heterogeneity is observed, which may be the reason for differences in sample size, year of study, and prevalence of diabetic neuropathy and peripheral artery disease.

Meta-analysis estimates were higher than those from Iran (39) and Saudi Arabia (3), where the prevalence rate of foot ulcers was 6.4 and 3.3%, respectively. This disparity could be attributed to a variation in research methodology. On the other hand, the prevalence of diabetic foot ulcers is lower than in the

TABLE 3 Univariable meta-regression analyses.

Variable	Beta (β)	*p*-value	95% CI	R^2%
Publication Year	0.0179	0.0007	0.0075–0.0282	49.07
Year of investigation	0.0144	0.0222	0.0021–0.0267	29.93
Methodology	0.0155	0.8986	−0.2226– 0.2536	0.00
Male ratio	0.0052	0.1884	−0.0026–0.0130	6.17
Sample size	0.00	0.9152	−0.0003–0.0001	0.00

research conducted in Ethiopia at 13% (40), Sudan at 18.1% (41), and Spain at 17% (37). This disparity could be attributed to a variation in research methodology.

According to our data, male diabetic patients (12.04%) had more significant diabetic foot ulcers than female diabetic patients (7.29%). Males' harder physical labor could be one explanation for this gender discrepancy (42). The findings are congruent with those of a similar global survey (1). Our findings revealed that Punjab had the highest prevalence of diabetic foot ulceration (16.13%), while Sindh had the lowest (5.86%). All studies conducted in Sindh were published before 2004, which might be the reason for the lower prevalence in Sindh than Punjab. The results also revealed that the duration of a patient's diabetic disease is one of the risk factors for the development of foot ulcers. The probability of developing a foot ulcer increases as a patient's duration with diabetes increases. This is due to the medical condition's proclivity to worsen over time if not appropriately managed. This finding is similar to previous research, which indicated that diabetic foot ulcers worsened when individuals lived with diabetes for longer periods of time (39, 40).

The study has several benefits and drawbacks. We deployed exhaustive search procedures, rigorous selection criteria, and a dual review procedure. We could generate reliable prevalence estimates since the included studies provided sufficient data. Our analysis identified no evidence of publication bias, indicating that we did not overlook any papers that could have altered the results of our meta-analysis. Furthermore, due to their superior methodological quality, all included studies exhibited a low or moderate risk of bias. According to the meta-regression analysis, the methodological quality of the studies did not affect the assessment of the overall prevalence.

There are some limitations to this study. The meta-analysis revealed significant variation in the estimated pooled prevalence, as expected. To address the issue of substantial heterogeneity, subgroup analysis and meta-regression with components added to the univariate model were used. The outcomes of this study should be regarded with caution due to the significant degree of heterogeneity. Second, we could not discover any research article published on Khyber Pakhtunkhwa or Baluchistan. As a result, the findings should be regarded with caution. Thirdly, the aim of the study was to estimate the foot ulcers prevalence in diabetic patients which is the reason it excluded the studies which did not provide prevalence estimates. Fourthly, in the subgroup meta-analyses and meta-regression models, the choice of important covariates (HbA1c, peripheral artery diseases, smoking, and diabetic neuropathy) was limited, on the basis of the restricted availability of primary data in the eligible studies. Finally, because the number of papers included in this review is limited, a univariate meta-regression analysis rather than a multivariable meta-regression model is employed to assess the importance of each covariate.

Conclusions

This study concludes with pooled estimates of foot ulcers among diabetic patients in Pakistan, indicating that diabetic foot is a substantial public health issue in Pakistan. The frequency of foot ulcers in the general population has increased over the past three decades, and this trend may continue in the future. Foot ulcer among diabetic patients is on the rise in Pakistan. Therefore, diabetic foot clinical centers are required for foot ulcer screening, identification, and management in urban as well as rural areas.

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

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

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.1017201/full#supplementary-material

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Global miRNA expression reveals novel nuclear and mitochondrial interactions in Type 1 diabetes mellitus

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Background: Considering the potential role of miRNAs as biomarkers and their interaction with both nuclear and mitochondrial genes, we investigated the miRNA expression profile in type 1 diabetes (T1DM) patients, including the pathways in which they are involved considering both nuclear and mitochondrial functions.

Methods: We analyzed samples of T1DM patients and control individuals (normal glucose tolerance) by high throughput miRNA sequencing (miRNome). Next, five miRNAs – *hsa-miR-26b-5p*, *hsa-let-7i-5p*, *hsa-miR-143-3p*, *hsa-miR-501-3p* and *hsa-miR-100-5p* – were validated by RT-qPCR. The identification of target genes was extracted from miRTarBase and mitoXplorer database. We also performed receiver operating characteristic (ROC) curves and miRNAs that had an AUC > 0.85 were considered potential biomarkers.

Results: Overall, 41 miRNAs were differentially expressed in T1DM patients compared to control. *Hsa-miR-21-5p* had the highest number of predicted target genes and was

associated with several pathways, including insulin signaling and apoptosis. 34.1% (14/41) of the differentially expressed miRNAs also targeted mitochondrial genes, and 80.5% (33/41) of them targeted nuclear genes involved in the mitochondrial metabolism. All five validated miRNAs were upregulated in T1DM. Among them, *hsa-miR-26b-5p* showed AUC>0.85, being suggested as potential biomarker to T1DM.

Conclusion: Our results demonstrated 41 DE miRNAs that had a great accuracy in discriminating T1DM and control group. Furthermore, we demonstrate the influence of these miRNAs on numerous metabolic pathways, including mitochondrial metabolism. *Hsa-miR-26b-5p* and *hsa-miR-21-5p* were highlighted in our results, possibly acting on nuclear and mitochondrial dysfunction and, subsequently, T1DM dysregulation.

KEYWORDS

type 1 diabetes, miRNAs, miRnome, nuclear target, mitochondrial target

1 Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease associated with failure in insulin production that occurs as a consequence of the pancreatic islet β-cells dysregulation mediated by T-cells (1). This type of Diabetes Mellitus (DM) can affect any age group, but the onset is more frequent in children and adolescents (2). Globally, 1.1 million individuals under the age of 20 years are affected by T1DM, with an annual increase of about 3% (3).

T1DM is an immune-based disease driven by the interaction between environmental, genetic, and epigenetic factors (4, 5). The presence of autoantibodies is the first sign of autoimmunity against β-cells (6). Currently, the standard method to identify individuals at risk for T1DM is to analyze the presence of autoantibodies against islet antigens, among them, against islet cells (ICA), glutamate decarboxylase (GADA), insulin (IAA), tyrosine phosphatases (IA-2 and IA-2β), and zinc transporter 8 (ZnT8) (7). Antibodies are the most common biomarkers of T1DM, but only a portion of the autoantibody-positive individuals develop the disease. Thus, new biomarkers are required to help the identification of T1DM patients (8).

Several microRNAs (miRNAs) – short non-coding RNAs (~22 nucleotides) that play important roles in the gene expression regulation (9) – have been reported in association with T1DM, affecting β-cell metabolism (10, 11), insulin secretion (12, 13), T-cell function (14, 15), biosynthesis and performance of autoantigens (16). Furthermore, it has been demonstrated that the miRNAs act on T1DM not only *via* nuclear, but also *via* mitochondrial pathways. The miRNA-181a, for example, is overexpressed in T1DM patients compared to control and it is able to inhibit hydrogen peroxide-induced cellular apoptosis, lead to disruption of mitochondrial structure, increase ROS (reactive oxygen species) production, and downregulate the expression of mitochondrial anti-apoptotic proteins (10, 17).

In this context, we explored miRNA expression profiles in T1DM patients through miRNome sequencing and investigated the pathways these miRNAs are involved considering both nuclear and mitochondrial functions.

Abbreviations: T1DM, Type 1 diabetes mellitus; DM, Diabetes Mellitus; ICA, against islet cells; GADA, glutamate decarboxylase; IAA, insulin; IA-2, tyrosine phosphatase; IA-2β, tyrosine phosphatase; ZnT8, zinc transporter 8; miRNAs, microRNAs; OXPHOS, oxidative phosphorylation; ADA, American Diabetes Association; NGS, Next-Generation Sequencing; DE, Differential Expression; Hg19, Human Genome Reference; RPKM, Reads per Kilobase per Million; RT-qPCR, Real-Time Quantitative Reverse Transcription-PCR; ROC, Receiver Operating Characteristics; AUC, Area Under the Curve; PBMCs, Peripheral Blood Mononuclear Cells; DDR2, Discoiding Domain Receptor 2; ETC, electron transport chain; ROS, reactive oxygen species; GH, growth hormone.

2 Material and methods

2.1 Ethics statement

This study was approved by the Institutional Review Board from João de Barros Barreto University Hospital (HUJBB, Belém, Pará, Brazil) (Protocol Number 005/12). All procedures performed involving human participants were conducted according to the ethical guidelines of the Declaration of Helsinki. Written informed consent was obtained from all study participants.

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2.2 Sample collection

Sixty patients with T1DM – diagnosed according to the American Diabetes Association (ADA) criteria (18) – and twenty-eight subjects with normal glucose tolerance (control individuals) were enrolled in the current study by the Endocrinology and Metabology/Diabetes Unit at HUJBB. T1DM group had mean age 26.93 ± 9.62 years, with individuals equally distributed between females and males. The mean age of the control group was 28.83 ± 6.85 years old, with predominance of females (75%). Peripheral blood samples were collected into a Tempus Blood RNA tube (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20°C until RNA extraction. A summary of our experimental workflow is presented in Figure 1.

2.3 Total RNA isolation and quantification

Total RNA was extracted using MagMAX™ RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's specification and quantified with NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA integrity was determined using Agilent RNA ScreenTape assay and 2200 TapeStation Instrument (Agilent Technologies, Santa Clara, CA, USA).

2.4 Library preparation and next-generation sequencing

A high throughput small RNA-sequencing experiment was conducted in 12 patients with T1DM and 4 control individuals. For library preparation, 1 µg of total RNA per sample was used with TruSeq Small RNA Library Preparation (Illumina, San Diego, CA, USA). The library was validated and quantified by DNA ScreenTape assay in a 2200 TapeStation Instrument (Agilent Technologies, USA) and by real-time PCR with a KAPA Library Quantification Kit (Roche, Basel, Switzerland). A total library pool of 4 nM was sequenced using a MiSeq Reagent Kit v3 (150 cycles) at the MiSeq System (Illumina).

2.5 Sequencing data processing and differential expression analysis

A pipeline of quality control to remove adapters and filter low quality reads was applied using Trimmomatic software (19). Resulting sequences were aligned with the human genome reference (Hg19) using STAR software (20). Mature miRNAs sequencing was quantified using miRbase human annotation and expression count was performed with HTSeq software (21).

The DE analysis was performed using the Bioconductor-DESeq2 package (22) in R software. Comparisons between patients with T1DM and control individuals were conducted. Adjusted values of $p \leq 0.05$ and a |log2 fold change| ≥ 1.5 were

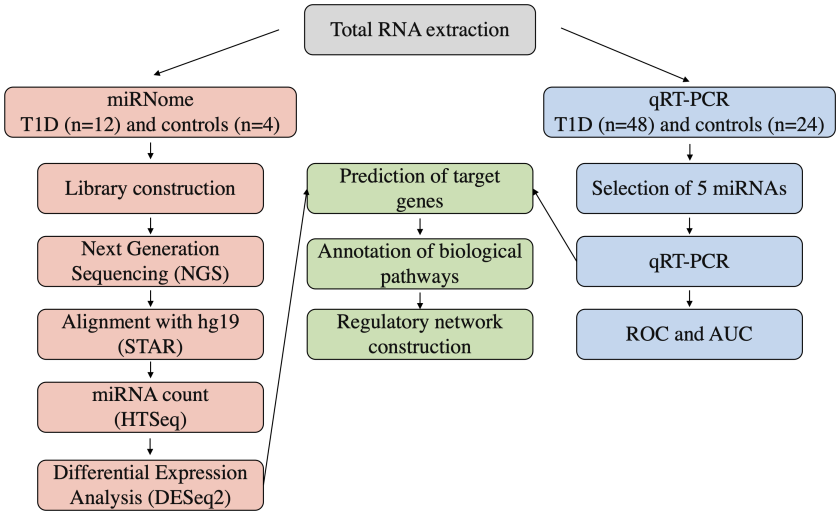

FIGURE 1
Experimental workflow of the analyses in the study.

considered statistically significant. Graphical analysis of miRNAs was normalized to RPKM (Reads per Kilobase per Million). Heatmap was used for hierarchical clustering of DE miRNAs.

2.6 Validation by quantitative real-time reverse transcription-PCR

Based on the NGS data, five miRNAs – *hsa-miR-26b-5p*, *hsa-let-7i-5p*, *hsa-miR-143-3p*, *hsa-miR-501-3p* and *hsa-miR-100-5p* – were selected for validation by Real-Time Quantitative Reverse Transcription-PCR (RT-qPCR). The experiment was conducted in 48 patients with T1DM and 24 control individuals. Total RNA was used in a reverse transcription reaction using miRNA 1st Strand cDNA Synthesis Kit (Agilent Technologies). The reverse transcription product was subject to amplification using PowerUp SYBR Green Master Mix in ABI 7500 Real Time PCR System (Thermo Fisher Scientific). The specific primers for mature miRNAs are listed in Supplement Table S1. All reactions were performed in triplicate, and the comparative Ct method was used to analyze the differences in the expression of each group. The expression levels of miRNAs were normalized by using the endogenous control small nucleolar RNA U6.

2.7 ROC and AUC analyses

To estimate the biomarker sensitivity for distinguishing groups, Receiver Operating Characteristics (ROC) and Area Under the Curve (AUC) analyses were used. These measures are effective in discriminating the true state of individuals, being a standard analysis for searching for biomarkers. In this study, the miRNAs that demonstrated AUC>0.85 were considered potentially useful for the diagnosis of T1DM.

2.8 Identification of target genes and functional enrichment analysis

Target genes of DE miRNAs were extracted from miRTarBase database (access in July 2021) (23) considering only those that were validated by strong evidence (report assay, western blot, and qPCR). Enrichment analysis of the target genes were conducted in both KEGG and Reactome pathways using the ReactomePA (24) and ClusterProfiler package (25) in R. Enriched terms with an FDR adjusted p-value < 0.05 were considered statistically significant. Interaction network of miRNA-target gene and target gene-biological pathways were constructed using Cytoscape (26). For the mitochondrial approach, we based our analyses on the human mitochondrial interactome (mitochondrial functions and their associated genes) present in the mitoXplorer platform (27).

2.9 Statistical analyses

Statistical analyses and graphing were performed using R software (28). Shapiro-Wilk test was used to evaluate the data distribution. To evaluate the statistical significance between the analyzed groups, we used Mann-Whitney U test. P value < 0.05 was considered statistically significant.

3 Results

3.1 Identification of differentially expressed miRNAs in type 1 diabetes

We identified 41 differentially expressed (DE) miRNAs in patients with T1DM in comparison to control individuals, including 36 downregulated and 5 upregulated miRNAs (Figure 2). Hierarchical clustering of normalized expression of these miRNAs provided a heatmap graph that clearly separated T1DM and control group (Figure 3).

3.2 Target genes identification

We identified 1,237 interactions with 777 target genes (Supplement Table S2). Only *hsa-miR-501-3p* had no predicted target genes with strong evidence. The *hsa-miR-21-5p* was by far the miRNA with the highest number of targets (Figure 4A). On the other hand, *PTEN* and *VEGFA* were the genes with the greater number of interactions with different miRNAs, followed closely by *BCL2*, *HMGA2*, *IGF1R* and *MYC* (Figure 4B).

3.3 Validation of miRNAs expression by RT-qPCR

The five most DE miRNAs, considering the lowest p-value and highest $\log_2$ fold change, were selected to be validated by RT-qPCR: *hsa-miR-100-5p*, *hsa-miR-501-3p*, *hsa-miR-143-3p*, *hsa-let-7i-5p* and *hsa-miR-26b-5p*. All of them were upregulated in T1DM in comparison to control (Figure 5). Only *hsa-miR-26b-5p* showed AUC>0.85, being highlighted as a potential biomarker to T1DM (Figure 6).

Interestingly, hsa-miR-100-5p, hsa-miR-143-3p, hsa-let-7i-5p, and hsa-miR-26b-5p regulate the genes IGF1, TLR4, CTGF, JAG1, PTGS2, NR2C2, IGF1R, MMP13 and AKT1. Only the IGF1R gene (Insulin Like Growth Factor 1 Receptor) was regulated by three of these miRNAs (hsa-miR-100-5p, hsa-miR-143-3p and hsa-miR-26b-5p), making it a central gene in T1DM regulatory network. Curiously, hsa-miR-26b-5p regulate both IGF1R and IGF1, which are genes related to insulin signaling and apoptotic events (Figure 7). The hsa-miR-501-3p did not have target genes of strong evidence, so it was removed from the analyses.

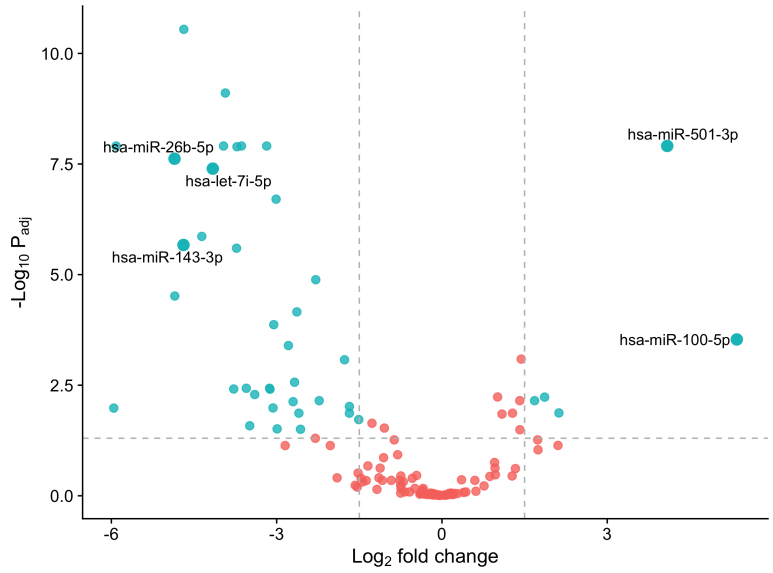

FIGURE 2
DE miRNAs in the T1DM patients in comparison to controls. Blue dots are considered DE miRNAs under the conditions of adjusted values of $p < 0.05$ and |log2 fold change| ≥ 1.5. Red dots are non-DE miRNAs. Note that miRNAs on right of figure are up-regulated, and on the left are down-regulated.

3.4 Functional enrichment analysis

To investigate the biological pathways that these 41 DE miRNAs play in the development of T1DM, we separated analyzes in miRNAs targeting nuclear genes and miRNAs targeting mitochondrial genes. These results are showed in the next sections.

3.4.1 Nuclear

To improve the interpretation of the results, we divided the functional enrichment of miRNAs for nuclear genes into genes regulated by downregulated miRNAs and those regulated by upregulated miRNAs.

The functional analysis of the upregulated miRNAs revealed that its target genes participate in 61 KEGG pathways (Supplement Table S3). Among these, we highlight 22 that are important for the development of T1DM (Supplement Figure S1), including apoptosis pathway and protein complex signaling (TGF-β, EGFR, Pi3K-Akt, HIF-1, TNF, mTOR, hippo, Notch etc.)

The investigation of the downregulated miRNAs presented interaction with 751 nuclear genes that are involved in 150 KEGG pathways (Supplement Table S4), of which at least 40 pathways are related to T1DM (Supplement Figure S2). In addition to pathways already associated with upregulated miRNAs, we found pathways associated with the immunological response and insulin signaling.

3.4.2 Mitochondrial

To better explore mitochondrial association with T1DM, we divided the miRNAs in two groups: miRNA targeting mitochondrially-encoded genes and miRNAs targeting nuclear genes involved in mitochondrial metabolism (NucGenMito).

In total, 14 miRNAs targeting mitochondrial genes were recognized. Curiously, eight of them were found interacting with *MT-COX2* (also known as *MT-CO2* and MT-*COII*; mitochondrially-encoded cytochrome c oxidase II) considering both strong and weak evidence interactions. Among these miRNAs are the three that were validated by RT-qPCR, although only the interactions with *hsa-miR-21-5p* and *hsa-miR-26b-5p* are of strong evidence in the global literature (in red) (Figure 8A). All mitochondrial genes targeted by miRNAs are expressed in the pancreas and whole blood from GTEx data (Figure 8B), reinforcing the potential role of these genes in T1DM. KEGG pathways and Reactome pathways are shown in Figures 8C,D, respectively, highlighting important mitochondrial mechanisms and some conditions that have been related to both nucleus and mitochondria.

Considering mitoXplorer database, 33 DE miRNAs targeting NucGenMito were reported, including those validated by RT-qPCR (Supplement Figure S3A). Most of these miRNAs are involved in Transcription (nuclear), Apoptosis and Mitochondrial Signaling (Supplement Figure S3B). *Hsa-miR-21-5p*, *hsa-miR-221-3p* and *hsa-miR-181a-5p* had the greater number of targets, over 10 target genes (Supplement Figure S3C). As 80.5% (33/41) of miRNAs targeting NucGenMito also targeted mitochondria-independent nuclear genes, functional analysis enriched for the same previously mentioned biological pathways (Supplement Figures S4, S1-S2).

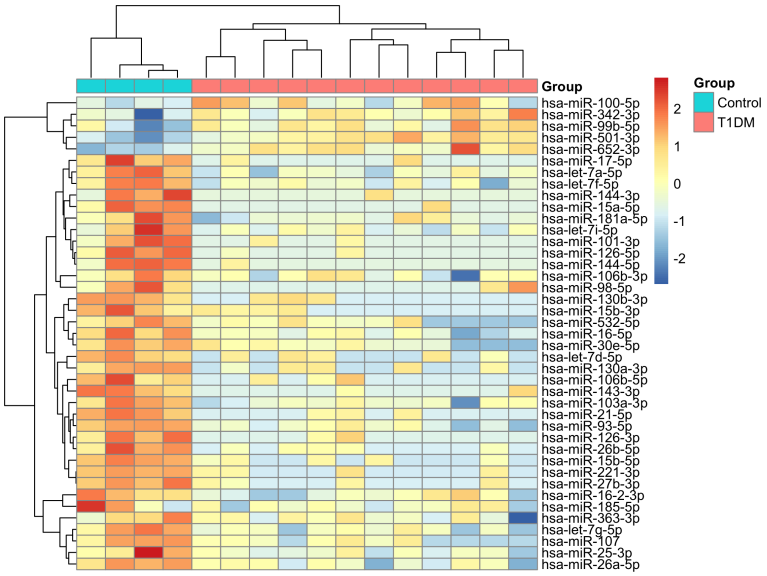

FIGURE 3
Heatmap with hierarchical clustering analysis of DE miRNAs in T1DM. Blue color in top bar represents control individuals and red colors represents T1DM patients. In the heatmap, dark-red color corresponds to high miRNA expression, and dark blue corresponds to low expression.

4 Discussion

Circulating miRNAs are strong candidates to be biomarkers for complex diseases, including diabetes mellitus. In a way, because they are stable, resistant to ribonuclease, can be easily collected, and their level can be measured using assays that are rapid, specific, and sensitive (7). Therefore, we investigated the profile of miRNAs expressed in the blood from a cohort of T1DM patients, looking for potential new biomarkers for this disease.

Here, 41 miRNAs were found to be dysregulated in T1DM patients in comparison to controls, suggesting a potential role in T1DM development. Among these, 10 miRNAs (*hsa-miR-99b-5p*, *hsa-miR-501-3p*, *hsa-let-7f-5p*, *hsa-miR-143-3p*, *hsa-miR-144-3p*, *hsa-miR-181a-5p*, *hsa-miR-126-5p*, *hsa-miR-144-5p*, *hsa-miR-16-5p*, and *hsa-miR-25-3p*) are the same found in the previously whole-blood miRNA sequencing in diabetes performed by Massaro et al. (2019) (29), reinforcing the involvement of these miRNAs with the diabetes process. In the last-mentioned study, these miRNAs were also related to diabetes complications (*i.e.*, neuropathy, retinopathy, and nephropathy) and were able to differentiate T1DM patients from controls. Nonetheless, there is still limited literature on these miRNAs and T1DM currently.

In our study, it should be noted that *hsa-miR-21-5p* had the highest number of predicted target genes. This miRNA – together with others such as *hsa-miR-181a-5p* – has been reported in plasma/serum and Peripheral Blood Mononuclear Cells (PBMCs) acting as potential circulating biomarker in T1DM (6). In a recent study with breast cancer, *hsa-miR-21-5p* was reported to be sublocated in mitochondria and able to interact with mitochondria-related differentially expressed genes in multiple mechanisms (30), including the collagen metabolism by Discoiding Domain Receptor 2 (DDR2), which, in turn, has been related to diabetic osteopenia (31). Here, *hsa-miR-21-5p* was associated, among so many other pathways, with insulin resistance, apoptosis, and diabetic cardiomyopathy (Supplement Tables S2, S4). Curiously, these three pathways not only have been notably present in our findings but have also been associated to mitochondrial functions in T1DM in previous studies (32–34).

Moreover, we highlight the association of multiple miRNAs to *MT-COX2* in our study, particularly *hsa-miR-21-5p* and *hsa-miR-26b-5p* that were predicted with strong evidence. The *MT-COX2* gene encodes a subunit of the Complex IV (also known as cytochrome c oxidase), one of the five protein complexes in the electron transport chain (ETC) repeatedly located in the mitochondrial cristae and responsible for the energy generation during OXPHOS (35). Importantly, mitochondrial dysfunction leading to imbalanced OXPHOS activity has been reported in T1DM, including the decreased activity of ETC complexes in T1DM heart (36), although the specific mechanisms affected in these processes have not yet been clarified. In addition, it should be noted that oxidative stress by the accumulation of mitochondrial ROS – mainly due to hyperglycemia-induced mitochondrial dysfunction and altered dynamics and biogenesis – has been described as a key factor to T2DM and some of the diabetic complications, including insulin resistance (37, 38).

Curiously, insulin resistance has been related to serum levels of the growth factor IGF1 and its receptor IGF1R – components of the growth hormone (GH) and energy

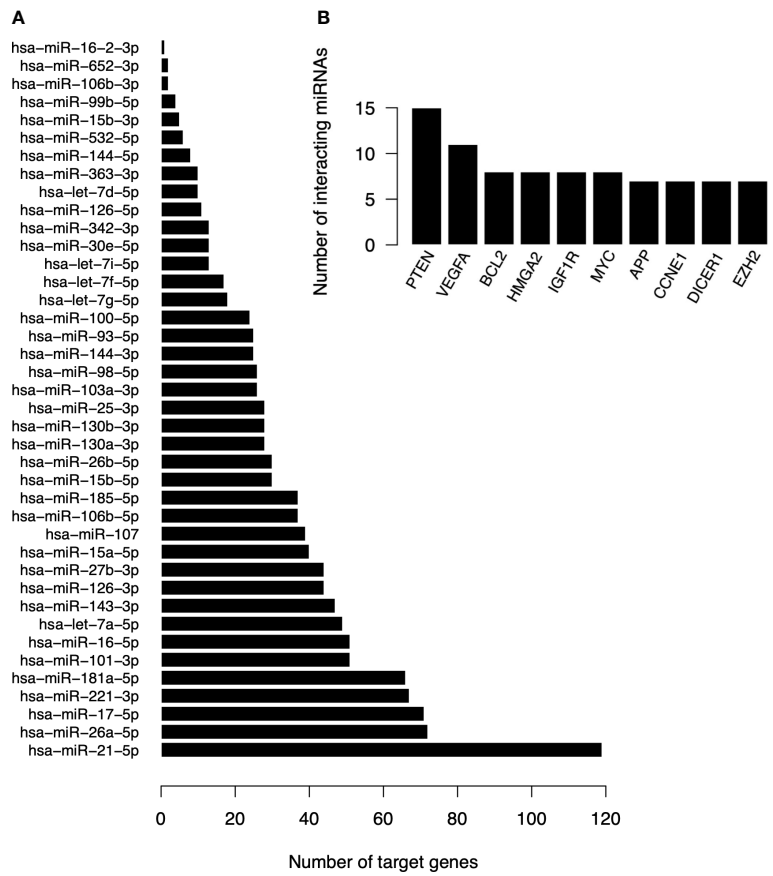

FIGURE 4
Quantitative of target genes of DE miRNAs in T1DM. **(A)** Number of target genes per DE miRNA. **(B)** Number of interacting miRNAs for the ten most frequently found genes.

metabolisms (39, 40). In fact, the dysregulation of IGF1 and IGF1R levels has been described in association to hyperglycemia in diabetes, including T1DM, and several diabetic complications (40–42). In our study, *hsa-miR-26b-5p, hsa-let-7i-5p, hsa-miR-100-5p* and *hsa-miR-143-3p* were found to be interacting with *IGF1R* and/or *IGF1* gene, suggesting that these miRNAs might play a role in this insulin resistance metabolism, in addition to diabetic complications. Surprisingly, all three miRNAs (*hsa-miR-26b-5p, hsa-miR-100-5p* and *hsa-miR-143-3p*) were shown here to interact with *MT-COX2* gene, which is particularly relevant considering that IGFR1 and the GH/IGF1 axis have been related to mitochondrial function and dynamics (43, 44).

In addition, *hsa-miR-26b-5p* was recently described to form a signaling pathway with *Mfn1* (mitofusin 1), an essential gene for mitochondrial fusion; this *hsa-miR-26b-5p/Mfn1* axis seems to affect mitochondrial dynamics and apoptosis in the context of myocardial infarction and cardiac microvascular dysfunction (45). Therefore, *hsa-miR-26b-5p* could be especially important to diabetic cardiomyopathy and, possibly, other diabetic complications. To the best of our knowledge, this is the first study to highlight a potential key role of *hsa-miR-26b-5p* in T1DM development and progression.

Here, we describe the main regulatory dysfunctions in the miRNA pathways associated with T1DM, including their role at the nuclear and mitochondrial levels. To strengthen our results, we recommend future investigations on these miRNAs in cellular and animal models to validate the regulatory network in which they are involved. Of note, a major limitation of this study was the sample size, so we also recommend the validation of miRNAs in a larger cohort to guarantee the veracity of the results, in addition to patients with different diabetic complications. Despite these limitations, our findings contribute to the knowledge of complex regulation of T1DM and identification of miRNAs as potential biomarkers.

Conclusion

In summary, we found differentially expressed miRNAs between T1DM patients and control individuals that clearly

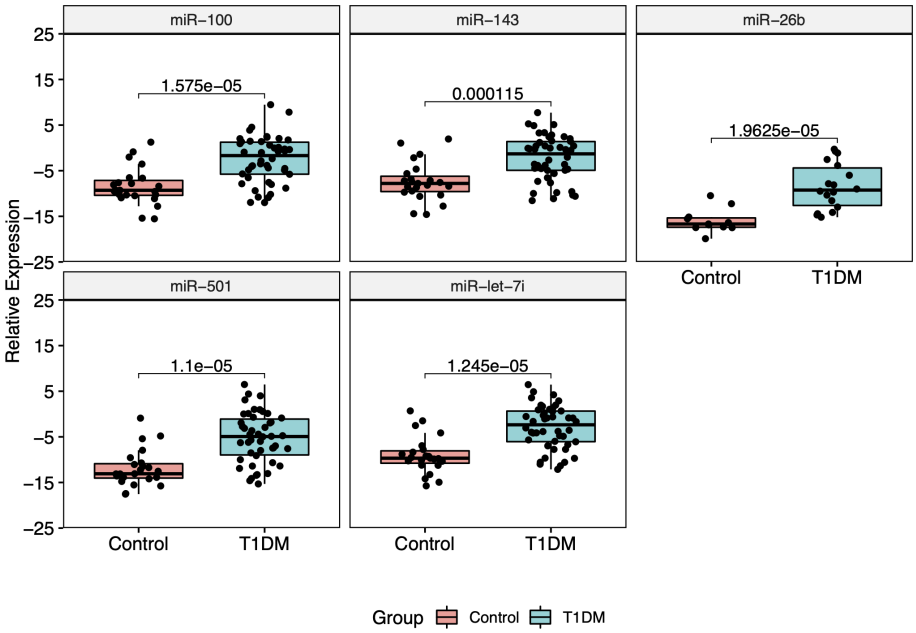

FIGURE 5
Relative expression of five RT-qPCR validated miRNAs. All of them showed to be upregulated in T1DM group.

separate both groups. These miRNAs seem to regulate multiple nuclear and mitochondrially-encoded genes, with emphasis on the validated *hsa-miR-26b-5p, hsa-miR-100-5p* and *hsa-miR-143-3p*, as well as *hsa-miR-21-5p* and its high number of target genes. Our findings reinforce some known pathways and suggest novel interactions that might be associated with T1DM and its complications, such as *hsa-miR-26b-5p* and the mitochondrial metabolism.

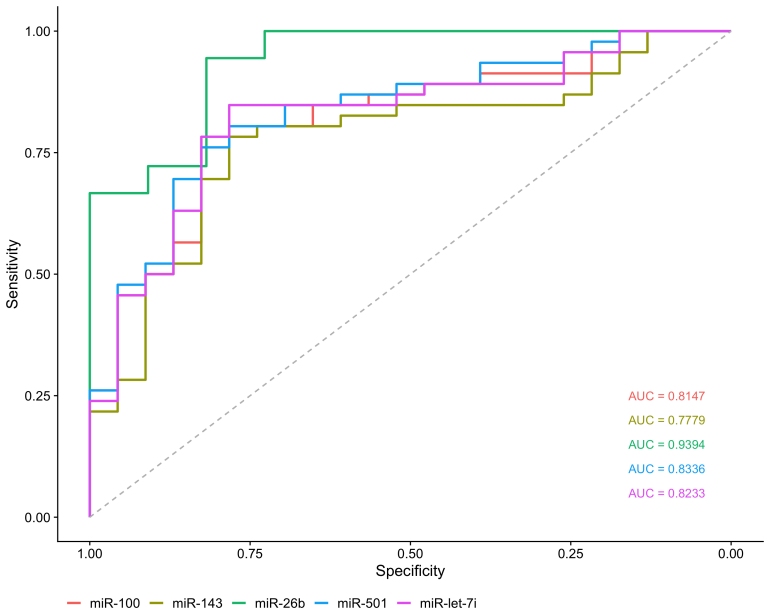

FIGURE 6
Analysis of biomarker sensitivity of five validated miRNAs in T1DM.

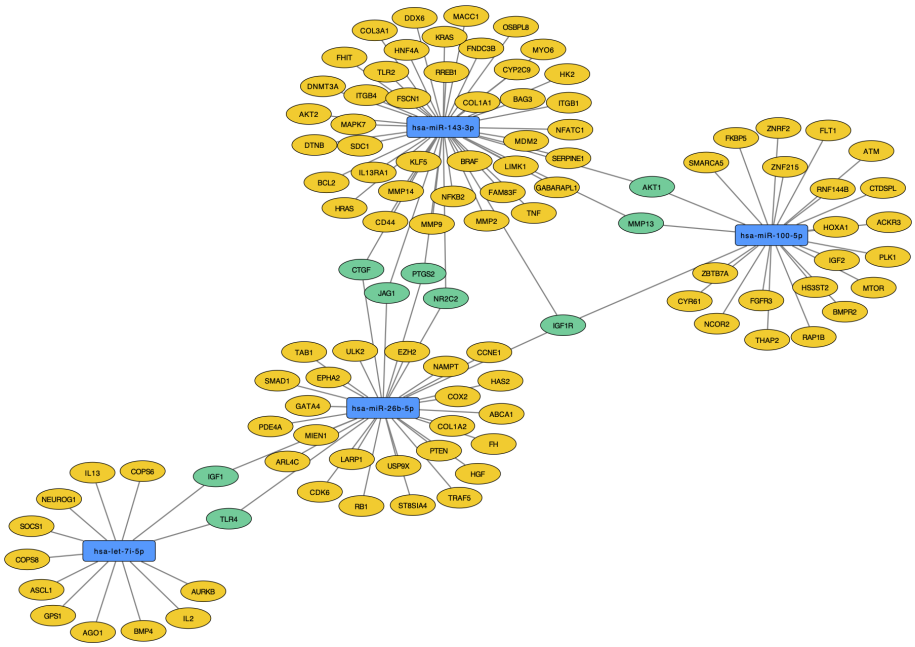

FIGURE 7
Target genes of validated miRNAs (blue) with strong evidence in T1DM. Those regulated by at least two of the miRNAs are pictured in green and those solely regulated by one of the validated miRNAs are picture in yellow.

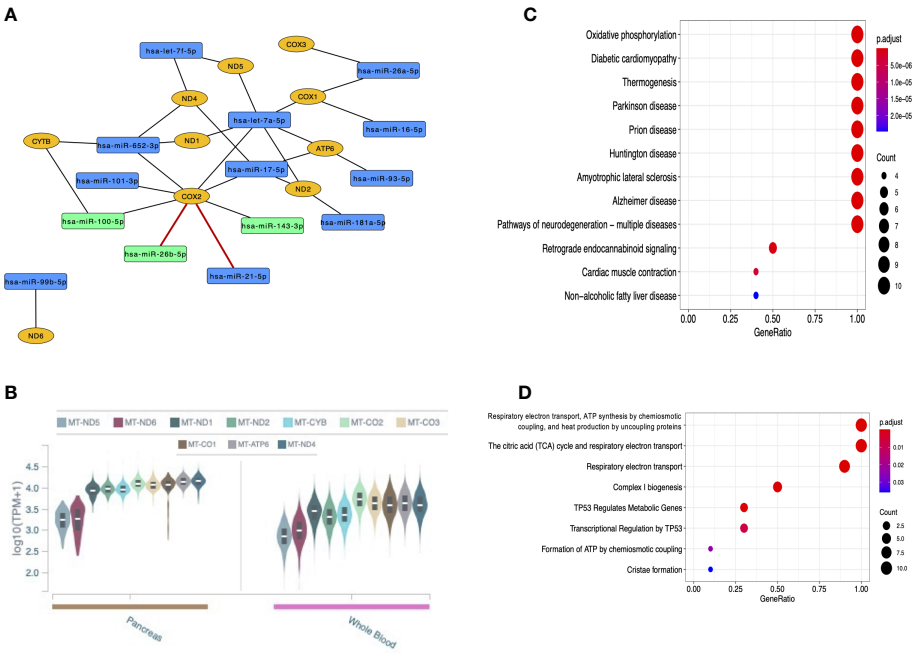

FIGURE 8
Analyses of the 14 DE miRNAs that target mitochondrially-encoded genes. **(A)** Network of these miRNAs (in blue or green, the latter being those validated in the current study) and the mitochondrial genes they target (in yellow); **(B)** GTEx data of mitochondrially-encoded gene expression in the pancreas and whole blood; **(C)** KEGG pathways of mitochondrial functions and some multifactorial conditions; **(D)** Reactome pathways of mitochondrial mechanisms.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJEB51173.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board from João de Barros Barreto University Hospital (HUJBB, Belém, Pará, Brazil) (Protocol Number 005/12). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, ÂR-d-S and JF. Data collection and clinical trial, FTCM, ACCBS, VSGL, PBBF, JFAN, LVM and GNL. Methodology, AV, TV-S and LM. Validation, LS, RC, CB-d-S and LR-d-M. Formal analysis, ÂR-d-S. Investigation, RF and GC. Data curation, ÂR-d-S and AP. Writing-original draft preparation, RF and GC. Writing-review and editing, RF, GC and ÂR-d-S. Visualization, RF and LM. Supervision, LS, NQ and KF. Project administration, ÂR-d-S and JF. Funding acquisition, ÂR-d-S and JF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1033809/full#supplementary-material

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Prevalence and clinical characteristics of T2DM patients with OTUD3 gene rs78466831 SNP at a single academic center in China

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Background: A novel, rare OTUD3 c.863G>A (rs78466831) in humans has been reported associated with diabetes, but the prevalence and clinical characteristics of T2DM patients with rs78466831 have not been reported before.

Objective: To investigate the prevalence and clinical characteristics of T2DM patients with rs78466831 and provide a basis for clinical diagnosis and treatment.

Methods: OTUD3 gene rs78466831 SNP was detected by Sanger sequencing in all the collected specimens of laboratory-confirmed T2DM patients and healthy people. Clinical characteristics indexes inconsisting of fasting blood glucose (FBG), glycosylated hemoglobin (HbA1c), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglyceride (TG) and a body mass index (BMI), T2DM-associated chronic complications (myocardial infarction, cerebrovascular disease, retinopathy, arterial plaque, peripheral neuropathy and nephropathy) were obtained from the clinical laboratory information systems and electronic medical record system. Clinical characteristic indicators were compared between the wild-type and variant (rs78466831) patients with T2DM.

Results: The prevalence of rs78466831 in the T2DM patients group was significantly higher than the healthy control in our academic center. The general characteristic indicators were not significantly different between the wild-type and rs78466831 patients with T2DM, except the family history of diabetes. Clinical laboratory indicators including HbA1c, FBG, OGTT, TC, HDL-

C, LDL-C and CP had no significant difference between the two groups. The therapeutic drug and target achievement rates were not significantly different between the two groups. The incidence of diabetic retinopathy in the variant group was significantly higher than the wild-type group.

Conclusions: The OTUD3 gene rs78466831 was associated with T2DM and may be a biological risk factor of diabetes retinopathy.

KEYWORDS

OTUD3, type 2 diabetes, clinical characteristics, gene, diabetes retinopathy

Introduction

The prevalence of type 2 diabetes mellitus (T2DM) has increased worldwide over the past several decades. T2DM is the most common form of diabetes, accounting for more than 90% of diabetes cases in China (1–3). The International Diabetes Federation (IDF) reported that in 2015, more than 400 million adults worldwide suffered from diabetes (4). The IDF estimates that this number will exceed 600 million by 2040. China currently has the largest number of T2DM cases worldwide. T2DM can lead to many different chronic complications that can reduce the quality of life and even induce premature death. T2DM has a multifactorial etiology, and genes play a key role in its pathogenesis (5). For example, Lee et al. found that the Gas6 gene rs8191974 SNP is associated with T2DM cases in Taiwan (6). The Gas6 polymorphism is associated with stroke (7). The Gas6/TAM system is involved in the pathogenic mechanism of diabetes-associated renal and cardiovascular complications (8). Moreover, low levels of AIM2 promoter total methylation might increase the risk of T2DM and AIM2 promoter total methylation or some loss of CpG methylation increase the risk of vascular complications in T2DM (9). Therefore, we can speculate that patients with T2DM may have different clinical characteristics due to various susceptibility genes. Genetic tests can not only reveal clinical subgroups but can also result in improved treatment outcomes for these patients. For example, combined multigene screening before therapy and LDL-C and sdLDL-C detection before and after therapy could well assist T2DM treatment (10). Brown et al. suggested that increased SLC4A4/NBCe1 in β cells in T2DM contributes to the promotion of β cell failure and should be considered as a potential therapeutic target (11).

In 2022, Zhou et al. reported that in humans, the novel, rare OTUD3 c.863G>A (rs78466831) mutation is associated with diabetes (12). They found that the wild-type genotypes in healthy controls were GG and all the variants were heterozygous GA. OTUD3 c.863 G>A reduced protein stability and DUB activity, which is important for the function of OTUD3 in humans. The data of that study suggested that the CREB-binding-protein-dependent OTUD3 (CBP–OTUD3) signaling pathway plays a key role in glucose and fatty acid metabolism. Glucose and fatty acids can stimulate CBP–OTUD3 acetylation, thus promoting nuclear translocation, wherein OTUD3 regulates various genes involved in glucose and lipid metabolism and oxidative phosphorylation by stabilizing peroxisome-proliferator-activated receptor delta (PPARd) (12).

However, the prevalence and clinical characteristics of T2DM patients with rs78466831 in different regions are still unknown. Clinical characteristics can provide useful information for the effective treatment and management of patients who suffer from diabetes. The comparison of clinical laboratory indicators, general characteristics, target achievement rates, selected hypoglycemic drugs, and associated complications needs further investigation. Therefore, in this study, we intend to explore the data from our academic center to provide a basis for clinical diagnosis and treatment.

Materials and methods

Patients

Cases of T2DM were diagnosed on basis of the 1999 WHO guidelines (13, 14). Patients with T2DM were diagnosed by two endocrinologists in the in-patient departments of the First Affiliated Hospital of Nanchang University. All patients were admitted voluntarily. The inclusion criterion for the patients with T2DM was either fasting plasma glucose level ≥ 7.0 mmol/L or 2 h oral glucose tolerance test glucose level ≥ 11.1 mmol/L. The exclusion criteria included type 1 diabetes, gestational diabetes mellitus, and other special types. The specimens for laboratory detection were collected from patients with type 2 diabetes who were hospitalized in the First Affiliated Hospital of Nanchang University. Blood samples for diagram construction were donated by one of the variant's family members with informed consent. The samples of healthy adults were

collected in the health examination center of our hospital. The inclusion criteria for healthy adults were as follows: 1. Clinical biochemical tests (liver function test, kidney function test, blood glucose test, and blood lipid test) within the normal reference range. 2. Routine blood test indexes within the normal reference range. 3. Routine urine tests within the normal reference range. The exclusion criteria were as follows: 1. History of diabetes. 2. Family history of T2DM. 3. Systemic diseases. 4. Renal and hepatic failure. 5. Cardiovascular disease. 6. Malignant tumors, infections, or other endocrine diseases. 7. Other types of diabetes.

DNA extraction and quality control

A DNeasy Blood & Tissue Kit (69506, Qiagen, Germany) was used to extract blood genomic DNA. NanoDrop ND-1000 (Thermo Fisher Scientific, USA) was applied to detect DNA concentration and quality. A A260/A280 ratio between 1.7–2.0 indicated high DNA purity. DNA was diluted to the working solution concentration of 20 ng/μL for further study.

Amplification

The primers for the detection of the G>A single-nucleotide mutation of the OTUD3 gene (rs78466831 SNP) were designed by the online software Primer-BLAST provided by the National Center for Biotechnology Information. The forward primer sequence of the OTUD3 rs78466831 SNP was GACTGAAGTAGGGACCCAGG, and the reverse primer sequence was ACTGTCACGGCATACACCAA. The length of the amplified fragment was 480 bp. The polymerase chain reaction (PCR) system had the total volume of 40 μL and contained 2 μL of 10 μmol/L Primer F, 2 μL of 10 μmol/L Primer R, 1 μL of 20 ng/μL template gDNA, 20 μL of 2× T8 High-Fidelity Master Mix, and 15 μL of ddH_2O. The reaction procedure was as follows: 98 °C for 2 min, 35 cycles of 98 °C for 10 s, 58 °C for 10 s, and 72 °C for 15 s then at 72 °C for 5 min. A PCR amplification instrument (A300, LONGGENE, China) was used.

Sanger sequencing (G–normal allele; A–variant allele)

The amplified PCR products were subjected to agarose gel electrophoresis (2 μL of sample + 6 μL of bromophenol blue) at the voltage of 300 V for 12 min. The gel map showed that the target band size was single. Then, the qualified PCR products were Sanger sequenced by a sequencer (ABI 3730XL, Thermo Fisher Scientific). The rs78466831 SNP was analyzed by using Sequencing Analysis 5.2 software.

Detection of clinical laboratory indicators

Fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and C-peptide were detected by using serum samples. The clinical biochemical indexes of the patients were determined by using a Hitachi 7600 automatic biochemical analyzer. The detection methods were as follows: fasting blood glucose (FBG): hexokinase method; TC: oxidase method; TG: enzymatic method; LDL-C: direct clearance method; and HDL-C: direct clearance method. All the above biochemical testing reagents were provided by Ningbo Meikang Biotechnology Co., LTD. C-peptide was detected through chemiluminescent immunoassay by using *in vitro* diagnostic kits and MAGLUMI chemiluminescence detector were produced by Shenzhen New Industry Biomedical Engineering Co., LTD. Glycosylated hemoglobin (HbA1C) was detected through high-performance liquid chromatography (TOSOH HLC-723G8).

Collection of general characteristic data

General characteristic information, such as sex, age, diagnose age of onset, body mass index (BMI = weight (kg)/(height $[m]^2$),blood pressure, smoking, drinking, associated chronic complications, family history of diabetes and hypoglycemic drug use of T2DM patients were collected through the Clinical electronic medical record system. And some incomplete medical records were supplemented by telephone questionnaires. The T2DM-associated chronic complications of the patients were judged by the diagnosis medical records and the abnormal results of diagnostic examinations. The patients were assumed to have CHD (coronary heart disease) if they had been diagnosed by the diagnostic examinations included coronary angiography or coronary artery computed tomography. Retinopathy was diagnosed according to the ophthalmologic test, arterial plaque was diagnosed by carotid ultrasonography and diabetic nephropathy was diagnosed as GFR (glomerular filtration rate) <60 mL/min/1.73 m^2 or urinary albumin to creatinine ratio>30 mg/g. Peripheral Neuropathy was diagnosed by Neuroelectrophysiological examination.

Therapeutic drug and target value achievement rate of patients with T2DM on admission

The standard treatment of the patients was based on the 2017 China guidelines for T2DM (14). The target values of treatment were set as follows: FBG 4.4–7.0 mmol/L, HbA1c < 7.0%, TC < 4.5 mmol/L, TG < 1.7 mmol/L, HDL-C > 1.0 mmol/L (men) or >1.3 mmol/L (women), LDL-C < 2.6 mmol/L (not

accompanied by CHD) or <1.8 mmol/L (accompanied by CHD), and BMI < 24 kg/m^2.

Statistical analysis

Data were analyzed by using SPSS 20.0 (SPSS Inc., Chicago, USA). P-values < 0.05 were statistically significant. Continuous variables were descriptively analyzed by using the mean and standard deviation, whereas categorical variables were summarized as counts and percentages in each category. The general characteristics and laboratory indicators were analyzed through t-tests (for normally distributed variables) and Mann–Whitney U test (for non-normally distributed variables). Chi-square test was applied to analyze T2DM-associated complications.

Results

Results of the prevalence of rs78466831 in our academic center

We found six variants (rs78466831) in 300 patients with T2DM and zero in the healthy controls (Table 1). All the genotypes of the variants were GA. The frequencies of allele A in patients with T2DM and healthy controls were 1% and 0%, respectively. In our academic center, the prevalence of rs78466831 in the patients with T2DM was significantly higher than that in the healthy controls.

Diagram of a variant family

All of the variants, except for one child (child-III-1), had type 2 diabetes (Figure 1A). One of the three patients in the family had an onset age earlier than 35 years old, and two had an onset age of greater than 35 years old. The genotypes of the patients were heterozygous mutations GA (Figure 1B), the other healthy adults were wild-type GG (Figure 1C). The arrow indicates the position of the mutant base (rs78466831 position).

General characteristics of the wild-type and rs78466831 patients with T2DM

Among 300 cases, 148 wild-type cases with complete data were selected as the control group and compared with the variant (rs78466831) group. The general characteristics (Table 2), including age, sex (M/F), diagnosed age of onset, diabetes, duration (years), hypertension, smoking (%), and alcohol (%) did not significantly differ between the two groups. However, the family history of diabetes significantly differed between the two groups. The variants all had a family history of diabetes at rates significantly higher than those in the control group.

Clinical laboratory characteristics of the two groups

Clinical laboratory indicators, including HBA1c, FBG, OGTT, CP, TC, HDL-C, TG, and LDL-C, did not significantly differ between the two groups (Table 3).

The selection of treatment drugs and the target achievement rate of the two groups at admission

In accordance with the 2017 China guidelines for T2DM, the therapeutic drug selections and target value achievement rate (Table 4) of the wild-type and rs78466831 patients with T2DM at admission did not significantly differ between the two groups. See the table below for details.

Comparison of T2DM-associated chronic complications between the two groups

T2DM-associated chronic complications, including nephropathy, cerebrovascular disease, cardiovascular disease, arterial plaque, and peripheral neuropathy did not significantly differ between the two groups (Table 5). However, the incidence rate of diabetic retinopathy (DR) was 100% in the variant group

TABLE 1 Different prevalence rates of rs78466831 between healthy controls and patients with T2DM in our academic center.

subjects	Genotypes			Allele	
	GG	GA	AA	G	A
Healthy	300	0	0	600	0
T2DM	294	6	0	594	6
OR			1.01		
P			0.041		
95% CI			1.002-1.018		

CI, Confidence Interval.

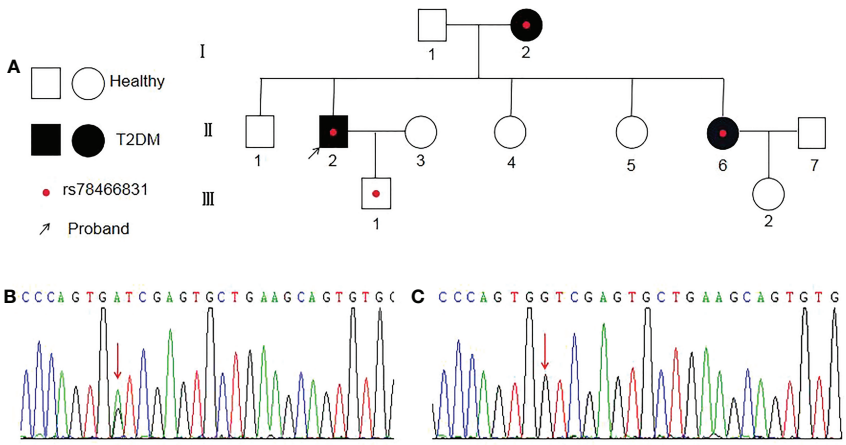

FIGURE 1
Diagram of a family with rs78466831. **(A)** Proband is indicated with an arrow. ◯ females; □ males; ● female with T2DM; ■ male with T2DM; ● with rs78466831. **(B)** Single-base substitution mutation (rs78466831) is indicated with a red arrow. **(C)** Normal base (red arrow).

TABLE 2 General characteristics of the wild-type and rs78466831 patients with T2DM.

Variables	Wide-type	rs78466831	P-Value
Age	59.07± 11.684	58.83±14.497	0.962
Sex(M/F)	94/54	5/1	0.422
Family history	30/148	6/6	<0.01
Diagnose age of onset	49.49±11.2	49.50±18.15	0.999
Diabetes duration (years)	9.57±7.13	9.33±7.9	0.936
Hypertension	101/148	2/6	0.879
Smoking (%)	35/148	3/6	0.16
Alcohol(%)	27/148	1/6	0.921

TABLE 3 Clinical laboratory characteristics of the wild-type and rs78466831 patients with T2DM.

Variables	Wide-type	rs78466831	P-Value
HBA1c(%)	9.103±2.798	8.333±1.405	0.504
FBG (mmol/L)	9.706±5.313	9.21±6.024	0.825
OGTT 1h (mmol/L)	13.353±5.423	10.966±2.116	0.315
OGTT 2h (mmol/L)	14.037±6.274	11.255±2.339	0.263
CP(ng/mL, 0h)	1.1097±1.03559	0.8700±0.922	0.578
CP (ng/mL,1h)	2.107±1.693	1.283±0.851	0.238
CP (ng/mL, 2h)	2.328±1.958	1.36±0.736	0.350
TC(mmol/L)	4.56±1.309	5.505±1.352	0.086
HDL-C (mmol/L)	1.206±0.40524	1.417±0.433	0.214
TG(mmol/L)	1.746±1.637	1.44±0.387	0.649
LDL-C(mmol/L)	2.848±1.175	3.618±1.22	0.118

HbA1c, Glycosylated hemoglobin; FBG, Fasting blood glucose; OGTT, Oral glucose tolerance test; TC, Total serum cholesterol; HDL-C, High-density lipoprotein cholesterol; HDL-C, Low-density lipoprotein cholesterol; CP, C peptide.

TABLE 4 Therapeutic drug and target value achievement rates of the wild-type and rs78466831 patients with T2DM.

Variables(%)	wide-type	rs78466831	P-value
Oral agents (hypoglycemic drug)	128/148	6/6	0.192
Insulin (hypoglycemic drug)	112/148	6/6	0.337
FBG(4.4-7.0 mmol)	46/148	3/6	0.383
HbA1c (<7.0%)	42/148	1/6	0.463
TC(<4.5 mmol/L)	75/148	2/6	0.681
HDL-C (>1.0 mmol/L men or >1.3 mmol/L women)	101/148	5/6	0.666
TG(<1.7 mmol/L)	89/148	2/6	0.227
LDL-C(<2.6 mmol/L ,not accompanied by CHD or <1.8 mmol/L, accompanied by CHD))	66/148	1/6	0.234
BMI (<24 kg/m^2)	90/148	5/6	0.255

and was significantly higher than that in the control group. In most variant cases, punctate hemorrhage and exudation can be seen in the retinas of both eyes.

Discussions

OTU-domain ubiquitin aldehyde-binding proteins (OTUs) are members of DUBs, which can reverse protein ubiquitination (15–17). DUBs are crucial for cellular functions and can be divided into six families, including ubiquitin C-terminal hydrolases, ubiquitin-specific processing proteases, Jab1/Pab1/MPN domain-containing metalloenzymes, OTU Ataxin-3/Josephin, and monocyte chemotactic protein-induced proteases (18). DUBs have been found to regulate many important cellular functions, such as DNA repair, gene expression, cell cycle progression, apoptosis, kinase activation, proteasome or lysosome-dependent protein degradation, and protein degradation prevention (19).

OTUD3 is a hot topic in studies on OTUs. Although OTUD3 has been well described as a key factor in tumorigenesis (20–24), its physiological functions still need further understanding. The variant SNP (rs78466831) found in a MODY-like family is a high-risk factor of diabetes. A novel regulatory mechanism wherein OTUD3 can regulate energy metabolism by blocking ubiquitin-dependent PPARd degradation was found. MODY is easily misdiagnosed as type 2 diabetes because its clinical features always largely overlap with those of type 2 diabetes (25–27). The data of the ALFA project (Release Version: 20201027095038), which provides aggregate allele frequency, showed that the allele frequency of this mutation varies by race and region. The variant allele A frequency of the mutation is significantly higher in East Asian populations (approximately 0.69%) than in other populations (almost zero). Therefore, in our study, we intended to explore the prevalence and clinical characteristics of T2DM patients with rs78466831, including laboratory indicators, age of onset, treatment, complications, and family history of diabetes, from a single academic center.

Our study further confirmed that the rs78466831 mutation was associated with type 2 diabetes in a province located in east China. The general characteristics, including age, sex (M/F), age of onset, duration (years), hypertension, smoking (%), and alcohol (%), but not family history of diabetes, did not significantly differ between the two groups. The variants all had a family history of diabetes at rates significantly higher than those in the control group. The family diagrams showed that all of the variants, except for one child (III-1), had type 2 diabetes. Type 2 diabetes is well known to be an age-related disease that is prevalent only in the adult population. One of the three patients in the family had an onset age earlier than 35 years old, and two had an onset age later than 35 years old. Age of onset may differ due to the varying diets, lifestyles, and environmental factors of individual patients (28). Therefore, we can infer that the rs78466831 gene plays an important role in the development of T2DM on the basis of the family history of diabetes and diagram.

TABLE 5 T2DM-associated chronic complications of the wild-type and rs78466831 patients with T2DM.

Variables	wide-type	rs78466831	p-value
Retinopathy (%)	32/148	6/6	<0.01
Nephropathy (%)	41/148	2/6	0.616
Cerebrovascular disease (%)	10/148	1/6	0.364
Cardiovascular disease (%)	16/148	0/6	0.246
Arterial plaque (%)	76/148	2/6	0.439
Peripheral Neuropathy (%)	85/148	3/6	0.720

Furthermore, we compared the laboratory characteristics of rs78466831 patients with T2DM with those of the wild-type patients with T2DM. Most laboratory indicators, including HBA1c, FBG, OGTT, CP, TC, HDL-C, TG, and LDL-C, did not significantly differ between the two groups. Therefore, distinguishing patients with rs78466831 on the basis of common laboratory indicators was difficult. Furthermore, the therapeutic drug selected and target value achievement rates did not significantly differ between the two groups on admission. However, the incidence of DR in the variant group was significantly higher than that in the wild-type group. Zhang et al. reported that OTUD3 restricts innate antiviral immune signaling. The acetylation-dependent deubiquitinase OTUD3 controls MAVS activation in innate antiviral immunity. IL-6, Tnf-a, IL-1b, and Nos2, which are critical NF-kB target genes activated by MAVS aggregation, are consistently and efficiently induced by SeV in OTUD3-deficient macrophages (29). Most of the inflammatory cytokines mentioned above have been reported to be associated with DR (30, 31). The positive effect of anti-inflammatory therapeutics in patients with DR have highlighted the central involvement of the innate immune system (32), and immune dysregulation has become increasingly identified as a key element of the pathophysiology of DR by interfering with normal homeostatic systems (33, 34). Therefore, we inferred that OTUD3 c.863 G>A leads to reductions in protein stability and DUB activity, which may result in the impaired function of the innate immune system and the higher frequency of retinopathy in the variant patients than in the wild-type patients. DR is recognized as the leading cause of visual impairment and acquired blindness among adults worldwide (35, 36). The incidence of DR in the wild-type group was consistent with that reported by other scholars. To illustrate, in a meta-analysis of approximately 23 000 people with diabetes worldwide, the prevalence of DR was approximately 36% (37). DR is reported to have genetic and acquired (environmental) factors (38, 39). For example, a study in Japan reported associations between long noncoding RNA RP1-90L14 and susceptibility to DR (40). Therefore, the high prevalence of DR in the variants suggested that rs78466831 may be a risk factor of DR. Additionally, given that DR may be asymptomatic for years even at an advanced stage (41–43), screening is crucial to identify, monitor, and guide the treatment of retinopathy. Currently, the diagnosis of DR status should be based on ophthalmoscopy or mydriatic or nonmydriatic retinal photography (44–46). Therefore, we suggest that T2DM patients with rs78466831 should be regularly screened for DR. This approach may help patients obtain accurate treatment and reduce the harm of DR.

Our research has some limitations. First, we only investigated the prevalence and clinical characteristics of T2DM patients with the OTUD3 gene rs78466831 SNP from a single academic center in China and not from multiple clinical research centers. Therefore, a large study is needed. Such a study may be costly, but important. Furthermore, the exact pathological mechanisms of the OTUD3 gene rs78466831 SNP in DR progression remain unknown. Finally, even if the susceptible gene of DR is identified, alterations in gene expression may occur because of environmental factors. Thus, epigenetics studies are very necessary.

To summarize, T2DM is a heterogeneous and broad-spectrum disease with many variations (47). The clinical characteristics of T2DM subtypes may vary depending on the genetic and environmental background (48–50). Our study confirmed that the mutation rs78466831 is associated with type 2 diabetes in a province located in east China. Most laboratory indicators did not significantly differ between the two groups. However, the incidence of DR in the variant group was significantly higher than that in the wild-type group. Therefore, rs78466831 can be a biomarker of DR. This finding will be helpful for the early treatment and management of DR in such patients.

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

This study had been approved by the Medical Research Ethics Committee of the First Affiliated Hospital of Nanchang University. Ethics No.2020 (8–42). Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

J-PL conceived the study and wrote the manuscript. A-PL revised the manuscript. A-PY analyzed the data. MY collected the samples and detected indicators levels. GL assisted in specimen collection. YP checked the patient's clinical data. All authors read and approved the final manuscript.

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Conflict of interest

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

Publisher's note

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Investigating the causal mediating effect of type 2 diabetes on the relationship between traits and systolic blood pressure: A two-step Mendelian randomization study

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Background: Type 2 diabetes mellitus (T2DM) and hypertension commonly coexist, and we presumed that T2DM might mediate the relationship between some shared risk factors and systolic blood pressure (SBP).

Methods: The causal association between T2DM and SBP was first confirmed using Mendelian randomization (MR) analyses, and a two-step MR design was then used to test the causal mediating effect of T2DM on the relationship between 107 traits and SBP using summary statistics from genome-wide association studies.

Results: T2DM was causally associated with SBP. The univariable MR of the two-step causal mediation analyses suggested that 44 and 45 of the 107 traits had causal associations with T2DM and SBP, respectively. Five of the 27 traits that were significantly associated with both T2DM and SBP could not be reversely altered by T2DM and were included in the second step of the causal mediation analyses. The results indicated that most of the investigated traits causally altered SBP independent of T2DM, but the partial causal mediating effect of T2DM on the association between fasting insulin and SBP was successfully identified with a mediation proportion of 33.6%.

Conclusions: Our study provides novel insights into the role of risk factors in the comorbidity of T2DM and high blood pressure, which is important for long-term disease prevention and management.

KEYWORDS

type 2 diabetes, blood pressure, hypertension, mediating effect, Mendelian randomization

Introduction

Type 2 diabetes mellitus (T2DM) and hypertension, commonly found to coexist (1–3), share many risk factors, including physical inactivity, alcohol consumption, and being overweight (4, 5). Additionally, an elevated risk of arterial stiffness, which contributes to the development of hypertension, is correlated with hyperglycemia and T2DM (6–8). Thus, we presumed that T2DM might mediate the relationship between shared risk factors and blood pressure. Determining these effects is important for understanding the underlying mechanisms of the comorbidities of T2DM and hypertension, as well as for long-term disease prevention and management.

Mendelian randomization (MR), a method implementing genetic instruments as a proxy for exposures, is a potent technique used for inferring the causality of exposures and outcomes free from bias due to residual confounding and reverse causality based on three core assumptions (9). Two-step MR is a novel strategy based on the well-established MR framework to improve causal inference for mediation analysis. The causal effect of exposure on outcome independent of (direct effect) or *via* (indirect effect) a mediator can be estimated in causal mediation analyses based on two-step MR (10, 11). No individual-level data are required by two-step MR analyses because they use genome-wide association studies (GWAS) summary statistics of traits and phenotypes, which are normally generated using populations with large sample sizes (12). Additionally, the accessibility to GWAS datasets facilitates the investigation of the mediating effect of T2DM on the association between many traits and blood pressure.

MR studies have been conducted to investigate the causal effect of risk factors on T2DM (13–15) and blood pressure alteration (16, 17). In addition, a two-step MR design has been successfully used to distinguish the direct effects of risk factors on atherosclerotic cardiovascular disease from those mediated by T2DM (18). However, to our knowledge, the potential causal mediating effect of T2DM on the relationship between risk factors and blood pressure is yet to be explored. Therefore, in the present study, we examined the causal association of T2DM with systolic blood pressure (SBP) and then performed causal mediation analyses based on two-step MR to systematically assess the potential mediating effect of T2DM on the causal association of risk factors with SBP.

Abbreviations: MR, Mendelian randomization; GWAS, genome-wide association studies; UKBB, UK Biobank; IVs, instrumental variables; LD, linkage disequilibrium; FDR, False discovery rate; SNPs, single nucleotide polymorphisms; OR, odds ratio; CIs, confidence intervals; IGF-1, insulin-like growth factor-1; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SHBG, sex hormone binding globulin; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2DM, type 2 diabetes mellitus.

Methods

Study design

A two-step MR design was used to test the causal mediation effect of T2DM (mediator) on the relationship between the traits (exposure) and SBP (outcome). First, the causal associations of 107 traits (Supplementary Figure 1) with T2DM and SBP (total effect) were studied using univariable MR as the first step of the two-step MR analyses. In addition, reverse univariable MR was conducted to examine whether these traits could be caused by T2DM because a reciprocal association between exposure and mediator was not allowed in the mediation analyses. Thus, only the traits that had a causal association with T2DM and SBP, but were not causally changed by T2DM, were included in the second step of the two-step MR analyses. Next, the direct effect of traits on SBP was calculated using multivariable MR, in which T2DM was set as the covariable. The indirect effects of traits on SBP were estimated by multiplying the beta coefficient from the causal association of traits with T2DM by those from the causal effect of T2DM on SBP with the adjustment of the trait as a covariable. In sum, the total, direct, and indirect effects in the causal mediation analyses were estimated using a two-step MR.

Data sources

The trait selection procedure (Supplementary Figure 1) was similar to that used in a recent publication (18), in which GWAS summary statistics datasets from European/mixed ancestry, both sexes, and the largest population in the IEU OpenGWAS database were used. Most GWAS summary statistics of exposure traits were from the United Kingdom Biobank (UKBB). For the mediator and outcome data, GWAS datasets of T2DM and SBP were obtained from the Diabetes Meta-analysis of Trans-ethnic Association Studies (DIAMANTE) Consortium (19) and International Consortium of Blood Pressure (20), respectively. Detailed information is provided in Supplementary Table 1.

Statistical methods

Instrumental variables (IVs) for exposure traits were selected according to several criteria in the univariable MR analyses. First, IVs should be strongly associated with exposure traits ($P < 5\times10^{-8}$). Second, IVs should be independent of each other, as quantified by linkage disequilibrium (LD) of $R^2 < 0.001$, which was achieved by clumping with a 10 Mb window. Third, the IVs for each trait should have at least 10 variants, and the single nucleotide polymorphisms (SNPs) should be biallelic. The inverse-variance weighted (IVW) method, weighted median

method, and MR-Egger were used in the univariable MR analyses, in which the IVW method was considered the main method because of its high statistical power when the selected IVs were valid (21). The MR-Egger intercept test was used to examine potential horizontal pleiotropy, and instrument strength was estimated using conditional F-statistics. Multiple comparisons were corrected using a 5% false-discovery rate (FDR). The code for two-step MR analyses was adapted from a published work (18), in which univariable and multivariable MR analyses were performed using the TwoSampleMR package and MVMR package in R, respectively.

Results

We used univariable MR to test the causal association between T2DM and SBP, and the results from MR analysis using the IVW method revealed a significant association of genetically predicted T2DM with SBP (beta, 95% confidence intervals [CIs] and *P* were 0.71, 0.49–0.93 and 1.80×10^{-10}, respectively) (Figure 1, Supplementary Table 2). MR sensitivity analyses with weighted median and MR-Egger methods indicated the same direction of association as the IVW method (Figure 1, Supplementary Table 2). Leave-one-out sensitivity analysis showed that the causal effect of T2DM on SBP was not driven by a specific SNP (Supplementary Figure 2). The evaluation of horizontal pleiotropy using the intercept term of the MR-Egger method suggested that horizontal pleiotropy was not significant ($P = 0.30$) in the analyses. MR-PRESSO also revealed a consistent causal association between T2DM and SBP after excluding potential outlier IVs ($P = 1.38\times10^{-17}$).

After performing the trait exclusion procedure according to the criteria listed in the flowchart (Supplementary Figure 1), a final set of 107 traits from the IEU open GWAS database was included in the current study. Additional information on these traits, as well as their respective GWAS summary datasets, is included in Supplementary Table 1. The univariable MR of the two-step causal mediation analyses suggested that 44 and 45 of the 107 traits had causal associations with T2DM and SBP, respectively (Supplementary Tables 3–5). In addition, the Venn diagram indicates that 27 traits were significantly associated with both T2DM and SBP (Figure 2). Bidirectional univariable MR revealed that 22 of these traits could be reversely caused by T2DM and thus were excluded in the second step of the causal mediation analyses, which is presented in Supplementary Tables 5. For the included five traits (i.e., fasting insulin, trunk fat percentage, hip circumference, standing height, aspartate aminotransferase), multivariable MR analyses showed similar direct and total effects, indicating that adjustment of T2DM as a covariable did not alter the significance of the association between these traits and SBP (Supplementary Tables 6, 7). Furthermore, the direction of the indirect effects of three traits favored a potential mediating effect of T2DM on the causal association between traits and SBP (Supplementary Table 7, Figure 3), and the proportion of the medicating effect by T2DM for the traits "fasting insulin," "aspartate aminotransferase," and "standing height" was 33.6%, 10.2%, and 6.9%, respectively (Supplementary Table 7). The conditional F-statistics of the investigated traits in the multivariable MR ranged from 9.2 to 43.0, representing good instrument strength. Thus, the causal medication analyses using a two-step MR design showed that most investigated traits causally altered SBP independent of T2DM, but the partial causal mediating effect of T2DM on the association between fasting insulin and SBP was successfully identified.

Discussion

In this study, univariable MR analyses indicated a causal relationship between T2DM and SBP, as well as the causal effects of numerous traits on T2DM and SBP. Many common risk factors for the two outcomes of interest were identified, including glycemic traits (e.g., fasting insulin and glycated

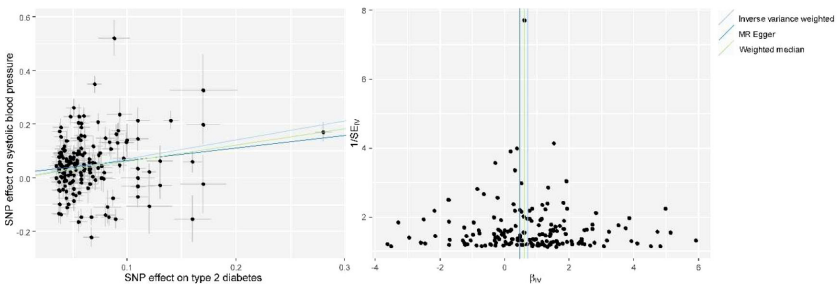

FIGURE 1
Scatter plot (*left*) and funnel plot (*right*) of Mendelian randomization (MR) analyses showing that T2DM is causally associated with increased systolic blood pressure (SBP). MR, Mendelian Randomization; SNP, single nucleotide polymorphism; IV, instrumental variable; T2DM, type 2 diabetes mellitus; SBP, systolic blood pressure.

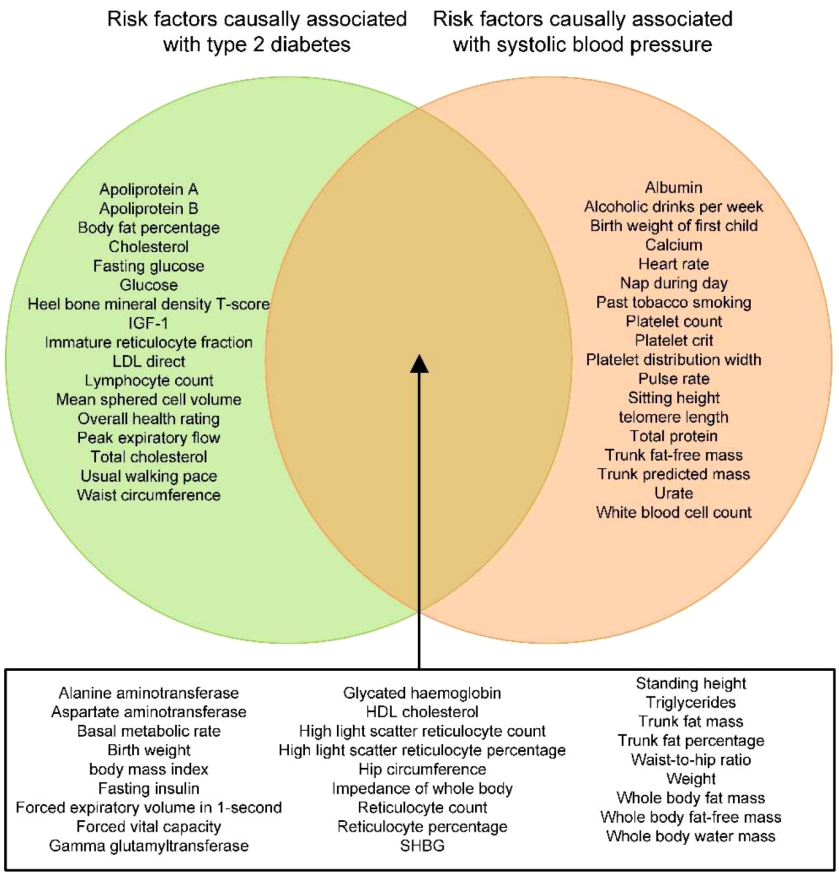

FIGURE 2
Venn diagram depicting the traits that are causally associated with type 2 diabetes mellitus (T2DM) and/or SBP. IGF-1, insulin-like growth factor-1; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SHBG, sex hormone binding globulin; T2DM, type 2 diabetes mellitus; SBP, systolic blood pressure.

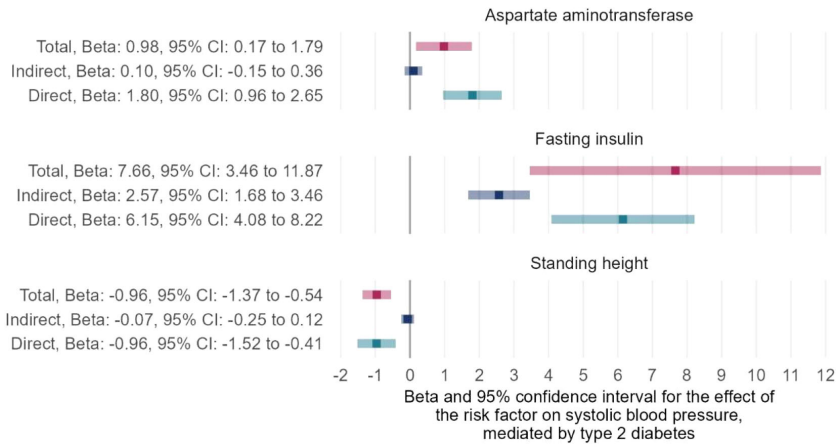

FIGURE 3
Two-step MR for mediation analyses showing the total, indirect (mediated by T2DM), and direct (independent of T2DM) effects of the traits on SBP. MR, Mendelian Randomization; T2DM, type 2 diabetes mellitus; SBP, systolic blood pressure.

hemoglobin), blood lipid indices (e.g., high-density lipoprotein [HDL] and triglycerides), anthropometric markers (e.g., body mass index, standing height, waist-to-hip ratio, and whole body fat mass), and pulmonary function indicators (e.g., forced vital capacity and forced expiratory volume in 1-second). However, most of these common risk factors (22 of 27 risk factors) of T2DM and SBP were not included in the second step of the two-step MR analyses because causal mediation analysis required no reciprocal causal association of the mediator (T2DM) with exposure (common risk factors). Two-step MR for mediation analyses suggested that three of the included five traits had indirect effects with a direction favoring a potential mediating effect of T2DM, and the causal association between fasting insulin and SBP could be partially mediated by T2DM with an estimated mediation proportion of 33.57%.

Diabetes and hypertension frequently occur together (22–24). For instance, patients with diabetes are twice as likely to have hypertension than that of non-diabetic individuals (25). Furthermore, a recent MR analysis revealed a causal relationship between T2DM and hypertension, in which a higher SBP, but not diastolic blood pressure (DBP), can be induced by T2DM (26). SBP refers to the peak blood pressure recorded during heart contraction, while DBP refers to the blood pressure recorded when the heart rests between beats. Since the Framingham study in 1980 showed that systolic hypertension is a more powerful indicator of cardiovascular events than diastolic hypertension (27), SBP has been of great importance (28–30). Moreover, the hypertension management guidelines by the American College of Cardiology (ACC) and American Heart Association (AHA) removed DBP from the assessment of cardiovascular risk in 2017 (31). Thus, we selected SBP as the outcome in the current study, and our findings provide consistent evidence supporting the causal effect of T2DM on SBP. Mechanistically, the natural course of diabetes promotes the development of high blood pressure. For example, hyperglycemia and hyperinsulinemia lead to peripheral artery resistance by vascular remodeling and narrowing and increase circulatory blood volume by sodium reabsorption and hyperosmolarity, which eventually elevates heart contractility and blood pressure (32, 33).

Diabetes and hypertension share a considerable number of common pathophysiological pathways as end results of the metabolic syndrome (22, 24, 34). These pathways involve multiple key players, such as the renin-angiotensin-aldosterone system (RAAS) (35), obesity (36, 37), inflammation (38, 39), oxidative stress (40) and insulin resistance (41), which interact with each other and form a vicious cycle. For instance, RAAS activity is inappropriately upregulated in obese individuals (42), which may induce insulin resistance through the regulation of Ang II type 1 receptor, resulting in increased oxidative stress in adipocytes, skeletal muscle, and cardiovascular tissue, aggravating the development of diabetes and hypertension (34, 35). Thus, diabetes and hypertension are expected to be associated with several common risk factors. Epidemiological data indicate that the waist-hip ratio and hip circumference are both closely associated with the risk of T2DM and blood pressure (43–46). MR studies further verified the clinical observation of the potent contributions of the waist-hip ratio and body mass index on diabetes or cardiovascular risk (e.g., high blood pressure) from a genetic perspective (47, 48). In addition, a review pointed out that unhealthy fat distribution in the body increases cardiometabolic risk, whereas a high amount of fat in the lower part of the body may play a protective role against T2DM and cardiovascular diseases (49). One plausible explanation is that visceral abdominal obesity, measured by waist-hip circumference, is detrimental to metabolic activity and the cardiovascular system (50). Another explanation is that subcutaneous adipose tissue in the lower part of the body has a less negative impact on metabolism than that in the viscera in the upper part of the body (51–53). In accordance with previous studies, we found evidence for several anthropometric markers as common risk factors that could lead to both T2DM and SBP, such as body mass index, hip circumference, waist-to-hip ratio, and whole-body fat mass. As for other traits, the association between lung function and diabetes (54) and blood pressure (55, 56), as well as the association of glycemic traits and lipids with blood pressure (57, 58), have been epidemiologically and clinically well-accepted. In the present study, we illustrated the causal relationship of 27 common risk factors with both T2DM and SBP, including anthropometric markers, pulmonary function indicators, glycemic traits, and blood lipid indices.

Insulin is a hormone that can significantly affect blood glucose levels, and the abnormal regulation of insulin contributes to the pathogenesis of diabetes. Fasting insulin is considered part of the clinical definition of T2DM and is an effective clinical tool for predicting prediabetes (59). In addition, insulin plays an important role in regulating SBP independently of diabetes, with evidence that insulin is correlated with SBP in non-diabetic individuals (60, 61). Moreover, an animal study demonstrated that excessive insulin increased heart function and significantly pushed up SBP (62). Biologically, insulin increases the activity of Na^+/K^+-ATPase to promote the transport of sodium ions into the blood vessels through renal tubule cells (63). Thus, insulin resistance with compensatory hyperinsulinemia facilitates sodium retention to elevate blood pressure, independent of diabetes (64). Along with the progression of diabetes induced by insulin deficiency, vascular fibrosis and stiffness and activated RAAS drive up blood pressure (33, 65, 66), which reflects the indirect effect of insulin on SBP *via* diabetes. Our results align with these mechanisms, indicating that the modulating effect of fasting insulin on SBP could be independent of (direct effects) or *via* (indirect effects) diabetes. In addition, considering a broad range of risk factors for blood pressure control in individuals with diabetes is necessary, given our results that most traits influence SBP independent of diabetes.

Two-step MR can infer causality when analyzing the mediating effect of T2DM on the association between various traits and SBP, which is a major strength of the current study because the biases caused by residual confounding and reverse causality are diminished by the MR design. In addition, the use of summary statistics from large GWAS for exposures, mediators, and outcomes increases the power of the statistical analyses. Moreover, multiple MR methods, including the weighted median method, MR-Egger, and multivariable MR, were used for sensitivity analyses, by which horizontal pleiotropy and instrument strength were estimated. However, the present study has several limitations. First, the relatively large number of traits included in this study increased the burden of multiple comparison correction, thereby altering the number of traits that could be passed on to the second step of the two-step MR analysis. Second, the potential mediating effect might have been underestimated because of the higher statistical power required for multivariable MR analyses. Third, horizontal pleiotropy is ubiquitous in MR analyses, which may have introduced bias in the current study.

Conclusion

T2DM causally increases SBP and partially mediates the causal association between fasting insulin and SBP. Other eligible traits included in the causal medication analyses altered SBP independent of T2DM. Our study provides novel insights into the role of risk factors in the comorbidity of T2DM and high blood pressure.

Data availability statement

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

Ethics statement

The GWAS summary statistics used in this study were approved by the relevant review board. Informed consent was also obtained from all participants.

Author contributions

XH and WL designed the study. XH, WL and RS performed the statistical analyses and drafted the manuscript. QW critically reviewed the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1090867/full#supplementary-material

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Association between hyperglycemia and adverse clinical outcomes of sepsis patients with diabetes

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Background: Hyperglycemia is one of the poor prognostic factors in critical ill sepsis patients with diabetes. We aimed to assess the interaction between admission glucose level and clinical endpoints in sepsis patients with diabetes admitted in the intensive care unit (ICU).

Methods: Data from the Medical Information Mart Intensive Care III database were used in this study. The study primary endpoint was 28-day mortality after ICU admission. Multivariate Cox regression models were used to explore the association between admission glucose level and the primary endpoint.

Results: We included 3,500 sepsis patients with diabetes. Of participants with no hyperglycemia, mild hyperglycemia, and severe hyperglycemia, no differences were evident in hospital mortality, ICU mortality, or 28-day mortality (all P >0.05). The multivariable Cox regression analysis demonstrated that severe hyperglycemia did not increase the risk of 28-day mortality (hazard ratio [HR]=1.06, 95% confidence interval [CI]: 0.86–1.31, P=0.5880). Threshold effects analysis identified the inflection points for 28-day mortality as 110 mg/dl and 240 mg/dl. The HRs for 28-day mortality were 0.980 in the <110 mg/dl and 1.008 in the >240 mg/dl. A short-term survival advantage was observed in the 110–240 mg/dl group compared with that in the <110 mg/dl group; meanwhile, no adverse hazard was detected in the >240 mg/dl group. In the stratified analyses, the association effect between the three glucose groups (<110 mg/dl, 110–240 mg/dl, and ≥240 mg/dl) and 28-day mortality was consistent in terms of different sequential organ failure assessment (SOFA) scores and infection sites. The 28-day mortality of the 110–240 mg/dl group with a SOFA score of ≥10 was lower than that of the <110 mg/dl group (HR=0.61, 95% CI: 0.38–0.98).

Conclusion: Admission hyperglycemia was not a risk factor for short-term prognosis in critical ill sepsis patients with diabetes; a lower admission blood glucose level was associated with increased risk of poor prognosis. The potential benefit of higher admission glucose level on 28-day mortality in patients with a more severe condition remains a concern.

KEYWORDS

critical care, diabetes, sepsis, glucose, prognosis

Background

Diabetes is a common comorbidity among critically ill sepsis patients and generally causes immune dysfunction and metabolic disorders, including hyperglycemia (1–3). In recent years, diabetes is developing swiftly as a global health epidemic and is one of the top ten causes of adult death (4). Hyperglycemia was closely related to endothelial cell injury, mitochondrial damage, and inflammation activation (5, 6). In terms of clinical research, Vught et al. revealed that severe hypoglycemia contributed to higher 90-day mortality in sepsis patients with diabetes (7). In another study, Vught et al. indicated that severe hyperglycemia was correlated with 30-day mortality in patients with sepsis, regardless of the presence or absence of diabetes (8). Subsequently, multiple studies that examined the glucose levels of this patient group reported different views, and some indicated the adverse effects of glycemic control (9–12). A previous large randomized trial found that a glucose level of 81–108 mg/dl was associated with adverse clinical outcomes of glycemic control compared with a glucose level of ≤180 mg/dl (2).

To our knowledge, evidence on how hyperglycemia affects the clinical outcomes in critical sepsis patients with diabetes remains limited and debatable. Considering that diabetes is consistently correlated with other diseases, the impact of admission glucose level in the outcome of sepsis patients should be explored, potentially determining better individualized glycemic control strategies. Consequently, we aimed to assess the interaction between admission glucose levels and clinical endpoints in sepsis patients with diabetes admitted in the intensive care unit (ICU).

Methods

Patient data

Data from the Medical Information Mart Intensive Care III (MIMIC-III) database were used in this study (13). The institutional review boards of Beth Israel Deaconess Medical Center and Massachusetts Institute of Technology Affiliates approved the access to the database (record identification numbers: 33460949 and 49780033). The requirement for obtaining informed consent was waived due to the use of anonymized data.

Adult (aged ≥18 years) patients diagnosed with sepsis based on the following criteria were included in the study: suspected infection and a sequential organ failure assessment (SOFA) score of ≥2 (14). We excluded patients with 1) multiple ICU admissions, 2) less than one day of follow-up, 3) hospital length of stay less than the ICU length of stay, 4) no diabetes, and 5) admission blood glucose level of <70 mg/dl. The first plasma glucose measurement obtained in patients admitted in the ICU was used in the study and grouped into the following categories: no hyperglycemia (≤139 mg/dl), mild hyperglycemia (140–199 mg/dl), and severe hyperglycemia (≥200 mg/dl) (7, 8). Along with the patient's baseline information (e.g., age and sex), therapeutic measures, and clinical endpoints for routine variables, we also extracted the data of patients' SOFA score, Elixhauser Comorbidity Index (SID30) (15), and specific comorbidities. The code for assisting in the investigation of MIMIC-III is openly available on the website (16).

Outcomes

The primary outcome was 28-day mortality after ICU admission, and the secondary outcome was ICU mortality.

Statistical analysis

The data were expressed as mean ± standard deviation or median (interquartile range) for continuous variables and as numbers and percentages for categorical variables. We compared the characteristics of participants between glucose groups using one-way analysis of variance for continuous variables and chi-square test for categorical variables. Initially,

we applied Cox regression models to explore the associations of admission glucose level with the 28-day mortality and logistic regression models to explore the association of admission glucose level with ICU mortality. We presented different adjusted models to assess the effect of admission glucose level on clinical endpoints in sepsis patients with diabetes. In model I, we adjusted for demographic characteristics (age and sex), disease severity (SOFA scores), comorbidity scores (SID30), infection site, and initial treatment (mechanical ventilation and renal replacement therapy on the first); in model II, we substituted the SID30 with the specific diseases (congestive heart failure, cardiac arrhythmias, etc.). Covariate screening was used to include covariates as potential confounders if they changed the estimates of admission glucose level on 28-day mortality by more than 10% or were associated significantly with 28-day mortality.

Subsequently, to explore whether a nonlinear relationship exists between glucose level and 28-day mortality, we performed the smoothed spline method using a Cox model to fit the 28-day mortality (generalized additive model for fitting ICU mortality). If it existed, segmental regression models constructed during the threshold effects analysis were used to detect the inflection points, and the differences were compared by log-likelihood ratio tests (17). Next, the admission glucose level was re-grouped by inflection points, and the different adjustment models described above were used to evaluate the clinical outcome. Finally, stratified analysis and interaction tests were conducted to explore the consistency of the relationship between the inflection point grouping of glucose and 28-day mortality in the patient subgroups based on SOFA scores (<5, 5–10, and ≥10) and infection site. All data were analyzed using EmpowerStats (www.empowerstats.com) and R (http://www.R-project.org). A *P*-value of <0.05 was considered significant.

Results

Participants' characteristics

A total of 3,500 sepsis patients with diabetes with a mean age of 66.79 years were enrolled in this study (Figure 1). Majority of the sepsis patients with diabetes were men (51.8% *vs.* 48.2%). No significant differences were observed between the three groups in terms of SID30, SOFA score, infection site, and need for mechanical ventilation or renal replacement therapy on the first day of ICU admission. Additional detailed results are presented in Table 1.

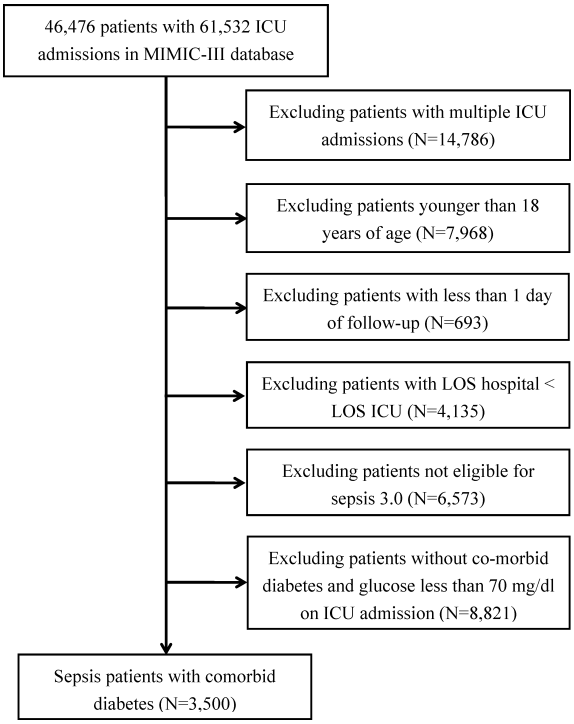

FIGURE 1
Flowchart of study participants. ICU, intensive care unit; LOS, length of stay.

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TABLE 1 Characteristics of participants.

Variables	All patients (N=3500)	No hyperglycemia (N=1271)	Mild hyperglycemia (N=1426)	Severe hyperglycemia (N=803)	*P*-value
Age (years)	66.8 ± 17.1	67.2 ± 16.6	66.1 ± 17.2	67.3 ± 17.3	0.176
Sex					0.387
Male	1814 (51.8%)	645 (50.7%)	759 (53.2%)	410 (51.1%)	
Female	1686 (48.2%)	626 (49.3%)	667 (46.8%)	393 (48.9%)	
Admission glucose (mg/dl)	165.4 ± 53.6	115.9 ± 17.2	166.0 ± 16.9	242.9 ± 41.0	<0.001
Infection site					0.629
Bloodstream	1507 (43.1%)	570 (44.8%)	596 (41.8%)	341 (42.5%)	
Pulmonary	238 (6.8%)	81 (6.4%)	102 (7.2%)	55 (6.8%)	
Abdominal	76 (2.2%)	25 (2.0%)	30 (2.1%)	21 (2.6%)	
Urinary tract	727 (20.8%)	258 (20.3%)	290 (20.3%)	179 (22.3%)	
Others	952 (27.2%)	337 (26.5%)	408 (28.6%)	207 (25.8%)	
Mechanical ventilation on first day	1746 (49.9%)	612 (48.2%)	729 (51.1%)	405 (50.4%)	0.287
Renal replacement therapy on first day	172 (4.9%)	69 (5.4%)	69 (4.8%)	34 (4.2%)	0.465
SOFA	5.0 (3.0-7.0)	5.0 (3.0-7.0)	5.0 (3.0-7.0)	5.0 (3.0-7.0)	0.280
Elixhauser Comorbidity index (SID30)	17.0 (8.0-26.0)	17.0 (8.0-26.0)	16.0 (8.0-25.0)	17.0 (9.0-26.0)	0.219
Length of ICU stay (days)	3.3 (1.8-7.8)	3.3 (1.8-7.9)	3.3 (1.8-7.8)	3.3 (1.8-7.8)	0.271
Length of hospital stay (days)	10.7 (6.3-18.5)	10.8 (6.3-19.1)	10.8 (6.2-18.0)	10.4 (6.2-18.4)	0.264
28-day mortality, n(%)	604 (17.3%)	226 (17.8%)	237 (16.6%)	141 (17.6%)	0.704
ICU mortality, n(%)	326 (9.3%)	105 (8.3%)	139 (9.8%)	82 (10.2%)	0.253
Hospital mortality, n(%)	520 (14.9%)	183 (14.4%)	213 (14.9%)	124 (15.4%)	0.804
Comorbidities, n(%)					
Congestive heart failure	1529 (43.7%)	585 (46.0%)	629 (44.1%)	315 (39.2%)	0.009
Cardiac arrhythmias	1327 (37.9%)	522 (41.1%)	535 (37.5%)	270 (33.6%)	0.003
Valvular disease	528 (15.1%)	240 (18.9%)	208 (14.6%)	80 (10.0%)	<0.001
Peripheral vascular disease	536 (15.3%)	237 (18.6%)	208 (14.6%)	91 (11.3%)	<0.001
Hypertension	2413 (68.9%)	896 (70.5%)	982 (68.9%)	535 (66.6%)	0.178
Other neurological diseases	409 (11.7%)	127 (10.0%)	178 (12.5%)	104 (13.0%)	0.059
Chronic pulmonary disease	805 (23.0%)	274 (21.6%)	334 (23.4%)	197 (24.5%)	0.259
Liver disease	357 (10.2%)	134 (10.5%)	134 (9.4%)	89 (11.1%)	0.396
Renal failure	1063 (30.4%)	425 (33.4%)	405 (28.4%)	233 (29.0%)	0.011
AIDS	17 (0.5%)	9 (0.7%)	4 (0.3%)	4 (0.5%)	0.280
Lymphoma	63 (1.8%)	21 (1.7%)	24 (1.7%)	18 (2.2%)	0.562
Metastatic cancer	163 (4.7%)	62 (4.9%)	75 (5.3%)	26 (3.2%)	0.084
Solid tumor	176 (5.0%)	56 (4.4%)	82 (5.8%)	38 (4.7%)	0.255

(Continued)

TABLE 1 Continued

Variables	All patients (N=3500)	No hyperglycemia (N=1271)	Mild hyperglycemia (N=1426)	Severe hyperglycemia (N=803)	*P*-value
Obesity	398 (11.4%)	145 (11.4%)	157 (11.0%)	96 (12.0%)	0.795
Fluid and electrolyte disorders	1558 (44.5%)	548 (43.1%)	583 (40.9%)	427 (53.2%)	<0.001
Alcohol abuse	162 (4.6%)	66 (5.2%)	58 (4.1%)	38 (4.7%)	0.377
Drug abuse	60 (1.7%)	24 (1.9%)	20 (1.4%)	16 (2.0%)	0.492
Depression	305 (8.7%)	102 (8.0%)	125 (8.8%)	78 (9.7%)	0.412

ICU, intensive care unit; SOFA, sequential organ failure assessment; AIDS, acquired immune deficiency syndrome.

Clinical outcomes of the participants

With regard to the clinical outcomes, the hospital mortality, ICU mortality, and 28-day mortality in sepsis patients with diabetes in the no hyperglycemia, mild hyperglycemia, and severe hyperglycemia groups were not significant (all P >0.05). No significant difference was found in the length of hospital or ICU stay among the three groups (all P >0.05).

TABLE 2 Association of admission glucose groups with primary and secondary outcomes.

28-day mortality	Groups	HR (95% CI)	*P*-value
Crude	No hyperglycemia	1.0	–
	Mild hyperglycemia	0.93 (0.78-1.12)	0.4346
	Severe hyperglycemia	0.99 (0.80-1.22)	0.9018
Model I	No hyperglycemia	1.0	–
	Mild hyperglycemia	0.98 (0.81-1.17)	0.7950
	Severe hyperglycemia	0.99 (0.81-1.23)	0.9625
Model II	No hyperglycemia	1.0	–
	Mild hyperglycemia	0.99 (0.82-1.19)	0.9097
	Severe hyperglycemia	1.06 (0.86-1.31)	0.5880
ICU mortality	Groups	OR (95% CI)	*P*-value
Crude	No hyperglycemia	1.0	–
	Mild hyperglycemia	1.20 (0.92-1.56)	0.1797
	Severe hyperglycemia	1.17 (0.85-1.61)	0.3346
Model I	No hyperglycemia	1.0	–
	Mild hyperglycemia	1.22 (0.94-1.60)	0.1412
	Severe hyperglycemia	1.16 (0.84-1.59)	0.3630
Model II	No hyperglycemia	1.0	–
	Mild hyperglycemia	1.24 (0.95-1.63)	0.1138
	Severe hyperglycemia	1.19 (0.87-1.65)	0.2805

Model I was adjusted by age, sex, SOFA, SID30, infection site, mechanical ventilation on first day, renal replacement therapy on first day.
Model II was adjusted by age, sex, SOFA, infection site, mechanical ventilation on first day, renal replacement therapy on first day, congestive heart failure, cardiac arrhythmias, valvular disease, peripheral vascular disease, hypertension, other neurological diseases, chronic pulmonary disease, liver disease, renal failure, AIDS, lymphoma, metastatic cancer, solid tumor, obesity, fluid and electrolyte disorders, alcohol abuse, drug abuse, and depression.
SOFA, sequential organ failure assessment; SID30, Elixhauser Comorbidity index; AIDS, acquired immune deficiency syndrome; HR, hazard ratio; OR, odds ratio; CI, confidence interval.

Associations between admission glucose level and clinical outcomes

The Cox regression analysis demonstrated that severe hyperglycemia did not increase the risk of 28-day mortality (crude hazard ratio [HR]=0.99, 95% confidence interval [CI] 0.80-1.22, *P* =0.9018). After adjusting for confounding factors, hyperglycemia remained a non-risk factor (Table 2). In model II, when compared with the no hyperglycemia group, the 28-day mortality rate in the severe hyperglycemia group did not significantly increase (HR=1.06, 95% CI: 0.86–1.31, *P*=0.5880). Similar findings were reported in the mild hyperglycemia group (HR=0.99, 95% CI: 0.82–1.19, *P*=0.9097). With regard to ICU mortality, the results similarly indicated no significant increase in ICU mortality in both the mild hyperglycemia and severe hyperglycemia groups compared with that of the no hyperglycemia group (Table 2).

Smooth splines showed a nonlinear relationship of admission glucose with 28-day and ICU mortality (Figures 2A, B). Threshold effect analysis identified the inflection points for 28-day mortality of 110 mg/dl and 240 mg/dl. For 28-day mortality, the HR was 0.980 for a glucose level of <110 mg/dl and 1.008 for a glucose level of >240 mg/dl (Table 3). Subsequently, the admission glucose level was divided into three categories according to the inflection point: <110 mg/dl, 110–240 mg/dl, ≥240 mg/dl (inflection point grouping of glucose); a Cox regression analysis was performed, and the results revealed a 26% significant reduction of 28-day mortality in the 110–240 mg/dl group compared with the <110 mg/dl group (HR=0.74, 95% CI: 0.59–0.93, *P*=0.0100); in the >240 mg/dl group, no substantial increase was observed in the risk of 28-day mortality rate (*P* >0.05) (Table 4). A considerable short-term survival advantage was observed in the 110–240 mg/dl group compared with that in the 110 mg/dl group; meanwhile, no remarkable adverse hazard was detected in the >240 mg/dl group (Figure 3).

In the stratified analysis, the association effect between the new glucose category and the risk of 28-day mortality was generally consistent in the different SOFA scores and infection site (Table 5). Furthermore, the 28-day mortality of the 110–240 mg/dl group with a SOFA score of ≥10 was lower than that of the <110 mg/dl group (HR=0.61, 95% CI: 0.38–0.98). Similarly, patients with bloodstream infection in the 110–240 mg/dl group experienced substantially lower 28-day mortality rate compared with those in the <110 mg/dl group (HR=0.70, 95% CI: 0.49–1.00).

Discussion

The present study explored the association between admission glucose level and clinical outcomes among critical sepsis patients with diabetes and found that the risk of 28-day mortality was not substantially increased in sepsis patients with diabetes who had an admission glucose level of ≥240 mg/dl compared with those who had an admission glucose level of <110 mg/dl; notably, the 28-day mortality rate was markedly reduced in the 110–240 mg/dl group (HR=0.74, 95% CI: 0.59–0.93). Furthermore, an elevated admission glucose level was significantly associated with a reduction in the 28-day mortality rate in the SOFA score ≥10 subgroup, which may imply that patients with serious conditions require a higher energy supply.

Currently, a number of studies have evaluated the glycemic control goals in sepsis patients; the Surviving Sepsis Campaign

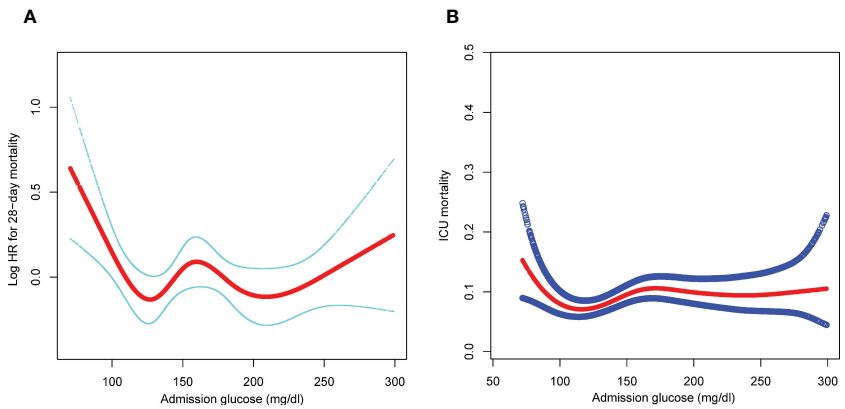

FIGURE 2
(A) Association of admission glucose level with 28-day mortality. **(B)** Association of admission glucose level with ICU mortality. adjusted by age, sex, SOFA, infection site, mechanical ventilation on first day, renal replacement therapy on first day, congestive heart failure, cardiac arrhythmias, valvular disease, peripheral vascular disease, hypertension, other neurological diseases, chronic pulmonary disease, liver disease, renal failure, AIDS, lymphoma, metastatic cancer, solid tumor, obesity, fluid and electrolyte disorders, alcohol abuse, drug abuse, and depression. SOFA, sequential organ failure assessment; AIDS, acquired immune deficiency syndrome; ICU, intensive care unit; HR, hazard ratio.

TABLE 3 Threshold effect analysis of glucose level and 28-day mortality rate using piece-wise linear regression.

Outcome: 28-day mortality			
Inflection point	HR	95% CI	*P*-value
< 110 mg/dl	0.980	0.968-0.990	0.0009
110-240 mg/dl	1.001	0.998-1.003	0.6563
> 240 mg/dl	1.008	1.002-1.013	0.0093

The log-likelihood ratio test: P <0.001
Adjusted by age, sex, SOFA, infection site, mechanical ventilation on first day, renal replacement therapy on first day, congestive heart failure, cardiac arrhythmias, valvular disease, peripheral vascular disease, hypertension, other neurological diseases, chronic pulmonary disease, liver disease, renal failure, AIDS, lymphoma, metastatic cancer, solid tumor, obesity, fluid and electrolyte disorders, alcohol abuse, drug abuse, and depression.
SOFA, sequential organ failure assessment; AIDS, acquired immune deficiency syndrome; HR, hazard ratio; CI, confidence interval.

similarly recommended a glycemic level of 8–10 mmol/L in glycemic management (18). To our knowledge, only a very few studies have investigated the effect of glucose on prognosis in critical sepsis patients with diabetes. A recent study by Zohar et al. included 1,527 patients with community-onset sepsis and found that admission hyperglycemia (>200 mg/dl) correlated with increased in-hospital mortality, 30-day mortality, and 90-day mortality; moreover, this adverse outcomes were more prevalent in patients with diabetes (19). In a study of 1,059 sepsis patients, Vught et al. similarly found that severe hyperglycemia (>200 mg/dl) upon admission did not increase the 30-day mortality rate in patients with sepsis; rather, hyperglycemia was strongly associated with increased 30-, 60-, and 90-day mortality rates in patients with sepsis without diabetes (20). Moreover, Tayek et al. searched the PubMed database for publications related to sepsis, diabetes, glycemia, and prognosis; nine studies were analyzed, which reported that hyperglycemia was not related to poor outcome in sepsis patients with diabetes; the opposite was true in hyperglycemic patients without diabetes, which was an independent hazard factor for ICU and in-hospital mortality (21). Stegenga et al. examined 830 patients with severe sepsis and suggested a measurable increase in 28- and 90-day mortality rates with hyperglycemia (>200 mg/dl) compared with admission glucose at or below 200 mg/dl in sepsis patients without diabetes. Although the authors did not explicitly analyze the admission glucose level in sepsis patients with diabetes, the curve fitting plots in the article indicated that admission hyperglycemia had a relatively slight effect on 28-day mortality in sepsis patients with diabetes (22). In addition, another relatively earlier research conducted by Freire et al. demonstrated that admission hyperglycemia was not appreciably associated with in-hospital mortality (23). All of the abovementioned studies showed results similar to those of our study; that is, in sepsis patients with diabetes, admission hyperglycemia was not an independent hazard factor for poor short-term prognosis. In our study, we further revealed a non-linear relationship between admission glucose level and 28-day mortality using smoothing spline

TABLE 4 Associations between inflection point grouping of glucose and 28-day mortality.

28-day mortality	Groups	HR (95% CI)	*P*-value
Crude	<110	1.0	–
	≥110, <240	0.71 (0.56-0.89)	0.0029
	≥240	0.86 (0.62-1.18)	0.3430
Model I	<110	1.0	–
	≥110, <240	0.71 (0.57-0.89)	0.0033
	≥240	0.82 (0.60-1.14)	0.2380
Model II	<110	1.0	–
	≥110, <240	0.74 (0.59-0.93)	0.0100
	≥240	0.93 (0.67-1.28)	0.6492

Model I was adjusted by age, sex, SOFA, SID30, infection site, mechanical ventilation on first day, renal replacement therapy on first day.
Model II was adjusted by age, sex, SOFA, infection site, mechanical ventilation on first day, renal replacement therapy on first day, congestive heart failure, cardiac arrhythmias, valvular disease, peripheral vascular disease, hypertension, other neurological diseases, chronic pulmonary disease, liver disease, renal failure, AIDS, lymphoma, metastatic cancer, solid tumor, obesity, fluid and electrolyte disorders, alcohol abuse, drug abuse, and depression.
SOFA, sequential organ failure assessment; SID30, Elixhauser Comorbidity index; AIDS, acquired immune deficiency syndrome; HR, hazard ratio; CI, confidence interval.

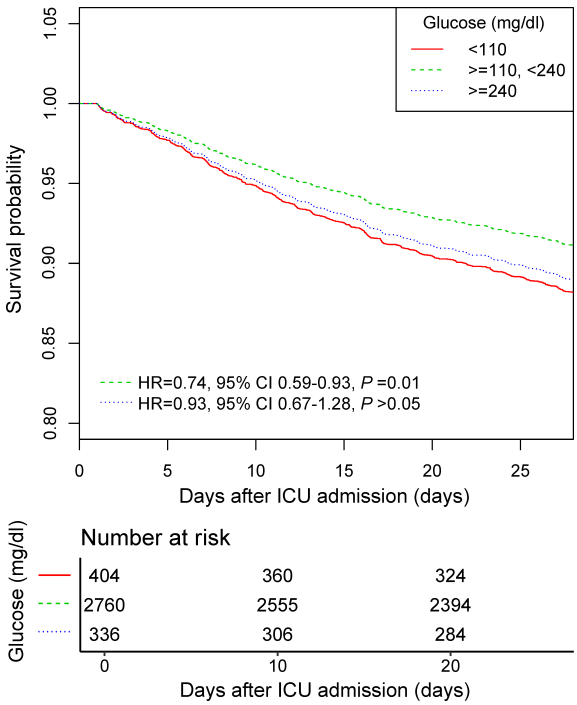

FIGURE 3
The 28-day survival curve of the Cox regression model for participants with inflection point grouping of glucose. Adjusted by age, sex, SOFA, infection site, mechanical ventilation on first day, renal replacement therapy on first day, congestive heart failure, cardiac arrhythmias, valvular disease, peripheral vascular disease, hypertension, other neurological diseases, chronic pulmonary disease, liver disease, renal failure, AIDS, lymphoma, metastatic cancer, solid tumor, obesity, fluid and electrolyte disorders, alcohol abuse, drug abuse, and depression. SOFA, sequential organ failure assessment; AIDS, acquired immune deficiency syndrome.

TABLE 5 Association of inflection point grouping of glucose with 28-day mortality stratified by different scores of SOFA and infection site.

28-day mortality	Crude		Adjusted model		
Admission glucose (mg/dl)	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	*P* interaction
SOFA: <5					0.5759
<110	1.0	–	1.0	–	
≥110, <240	0.89 (0.59-1.34)	0.5809	0.91 (0.60-1.38)	0.6460	
≥240	1.21 (0.70-2.08)	0.4949	1.14 (0.65-1.99)	0.6445	
SOFA: ≥5, <10					
<110	1.0	–	1.0	–	
≥110, <240	0.69 (0.48-0.99)	0.0427	0.75 (0.52-1.09)	0.1284	
≥240	0.87 (0.54-1.42)	0.5832	1.03 (0.63-1.68)	0.9172	
SOFA: ≥10					
<110	1.0	–	1.0	–	
≥110, <240	0.54 (0.35-0.83)	0.0046	0.61 (0.38-0.98)	0.0399	
≥240	0.47 (0.22-1.01)	0.0521	0.55 (0.24-1.22)	0.1413	
Infection site: Bloodstream					0.7049

(Continued)

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TABLE 5 Continued

28-day mortality	Crude		Adjusted model		
Admission glucose (mg/dl)	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	*P* interaction
<110	1.0	–	1.0	–	
≥110, <240	0.75 (0.53-1.06)	0.1045	0.70 (0.49-1.00)	0.0474	
≥240	1.00 (0.62-1.62)	0.9977	1.05 (0.64-1.71)	0.8470	
Infection site: Pulmonary					
<110	1.0	–	1.0	–	
≥110, <240	0.39 (0.18-0.87)	0.0205	0.48 (0.21-1.08)	0.0776	
≥240	0.45 (0.13-1.48)	0.1873	0.47 (0.14-1.63)	0.2351	
Infection site: Abdominal					
<110	1.0	–	1.0	–	
≥110, <240	0.65 (0.21-1.95)	0.4396	0.54 (0.09-3.37)	0.5131	
≥240	1.41 (0.32-6.32)	0.6508	3.05 (0.18-52.01)	0.4410	
Infection site: Urinary tract					
<110	1.0	–	1.0	–	
≥110, <240	0.63 (0.38-1.03)	0.0650	0.65 (0.39-1.08)	0.0987	
≥240	0.62 (0.31-1.26)	0.1866	0.62 (0.30-1.27)	0.1892	
Infection site: Others					
<110	1.0	–	1.0	–	
≥110, <240	0.84 (0.52-1.36)	0.4809	0.93 (0.56-1.52)	0.7646	
≥240	0.98 (0.50-1.92)	0.9629	0.99 (0.50-1.99)	0.9834	

Adjusted by age, sex, SOFA, infection site, mechanical ventilation on first day, renal replacement therapy on first day, congestive heart failure, cardiac arrhythmias, valvular disease, peripheral vascular disease, hypertension, other neurological diseases, chronic pulmonary disease, liver disease, renal failure, AIDS, lymphoma, metastatic cancer, solid tumor, obesity, fluid and electrolyte disorders, alcohol abuse, drug abuse, and depression except for the subgroup variable.
SOFA, sequential organ failure assessment; AIDS, acquired immune deficiency syndrome; HR, hazard ratio; CI, confidence interval.

curves, with the lowest 28-day mortality in the admission glucose range of 110–240 mg/dl, which was different from those reported in other studies and was one of the highlights of our study. In the subgroup analysis, we found that higher admission glucose level was significantly associated with lower 28-day mortality rate in the SOFA ≥10 subgroup; whether this means that critically ill patients require higher energy supply deserves further investigation. These results, contrary to our common knowledge of the devastating consequences of diabetes and hyperglycemia, suggest the need for an individualized glycemic control strategy for sepsis patients with diabetes that differs from other critically ill patients since they may be able to benefit from hyperglycemia.

In the light of the available studies, however, it seems that the clinical benefit of hyperglycemia and sepsis with co-existent diabetes remains a topic that cannot be thoroughly elucidated. From the clinical point of view, diabetes can cause immune dysfunction and metabolic disorders, which inevitably induce the organism's ability to defend against infection, in turn with catastrophic consequences. Physiologically, part of the potential mechanism can be attributed to the metabolic requirements and maintenance of the function of immune cells by glucose, with an equally critical role played by the synthetic action of immunomodulators (24, 25). Furthermore, patients with diabetes have a tolerance to hyperglycemia as a consequence of persistent high blood glucose concentrations, converting the detrimental elevated glucose into an energy reservoir (26). Additionally, the therapies administered to diabetic patients, including sulfonylureas, metformin, thiazolidinediones, and insulin, as well as the effects of diabetes on the immune system, may potentially affect the host's response to sepsis and clinical endpoints. Therefore, further investigations are imperatively needed to comprehensively address which mechanisms contribute to the overall impact of diabetes on the outcomes of sepsis.

Even with the relatively large sample size included in our study, the limitations should not be overlooked. First, we did not account for the effect of diabetes type and diabetes medications

like insulin and metformin; thus, we were unable to assess whether medications and diabetes type have an effect on outcomes at this point. Next, we cannot exclude the possibility of new-onset diabetes since data on HbA1c levels are not available. Moreover, we were not able to obtain information about the duration and severity of diabetes; thus, it was impossible to measure the effect of these factors on the outcome as well. Third, we used the first blood glucose measurement obtained after admission to the ICU for the purpose of eliminating the effect of medical therapies in the ICU, and the results were different from those of studies investigating glycemic control, although our results may help identify appropriate glycemic control strategies to some extent. Finally, we should interpret these results with caution, as the association analysis should not be mistaken for causality. Therefore, further in-depth basic and clinical studies are warranted to enrich the category of findings.

Conclusion

Admission hyperglycemia was not a risk factor for short-term prognosis in critical ill sepsis patients with diabetes; rather, a lower blood glucose level was associated with increased risk of poor prognosis. Notably, an elevated admission glucose level was significantly associated with a reduction in 28-day mortality rate in the SOFA score ≥10 subgroup; whether this implies that patients with severe illness require a higher energy supply deserves further research.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: The raw data itself is from a third-party dataset available from MIMIC-III, a freely accessible critical care database. Reproduction of their data is not permitted according to the Data Use Agreement of the database but access can be requested here: https://mimic.physionet.org/gettingstarted/access.

Ethics statement

The access of the database has been approved by the institutional review boards of both Beth Israel Deaconess Medical Center and Massachusetts Institute of Technology Affiliates (Record ID: 33460949). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

SL and DL designed the study and wrote the draft of this manuscript, WH mainly performed data extraction and statistical analysis, SL revised this manuscript. All authors are involved in data correction. Written consent to publication was obtained.

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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Diverse nanocomposites as a potential dressing for diabetic wound healing

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Wound healing is a programmed process of continuous events which is impaired in the case of diabetic patients. This impaired process of healing in diabetics leads to amputation, longer hospitalisation, immobilisation, low self-esteem, and mortality in some patients. This problem has paved the way for several innovative strategies like the use of nanotechnology for the treatment of wounds in diabetic patients. The use of biomaterials, nanomaterials have advanced approaches in tissue engineering by designing multi-functional nanocomposite scaffolds. Stimuli-responsive scaffolds that interact with the wound microenvironment and controlled release of bioactive molecules have helped in overcoming barriers in healing. The use of different types of nanocomposite scaffolds for faster healing of diabetic wounds is constantly being studied. Nanocomposites have helped in addressing specific issues with respect to healing and improving angiogenesis. Method: A literature search was followed to retrieve the articles on strategies for wound healing in diabetes across several databases like PubMed, EMBASE, Scopus and Cochrane database. The search was performed in May 2022 by two researchers independently. They keywords used were "diabetic wounds, nanotechnology, nanocomposites, nanoparticles, chronic diabetic wounds, diabetic foot ulcer, hydrogel". Exclusion criteria included insulin resistance, burn wound, dressing material.

KEYWORDS

nanocomposites, scaffolds, hydrogen-based scaffolds, Chitosan-based scaffolds, diabetic wound healing

Abbreviations: AgNP, Silver Nanoparticle; PVA, Polyvinyl alcohol; HA, Hydroxyapatite; VEGF, Vascular Endothelial Growth Factor; CD31, Cluster of differentiation 31; CuNP, Copper nanoparticle; MnO_2, Manganese dioxide; PCL, Polycaprolactone; ROS, Reactive Oxygen Secies; MMP, Matrix Metalloproteinases; MAPk, Mitogen-Activated Protein kinases.

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Introduction

Nanomedicine is one of the fastest-growing fields offering several avenues for therapy, diagnostics, delivery systems and improving efficiency (1). The superior properties of the 'nano' components have been used in tissue engineering for the repair and regeneration of several organs and tissues. Wound healing is a normal process involving a series of steps; however it is affected by a number of variables like age, obesity, stress, diseases, habits, infections, trauma etc. (2). But in certain conditions, healing is halted at the second phase, i.e., the inflammatory phase which could be due to chronic conditions. Many diseases that cause impaired blood flow, such as in the case of diabetic foot ulcers or pressure ulcers are the contributing factors. Common wound pathogens, nosocomial infections are also known to hinder the progression of healing to the third phase, which is proliferation. Several factors are attributed to patients with diabetes mellitus such as the improper function of macrophages and growth factors, and low blood circulation; the major factor for delayed wound healing (3). The incidence of diabetes is seen to be increasing at a steady rate globally, with a mortality rate of 1.5 million deaths in 2019. The indirect death due to diabetes was 460,000 due to kidney disease, and 20% due to cardiovascular complications (4). Diabetic patients are prone to develop diabetic foot ulcers and the percentage affected is more than 20% (5). To treat such chronic wounds, newer therapies such as cell/gene therapy, and engineered biomaterials are sought after due to unsuccessful treatment modalities. Tissue engineering has led researchers to explore several new skin substitutes using natural, synthetic, and semi-synthetic polymers. They are often used in combination with biomolecules, proteins, and polysaccharides (6). To overcome the existing limitations, they have been combined with nanomaterial to form a highly functional, multi-modal, smart nanocomposite to treat chronic wounds such as in the case of diabetes (7). The major advantages of nanotherpay are due to the charge, and large surface area to volume ratio that enhances the interaction with the target area (8). The ability to encapsulate and control the drug release by attaining a sustained release of the desired biomolecules leads to accelerated healing (9). Figure 1 represents the types of nanocomposites and its advantages in wound healing.

Various factors, including pH, temperature, blood sugar level, and oxygen saturation, are important in the healing of wounds. Scaffolds have attracted interest recently as a novel dressing and provide an innovative perspective on tissue regeneration (10, 11). Researchers state that the design of the dressing material spatially is of utmost importance for a biomaterial to function as an effective regenerative scaffold, which is now possible through nanocomposites which have been summarised in this review.

Nanoparticle based composite scaffold for enhanced healing

While researchers are experimenting with effective and scarless wound healing strategies, wound management in diabetes itself is a tedious process for the patients and the healthcare sector. So, several advanced techniques and technology have been employed in the remedial measures for diabetic wounds.

Several types of metal nanoparticles, metal oxide nanoparticles, nanotubes, and polymeric nanoparticles have been used in wound healing. Because of their innate antimicrobial property, the silver nanoparticle has been extensively used in wound care management. It is strikingly important to note that silver nanoparticles are effective against nosocomial infections and multidrug-resistant pathogens (12, 13). At a concentration of 50 mg/ml silver nanoparticles were observed to destruct the bacterial cell membrane and kill *S. aureus* and *E. coli* (14).

A nanocomposite was fabricated with polyethylene glycol diacrylate, silica, bioactive glass nanoparticles, sodium alginate and copper. This silica-based nanocomposite was found to be an excellent injectable with elastomeric, biomimetic, and antibacterial properties. The regeneration of blood vessels was observed with high collagen deposition, and VEGF expression in a full-thickness diabetic wound model (15). Table 1 indicates the different types of nanocomposites employed for diabetic wound healing.

Nitric oxide (NO) induces the formation of blood vessels and the migration of endothelial cells by eNOS or MAPk pathways. Zinc oxide is known to induce NO production, hence several scaffolds for wound healing have incorporated ZnO NP's nanofibers fabricated using poly-caprolactone with ZnO NP's which exhibited high proliferation of fibroblast cells. A higher rate of vascular regeneration was observed because of the expression of VEGF and FGF (27). Cerium oxide nanoparticles were used in combination with microRNA (miR-146a) for faster healing in diabetic wounds. The synergistic role of scavenging the free radicals and modulating the inflammatory pathway proved to increase the synthesis of collagen, thereby higher rate of angiogenesis and low inflammation; this aided in a significantly higher rate of wound closure (28).

Poly-N-acetyl-glucosamine based nanofibrous scaffold was prepared to overcome the limitations in treating a diabetic wound. This bioactive scaffold was found to enhance cell metabolism, and migration of endothelial cells with a higher rate of wound closure in a full- thickness diabetic mice model. The gene expression of uPAR, VEGF, Il-1 and MMP responsible for migration, angiogenesis, inflammatory activity, and matrix

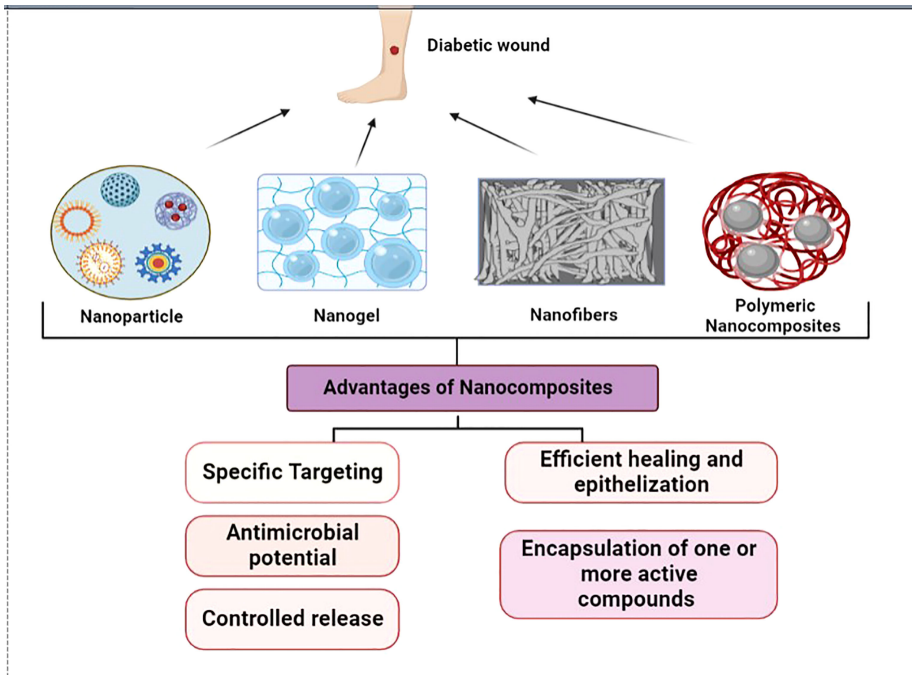

FIGURE 1
Types of nanocomposites and its advantages as a wound dressing.

TABLE 1 Types of nanocomposites employed for diabetic wound healing.

Nanocomposite	Role in wound healing	Reference
Polyurethane nanoparticles	Induces angiogenesis, cell proliferation	(16)
AuNPs with epigallocatechin and lipoic acid	Regulated angiogenesis and inflammation to accelerate faster healing	(17)
Bioactive glass with Cu	Stimulation of CD31, HIF-1α, VEGF expression. Antibacterial activity	(18)
Silicate Bioglass NPs	Increased proliferation of epithelial cells and nitric oxide expression that enhanced angiogenesis	(19)
45S5 bioglass with Strontium and Copper	Aided the differentiation of stem cells to vascular endothelial cells, formation of tubular vein endothelial cells.	(20)
CuNPs in carbon nanofibers	Upregulation of placental growth factor, VEGF, hypoxia inducible growth factor. Increased vascularisation and wound closure rate	(21)
CuNPs in hyaluronic acid hydrogel	Upregulation of the growth factor, VEGF. Promoted angiogenesis and collagen deposition	(22)
PCL nanofibers with curcumin	Distinct granulation tissue formation. Increased fibroblast proliferation, collagen content, and faster regeneration.	(23)
AgNPs in hyaluronic acid nanofibers	Antibacterial activity. Accelerated healing in wounds	(24)
Cellulose nanocrystals in PLGA fibers	Inflammatory cytokines, IL-1 and IL-6 were reduced. Higher rate of epidermal and dermal regeneration.	(25)
Chitosan in PVA nanofibers	Upregulation of HIF-1 and VEGF. Improved interaction among endothelial cells and fibroblasts	(26)

remodelling was observed (29). Another study found that short-fibre poly-N-acetyl glucosamine nanofibers were used alongside the vacuum-assisted closure of complex wounds. This aided in controlling the blood loss by acting as a hemostatic agent activating platelets and better granulation. The presence of collagen I and the wound contraction rate was significant in the treated groups (30).

Stimuli-responsive scaffold for modulated healing process

The ulcers in diabetic wounds are caused by oxidative stress, so researchers prepared Prussian blue nanoparticles (PBNP's) to scavenge the free radicals generated at the wound site. This PBNP was encapsulated in a heat-sensitive gel using poly (d, L-lactide)-poly (ethylene glycol)-poly (d, L-lactide) (PDLLA-PEG-PDLLA) hydrogel (PLEL). It was confirmed that the nanoparticle was able to protect the cells and mitochondria against reactive oxygen species (ROS). In an animal model, it was found to progress diabetic wound healing at a faster rate, reduce ROS production, and enhance cell survival and growth simultaneously reducing the interleukin and tumor necrosis factor (31).

A pH-responsive scaffold was developed which aided in faster healing with less scar formation. This injectable scaffold was prepared with polysaccharides and exhibited antibacterial activity against multi-drug resistant bacteria. *In vivo* studies showed that the exosome released promoted angiogenesis in the full-thickness wound (32). A dual responsive scaffold that modulates the release based on pH and metformin release was prepared using PEG. The active components encapsulated were phenylboronic acid, benzaldehyde, L-arginine, and chitosan which exhibited anti-inflammatory effects and promoted angiogenesis. The synergistic healing of metformin and graphene oxide was observed in a rat model with type II diabetic foot ulcer. Based on the stimulus it was found to release the drug, metformin which was faster healing in chronic diabetic athletic wounds (33).

Silver nanoclusters were conjugated with vancomycin in a gelatin-based hydrogel along with nimesulide that is pH sensitive. This complete biomaterial containing phenylboronic acid and polyvinyl alcohol also contained ROS and exhibited anti-inflammatory action. It was found to be biocompatible, with excellent cell-adhesive behaviour and aided healing in wounds with infection. Because of its sensitive and dual-responsive properties, hydrogel was found to be good for treating chronic diabetic wounds (34). A thermos-responsive scaffold that is skin-friendly and designed for infants and diabetic patients with sensitive skin was attempted by researchers. This non-irritable hydrogel patch was designed with a protein-polyphenol complex that was activated upon reaction to the body temperature upon application. This was found to be skin-friendly and gentle even for a prolonged period of use because of its immune-modulatory action (35).

Hydrogel-based scaffold

Hydrogels are the most preferred dressing agent for wound healing owing to their capability to retain moisture at the site of wounds, agent because hydrogels are designed to hold moisture at the wound surface, and create the best setting for healing, balancing skin hydration and in the removal of necrotic tissue. They could be prepared with ease providing sustained drug release. Both natural and synthetic polymers could be used in the preparation of hydrogels. These may include, fibrin, hyaluronic acid, cellulose derivatives, copolymers and others (36).

Hydrogels are exceptional in providing a humid atmosphere for the healing of wounds and ensure permeable water vapours with microbial entry prevention at the wound site. A heparinised PVA-based hydrogel formulated demonstrated significant antibacterial activity without any cellular toxic effects (37). Another hydrogel containing coumestrol/hydroxypropyl-β-cyclodextrin was developed using hydroxypropyl methylcellulose. The insoluble coumestrol (helps with photoaging; improves elasticity of skin during menopause) was solubilized using hydroxypropyl-β-cyclodextrin to obtain a hydrogel which led to faster wound healing process through the better propagation of cells. This also demonstrated good cell adhesion and compatibility as observed through Wistar rats (38). A gel-based hydrogel was formulated using adipose-derived stem cells as a suitable wound healing agent that was obtained from both mouse and porcine models. These *in vivo* models demonstrated excellent healing of wounds (39). Topic nitric oxide helps in the healing process of acute and chronic wounds. An antibacterial peptide was developed based on this, which could self-assemble with respect to changes in pH, and could lead to the development of hydrogel with improved bactericidal activity (40). A ZnO-based nanocomposite hydrogel demonstrated significant antibacterial properties and was found biocompatible and safe with a faster rate of wound healing (41). Though there are many ongoing research on hydrogels related to skin repair, there is another group of researchers who developed hydrogels containing HA and carboxylated CS that mimics skin with high mechanical strength. The *in vitro* studies on L929 cells demonstrate superior biocompatibility with improved cell proliferation. Further, *in vivo* studies also demonstrated a faster healing process and suggested that this hydrogel as an ideal candidate suited for wound recovery and healing (42). Though there are many ongoing research on hydrogels used as wound dressing agent, we would like to identify the importance of hydrogels as a potential wound dressing agent with reference to diabetic wounds.

Diabetes being a chronic disease is yet challenging to cure and the medical requirements are inadequate (43). The skin wounds

caused by diabetes do not get completely healed due to limited blood supply and deprived antimicrobial capability with the poor inflammatory response (44). Among 750,000 emerging cases of diabetic foot ulcer in America, nearly 10% of cases involved amputation of limbs every year (45). Many measures are taken for treating wound healing due to diabetes such as growth factor and cellular-based therapy but the cost was too high (46–48). So, there has been an increasing interest in bioactive biomaterials as a potent wound dressing agent for treating in case of diabetic-based wounds (49). Some of the biomaterials have progressed to clinics such as biomedical hydrogels, films and ointments, and others (50). On the other hand, multifunctional biomaterials are developed with potent antioxidants, antibacterial activity and hemostasis (51–53). Hydrogel biomaterial-based dressings are also developed with their property similar to that of the extracellular matrix and this demonstrated good wound healing (54–58).

Due to vascular impairment, diabetes-related wound healing and skin regrowth remain a major concern. To overcome this, a silica-based nanocomposite hydrogel scaffold that could promote both wound healing and skin regeneration in diabetic conditions was developed by enhancing early angiogenesis with no bioactive factors. This injectable nanocomposite exhibits an excellent healing pattern with superior antibacterial properties. Also, enables viability, growth, and angiogenesis of endothelial progenitor cells through *in vitro* studies. *In vivo* studies demonstrated restoration of blood vessels through HIF-1α/VEGF and collagen deposition in diabetic wound. It was also suggested to have its application in regenerative medicine (15). Several tissue engineering strategies using nanobiomaterials for vascular regeneration have been reported (59). A multifunctional sprayable cross-linking bioadhesive hydrogel-based nanocomposite was developed for diabetic wound healing. Here, Kappa-carrageenan being the hydrogel matrix, different concentrations of modified ZnO nanoparticles were incorporated to improve their mechanical properties with good antibacterial activity. To this, L-glutamic acid was also loaded into this network to enhance the rate of wound healing. This biocompatible nanocomposite also demonstrated elasticity similar to human skin with adhesive nature and clotting capability. The *in vivo* studies further demonstrated significant wound healing at a faster rate without any infection (59). A 2-D nanoclay (Laponite RD)/polymer-based nanocomposite hydrogels were developed as a substitute for treating foot ulcers due to diabetes. It was also suggested that enzymes or active compounds loaded to the hydrogel could help in the healing of diabetic foot ulcers through their antibacterial activity (60). Another research on zwitterionic poly (sulfobetaine acrylamide) nanocomposite that was composed of hectorite nanoclay demonstrated as a potent chronic wound dressing agent. This hydrogel exhibited insignificant cytotoxicity against NIH-3T3 fibroblast and was resistant against the adsorption of BSA and certain bacterial strains. *In vivo* studies on both normal and diabetic wounds were conducted in mice in comparison with commercially available dressings. Histology confirmed significant re-epithelialization and faster healing of diabetic wounds than the commercial products (61). A bioactive HQB nanocomposite hydrogel was developed through the cross-linking of modified hyaluronic acid with quaternized chitosan coated with bioactive glasses. This demonstrated superior wound healing properties in diabetic-induced rats and suggested it to have a good prospect in clinical application (62). A cost-effective and simple dual-network hydrogel comprised of MnO2 nanosheets was developed from silk fibroin and carboxymethyl cellulose. This helped in angiogenesis, reduced inflammation, and had remarkable healing rates comparable to commercial dressing through *in vivo* studies (63). An alginate and Eudragit nanoparticle-based nanocomposite hydrogel comprising edaravone was produced for the highest ROS sequestration to overcome chronic inflammation and delayed wound healing in diabetes. A lower dosage of this hydrogel enhanced wound healing, and a higher dosage impeded the healing process in diabetic mice and suggested dosage levels played a key role in the healing process (64). Some examples of nanoparticle-based wound dressing materials for which clinical trials are undertaken is listed in Table 2.

Chitosan-based scaffolds

The major risk associated with patients affected with diabetes includes delayed wound healing and amputation. This is mainly due to the reduced tissue blood circulation causing hypoxia and the associated risks. A PVA/Chitosan-based nano fibre wound dressing was developed with high antimicrobial activity, improved vapour transmission rate, good odour-absorbing capacity and no cytotoxic effects; and was proved to accelerate the diabetic wound healing when tested in both diabetic and non-diabetic rats (72). A safe, cyto-compatible, epidermal growth factor-modified curcumin-incorporated chitosan nano-spray was developed that demonstrated accelerated wound healing properties, improved angiogenesis, and re-epithelialization with superior antibacterial effects in rats. It was further suggested that this nano-spray could

TABLE 2 List nanocomposite scaffolds undergoing/completed clinical trials.

Nanocomposite	Wound type	Reference
Hydrogel/Nano Silver-based Dressing	Diabetic foot wound	(65)
Wound Dressing FibDex (Nanofibrillar cellulose)	Dermal burn	(66)
AgNP	Partial thickness burns	(67)
Ag nylon	Surgical wound	(68)
AgNP- Acticoat	Fresh burn	(69)
Hydrofibre of Ag	Pilonidal sinus	(70)
Nanocrystalline silver	Leg ulcer	(71)

help in the treatment of diabetic wounds and other skin injuries (73). A formulation composed of poly lactic acid/chitosan nanoscaffolds encapsulating cod liver oil was developed and characterized which demonstrated significant wound healing property to be used in the treatment of the most complicated disorder, diabetic foot ulcers, seen in diabetic patients (74). A topical formulation of lecithin-chitosan nanoparticles incorporated with melatonin was developed with desirable properties such as fibroblast induction, collagen deposition and promotion of angiogenesis. The formulation demonstrated4 accelerated wound closure in diabetic rats (75). A nanocomposite sponge comprised of chitosan, hyaluronic acid and nano-silver was developed against many antibiotic-resistant bacteria including methicillin-resistant *S. aureus*. The excellent antibacterial action exhibited by this nanocomposite sponge made it a suitable dressing agent for diabetic foot ulcers with mild toxicity towards mammalian cells (76). A hydrogel membrane composed of polyvinyl alcohol, starch, chitosan and nano zinc oxide was prepared and was found effective as a potent wound dressing agent in initial wound healing stages through *in vivo* studies in rats and exhibited wide-spectrum antibacterial action through *in vitro* studies (77). An injectable nanocomposite composed of curcumin, chitosan and alginate was identified as a promising wound dressing agent for wound recovery. The *in vivo* studies in rats demonstrated that the nano-curcumin based nanocomposite showed significant collagen deposition and epidermis re-epithelialization in wounds (78).

Although there is a gap in the translation of nanomedicine, the use of computer-aided analysis has become evident in this area. This has led researchers get a clear vision on the behaviour and application of nanoparticle- based therapy in reproductive biology (79), transporting drugs across biological barriers like the blood-brain barrier (80, 81). Another important aspect with respect to the design and use of nanotherapeutics is the toxicity; the understanding of which has been highly enhanced using computational biology (82). Several machine-learning approaches have been explored by the researchers that gives a magnified view of the interaction of the nanomaterial with the cells. This enables tailor-made and non-toxic application of nanomedicine to improve healthcare (83).

Conclusion and future perspective

Several impeding factors in healing chronic wounds exist using conventional treatment methods. Novel strategies have been designed to overcome them using nanotechnology has proved to be promising. The advanced biomaterials developed with cellular and acellular scaffolds in conjunction with nanomaterials of suitable nature would prove to be an efficient wound care management in diabetic ulcers. The ability to modulate and control the release of active compounds and drugs has added advantage of controlling infection in these wounds significantly shortening the stay at the hospital for patients. An in-depth analysis of the factors that promote angiogenesis and wound closure at a faster rate by the use of nanocomposite biomaterials would help in translating these products to patients. Production of such tailor-made biomaterial constructs with specific factors and design would be the desired wound treatment strategy specifically in chronic wounds. With the recent advancements in the field of artificial intelligence the design of scaffolds could be customised to evade toxicity and meet the scrupulous needs that exist in regenerative therapy. This would help researchers validate and predict the outcome of their research without the sacrifice of many animals, avoid the strain of extraneous tasks involving the toxicological assessment. So, by means of integrating artificial intelligence and the lab-scale studies will yield effective translation of nanotherapeutics in wound care management.

Author contributions

RRR and DV conceptualized the manuscript and performed the literature search and AJ and SY drafted the manuscript. RR, DU and AS reviewed and modified the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

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Combined effect of diabetes and frailty on mortality among Chinese older adults: A follow-up study

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Background: Frailty and diabetes are two important health problems associated with aging in older individuals. This paper seeks to analyze the frailty in older adults suffering from diabetes and the combined effect of diabetes and frailty on mortality risk.

Methods: The frailty index (FI) model was employed when evaluating frailty among the older adults based on the baseline data conducted in 2009; and death as outcome variables collected in 2020 were analyzed. The influence of diabetes on age-related changes in frailty in the older adults and resulting mortality rates was analyzed. Cox regression and Kaplan-Meier curves were applied to evaluate the influence on the risk of death and the 11-year survival of the older adults with varying diabetes and frailty statuses.

Results: Ultimately, 1,213 older people aged between 60 and 101, with an average age of (74.79 $\pm$ 8.58) at baseline, were included in the analysis. By 2020, there had been 447 deaths with mortality at 36.9% (447/1,213); there were 271 cases of diabetes, with a prevalence of 22.3% (271/1,213). The mean FI value for older adults with diabetes was higher than that of those without regardless of age, and the average annual relative growth rate of the FI value for older adults with diabetes was higher than that of those without diabetes (β = 0.039 *vs.* β = 0.035, t = 8.367, P < 0.001). For all FI value levels, the mortality rate among older adults with diabetes was higher than that of those without. The Cox Regression analysis showed that, compared with those suffering from neither diabetes nor frailty, older adults with both had the higher mortality risk (*HR* = 1.760. P < 0.001), followed by older adults suffering from frailty alone (*HR* = 1.594, P = 0.006), and then by older adults suffering from only diabetes (*HR* = 1.475, P = 0.033). The survival analysis showed that the median survival of those suffering from diabetes and frailty to be the shortest at just 57.23 (95% *CI*: 54.05 to 60.41) months, lower than the 83.78 (95% *CI*: 79.33 to 88.23) months in those suffering from frailty alone, and 119.93 (95% *CI*: 113.84 to 126.02) months

in those with only diabetes, and 124.39 (95% *CI*: 119.76 to 129.02) months in older adults with neither diabetes nor frailty ($P < 0.001$).

Conclusion: Frailty is common among older adults suffering from diabetes, and there is an increased risk of poor health outcomes, such as death, among older adults suffering from diabetes and frailty. When diagnosing, treating, and dealing with older adults with diabetes, attention should be paid to screening and assessing frailty in hopes of identifying it early so that appropriate measures of intervention can be taken to avoid or delay the resulting adverse effects.

KEYWORDS

frailty, diabetes, mortality, elderly, follow-up study

Introduction

The ageing of the population has been accompanied by a dramatic increase in the prevalence of diabetes (1). Diabetes is associated with a variety of complications that include cardiovascular disease, retinopathy, renal failure, and peripheral vascular disease, all of which are capable of seriously affecting quality of life in older adults. China ranks first in diabetes, with more than 140 million patients suffering from the disease in 2021, and cases expected to rise to 174 million by 2045, of which 30% are older adults (2). Studies have shown diabetes to be accompanied by complications, disability, and something known as frailty syndrome (3). Frailty syndrome is characterized clinically by declined physiological reserve, multiple system disorders, increased susceptibility to internal stress, and decreased internal stability. Diabetes has been found to be a risk factor for frailty, and the two have been shown to interact with one another: on the one hand, long-term imbalance in blood glucose regulation increases protein and fat decomposition, and decreases muscle mass and strength resulting in frailty with such manifestations as fatigue and body mass reduction, while complications from diabetes also reduce immunity and mobility, ultimately leading to the development of frailty; on the other hand, frailty threatens to change glucose-insulin metabolism, while reduced energy intake and malnutrition aggravate the risk of hypoglycemia, to say nothing of the impact on the selection and treatment of blood glucose control drugs (4, 5). In comparison to the older adults not suffering from diabetes, older adults with diabetes experience an increased risk of frailty of approximately 60% (6); in addition, older adults suffering from diabetes and frailty tend to experience serious adverse health outcomes that include higher rates of mortality, disability, and readmission and a significant decrease in daily activities (3, 7). Consequently, the early identification of frailty and subsequent interventions in patients with diabetes are important for helping to avoid or delay adverse effects.

Studies have shown frailty to be an important factor in influencing blood glucose management in older adults suffering from diabetes (8). The International Position Statement on the Management of Frailty in Diabetes Mellitus emphasizes the importance of including the identification and assessment of frailty in the routine management of patients suffering from diabetes (9, 10). In developed countries, researchers attach importance to the monitoring, evaluation, and prevention of frailty in patients with diabetes and have carried out a large number of studies on how diabetes can be complicated by frailty. In China, older adults with diabetes can be found more and more often in the community, and so the management of their health has become a focus of many community health services; however, frailty has still yet to be included in routine screening for them (11). As of now, there continue to be few studies on the situation of older adults with diabetes and frailty in Chinese communities and on the influence of diabetes with frailty on long-term risks of mortality. This study chose to focus on the older adults in the urban communities of Beijing and to analyze the prevalence of diabetes and frailty in the older adults and its influence on the risk of mortality as a basis for the management of frailty among the older adults with diabetes and corresponding measures to reduce the resulting adverse health effects.

Materials and methods

Survey sites and subjects

This is a secondary analysis of the Health Status and Fall Status Follow-up Survey database, a representative cohort of urban community dwelling elder people aged 60 years and older in Beijing. In this study, the baseline survey population in 2009 was

used as samples, and death events from this cohort collected in the follow-up survey in 2020 were used as the outcome variables. The baseline survey was conducted in 2009 in a community under the jurisdiction of a sub-district office in Dongcheng District, Beijing. In 2009, the proportion of elder people aged 60 years and older in this community was similar to that in the whole country at that time (13.9% vs. 12.5%), which could well represent the situation of the older adults in China (12). A total of 4 community neighborhood committees were randomly selected from the sub-district office, after which older subjects aged 60 years and older under the jurisdiction of the selected communities were selected on the basis of random cluster sampling for further analysis. Criteria for being included: resident individuals aged 60 years and older in the surveyed communities. Criteria for being excluded: individuals suffering from extreme frailty and unable or unwilling to complete the questionnaires. A total of 1,578 older adults met the survey requirements for 2009 as baseline samples. During the survey, 37 older adults refused to be interviewed, and 63 older adults could not be followed-up (could not be found in two visits during the survey), as a result of which 1,478 older adults were included in the end. By 2020, 232 older adults could not be followed-up, accounting for 15.7% (232/1,478), either as a result of having left or moved from the locale of the survey. Among the older adults who could not be followed-up, 108 (46.6%) were male and 124 (53.4%) were female, with an average age of (68.24 ± 3.58) years. In the end, follow-up data were available for 1,246 older adults, including 519 males (41.7%) and 727 females (58.3%), with an average age of (72.05 ± 4.52) years. Although the average age of those who could not be followed-up is lower than that of the older individuals included in the study (t = 12.148, P <0.001), the gender difference between these two population is not statistically significant (χ^2 = 1.921, P = 0.166). This study was approved by the ethics committee of Beijing Hospital (No.2020BJYYEC-134-02). All subjects signed the informed consent form.

Survey content

This study used validated standard questionnaires selected following several rounds of expert discussion to investigate the target population, covering such content as demographic characteristics (age, gender, educational level, marital status), family support (whether they lived alone and whether they enjoyed positive family relations), social support [number of friends able to offer support (help), frequency of participation in group activities)], economic level, lifestyle (smoking, alcohol consumption, exercise), health and physical performance status (vision and hearing, walking balance), diseases (including diabetes) and medications, activities of daily living (ADL) and instrumental activities of daily living (IADL) (13), cognition and emotion [memory loss, emotional instability, Mini Mental State Examination (MMSE)] (13), depression [The Center for Epidemiological Studies Depression Scale (CES-D)] (13), and comprehensive geriatric assessment (falls, urinary incontinence, pain, constipation, weight loss, sleep disorder, usage of sleep aids), etc. Diseases needed to be diagnosed by a hospital at or above the county level, while those experience symptoms deemed to be subjective and lacking definite diagnosis were not included in the statistics.

Assessment of frailty

The frailty index (FI) model developed by a team led by Professor Kenneth Rockwood, a Canadian geriatric expert, was used to quantitatively describe degree of frailty on the basis of the accumulation of health deficits (14). The FI calculation formula consists of the number of health deficits present in an individual/ the total number of items considered health deficits. The FI value ranges between 0 and 1, with larger values indicating more serious degrees of frailty (15). Based on the content of the survey questionnaires, a total of 36 variables were selected as health deficit items according to the conditions for constructing the FI health deficit variables (15), including comprehensive geriatric assessment (7 items), vision and hearing (2 items), walking balance function (6 items), disease and medication (15 items), activities of daily living (2 items), cognition and emotion (3 items), and depression (1 item). Meanwhile, each variable was assigned a value according to its type. See Appendix 1 for each specific variable and its assignment. Using the grading method recommended by Searle et al. (15), a frail individual was defined as one having a FI of 0.2 or more.

Definition of follow-up outcomes

Mortality among the follow-up survey subjects was used as outcome variable and included death (yes or no) and time to death. Information concerning death was collected or obtained by staff through the relatives of each subject, local neighborhood committees (for those without relatives), or local public security organs (for those without relatives and not belonging to any neighborhood committee). A precise method was used to calculate follow-up duration. If a subject died during the follow-up, the follow-up duration was calculated as (death date - baseline date)/12; if a subject was still alive, it was calculated as (last follow-up date - baseline date)/12.

Statistical methods

SPSS 24.0 and Matlab 2020 software were used for data analysis and plotting, and any missing data values were imputed by Markov Chain Monte Carlo (MCMC) method, a multiple imputation method (16). Shapiro-Wilk test was used to check normal distribution for continuous variables. The normally distributed

continuous variables were expressed as x ± s, an independent sample t-*test* was used for comparison between two groups, and analysis of variance (ANOVA) was used for comparison among multiple groups; non-normal variables were expressed as median and quartile *[M(Q1,Q3)]*, and Kruskal-Wallis *H* test was used for comparison among multiple groups; enumeration data were expressed in the number (or percentage) of cases, and χ^2 test was used for comparison between groups; nonlinear regression techniques were used to fit age-specific frailty index values as a function of age (an exponential function) and to fit the probability of death as a function of the frailty index (a logistic function) between older adults with and without diabetes*;* the Cox multivariate regression model was used to evaluate the hazard ratio (HR) of diabetes (yes or no) and frailty (yes or no) on the death of older adults, the Kaplan-Meier method was used to plot the survival curve to analyze the influence of different diabetes and frailty statuses on the survival time of older adults, and the log-rank method was used for testing. $P < 0.05$ was considered statistically significant.

Results

Comparison of baseline status of the older adults with or without diabetes and/or frailty

Of the 1,246 older individuals involved, 33 individuals suffering from subjective symptoms and lacking a definite diagnosis were excluded, with 1,213 subjects ultimately being included in the analysis. These 1,213 older adults were aged between 60 and 101, with an average age of (74.79 ± 8.58) at baseline, including 486 males, with an average age of (74.88 ± 8.88), and 727 females, with an average age of (74.73 ± 8.38). By 2020, there had been 447 deaths with mortality at 36.9% (447/1,213), including 198 (40.7%) males and 249 (34.3%) females - the mortality rate of males higher than that of females ($\chi^2 = 5.273$, $P = 0.022$). Of the 1,213 older individuals, 271 had diabetes, with a prevalence of 22.3% (271/1,213) for all genders, 25.1% (122/486) for males and 20.5% (149/727) for females, and without statistically significant difference between the two genders ($\chi^2 = 3.564$, $P = 0.059$); 156 had frailty, with a prevalence of 12.9%, including 43 cases in males (8.8%) and 113 cases in females (15.5%), indicating a higher proportion in females ($\chi^2 = 11.652$, $P = 0.001$*);* the prevalence of frailty in older adults with diabetes was 16.6% (45/271), higher than that among those without (11.8%, 111/942) ($\chi^2 = 4.366$, $P = 0.037$). A comparison of baselines for older individuals with or without diabetes and/or frailty showed a higher proportion of the older adults for frailty and diabetes combined with frailty in older adults, females, lower education levels, and widowed older individuals; compared to those not suffering from diabetes or frailty, those suffering from frailty and those with both diabetes and frailty trended towards having 3 or more chronic diseases and taking multiple medications, a significant decrease in activities of daily living (decreased ADL score, increased IADL score) and cognitive function (decreased MMSE score), and increased CES-D score, as well as increased mortality rate ($P < 0.05$ for all). See Table 1.

Influence of diabetes on age-related changes in frailty in the older adults

An analysis of age-related changes to FI values in the older adults with or without diabetes showed that the FI value increased exponentially with age regardless of diabetes status as expressed in the formula In (FI) = A + B× age, including In (FI) = -4.946 + 0.035 × age ($r = 0.942$, $P < 0.001$) for older adults without diabetes and In (FI) = -4.855 + 0.039 × age ($r = 0.934$, $P < 0.001$) for older adults with diabetes. The FI value was higher for older adults with diabetes than for those without diabetes, i.e., the prevalence of diabetes aggravated the degrees of frailty among the older adults. As the logarithmic coordinates demonstrate, the average annual relative age-related growth rate of health deficits and FI value in older adults with diabetes was higher than in those without diabetes ($\beta = 0.039$ *vs.* $\beta = 0.035$, $t = 8.367$, $P < 0.001$*), i.e.*, the speed of cumulative health deficits was faster for older adults with diabetes than for those without diabetes. See Figure 1.

Influence of diabetes on mortality among older adults with varying degrees of frailty

The relationship between FI value and mortality among older adults with or without diabetes was analyzed using a Logistic regression curve according to the literature (17). The results showed that the mortality among older adults with or without diabetes rose with the increase of FI value, and mortality was higher among older adult with diabetes than those without diabetes at any FI value level. An analysis of the difference in mortality rates between older adults with or without diabetes revealed a peak in the range of FI values between 0.1 and 0.3, with the difference diminishing in accordance with increasing frailty. See Figure 2.

Multivariate cox regression analysis of the influence of the presence or absence of diabetes and frailty on mortality risk among the older adults

With death and time to death as dependent variables, and adjusting for variables, including age, gender (Male = 0, Female = 1), education level (Primary high school = 1, Junior high school = 2, Senior high school or above = 3), marital status (Married or cohabiting = 1, Others = 2), it was possible to

TABLE 1 Comparision of characteristics of the sample as separated by different status of diabetes and frailty.

Variables	No diabetes and no frailty(n=831)	Diabetes and no frailty(n=226)	No diabetes but frailty(n=111)	Diabetes and frailty(n=45)	$F/x^2/H$	P-value
Age(x ± s)	73.53 ± 8.08	74.40 ± 8.74	82.27 ± 7.31	82.90 ± 6.12	51.624	<0.001
Sex[n(%)]					14.574	0.002
Men	336 (40.4)	108 (46.6)	28 (25.2)	14 (35.9)		
Women	495 (59.6)	124 (53.4)	83 (74.8)	25 (64.1)		
Education[n(%)]					89.572	<0.001
Primary school	257 (30.9)	72 (31.0)	79 (71.2)	26 (66.7)		
Junior high school	237 (28.5)	74 (31.9)	17 (15.3)	5 (12.8)		
Senior high school or above	337 (40.6)	86 (37.1)	15 (13.5)	8 (20.5)		
Marital Status [n(%)]					44.788	<0.001
Married or cohabiting with spouse	605 (72.8)	159 (68.5)	49 (44.1)	19 (48.7)		
Others[a]	226 (27.2)	73 (31.5)	62 (55.9)	20 (51.3)		
Employment status [n(%)]					3.011	0.390
Working	13 (1.6)	5 (2.2)	0 (0.0)	0 (0.0)		
Retied	818 (98.4)	227 (97.8)	111 (100.0)	39 (100.0)		
≥ 3 types of Chronic Diseases	297 (35.7)	101 (43.5)	64 (57.7)	30 (76.9)	43.708	<0.001
Types of Medication [n(%)]					70.497	<0.001
0	229 (27.6)	60 (25.9)	7 (6.3)	1 (2.6)		
1-3	538 (64.7)	154 (66.4)	75 (67.6)	28 (71.8)		
≥4	64 (7.7)	18 (7.8)	29 (26.1)	10 (25.6)		
ADL Score *[M(Q1,Q3)]*	100 (100,100)	100 (100,100)	90 (65,100)	75 (50,95)	626.120	<0.001
IADL Score *[M(Q1,Q3)]*	0 (0,0)	0 (0,0)	3 (0,8)	5 (3,11)	497.485	<0.001
MMSE Score *[M(Q1,Q3)]*	26 (22,28)	24 (21,26)	23.00 (19.50,24.25)	20 (18,24)	63.496	<0.001
CES-D Score *[M(Q1,Q3)]*	5 (2,8)	5 (2,9)	8.5 (5.0,13.5)	13 (8,21)	147.992	<0.001
FI *[M(Q1,Q3)]*	0.08 (0.05,0.11)	0.09 (0.06,0.13)	0.27 (0.23,0.33	)0.31 (0.24,0.37)	405.930	<0.001
N of Death[n(%)]	259 (31.2)	87 (37.5)	72 (64.9)	29 (74.4)	72.588	<0.001

ADL, Activities of daily living; IADL, Instrumental activity of daily living; MMSE, Mini-mental status examination; CES-D, Center for epidemiologic studies depression scale; FI, frailty index; [a]Including single, separated, divorce and widowed.

ascertain from the statistical results that the mortality risk in the older adults increased with age, while the mortality risk in women and older adults who were married or cohabiting was lower than that in the control group. Moreover, compared with the older adults without diabetes and frailty, the highest mortality risk was discovered among those with diabetes and frailty (*HR* = 1.760, 95%*CI:* 1.622 to 1.909, *P* <0.001), followed by those with only frailty (*HR* = 1.594, 95%*CI*: 1.143 to 2.222, *P* = 0.006), and then those with only diabetes (*HR* = 1.475, 95% *CI:*1.238 to 2.766, *P* = 0.033). Furthermore, statistical results by age group revealed that the influence of frailty or diabetes alone on the mortality risk decreased gradually with age, with no statistically significant influence on death in the older adults aged 70- and ≥80 years (all *P* > 0.05). However, the mortality risk among older adults with diabetes and frailty did increase in all age groups (*P* <0.001). See Table 2.

Comparison of survival curves for older adults with or without diabetes and frailty

The survival analysis showed that the median survival of those suffering from diabetes and frailty to be the shortest at

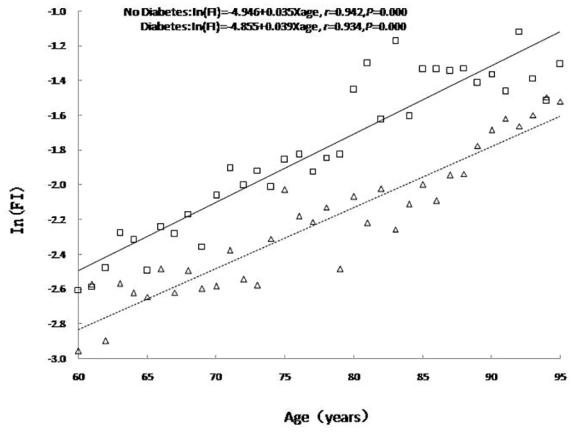

FIGURE 1
The relationship between age and the mean value of FI. No diabetes: triangle and dashed line; Diabetes: square and solid line.

just 57.23 (95% *CI:* 54.05 to 60.41) months, lower than the 83.78 (95% *CI:* 79.33 to 88.23) months in those suffering from frailty alone, and 119.93 (95% *CI:* 113.84 to 126.02) months in those with only diabetes, and 124.39 (95% *CI:* 119.76 to 129.02) months in the older adults with neither diabetes nor frailty (P <0.001). A further comparison of survival curves of older adults s with or without diabetes and frailty in different age groups revealed that the survival rates of older adults in the 60-, 70-, and ≥80-year-old age groups decreased with the prevalence of diabetes and frailty (all P<0.001). The results of a pairwise comparison of the survival rate of older adults with different diabetes and frailty status in all age groups showed statistically significant differences, only except for the survival rate between those with only diabetes and with neither diabetes nor frailty in the ≥ 80-year-old age group (P = 0.346). See Figures 3–6.

Discussion

Frailty and diabetes are two important health problems associated with aging in older adults. Meanwhile, both conditions frequently co-occur and are increasingly prevalent among older adults. The results of this study revealed that the prevalence of diabetes among older adults in Beijing was 22.3%, which was consistent with the results of the Chinese diabetes survey. In the survey by Li et al. (2) and Wang et al. (18), the prevalence of diabetes in the Chinese population aged 60 years or more was 20.9% and 30.0% in 2013 and 2017, respectively, with an awareness rate of approximately 30.0%. Comparison at baseline in this study showed that older adults, females, individuals with lower education levels, and widowed older adults were associated with a higher proportion of frailty and diabetes with frailty. Among older adults with diabetes and frailty, there was an increased proportion with 3 or more chronic diseases and multiple medications, a significant decrease in the activities of daily living and cognitive functions, and an increase in depression scores, all of which were consistent with previous studies (19–21). Therefore, targeted intervention would be desirable in the management of older adults with diabetes and frailty to delay the course of diseases, relieve harm resulting from comorbidities, reduce the risk of adverse outcomes, and improve the level of diabetes management overall.

The results of this study revealed that the prevalence of frailty in older adults with diabetes was 16.6%, which was higher than in those without diabetes (11.8%). Moreover, the FI value of older adults with diabetes was higher than that of those without diabetes at any age, *i.e.*, the prevalence of diabetes aggravated the degrees of frailty in older adults. As the logarithmic coordinates demonstrate, the average annual relative growth rate of health defects and FI value over age in older adults with diabetes was higher than among older adults without diabetes (β= 0.039 *vs.*

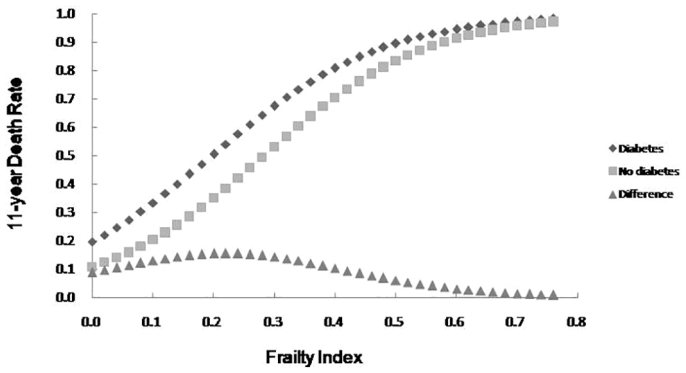

FIGURE 2
The 11-year death rate as a function of the FI and the mortality difference between older adults with diabetes and no diabetes.

TABLE 2 Multivariate Cox regression analysis of the impact of diabetes and frailty on mortality in the older adults of different agegroups.

Variables	Overall(n=1213)			60-(n=385)			70-(n=421)			≥80(n=407)		
	β-value	HR(95%CI)	P-value	β-value	HR(95%CI)	P-value	β-value	HR(95%CI)	P-value	β-value	HR(95%CI)	P-value
Age	0.049	1.050 (1.044~1.057)	<0.001	0.078	1.081 (1.053~1.110)	<0.001	0.045	1.046 (1.024~1.068)	<0.001	-0.003	0.997 (0.956~1.040)	0.896
Sex	-0.324	0.723 (0.663~0.788)	<0.001	-0.483	0.617 (0.520~0.731)	<0.001	-0.309	0.734 (0.646~0.834)	<0.001	-0.145	0.865 (0.734~1.020)	0.085
Education	-0.050	0.951 (0.885~1.022)	0.170	-0.106	0.900 (0.788~1.027)	0.118	-0.080	0.923 (0.831~1.025)	0.136	-0.099	0.906 (0.855~1.276)	0.179
Marital Status	0.310	1.363 (1.211~1.533)	<0.001	0.313	1.368 (1.149~1.628)	<0.001	0.234	1.263 (1.054~1.515)	0.012	0.455	1.576 (1.075~2.310)	0.020
Status of diabetes and frailty[a]												
Diabetes and no frailty	0.389	1.475 (1.238~2.766)	0.033	0.275	1.617 (1.120~4.654)	0.018	0.478	1.512 (0.667~3.897)	0.289	0.586	1.497 (0.444~7.268)	0.411
No diabetes but frailty	0.466	1.594 (1.143~2.222)	0.006	0.626	1.871 (1.150~3.044)	0.012	0.499	1.647 (0.949~2.857)	0.076	0.142	1.152 (0.511~2.599)	0.733
Diabetes and frailty	0.565	1.760 (1.622~1.909)	<0.001	0.657	1.929 (1.644~2.262)	<0.001	0.602	1.826 (1.624~2.054)	<0.001	0.403	1.496 (1.284~1.743)	<0.001

FI,frailty index; [a] No diabetes and no frailty as a reference.

β= 0.035), *i.e.*, the speed of cumulative health defects was greater when diabetes was present. As reported in the study of Kong et al. (6), the overall prevalence of older adults with diabetes experiencing conditions of frailty and pre-frailty in Chinese communities was 20.1% and 49.1%, respectively, with older adults suffering from diabetes more likely to develop frailty than those without diabetes (*OR* = 1.61, 95% *CI*: 1.47-1.70, $P < 0.001$). The Beijing Longitudinal Study of Aging II (BLSA-II) (22) revealed that the prevalence and incidence of frailty in older adults with diabetes were significantly higher than in those without diabetes (19.32% *vs.*11.92%, and 12.32% *vs.* 7.04%). Patients with diabetes at 65 years or older are more prone to frailty than those without diabetes. Angulo et al. (23) claimed the co-occurrence of diabetes and frailty in older adults not to be surprising as both age-related conditions share a common underlying pathophysiological mechanism, which may include premature aging of the organ system in a hyperglycemic state, chronic inflammation, increased oxidative stress, accumulation of advanced glycation end products, and insulin resistance (24). In recent years, some achievements have been made in the exploration of the common mechanism of diabetes and frailty at

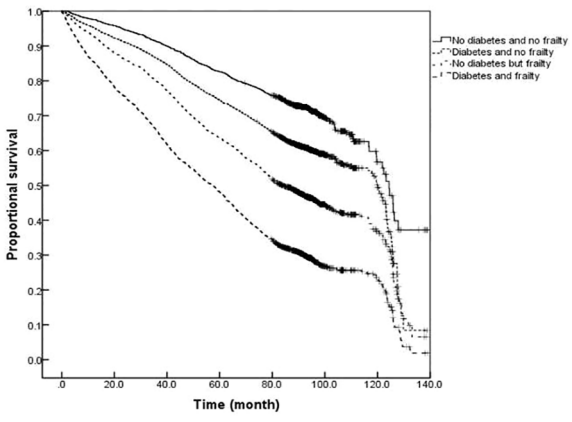

FIGURE 3
Kaplan-Meier curves for the proportional survival of total population with different diabetes and frailty status.

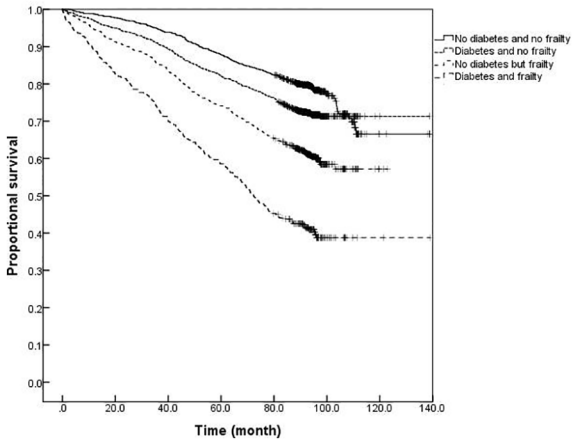

FIGURE 4
Kaplan-Meier curves for the proportional survival of older adults aged 60- years with different diabetes and frailty status.

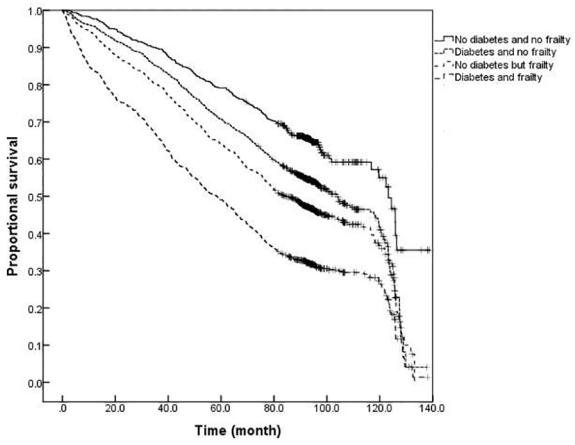

FIGURE 5
Kaplan-Meier curves for the proportional survival of older adults aged 70- years with different diabetes and frailty status.

various levels of genes, protein molecules, cells, tissues, and organs, mainly including insulin resistance, arteriosclerosis, chronic inflammation, oxidative stress, cell damage, and mitochondrial dysfunction among other theories. For example, C-reactive protein and interleukin-6, typical inflammatory factors, were present at a high level in patients with diabetes and frailty (25). Amino acid metabolism disorders may be a common pathway for dysfunction in patients with diabetes and frailty. Calvani et al. (26) investigated the amino acid metabolism profile of older adults with diabetes and frailty and discovered that the levels of some characteristic metabolites such as serum 3-methylhistidine were higher. A structural magnetic resonance study found that, in patients with

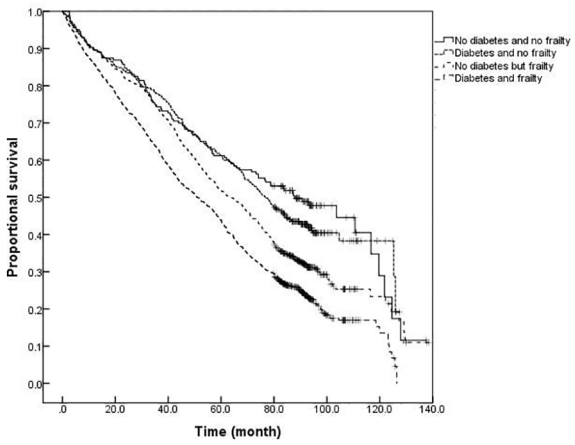

FIGURE 6
Kaplan-Meier curves for the proportional survival of older adults aged ≥80 years with different diabetes and frailty status.

diabetes and frailty, the decreased size of gray matter involved in motor control was linked to decreased muscle size and strength (27). In addition, metabolites of the intestinal microbiota and peripheral inflammation affected the decomposition and synthesis of muscle proteins through various signal pathways regulated by inflammation and insulin sensitivity, which also indirectly impacted food intake, resulting in decreased protein synthesis and body frailty (28).

The results of this study also demonstrated that mortality was higher among older adults with diabetes than those without diabetes at any degree of frailty. The difference in mortality between older adults with or without diabetes peaked in the range of FI values between 0.1 and 0.3, with the difference gradually narrowing with an increasing degree of frailty. In order to further illustrate the influence of diabetes and frailty on the mortality risk, the Cox regression analysis was performed in this study after adjusting for confounding factors, such as age, gender, and education level, and the results revealed the highest mortality risk among older adults with diabetes and frailty. Further statistical results by age group presented that the mortality risk for older adults with diabetes and frailty was increased in all age groups and exerted a greater influence on the mortality risk than for those suffering from only frailty or diabetes. A follow-up study found frailty to be helpful when seeking to identify diabetic patients at high risk of mortality (29). Patients with diabetes and frailty also suffered high rates of hospitalization and all-cause mortality (30). Combined with the results of this study, it is suggested that healthcare professionals should pay greater attention to the screening and assessment of frailty conditions in older adults with diabetes, especially among those with pre-frailty or mild frailty (FI value: 0.1- 0.3) who can receive more benefit. Japanese scholars have suggested that the management of older adults with diabetes should shift its focus from the prevention of metabolic syndrome to the prevention of frailty (31). It has also been proposed that the assessment of frailty should be conducted in all older adults with diabetes as early identification of frailty, assessment of its degree, and timely intervention can greatly delay the progression of diabetes and related complications in the management of diabetes (32). At present, the frailty assessment scale for the general population is still used in the assessment of frailty in individuals with diabetes, while the FI model was adopted to assess the frailty of the older adults in this study. The FI model is currently one of the most commonly used methods for the assessment of frailty in the older adults, and several studies have confirmed its satisfactory reliability and validity (33, 34). Additionally, the results of the systematic review study have suggested that FI is the only assessment tool capable of covering all frailty-related factors, and FI is also the most useful assessment tool for frailty in conventional care and community settings (34). Consequently, we employed the FI model to assess the frailty of the older adults in the community. Furthermore, the Comprehensive Frailty Assessment Instrument (CFAI) and the Tilburg Frailty Index have both been recommended for the

screening of early frailty in the older adults with chronic diseases such as diabetes and hypertension in primary care institutions, and at the same time, the Frailty Index (FI-CGA) based on the Comprehensive Geriatric Assessment can better assess the comprehensive conditions of hospitalized patients and quantify the degrees of frailty, all of which are of great significance in guiding diabetic patients in blood glucose control and drug selection in relation to degrees of frailty. As regards the management of older adults with diabetes and frailty, studies have disclosed that frailty, as an unfavorable factor causing severe hypoglycemia in diabetic patients experiencing intensive glycemic control treatment, is capable of compromising the efficacy of intensive treatment. For instance, Nguyen et al. (8) observed patients with type 2 diabetes who received intensive glucose-lowering therapy and found that the incidence of severe hypoglycemia in patients with frailty was significantly higher than that in those without frailty. Hence, the Expert Consensus Statement on the Management of Older Adults with Type 2 Diabetes recommends that the glycosylated hemoglobin (HbA1c) be controlled at 6%-7.5% in healthy and mild frailty patients, appropriately relaxed to 8.0% in moderate frailty patients, and not more than 9.0% in severe frailty patients, such as loss of independence or a combination with serious complications (32). Besides, the American Diabetes Association Professional Practice Committee (35) recommends that the target value of HbA1c control be relaxed to 8.0% in patients with varying degrees of functional dependence, and overall, a looser target value of glucose control is suggested for patients with diabetes and frailty after sufficient assessment of individual conditions (35). Furthermore, the results of the survival analysis in this study indicated that patients with diabetes and frailty suffered the shortest survival time, followed by those only with frailty, those only with diabetes, and those without diabetes and frailty, which further verified the conclusion of the regression model for the mortality risk.

The following limitations of this study should be noted. Baseline data was acquired based on a questionnaire survey and some key confounders (such as comorbidities) were not included, with a potential for information bias. In addition, as a prospective study, older adults lost to follow-up were relatively young individuals who had left or moved away from the place of the survey, and the possible loss to follow-up bias may have a certain impact on the study results. Moreover, the causes of death in the older adults were not collected in this study, and the impact of other causes of death on the study results cannot be excluded, so it would be necessary to further improve the questionnaire and supplement related information for a more profound analysis in the future.

Conclusions

Frailty is common among older adults suffering from diabetes, and there is an increased risk of poor health outcomes, such as death, among older adults suffering from diabetes and frailty. Given the interaction between diabetes and frailty, it would be advisable to strengthen our knowledge of frailty, promote the assessment of frailty, identify it early, and apply targeted interventions during the diagnosis and management of older adults with diabetes, so as to avoid or postpone the adverse effects caused by frailty and ease the medical burden.

Data availability statement

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

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JS conducted the survey, performed statistical analysis the data and drafted the paper. YT and LW helped with the analysis and assisted with manuscript preparation. SC, ZZ, LM and BZ conducted the survey and collected the data. CD assisted data analysis and result interpretation. HX and PY initiated and designed the study, revised the paper and finally approved the version to be published. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1105957/full#supplementary-material

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Clinical characteristics and genetic analysis of a Chinese pedigree of type 2 diabetes complicated with interstitial lung disease

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Purpose: Diabetes mellitus is a systemic metabolic disorder which may target the lungs and lead to interstitial lung disease. The clinical characteristics and mechanisms of type 2 diabetes mellitus (T2DM) complicated with interstitial lung disease (ILD) have been studied. However, little work has been done to assess genetic contributions to the development of T2DM complicated with ILD.

Method: A pedigree of T2DM complicated with ILD was investigated, and the whole genome re-sequencing was performed to identify the genetic variations in the pedigree. According to the literature, the most valuable genetic contributors to the pathogenesis of T2DM complicated with ILD were screened out, and the related cellular functional experiments were also performed.

Results: A large number of SNPs, InDels, SVs and CNVs were identified in eight subjects including two diabetic patients with ILD, two diabetic patients without ILD, and four healthy subjects from the pedigree. After data analysis according to the literature, *MUC5B* SNP rs2943512 (A > C) was considered to be an important potentially pathogenic gene mutation associated with the pathogenesis of ILD in T2DM patients. *In vitro* experiments showed that the expression of MUC5B in BEAS-2B cells was significantly up-regulated by high glucose stimulation, accompanied by the activation of ERK1/2 and the increase of IL-1β and IL-6. When silencing *MUC5B* by RNA interference, the levels of p-ERK1/2 as well as IL-1β and IL-6 in BEAS-2B cells were all significantly decreased.

Conclusion: The identification of these genetic variants in the pedigree enriches our understanding of the potential genetic contributions to T2DM complicated with ILD. *MUC5B* SNP rs2943512 (A > C) or the up-regulated MUC5B in bronchial epithelial cells may be an important factor in promoting ILD inT2DM patients, laying a foundation for future exploration about the pathogenesis of T2DM complicated with ILD.

KEYWORDS

type 2 diabetes mellitus, interstitial lung disease, MUC5B, pedigree, whole genome re-sequencing

1 Introduction

Diabetes mellitus (DM) is a systemic metabolic disorder characterized by chronic hyperglycemia due to insulin deficiency or resistance (1). The long-term effects of DM include neurological, micro-vascular and macro-vascular complications. The lungs are particularly susceptible targets of diabetic micro-vascular damage and non-enzymatic glycation as a result of their large alveolar-capillary network and abundant connective tissue. Diabetic patients frequently report respiratory symptoms, and diabetes related lung injuries have been observed in several studies (2, 3). Recently, epidemiological studies have suggested that type 2 diabetes (T2DM) is an independent risk factor for interstitial lung disease (ILD) (4–6).

Pulmonary function tests of patients with T2DM show restrictive ventilatory dysfunction and decreased diffusion capacity, including a reduction in forced expiratory volume in one second. High-resolution computed tomography (HRCT) images of the lungs from patients with T2DM are prone to show fibrotic pathological changes, such as a usual interstitial pneumonia pattern (7). However, there is still not enough HRCT data in patients with T2DM to generalize these results. Pathologically, nodular deposition of collagen in the middle of the alveolar walls, and increased thickness of the alveolar epithelial and endothelial capillary basal lamina have been reported to be the features of lung tissue in diabetic patients and animal diabetic models (8–10). A previous study reported significant increases in the cytokines in the bronchoalveolar lavage fluid of patients with T2DM compared to the controls (11), indicating that T2DM could induce inflammation which might promote pulmonary interstitial changes in the lungs. Altogether, clinical and experimental data from numerous studies have revealed that hyperglycemia or T2DM could promote the development of ILD. However, the pathogenic mechanisms involved in the association between T2DM and ILD haven't been well understood. Previous studies considered unbalanced oxidative stress (12–14), overproduction of advanced glycation end-products (AGEs) and their receptors (15–18), epithelial to mesenchymal transition (EMT) (7, 19, 20), endoplasmic reticulum (ER) stress (21–24), and defects in bronchiolar surfactant layer (25–27) as mechanisms underlying T2DM complicated with ILD. In genetics, abundant variants associated with DM have been identified by genome-wide association studies (GWAS) (28, 29). Additionally, the genetic contributors to the development of ILD have also been identified, especially in cases of familial interstitial pneumonia (FIP) (30). However, it has not yet been determined about the genetic variations accounting for the development of ILD in T2DM.

In this study, a pedigree with T2DM complicated with ILD was investigated, and the whole genome re-sequencing of eight subjects from the pedigree was performed respectively to identify the genetic variations. After the analysis of genetic data based on literature, we proposed a non-synonymous single nucleotide variant (A>C; rs2943512) in *MUC5B* gene or the over-expressed MUC5B in bronchial epithelial cells might be an important factor in promoting ILD in T2DM patients. To our knowledge, this study is the first one presenting potential pathogenic genetic variants in a pedigree of T2DM complicated with ILD.

2 Materials and methods

2.1 Subject recruitment and information

The subjects in this study were recruited from a pedigree with T2DM and interstitial lung disease in the Jilin Province of China (Figure 1). T2DM was diagnosed with the following criteria established by the American Diabetes Association: fasting plasma glucose concentration ≥ 126 mg/dl (7.0 mM), 2-hour post-load plasma glucose ≥200mg/dl (11.1 mM) after the oral glucose tolerance test, history of T2DM and/or on prescribed medication for diabetes. In addition, the tests of antibodies for T1DM were negative in each subject. Interstitial lung disease was collectively diagnosed by two radiologists and three respiratory physicians. Inclusion criteria for the subjects were as follows: 1) absence of systemic and metabolic disease other than obesity and T2DM; 2) absence of malignancy, infection, hepatic diseases, renal diseases, neurological diseases, cardiovascular events and endocrine dysfunction; and 3) absence of history of drug or alcohol abuse, defined as >80 g/day in men and >40 g/day in women. Recruited subjects are tagged in Figure 1. All subjects were informed of the purpose of the study and signed the consent. This study was approved by the Ethics Committee of the Second Hospital of Jilin University. Plasma of these subjects was withdrawn and stored at −80°C until analysis.

2.2 Genetic testing and data analysis

Whole-genome re-sequencing was performed in the HiSeq X ten PE150 NovaSeq 6000 system in Shanghai Genechem Company Limited on genomic DNA to identify copy number variations (CNVs), single nucleotide polymorphisms (SNPs), insertion-deletion (InDels), and chromosomal structural variations (SVs) in eight subjects from this pedigree. All data were processed using FastQC software developed by Babraham Bioinformatics. Sequence alignment was performed using Burrows-Wheeler Aligner (BWA) software (31). HaplotypeCaller software was applied for mutation detection (32). Variants were identified through SNV calling, SV calling and SNP calling. Annovar software was used to annotate any detected mutations (19).

2.3 Gene function enrichment analysis

Genes affected by CNVs, SVs, SNPs or InDels were selected for annotation by comparing with the reference genome. Genes or the corresponding proteins were uploaded to FunRich (Version 3.1.4) for GO classification, including cell component (CC), biological process (BP), molecular function (MF) and biological pathway enrichment analysis. The go-CC, GO-BP, GO-MF and biological pathway with $P < 0.05$ were identified as significant. Meanwhile, proteins were mapped using the online Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org/version11.5) to construct the PPI network and identify possible relationships between proteins. The PPI network was constructed by setting the minimum required interaction score to medium confidence (0.4). The active interaction

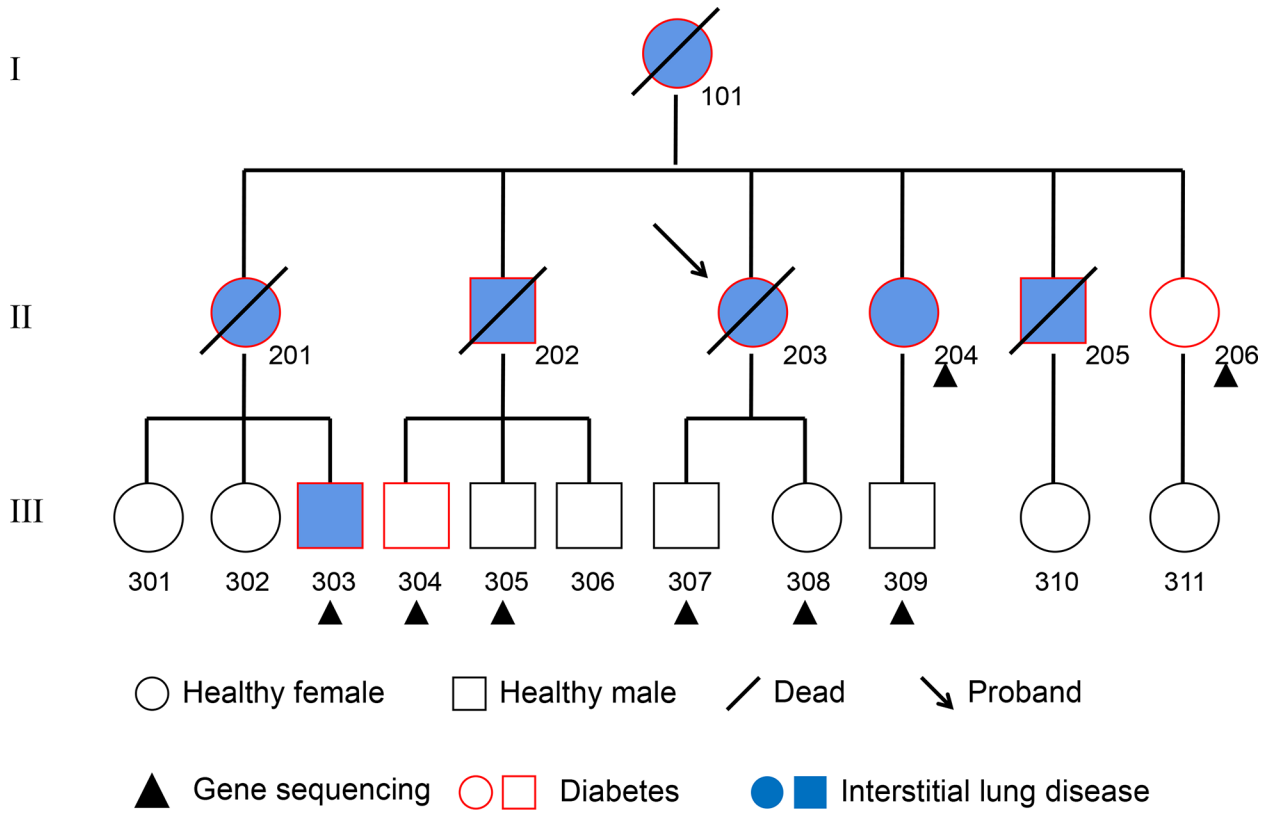

FIGURE 1
Pedigree Chart. The 8 subjects from the second and third generation who received whole-genome re-sequencing are marked with a black triangle. Squares represent males, circles represent females. The subjects affected by pulmonary fibrosis are painted blue, and the subjects affected by T2DM are indicated with a red border. Deceased individuals are labeled with a black slash.

sources included were "texming", "experiments", "database", "co-expressing", "neighborhood", "genefusion" and "co-occurence".

2.4 Cell culture and treatment

Human bronchial epithelial cells(BEAS-2B) were from National Collection of Authenticated Cell Cultures (Shanghai, China). The cells were cultured in RPMI 1640 medium (ThermoFisher Scientific, USA) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS; Hyclone Laboratories, USA). The cells were grown at 37°C in 5% CO_2 fully humidified air and were subcultured twice weekly. The cells were seeded in wells of a 6-well plate at 1×10^5 cells/well. When growth was confluent, the cells were incubated in RPMI 1640 medium containing certain concentrations of D-Glucose (Sigma-Aldrich, USA) for the indicated times. The cell proliferation and viability of BEAS-2B cells was quantified by CCK-8 Kit (Beyotime Biotechnology, China).

2.5 Cell viability assays

BEAS-2B cells were seeded into a 96-well plate at $1x10^4$ cells/well with 100 μl of 10% FBS RPMI1640 medium. After overnight incubation, the complete medium containing different concentrations of glucose (15 mM, 20 mM, 25 mM, 30 mM) replaced the original medium of each group for 12h, 24h, 48h and 72h. Then, 10 μl of CCK-8 solution was added to the medium of each group. After the cells were incubated in the dark at 37°C for an additional 1 h, the absorbance at a 450nm wavelength was detected. Then cell viability of each group was calculated.

2.6 RT-PCR analysis of MUC5B mRNA

Isolation of total RNA from the cultured cells was performed according to the manufacturer's instructions of cell total RNA isolation kit (ForeGene, China). Each sample was reverse transcribed into cDNA using the Prime Script RT Regent Kit (Takara, Japan). The primer sequences used in the PCR were 5′-GCCCACATCTCCACC TATGAT-3′ (sense) and 5′-GCAGTTCTCGTTGTCCGTCA-3′ (antisense) for *MUC5B*. Real-time PCR was performed with the SYBR Green Realtime PCR Master Mix Kit (Solarbio, China). Data were normalized versus *GAPDH*. According to the *Ct* values, the expression of *MUC5B* relative to *GAPDH* was calculated by using $2^{-\Delta\Delta Ct}$ formula.

2.7 Enzyme-linked immunosorbent assay

The protein levels of MUC5B, IL-1β and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA). The standard

substance and samples of cell supernatants were prepared, and incubated at 37°C in the 96-well plate for 2 hours. The standard substance and samples were discarded, and then the plate was blocked with biotin-labeled antibody for 1 hour at 37°C. Wells were then washed three times with the washing buffer, and horseradish peroxidase(HRP)-conjugated secondary antibody was added to wells. 1 hour later at 37°C, wells were washed three times with the washing buffer, and the substrate solution was added to wells, followed by the incubation at 37°C for 20min from light. Color was developed using stopping solution. Optical densities were measured using an ELISA reader (BioTek Instruments, USA) at 450 nm and 570nm. According to the standard curve, the corresponding concentration of each sample was calculated.

2.8 Western blot

Human BEAS-2B bronchial epithelial cells were seeded in a 6-well plate and treated with glucose for the indicated times and concentrations. The cells were then washed with cold PBS, and then exposed to the cold lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and bromophenol blue). The proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The memebrane was then blocked with 5% nonfat dry milk in 25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20, and then incubated with the indicated primary antibody of p-JNK (#4668, Cell Signaling Technologies, USA), p-ERK1/2 (#4370, Cell Signaling Technologies, USA), ERK1/2 (#4695, Cell Signaling Technologies, USA), p-p38 (#4511, Cell Signaling Technologies, USA), p-IκBα (#2859, Cell Signaling Technologies, USA), and β-actin (#4970, Cell Signaling Technologies, USA), JNK (#9252, Cell Signaling Technologies, USA), P38 (#9212, Cell Signaling Technologies, USA), IκBα (#4812, Cell Signaling Technologies, USA) overnight at 4°C. Subsequently, the membrane was washed and incubated for 2 hour with secondary antibody conjugated to HRP (#7074,Cell Signaling Technologies, USA). Finally, the membrane was developed using an chemiluminescence reagent kit (Bio-Rad, USA) and exposed to the imager.

2.9 Cell transfection with shRNA for MUC5B

The *MUC5B* shRNA knockout plasmid (Psuper-MUC5B-SH) and the negative control shRNA-NC were designed and synthesized by Jiangsu Cencefe Co., Ltd. ShRNA sequence was designed according to the target gene sequence (5'-GGGAAGTCATCT ACAATAAGACC-3') as follows: Top-Bgl II(60bp) 5'-gatccccGAAGTCATCTACAATAAGATTCAAGAGATCTTAT TGTAGATGACTTCTTTTTa-3'; Bottom-Xho I(60bp), 5'-tcgat AAAAAGAAGTCATCTACAATAAGATCTCTTGAATC TTATTGTAGATGACTTCggg-3'.Plasmid transfection was carried out using the transfection reagent Lipofectamine 2000 (Invitrogen, USA), and the procedure was as follows according to the kit instruction (Invitrogen). Briefly, BEAS-2B cells were seeded in wells of a 6-well plate at 2×10^{5} cells/well and incubated in RPMI 1640 medium. When the cells were confluent to 80%, MUC5B shRNA and Lipofectamine 2000 were incubated together in RPMI 1640 medium without serum to form a MUC5B shRNA-Lipofectamine complex. After the cells were washed with PBS, the complex-containing medium was then added to each well. After 48 hours of transfection with MUC5B shRNA, the cells were harvested for RT-PCR analysis of MUC5B mRNA. The same procedure was performed with control shRNA.

2.10 Statistical analysis

The results were expressed by mean ± standard deviation, analyzed and plotted by GraphPad Prism 6. The data were normally distributed by Pearson test. Comparisons were made using the Student *t*-test between two groups, one-way ANOVA test between multiple groups. Student-Newman-Keuls *post hoc* test was applied. For all tests, *P*-value less than 0.05 was considered statistically significant.

3 Results

3.1 Clinical data

The introduction of this pedigree:

NO.203, the proband of the pedigree, female, 61 years old, homemaker. In December 2015, the patient was first admitted to the outpatient department of our hospital for dyspnea, and diagnosed as "ILD" (Figure 2A) and "T2DM". However, the patient did not follow the doctor's advice for treatment. Since then, her dyspnea had been progressively aggravated. In May 2016, the patient was admitted to the inpatient department for severe dyspnea. Past medical history: coronary heart disease for 20 years, T2DM for 30 years. She denied the history of long-term smoking, and the exposure to special drugs, dust and poison. Her deceased mother also suffered from T2DM and ILD. Physical examination on admission: tachypnea, cyanosis, right jugular vein swelling, crackles rale (Velcro) in both lower lungs by auscultation. Arterial blood gas analysis: pH 7.35, PCO2 35mmHg, PO2 57mmHg, SaO2 86%. Sinus tachycardia, pulmonary P wave and right ventricular high voltage could be detected in her electrocardiogram. Right ventricular hypertrophy, severe pulmonary hypertension, and left ventricular ejection fraction 70% were shown in her color doppler echocardiography. Chest CT images were shown in Figure 2B. Laboratory tests: WBC 11.0×10^{9}/L, neutrophil percentage 80%, hemoglobin 170g/L, platelet was normal in the blood routine test; the urine routine, liver and kidney function tests, D-dimer, procalcitonin, and fungal-D-glucan were all at normal level; brain natriuretic peptide (BNP) 126pg/mL; the antinuclear antibody spectrum, anti-neutrophil cytoplasmic antibodies, serum complements, rheumatoid factor, cyclic citrullinated peptide, immuno-globulins and anti-cardiolipin antibody were all negative or normal. Besides, no abnormalities were found in the tumor markers. The patient was given antibiotics and intravenous glucocorticoid, however, her condition improved slightly. Unfortunately, the patient died at home in late 2016.

NO.101, who had been deceased when the pedigree was investigated, suffered from T2DM and ILD.

NO.201, who had been deceased at the age of 38, suffered from T2DM and ILD.

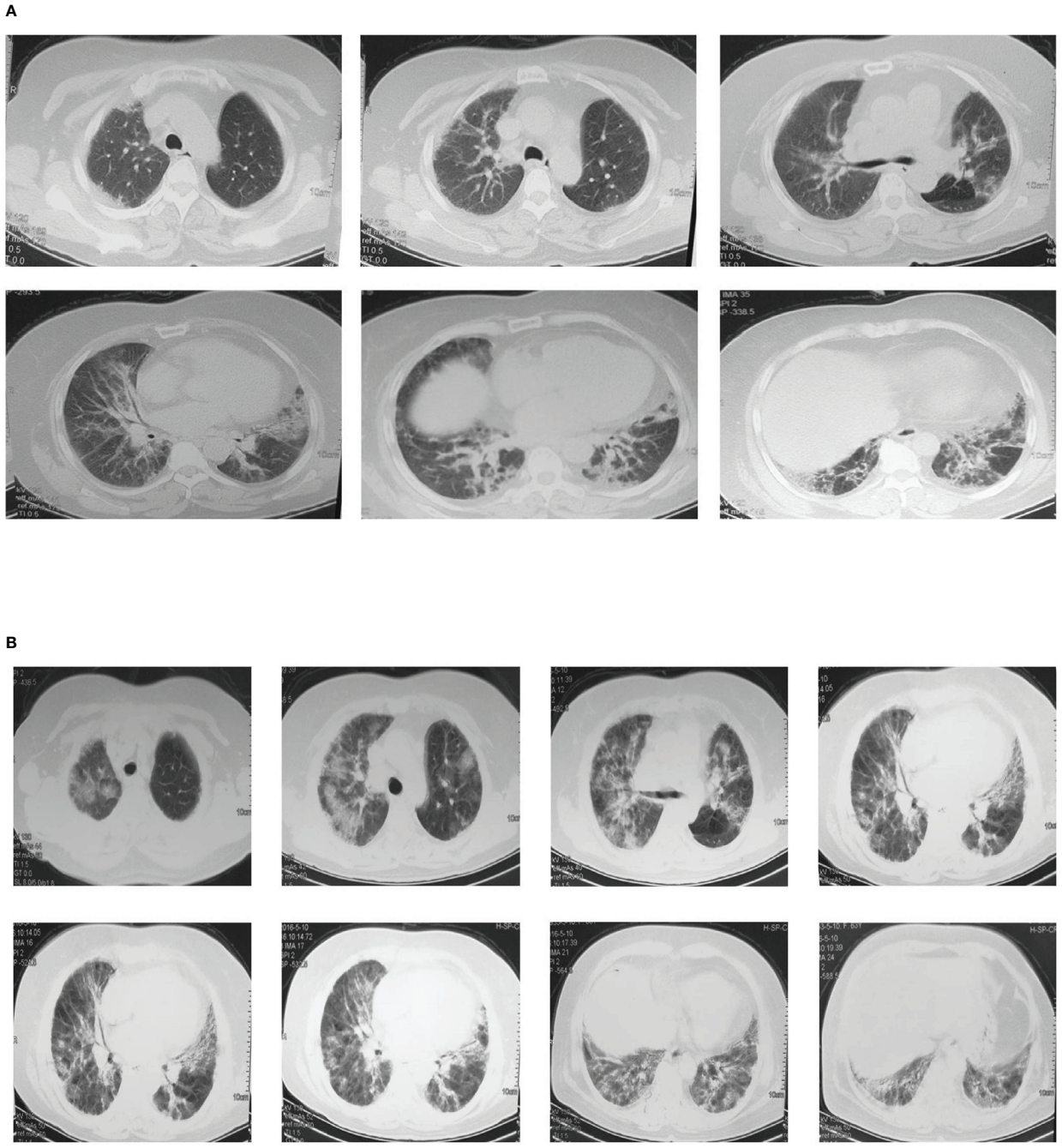

FIGURE 2
Chest CT images of the proband. **(A)** Multiple ground-glass and grid shadows distributing around the lungs and under the pleura were found in both lungs In December 2015. **(B)** Multiple patchy shadows, grid shadows and cable shadows were diffusely distributed in both lungs, and some of the shadows fused into large areas in May 2016.

NO.202, who had been deceased at the age of 40 when the pedigree was investigated, suffered from T2DM and ILD.

NO.204, who was still alive when the pedigree was investigated, had a history of T2DM for 8 years and ILD for 2 years. The patients once received more than 1 year of oral glucocorticoid treatment, which had been stopped when the pedigree was investigated. The insulin was still regularly used to treat her diabetes.

NO.205, who had been deceased at the age of 53 when the pedigree was investigated, suffered from T2DM and ILD.

NO.206, who had a history of T2DM for 5 years and denied any other diseases, was receiving insulin treatment when the pedigree was investigated. No abnormal changes showed in her chest HRCT images.

NO.303, who had a history of T2DM for 5 years, ILD for 1 year, was receiving regular oral glucocorticoid and insulin treatment when the pedigree was investigated.

NO.304, who had a history of T2DM for 6 years and denied any other diseases, was receiving regular insulin treatment when the

pedigree was investigated. No abnormal changes showed in his chest HRCT images.

NO.307, 308, 301, 302, 311, 305, 306, 309 and 310: They didn't have diabetes, cough, dyspnea and other respiratory symptoms. No abnormal changes showed in their chest HRCT images.

According to the inclusion criteria and informed consent, NO.204 (the proband's younger sister), NO.206 (the proband's youngest sister), NO.303 (the son of the proband's eldest sister), NO.304 (the eldest son of the proband's eldest brother), NO.305 (the second son of the proband's eldest brother), NO.307 (son of the proband), NO.308 (daughter of the proband) and NO.309 (the son of the proband's younger sister) of the pedigree map were included in this study. DNA was extracted from the blood of each subject, and the eight samples were re-sequenced to obtain the data of CNVs, SVs, SNPs and InDels from each sample.

3.2 Genetic variation data

3.2.1 CNV findings

We compared the sequencing data of each DNA sample with that of a control sample, and detected CNVs through the different distribution of reads. In this study, 23 genes existing in at least 7 subjects (> 80%) that might be affected by CNVs were selected out as shown in Table 1.

GO analysis and biological pathway analysis about the 23 genes were carried out. About cellular components, genes related to the nucleus accounted for the largest proportion. The molecular functions of 21.1% of the genes were unknown, while the rest genes were related to transcription factor activity, receptor activity, transferase activity and DNA binding. About biological processes, genes related to the metabolism of base, nucleoside, nucleotide and nucleic acid were the most abundant, followed by genes related to signal transduction and cell communication. The 23 genes were involved in many signaling pathways, such as ARF6 signaling pathway, PI3K signaling pathway, mTOR signaling pathway, ErbB signaling pathway, S1P1 signaling pathway, etc. Further analysis in STRING database didn't detect significant enrichment.

According to the literature, the relevant studies on the 17 genes affected by CNV, including *SH3RF3, UGT2B15, TRIM31, MICB, TRIM40, C6orf10, PGBD2, CHAMP1, PDPR, ZSCAN18, ERICH2, SIRPB1, APOBEC3B, RPL23AP82, CYB561D2, EPHA6* and *HCG17*, were not found in T2DM or ILD. The other 6 genes, including *CREM, GCSH, KALRN, ECE2, HCG18* and *GPSM*1, had a certain role in the pathogenesis of T2DM and its complications. However, their roles in the development of ILD had not been studied yet.

3.2.2 SV findings

SVs were detected by comparing the sequencing data of each sample with that of a control sample. In this study, 190 genes existing in at least 7 subjects (> 80%) that might be affected by SVs were selected out as shown in Table 2.

The 190 genes existing in at least 7 subjects which were affected by SVs all distributed on autosomes. About cell components, genes related to extracellular components accounted for the highest proportion. About cell function, the percentages of genes related to cell adhesion molecule activity, extracellular matrix composition and growth factor activity ranked among the top three. According to the biological process, the proportion of genes related to cell signal transduction and cell communication ranked among the top two. By biological pathway analysis, we found that the main pathways in which these genes were involved included neuronal system biological processes, EMT, chemical synaptic and postsynaptic transmission signaling. Further, the PPI network (Figure 3) of the proteins corresponding to the genes affected by SVs was constructed in the STRING database. The PPI network revealed that 15 proteins were associated with extracellular matrix components, 42 were involved in cell signal transduction, 54 belonged to glycoproteins, and 28 belonged to secretory proteins. The hub nodes and the four-color nodes in the PPI (the four-color nodes represented the proteins belonging to secretory protein, glycoprotein and extracellular matrix component at the same time, and also participating signal transduction) were analyzed, including PLG, MATN1, ANGPT1, MEPE, COL4A2, NID2, IMPG2, GDF10, SFRP1, ABI3BP, SBSPON, CDH2, SPP1, PTPRD and NRCAM. Whether each node played a role in the development of T2DM or ILD was still unclear. In addition, whether the genes corresponding to these nodes would form fusion genes, affect gene expression or change their phenotypes after being affected by SV had not been studied yet.

TABLE 1 Summary of common genes affected by CNVs.

CNV Region	Type	Gene
chr2:109745796-110262413	Gain	*SH3RF3*
chr4:69512114-69536694	Gain	*UGT2B15*
chr6:1582595-1593195	Gain	*TRIM31*
chr6:2747876-2764695	Gain	*MICB*
chr6:1485581-1498602	Gain	*TRIM40*
chr6:3521493-3600757	Gain	*C6orf10*
chr1:249200241-249213545	Loss	*PGBD2*
chr10:35415568-35502086	Loss	*CREM*
chr13:115079764-115093003	Loss	*CHAMP1*
chr16:70147328-70195384	Loss	*PDPR*
chr16:81115351-81130180	Loss	*GCSH*
chr19:58595008-58629993	Loss	*ZSCAN18*
chr2:171626991-171655681	Loss	*ERICH2*
chr20:1544828-1600889	Loss	*SIRPB1*
chr22:39378203-39388984	Loss	*APOBEC3B*
chr22:51195313-51238265	Loss	*RPL23AP82*
chr3:50387925-50405828	Loss	*CYB561D2*
chr3:96533224-97467986	Loss	*EPHA6*
chr3:123813357-124440236	Loss	*KALRN*
chr3:183967244-184011019	Loss	*ECE2*
chr6:1550103-1642179	Loss	*HCG17*
chr6:1603064-1643201	Loss	*HCG18*
chr9:139221731-139254257	Loss	*GPSM1*

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TABLE 2 Summary of common genes affected by SVs.

chrA	posA	ortA	chrB	posB	ortB	Type	GeneNameA	GeneNameB
chr1	30878819	–	chr1	30878513	+	ITX	LOC101929406,MATN1	LOC101929406,MATN1
chr1	53595128	+	chr1	53595604	+	DEL	SLC1A7	SLC1A7
chr1	96945545	+	chr3	114915084	–	CTX	LOC101928241,PTBP2	ZBTB20,GAP43
chr1	145092948	+	chr1	145097082	+	DEL	NBPF20,NBPF9	NBPF20,NBPF9,SEC22B
chr1	187466730	–	chr1	187466476	+	ITX	LINC01036,NONE	LINC01036,NONE
chr2	37453432	+	chr19	29855782	–	CTX	CEBPZ	LOC284395
chr2	41973156	+	chr2	41975866	+	DEL	SLC8A1,LOC388942	SLC8A1,LOC388942
chr2	119653329	+	chr2	119659369	+	DEL	EN1,MARCO	EN1,MARCO
chr2	123364878	+	chr2	123365377	+	DEL	TSN,CNTNAP5	TSN,CNTNAP5
chr2	138245304	+	chr2	138245633	+	DEL	THSD7B	THSD7B
chr2	173616807	+	chr2	173616766	+	INS	RAPGEF4	RAPGEF4
chr2	191002633	+	chr2	191002548	+	INS	C2orf88	C2orf88
chr2	236818929	+	chr2	236818861	+	INS	AGAP1	AGAP1
chr3	12696232	+	chr10	101851839	+	CTX	RAF1	CPN1,ERLIN1
chr3	31881392	+	chr14	29261491	+	CTX	OSBPL10	LINC01551
chr3	99940769	+	chr1	79582083	+	CTX	TMEM30C,TBC1D23	ELTD1,LOC101927412
chr3	100868474	+	chr3	100868430	+	INS	ABI3BP,IMPG2	ABI3BP,IMPG2
chr3	144693111	+	chr3	144693237	+	DEL	C3orf58,PLOD2	C3orf58,PLOD2
chr4	53155166	+	chr4	53155079	+	INS	SPATA18,USP46	SPATA18,USP46
chr4	78199225	+	chr4	78199274	+	DEL	CCNG2,CXCL13	CCNG2,CXCL13
chr4	88858700	–	chr4	88847164	+	ITX	MEPE,SPP1	MEPE,SPP1
chr4	162776134	+	chr4	162776213	+	DEL	FSTL5	FSTL5
chr4	187386699	+	chr4	187386636	+	INS	F11-AS1	F11-AS1
chr4	86442606	+	chr4	86442559	+	INS	ARHGAP24	ARHGAP24
chr4	157581838	+	chr4	157581700	+	INS	CTSO,PDGFC	CTSO,PDGFC
chr4	80894475	+	chr5	21207714	+	CTX	ANTXR2	CDH18,GUSBP1
chr5	152272036	+	chr14	52667761	+	CTX	LINC01470	NID2,PTGDR
chr5	28934877	–	chr5	28932856	+	ITX	LSP1P3,LOC101929645	LSP1P3,LOC101929645
chr5	91480787	+	chr5	91481129	+	DEL	ARRDC3-AS1,NR2F1-AS1	ARRDC3-AS1,NR2F1-AS1
chr5	114739725	–	chr8	117398602	+	CTX	CCDC112,FEM1C	LINC00536,EIF3H
chr5	143413889	+	chr10	127633806	+	CTX	HMHB1,YIPF5	FANK1
chr5	143512867	+	chr5	143515048	+	DEL	HMHB1,YIPF5	HMHB1,YIPF5
chr6	6034770	+	chr5	7411901	+	CTX	NRN1,F13A1	ADCY2
chr6	8902225	+	chr6	8902168	+	INS	LOC100506207,TFAP2A	LOC100506207,TFAP2A
chr6	43655533	+	chr9	33130549	+	CTX	MRPS18A	B4GALT1
chr6	44264732	+	chr6	44264682	+	INS	TCTE1	TCTE1
chr6	57284911	+	chr6	57289357	+	DEL	PRIM2	PRIM2
chr6	120764923	+	chr6	120764817	+	INS	LOC285762,TBC1D32	LOC285762,TBC1D32
chr6	136582615	+	chr6	136589300	+	DEL	BCLAF1	BCLAF1
chr6	151777322	+	chr19	29855783	+	CTX	C6orf211	LOC284395

(Continued)

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TABLE 2 Continued

chrA	posA	ortA	chrB	posB	ortB	Type	GeneNameA	GeneNameB
chr7	22853488	+	chr18	38517914	+	CTX	TOMM7	LINC01477,KC6
chr7	67120984	+	chr7	67121048	+	DEL	LINC01372,LOC102723427	LINC01372,LOC102723427
chr7	113416176	+	chr7	113422208	+	DEL	LINC00998,PPP1R3A	LINC00998,PPP1R3A
chr7	158180267	+	chr7	158180207	+	INS	PTPRN2	PTPRN2
chr8	23407223	+	chr8	23407860	+	DEL	SLC25A37	SLC25A37
chr8	24779109	+	chr13	101172615	+	CTX	NEFM,NEFL	PCCA
chr8	25327221	+	chr8	25327279	+	DEL	CDCA2	CDCA2
chr8	41270459	–	chr8	67615798	+	ITX	SFRP1,GOLGA7	C8orf44-SGK3
chr8	70839998	+	chr8	70840048	+	DEL	SLCO5A1,PRDM14	SLCO5A1,PRDM14
chr8	74044179	+	chr8	74044096	+	INS	SBSPON,C8orf89	SBSPON,C8orf89
chr8	92153187	+	chr11	104786600	–	CTX	LRRC69	LOC643733
chr8	107207882	–	chr1	11096278	+	CTX	ZFPM2-AS1,OXR1	MASP2
chr8	108569293	+	chr8	108569363	–	ITX	ANGPT1,RSPO2	ANGPT1,RSPO2
chr9	9413418	+	chr13	90947648	+	CTX	PTPRD	MIR622,LINC01049
chr9	88661978	+	chr9	88661918	+	INS	GOLM1	GOLM1
chr9	140772669	+	chr9	140773505	+	DEL	CACNA1B	CACNA1B
chr10	8717568	+	chr10	8717528	+	INS	LINC00708,LOC101928272	LINC00708,LOC101928272
chr10	48419946	–	chr5	100389387	+	CTX	GDF2,GDF10	ST8SIA4,SLCO4C1
chr10	54941242	+	chr10	54941198	+	INS	MBL2,PCDH15	MBL2,PCDH15
chr10	60984921	+	chr10	9972888	+	INS	PHYHIPL	LOC101928272,LOC101928298
chr10	67032386	+	chr10	67032802	+	DEL	ANXA2P3,LINC01515	ANXA2P3,LINC01515
chr10	74842608	+	chr10	74842833	+	DEL	P4HA1	P4HA1
chr12	50973716	+	chr12	50975506	+	DEL	DIP2B	DIP2B
chr12	74014507	+	chr7	125264207	+	CTX	LOC101928137,LOC100507377	LOC101928283,GRM8
chr13	87392949	–	chr7	44495374	+	CTX	SLITRK6,MIR4500HG	NUDCD3
chr13	73169686	+	chr7	127929321	+	CTX	DACH1,MZT1	LEP,MGC27345
chr13	111076796	–	chr13	41271120	+	ITX	COL4A2	FOXO1,MIR320D1
chr14	58871151	+	chr7	82430515	+	CTX	TOMM20L	PCLO
chr14	106484225	+	chr15	22486821	+	CTX	ADAM6,LINC00226	OR4N3P,REREP3
chr15	40854180	–	chr7	26241365	+	CTX	C15orf57	CBX3
chr15	62947784	+	chr15	62947921	+	DEL	TLN2	TLN2
chr15	91214612	+	chr19	29956114	+	CTX	LOC101926895	LOC284395
chr16	8739884	+	chr16	8739844	+	INS	METTL22	METTL22
chr16	22908973	+	chr16	22909252	+	DEL	HS3ST2	HS3ST2
chr16	54454410	+	chr4	42088050	–	CTX	IRX3,CRNDE	SLC30A9
chr17	29729944	+	chr12	46175163	+	CTX	RAB11FIP4	ARID2
chr17	30893853	–	chr6	161181867	+	CTX	MYO1D	PLG,MAP3K4
chr17	68624947	+	chr20	8670606	+	CTX	KCNJ2,CASC17	PLCB1
chr17	10886858	+	chr17	10895732	+	DEL	PIRT,SHISA6	PIRT,SHISA6
chr18	11572230	+	chr18	11572179	+	INS	LINC01255,SLC35G4	LINC01255,SLC35G4

(Continued)

TABLE 2 Continued

chrA	posA	ortA	chrB	posB	ortB	Type	GeneNameA	GeneNameB
chr18	25871823	+	chr20	32335134	+	CTX	CDH2,MIR302F	ZNF341
chr18	35306060	+	chr18	35306633	+	DEL	MIR4318,LINC00669	MIR4318,LINC00669
chr18	54706185	+	chr8	96730502	+	CTX	WDR7,LINC-ROR	C8orf37-AS1
chr18	66572496	–	chr6	76031227	+	CTX	CCDC102B	FILIP1
chr18	75266998	+	chr18	75268160	+	DEL	GALR1,LINC01029	GALR1,LINC01029
chr19	46175262	–	chr6	24384210	+	CTX	GIPR	DCDC2
chr20	6302495	+	chr7	107829261	–	CTX	FERMT1,CASC20	NRCAM
chr20	42300915	+	chr20	42300870	+	INS	MYBL2	MYBL2
chr20	53292970	+	chr2	115390731	–	CTX	DOK5,LINC01441	DPP10
chr20	61661208	+	chr20	61661143	+	INS	LOC63930	LOC63930
chr20	62709634	+	chr20	62709531	+	INS	RGS19	RGS19
chr20	6302495	+	chr7	107829261	–	CTX	FERMT1,CASC20	NRCAM

ChrA= the chromosome on one side of SV; PosA=the position on one side of SV; ortA= the plus or minus strand of this position ChrB= the chromosome on the other side of SV; PosB= the position on the other side of SV; ortB= the plus or minus strand of this position Type= Deletion (DEL),Insertion (INS), Inversion(INV), Intrachromosomal translocation (ITX), Interchromosomal translocation (CTX); GeneNameA= the name of the gene on one side of SV GeneNameB= the name of the gene on the other side of SV.

3.2.3 SNPs and InDels findings

20263-20507 SNPs in 3'-UTR, 5'-UTR and exonic regions were genotyped in each subject, including synonymous, non-synonymous, stop-gain and stop-loss. 510-587 InDels were genotyped in each subject, including non-frameshift deletion, non- frameshift insertion, frameshift deletion, frameshift insertion, stop-gain, and stop-loss.

70 common InDels distributed in the coding region on the autosomes and X chromosomes in at least 7 subjects, including 40 frameshift deletions and frameshift insertions. The genes with frameshift deletions are shown in Table 3, and the genes with frameshift insertions are shown in Table 4.

GO classification and biological pathway analysis were performed about the 40 genes with frameshift deletion. About the cell components, the genes related to cell membrane components were in the majority. About the molecular functions, the function of 26.5% of the genes were unknown, while the rest were related to G protein coupled receptor activity and cellular structural molecular activity. In the biological process, the biological process of 26.5% genes was unknown, while the rest were mostly related to cell signaling transduction and cell communication. The signaling pathways in which these genes were involved are numerous, including ARF6 signaling pathway, PI3K signaling pathway, mTOR signaling pathway, ErbB signaling pathway, S1P1 signaling pathway, and IGF1 signaling pathway, etc. Further analysis in STRING database did not detect significant enrichment of the corresponding proteins. According to the literature, only the variants of *SLC22A1, TBP, ORAI1, SARM1* and *COL18A1* were found to play a certain role in the development of T2DM or be the risk factors of T2DM. However, the remaining 35 genes were rarely studied in the field of T2DM or ILD.

GO classification and biological pathway analysis were carried out about the 30 genes with frameshift insertion. About cellular component, genes related to cytoplasm and nucleus accounted for a large proportion. The molecular function of 45.8% of the genes were unknown, while the rest were related to immune protein activity, transcription factor activity, and G-protein-coupled receptor activity. In biological process, the biological process of 33.3% of the genes were unknown, while the remaining related to cell signalling transduction and cell communication accounted for a large proportion. The 30 genes were involved in numerous signaling pathways, such as IL-3-mediated signaling pathway, IL-5-mediated signaling pathway, PEGFR signaling pathway, GMCSF-mediated signaling pathway, ErbB signaling pathway, S1P1 signaling pathway, IGF1 signaling pathway, etc. Further analysis in STRING database did not detect significant enrichment of the corresponding proteins. 5 mutated genes including *GIGYF2, ATG3, SRA1, WNK1* and *CLECL1* were found to be involved in the development of T2DM and its complications, or be the risk factors for T2DM, according to the very few related studies. The remaining 25 genes had not yet been studied in T2DM or ILD.

The number of SNPs detected by each subjects was about 20,000. In order to simplify the search scope and effectively query the SNPs that may be relevant to the T2DM complicated with ILD, we searched according to the mutated genes which had been identified to be associated with the development of ILD (including familial pulmonary fibrosis) in previous studies, including *AKAP13, ATP11A, CDKN1A, DPP9, DSP, ELMOD2, FAM13A, HLA-DRB1, IL1RN, IL8, MAPT, MDGA2, MUC2, MUC5B, OBFC1, SPPL2C, TERC, TERT, TGFB1, TLR3, TOLLIP and TP53* (30). 38 SNPs within the specific genes in at least 7 subjects were summarized in Table 5.

The 38 SNPs were found in 10 genes including *AKAP13, ATP11A, DSP, FAM13A, IL1RN, MAPT, TP53, MUC2, MUC5B, OBFC1* and *SPPL2C*. According to the literature, the roles of *AKAP13* SNPs rs8110, rs13225, rs3169121, rs2542604 and rs1808339, *ATP11A* SNPs rs7985702 and rs1046790, *IL-1RN* SNP RS315951, *TP53* SNP rs2909430, *MUC2* SNPs rs41411848, rs41345745 and rs57737240, *MAPT* SNP rs2258689, *OBFC1* SNPs rs10786775, rs2487999, rs4917405 and rs911547, as well as *SPPL2c* SNPs rs242944 and

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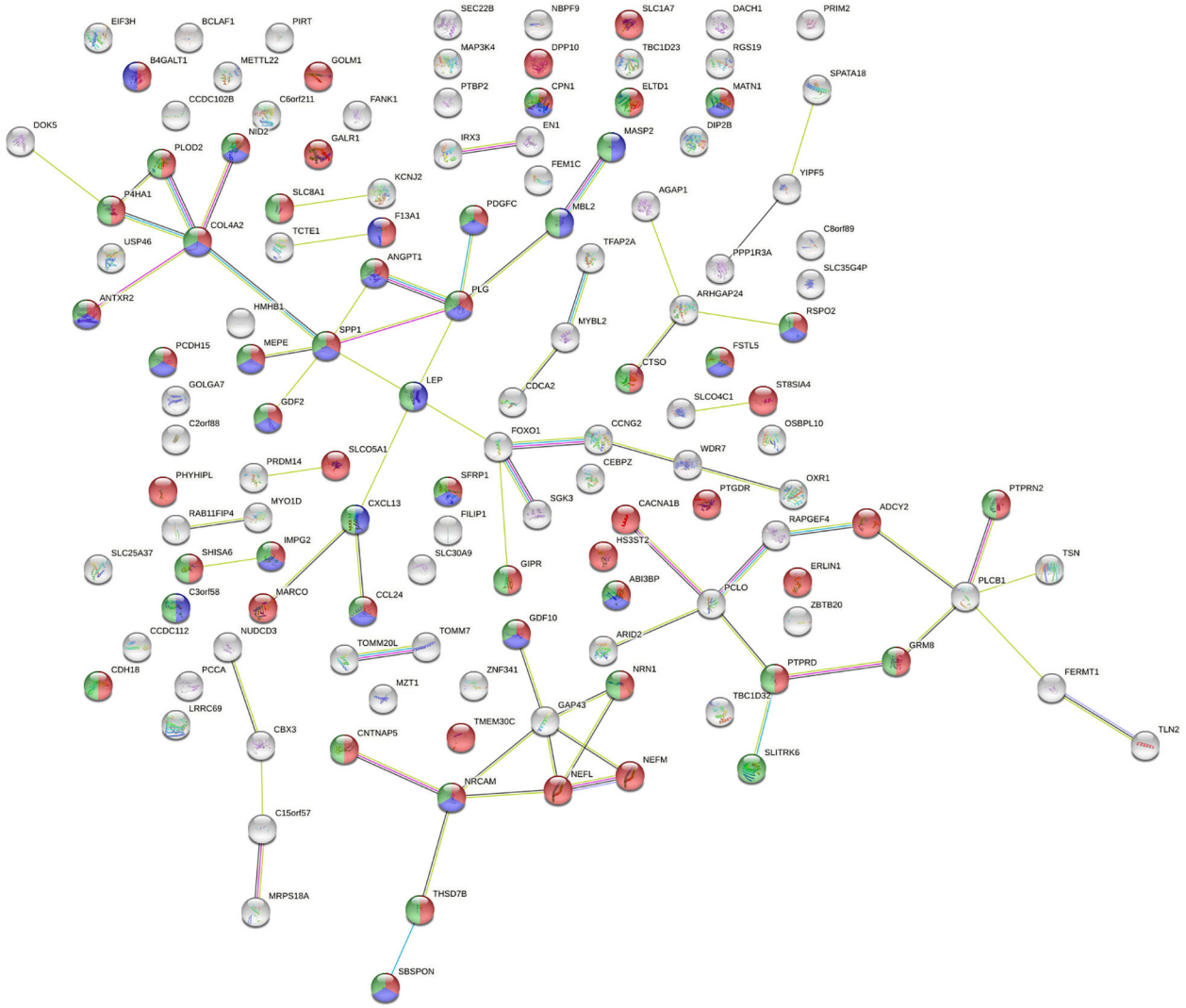

FIGURE 3
Analysis of protein-protein interaction network (PPI) affected by SVs. The number of nodes was 130, with each node representing a protein, 53 red nodes representing glycoproteins, 41 green nodes representing cell signal transduction, and 28 blue nodes representing secretory proteins. There were a total of 68 lines between nodes, each line representing the interaction between two proteins. Red line – gene fusion, green line – gene neighborhood, blue line – gene co-occurrence, purple line – experimentally determined, yellow line – textming, light blue line – from curated databases, black line – co-expression. PPI enrichment $P = 1.57x10^{-4}$.

rs171443 in the pathogenesis of T2DM or ILD were unclear; *OBFC1* SNP rs4387287 might be closely related to the susceptibility of T2DM, but its role in ILD remained unclear; *DSP* rs2076295 and *FAM13A* rs2609255 had been confirmed to be associated with some types of ILD, but their roles in ILD remained unclear; *MUC5B* SNP rs2943512 had been identified to be significantly associated with the susceptibility of T2DM, and the over-expressed MUC5B in the distal airway and alveolar cavity had been confirmed to be closely related to the development of ILD.

To sum up, the roles of most variants in the pathogenesis of T2DM or ILD were unclear. Nevertheless, *MUC5B* SNP rs2943512 (A > C) was considered to be a potentially pathogenic mutation associated with T2DM complicated with ILD. Next, the function experiment of MUC5B in bronchial epithelial cells was carried out, laying a foundation for the mechanism exploration of T2DM complicated with ILD.

3.3 The effects of high glucose on the expression of MUC5B in bronchial epithelial cells

3.3.1 High glucose affects viability of BEAS-2B cells

The viability of BEAS-2B cells stimulated by high glucose was detected by CCK-8 assay. The results demonstrated that high glucose 25mM (48h), 30mM (48h), 20mM (72h), 25mM (72h) and 30mM (72h) could inhibited cell growth significantly (***P<0.001) (Figure 4).

3.3.2 Effects of high glucose on the expression of MUC5B in BEAS-2B cells

The BEAS-2B cells in normal RPMI1640 medium (D-glucose concentration 11.11mM) was regarded as control, while the cells in medium containing 20mM, 25mM and 30mM glucose for 72h were

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TABLE 3 The common genes with frameshift deletions.

Chr	Region	Deleted sequence	Gene name
chr1	1q44	A	*OR2B11*
chr1	1q44	CAGCACG	*OR2T35*
chr1	1p36.11	CC	*UBXN11*
chr3	3p25.1	C	*SLC6A6*
chr3	3p12.3	C	*ZNF717*
chr4	4q31.3	TTTG	*DCHS2*
chr4	4q31.1	C	*MAML3*
chr4	4q31.1	GCTGCTGCTGC	*MAML3*
chr5	5q32	T	*TIGD6*
chr6	6q25.3	TGGTAAGT	*SLC22A1*
chr6	6q27	GC	*TBP*
chr7	7q11.23	A	*POMZP3*
chr8	8q24.3	GGGGGTGCAAGGTGA	*ADCK5*
chr8	8p23.1	AAC	*ERI1*
chr8	8p21.3	C	*NUDT18*
chr9	9p11.2	G	*CNTNAP3B*
chr9	9q31.1	GC	*OR13C2*
chr9	9q31.1	GTTA	*OR13C2*
chr9	9q31.1	T	*OR13C5*
chr11	11q12.2	TT	*MS4A14*
chr11	11p15.4	G	*OR52B4*
chr12	12q13.3	AT	*PTGES3*
chr12	12q24.31	CCGCCA	*ORAI1*
chr14	14q32.33	GACGGGCAG	*C14orf180*
chr15	15q13.2	CA	*CHRFAM7A*
chr15	15q11.2	T	*GOLGA6L2*
chr16	16q24.3	GGTGTG	*CTU2*
chr16	16q23.2	TT	*PKD1L2*
chr16	16q24.2	GA	*ZFPM1*
chr17	17q21.2	A	*KRT24*
chr17	17p13.2	G	*P2RX5*
chr17	17q11.2	A	*SARM1*
chr19	19p13.3	AGCTGGCCGGGGAGG	*HDGFRP2*
chr19	19q13.41	TG	*ZNF480*
chr21	21q22.3	GGCCCCCCA	*COL18A1*
chr21	21q22.3	TC	*KRTAP10-1*
chr21	21q22.11	G	*KRTAP19-6*
chr21	21q22.11	A	*SON*
chrX	Xp11.22	TCCTCGAGGCAGCC	*NUDT11*
chrX	Xq22.3	A	*TEX13A*

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TABLE 4 The common genes with frameshift insertions.

Chr	Region	Inserted sequence	Gene name
chr1	1q23.1	AC	*GPATCH4*
chr2	2q37.1	CG	*GIGYF2*
chr3	3q13.2	A	*ATG3*
chr5	5q31.3	GT	*SRA1*
chr5	5q33.3	C	*CYFIP2*
chr7	7q11.23	C	*SRRM3*
chr7	7q36.1	C	*SSPO*
chr9	9q33.2	A	*OR1B1*
chr10	10q25.3	TT	*ATRNL1*
chr11	11p15.4	AC	*C11orf40*
chr12	12p13.33	C	*WNK1*
chr12	12q13.11	A	*OR10AD1*
chr12	12p13.31	TAAGT	*CLECL1*
chr13	13q22.3	GG	*SLAIN1*
chr14	14q32.12	G	*ATXN3*
chr16	16q24.3	GTGA	*ZNF778*
chr17	17p13.1	G	*C17orf100*
chr17	17q11.2	G	*SARM1*
chr17	17q25.1	T	*LOC100134391*
chr17	17q25.1	T	*LOC100134391*
chr19	19q13.41	G	*VSIG10L*
chr19	19q13.42	C	*SBK3*
chr20	20p13	GCCCC	*GNRH2*
chr21	21q22.11	A	*SON*
chr21	21q22.3	G	*PRDM15*
chr21	21q22.3	TG	*KRTAP10-1*
chr22	22q11.21	C	*CLTCL1*
chr22	22q11.21	G	*SCARF2*
chrX	Xq22.1	G	*TCEAL6*
chrX	Xq25	G	*GRIA3*

experimental groups. MUC5B mRNA and MUC5B protein in the supernatant in these groups were detected, respectively. The results showed that compared with the control group, MUC5B mRNA in 20mM, 25mM and 30mM high-glucose groups were statistically increased at 72h (***$P < 0.001$) (Figure 5A), and MUC5B in the supernatant of 25mM and 30mM high-glucose groups were statistically increased at 72h (***$P < 0.001$) (Figure 5B). Finally, 30mM glucose stimulation for 72h was chosen as the subsequent experimental conditions.

3.3.3 Effects of MUC5B on cytokine production in BEAS-2B cells stimulated by high glucose

To identify the effects of MUC5B on the production of cytokines in BEAS-2B cells, the cells were cultured in medium containing 30mM glucose for 72 h following the transfection of *MUC5B* shRNA into the cells (Figure 6A). Subsequently, IL-1 β and IL-6 in the supernatant were detected by ELISA, respectively. (Figures 6B, C). The results showed that the concentrate ions of IL-1β and IL-6 in high glucose group were both significantly increased compared to the control (***$P < 0.001$). While, compared to the high glucose group, the concentrations of IL-1β and IL-6 were both significantly decreased when *MUC5B* was knockdown even stimulated by high glucose (**P <0.01, ***$P < 0.001$).

3.3.4 Effects of MUC5B on ERK1/2 activation in BEAS-2B cells stimulated by high glucose

After culturing BEAS-2B cells in the medium containing 30mM glucose for 15min, 30min, 1h, 3h and 6h, p-ERK1/2, p-P38, p-JNK,

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TABLE 5 The common SNPs within the genes associated with IPF.

Gene	Region	SNP	Alteration	Type
AKAP13	3'-UTR 3'-UTR 3'-UTR 3'-UTR 3'-UTR	rs8110 rs13225 rs3169121 rs2542604 rs1808339	A>T C>G T>G C>G A>C	N/A N/A N/A N/A N/A
ATP11A	3'-UTR 3'-UTR	rs7985702 rs1046790	T>C T>C	N/A N/A
DSP	exonic exonic exonic exonic	rs2806234 rs2076304 rs1016835 rs2744380	T>G G G>A G>C	synonymous synonymous synonymous synonymous
FAM13A	5'-UTR	rs2305934	T>C	N/A
IL1RN	exonic	rs315952	T>C	synonymous
	3'-UTR	rs315951	C>G	N/A
MAPT	exonic	rs2258689	T>C	non-synonymous
TP53	5'-UTR	rs2909430	C>T	N/A
MUC2	exonic exonic exonic exonic exonic exonic exonic exonic exonic exonic	rs7944723 rs10794292 rs6421972 rs7480563 rs41411848 rs41345745 rs57737240 rs10794288 rs10902088 rs10794291	C>G A>C T>C T>C T>C G>C G>C T>C C>T C>T	synonymous synonymous synonymous synonymous non-synonymous non-synonymous non-synonymous synonymous synonymous synonymous
MUC5B	exonic exonic exonic exonic exonic	rs2075859 rs7116614 rs4963031 rs2943531 rs2943512	C>T C>T T>C A>G A>C	synonymous synonymous non-synonymous non-synonymous non-synonymous
OBFC1	3'-UTR 3'-UTR exonic exonic 5'-UTR	rs4917405 rs911547 rs10786775 rs2487999 rs4387287	T>C G>A G>C T>C A>C	N/A N/A non-synonymous non-synonymous N/A
SPPL2C	exonic exonic	rs242944 rs171443	G>A A>G	synonymous non-synonymous

N/A, Not applicable.

and p-IκB in each group were detected. The results showed that compared with the control group, p-ERK1/2 was up-regulated when stimulated by the high glucose for 15min and 30min (Figure 7A,*P <0.05, ***P < 0.001). However, no change of p-P38, p-JNK or p-IκB was detected at each point from 15min to 6h (Figure 7B).

To confirm our suppose that over-expressed MUC5B could promote the synthesis of IL-1β and IL-6 by activating ERK1/2, BEAS-2B cells were stimulated by 30mM glucose for 30min following the silencing of *MUC5B* gene. Subsequently, p-ERK1/2 in each group was detected by western blot. The results showed that compared with the high glucose group, p-ERK1/2 in the *MUC5B* knockdown cells stimulated by high glucose was significantly down-regulated (Figure 8, **P <0.01), suggesting over-expressed MUC5B could promote ERK1/2 activation in BEAS-2B cells when stimulated by high glucose.

4 Discussion

T2DM and ILD both belong to complex diseases. In recent years, genetic studies about the both diseases have been gradually extensive, and mutations related to the risks or pathogenesis of the two diseases have been constantly revealed. With further studies, it has been recognized that ILD could be a complication of T2DM. However, the relevant studies about genetic variations promoting ILD in T2DM patients are still rare.

In this study, a pedigree with T2DM complicated with ILD including three generations was selected. Familial T2DM is not rare in clinical practice, however, pedigree with both T2DM and ILD are indeed rare. In this pedigree, almost all the first and second generation members had both T2DM and ILD. 8 living members of the pedigree were included as subjects. By conducting the whole-genome re-sequencing of each subject's blood DNA sample, it was found that

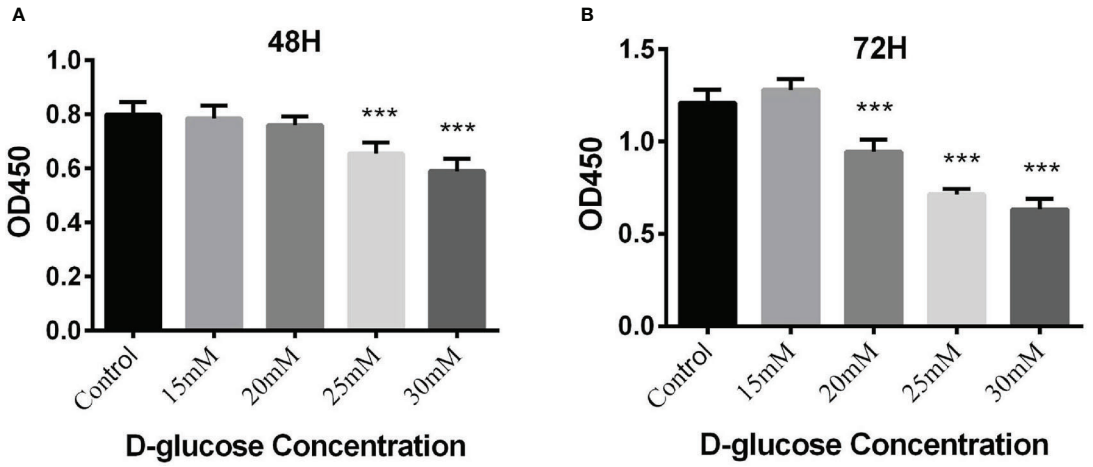

FIGURE 4
(A) Proliferation of BEAS-2B cells cultured in the medium containing different concentrations of glucose for 48 hours by CCK-8 assay; **(B)** Proliferation of BEAS-2B cells cultured in the medium containing different concentrations of glucose for 72 hours by CCK-8 assay (***$P < 0.001$).

the healthy subjects from the third generation also had the same potential pathogenic genetic variants as the patients from the second and third generation. It suggested that T2DM was relevant to the development of ILD, and T2DM complicated with ILD might be heredofamilial.

The common genetic variants of at least 7 subjects were further screened out after gene sequencing, involving numerous SVs, CNVs, SNPs and InDels. Previous studies considered that the susceptibility of many diseases was basically attributed to SNPs, however, SNP can only explain a part of the heritability of diseases. Our study also provided genes which were affected by InDels, SVs and CNVs, so as to provide more genetic information for the mechanism research of ILD in diabetic patients. Up to now, the roles of most variants in T2DM or ILD remained unclear, and only a small part might be involved in the development of T2DM or ILD. While, *MUC5B* variation was the specific one found to be related to both T2DM and ILD in the study, and *MUC5B* SNP rs2943512 (A > C) was considered to be a potentially pathogenic mutation associated with T2DM complicated with ILD. Meanwhile, the over-expressed MUC5B protein in the distal airway and alveolar cavity had also been considered to be closely related to the development of idiopathic pulmonary fibrosis (IPF) (33, 34). Therefore, it was of great significance to investigate the role of MUC5B SNP rs2943512 (A > C) or abnormal MUC5B protein in the development of T2DM complicated with ILD.

MUC5B encodes mucin 5B protein, which is a glycosylated macromolecular component of mucus and produced by mucinous cells in bronchial submucosal glands and type II alveolar epithelial (ATII) cells. Normally, MUC5B plays the physiological roles of maintaining airway homeostasis (35), involving capturing inhaled particles and bacteria which are transported out of the airway by cilia

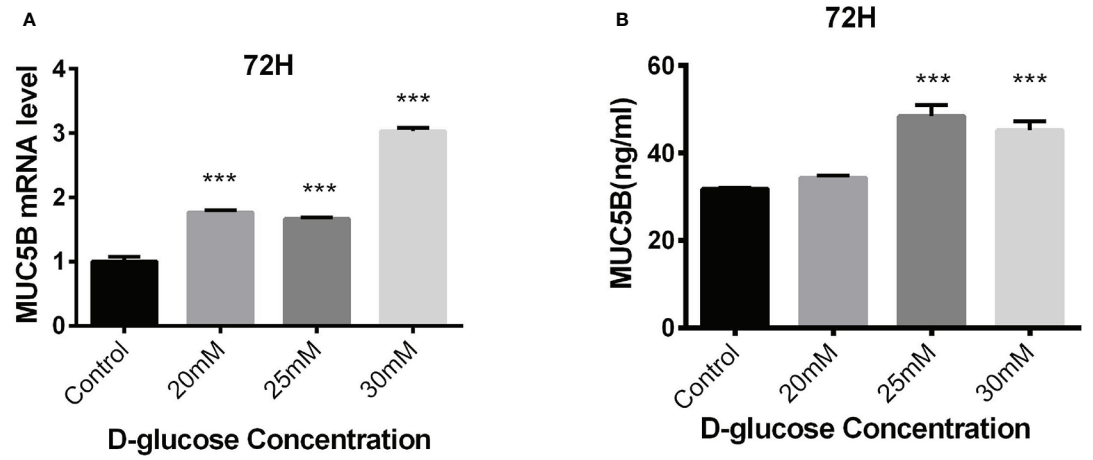

FIGURE 5
Effects of high glucose on the expression of MUC5B in BEAS-2B cells. **(A)** Compared with the control group, MUC5B mRNA in BEAS-2B cells were significantly increased after 20mM, 25mM and 30mM high-glucose stimulation for 72 hours (***$P < 0.001$). **(B)** Compared with the control group,MUC5B in the supernatant were significantly increased after 25mM and 30mM high-glucose stimulation for 72 hours (***$P < 0.001$).

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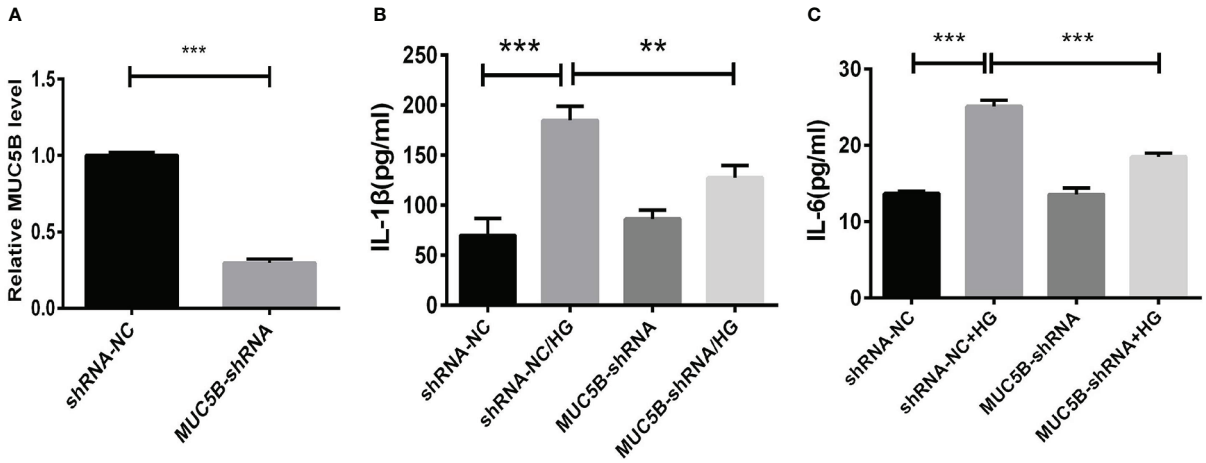

FIGURE 6
Effects of MUC5B on cytokine production in BEAS-2B cells stimulated by high glucose. **(A)** The efficiency of *MUC5B*-ShRNA transfection into BEAS-2B cells was detected by RT-PCR. After MUC5B knockdown, BEAS-2B cells were stimulated by 30mM glucose for 72 hours. **(B)** IL-1 β and **(C)** IL-6 in the supernatant were both significantly increased compared to the control (**$P < 0.01$, ***$P < 0.001$).

oscillating or coughing. In addition, MUC5B could also helps remove endogenous debris, including dead epithelial cells and white blood cells. When MUC5B is over-expressed by stimulus, the capacity of mucociliary clearance will be impaired, resulting in excessive retention of inhaled particles, microorganisms or endogenous inflammatory debris, and mediating the reactive fibrosis in the bronchoalveolar region, promoting the interstitial lesions (33, 34). Although the mechanisms of MUC5B mediating the interstitial fibrosis are still unknown, it is speculated that it may be related to the injury of ATII cells caused by the excessive MUC5B.

Both the endogenous factors (such as genetics and aging) and environmental factors have been implicated in ATII damages. Infection, drugs, poisons, etc., all cause a certain degree of damages to ATII in ILD with known etiology. In T2DM, the accompanied inflammation is not only closely related to the development of diabetic complications (36), but also could cause the impairment of lung function due to significantly elevated cytokines such as TNF-α, IL-1β and IL-6 (37). However, whether high glucose promotes the development of ILD by causing inflammatory injury of ATII cells needs to be further studied.

In our study, we identified *MUC5B* rs2943512 (A>C) in the pedigree. However, the effects of this SNP on the expression of MUC5B in ATII cells is unclear. The previous study showed that the expression of MUC5B was significantly elevated in pancreatic tissue of patients with T2DM compared to those without T2DM (38), therefore, we speculated that *MUC5B* rs2943512 (A>C) might cause the over-expression of MUC5B in lung tissue of T2DM patients. Even beyond the potential influence of the genetic factor of *MUC5B* SNP rs2943512, whether high glucose itself could cause the over-expression of MUC5B to mediate ATII cells injury and thus trigger pulmonary fibrosis is unclear. Therefore, in order to clarify the relationship between high glucose, MUC5B and ATII injury, this study selected human bronchial epithelial cells BEAS-2B to be stimulated by high glucose in this experiments, to simulate ATII cells (39) in diabetic patients. The results showed that the transcriptional and translational levels of MUC5B in BEAS-2B cells were significantly up-regulated, suggesting that MUC5B existed in the distal airways and ATII cells in the lung tissues of T2DM patients. While this hypothesis needs to be confirmed by *in vivo* experiments in the future.

To explore the potential mechanisms that over-expressed MUC5B promoting fibrosis in alveolar region, we started the experiment from the perspective of inflammatory injury of ATII cells, and detected the cytokines in BEAS-2B cells under high glucose stimulation. The results showed the increased IL-1β and IL-6 was accompanied by the over-expressed MUC5B. Numerous studies have confirmed that IL-1β could cause apoptosis in different types of cell including ATII. Moreover, IL-1β could also play a pro-fibrotic role in certain pathological conditions (40–43). *In vivo* experiments have shown that IL-1β induces progressive pulmonary fibrosis through long-term activation of TGF-β signaling (40). *In vitro*, IL-1β could promote EMT by activating TGF-β in bronchial epithelial cells (44, 45). In addition, IL-1β also stimulates the release of IL-6, which not only play the pro-inflammatory role, but also aggravates the pulmonary fibrosis by activating STAT3 pathway (46).

To clarify the interaction between MUC5B and the cytokines, RNA interference was applied to silence *MUC5B* before BEAS-2B cells were stimulated by high glucose. It was found that IL-1β and IL-6 were significantly decreased in MUC5B knockdown BEAS-2B cells. This finding had rarely been reported in previous studies, and suggested that MUC5B could promote the production of IL-1β and IL-6 in bronchial epithelial cells when stimulated by high glucose, and the over-expressed MUC5B causing inflammatory damage to ATII cells might initiating the pulmonary interstitial fibrosis.

This study also explored the mechanisms of over-expressed MUC5B promoting the synthesis of IL-1β and IL-6 in BEAS-2B cells. It was found that high glucose could cause the activation of ERK1/2 in

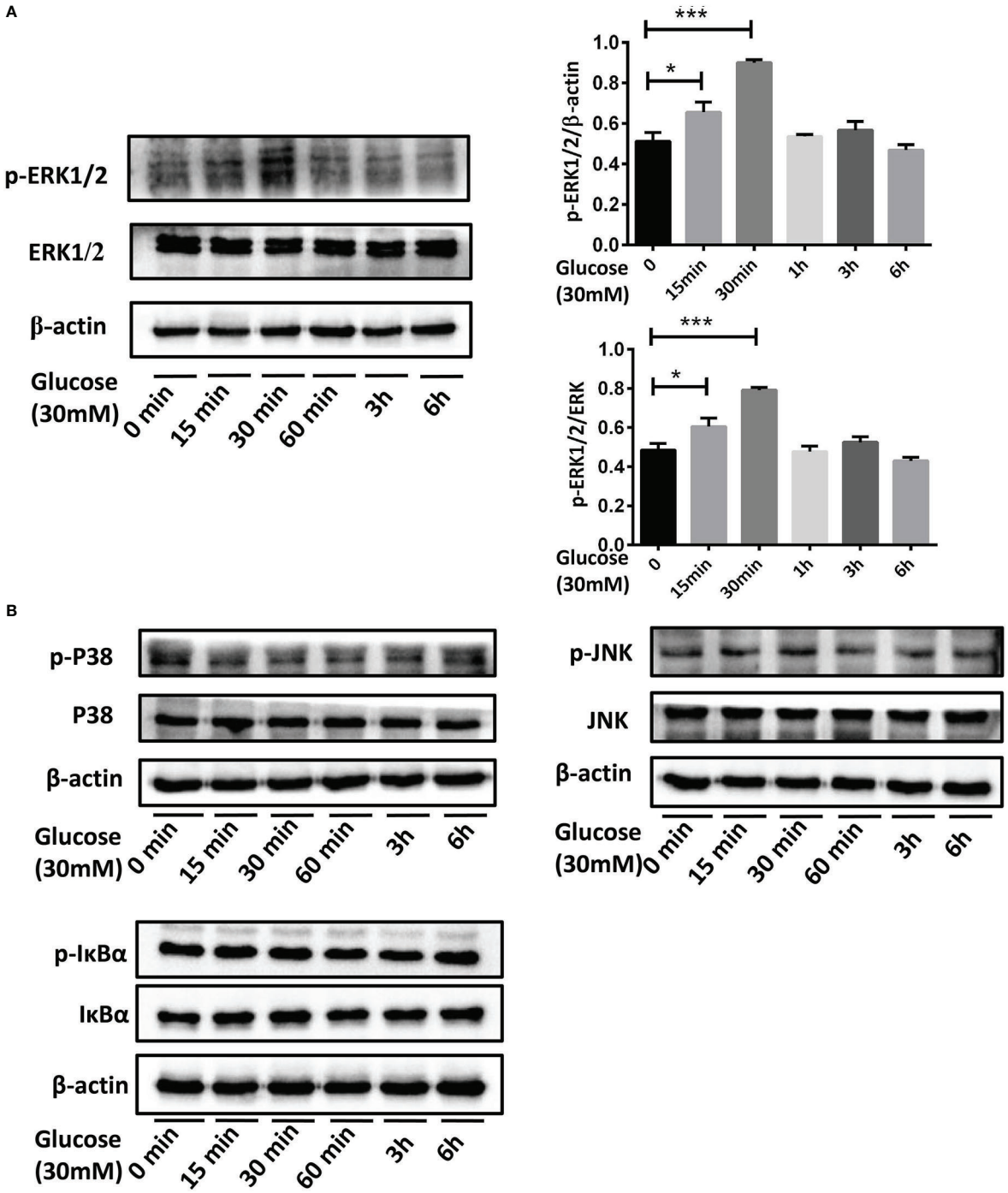

FIGURE 7
The activation of MAPK and NF-κB pathways in BEAS-2B cells stimulated by high glucose. **(A)** Compared with the control group, p-ERK1/2 in BEAS-2B cells was up-regulated when stimulated by 30mM glucose for 15 minutes and 30 minutes. **(B)** P38, JNK or IκB didn't activate at each point from 15 minutes to 6 hours when stimulated by 30mM glucose. The blots and densitometry analysis data are representative of three independent experiments. **P* <0.05, and ****P* < 0.001.

BEAS-2B cells, while the activation was significantly decreased after *MUC5B* silencing, suggesting that the changes in *MUC5B* transcriptional or translational level might affect ERK1/2 activation, which at least partly explained the changes in IL-1β and IL-6. However, whether MUC5B also affect the synthesis of IL-1β and IL-6 through other pathways remains to be explored. In addition, the molecular mechanisms by which high glucose promotes the over- expression of MUC5B as well as MUC5B promotes the activation of ERK1/2 are both unclear at present, which are needed to be studied in the future.In addition,the lack of further experiments to validate the implications of *MUC5B* site-specific mutation for the functions of BEAS-2B cells and for the aggravation of T2DM and ILD is also one of the limitations of

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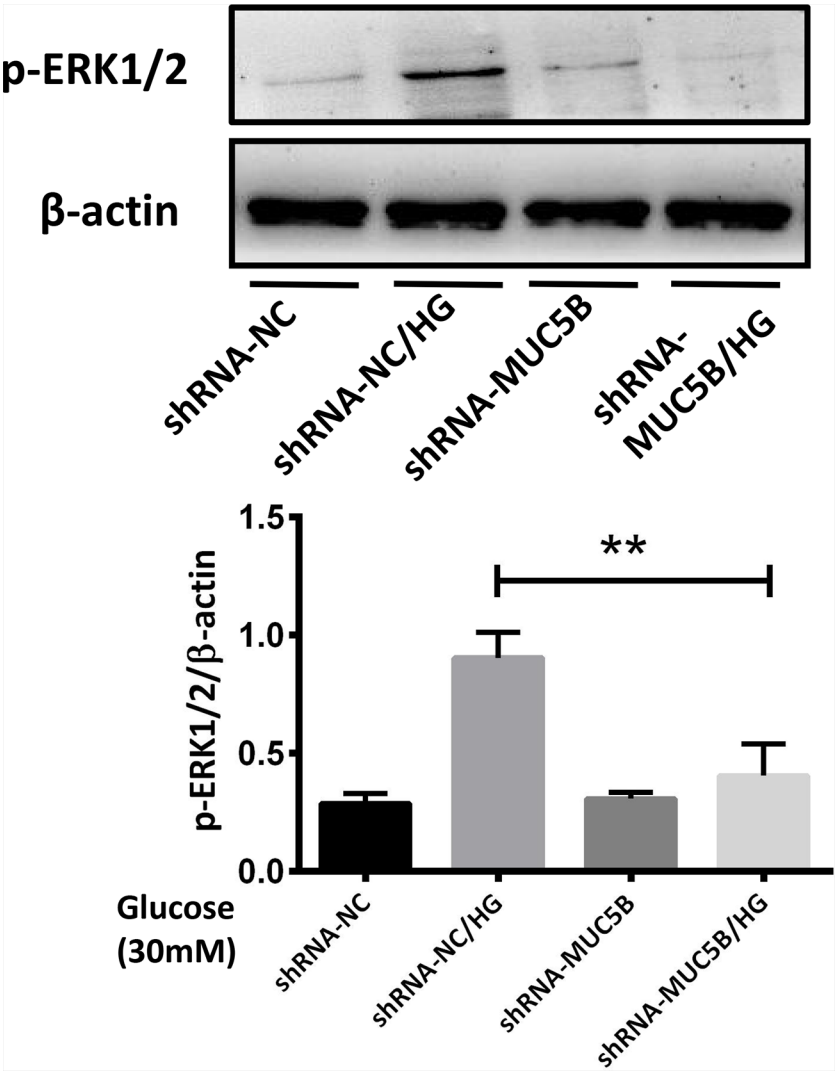

FIGURE 8
MUC5B promoted ERK1/2 activation in BEAS-2B cells stimulated by high glucose. Compared with high glucose group, p-ERK1/2 in the *MUC5B* knockdown cells stimulated by 30mM glucose for 30min was significantly down-regulated (***P* <0.01). The blots and densitometry analysis data are representative of three independent experiments. ***P* <0.01.

this study. Therefore, we will also focus on the cell and animal experiments related to *MUC5B* site-specific mutation in the next step.

5 Conclusion

In this study, a pedigree with T2DM complicated with ILD was selected, and 8 members of the pedigree were included as subjects. Whole-genome resequencing of each subject's blood was conducted, and the common genetic variants of at least 7 subjects were further screened out, involving numerous SVs, CNVs, SNPs and InDels. The identification of these genetic variants in the pedigree enriches our understanding of the potential genetic contributions to T2DM complicated with ILD. *MUC5B* SNP rs2943512 (A > C) or the up-regulated MUC5B in bronchial epithelial cells may be an important factor in promoting ILD in T2DM patients, making MUC5B a potential biological marker for the development of ILD in diabetic patients, and laying a foundation for future exploration about the pathogenesis of T2DM complicated with ILD.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA919498.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of The Second Hospital of Jilin

University, China. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

QZ and YW collected, analyzed and interpreted the data, drafted and revised the manuscript. CT conducted the cellular experiments, and helped revise the manuscript. JYu and YL helped process and analyze the data. JYa conceived, designed and supervised the study, and guided the revision of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

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

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Relationships of adiponectin to regional adiposity, insulin sensitivity, serum lipids, and inflammatory markers in sedentary and endurance-trained Japanese young women

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Introduction: This study aims to compare the differences in circulating adiponectin levels and their relationships to regional adiposity, insulin resistance, serum lipid, and inflammatory factors in young, healthy Japanese women with different physical activity statuses.

Methods: Adipokines (adiponectin and leptin), full serum lipid, and inflammatory factors [white blood cell counts, C-reactive protein, tumor necrosis factor-α, tissue plasminogen activator inhibitor-1 (PAI-1)] were measured in 101 sedentary and 100 endurance-trained healthy Japanese women (aged 18–23 years). Insulin sensitivity was obtained through a quantitative insulin-sensitivity check index (QUICKI). Regional adiposity [trunk fat mass (TFM), lower-body fat mass (LFM), and arm fat mass (AFM)] was evaluated using the dual-energy X-ray absorptiometry method.

Results: No significant difference was observed between the sedentary and trained women in terms of adiponectin levels. The LFM-to-TFM ratio and the high-density lipoprotein cholesterol (HDL-C) were the strong positive determinants for adiponectin in both groups. Triglyceride in the sedentary women was closely and negatively associated with adiponectin, as well as PAI-1 in the trained women. The QUICKI level was higher in the trained than sedentary women. However, no significant correlation between adiponectin and insulin sensitivity was detected in both groups. Furthermore, LFM was associated with a favorable lipid profile against

cardiovascular diseases (CVDs) in the whole study cohort, but this association became insignificant when adiponectin was taken into account.

Conclusions: These findings suggest that adiponectin is primarily associated with regional adiposity and HDL-C regardless of insulin sensitivity and physical activity status in young, healthy women. The associations among adiponectin, lipid, and inflammatory factors are likely different in women with different physical activity statuses. The correlation of LFM and a favorable lipid profile against CVD and adiponectin is likely involved in this association.

KEYWORDS

adiponectin, physical activity, regional adiposity, insulin resistance, diabetes

1 Introduction

Energy over intaking and physical inactivity are two major risk factors for the development of obesity, type 2 diabetes, and many aspects of metabolic syndrome, which are attributed to insulin resistance (1). Moderate physical activity is currently recommended for obese or overweight individuals to reduce the risk of type 2 diabetes and metabolic syndrome (2). However, the mechanisms through which physical activity improves insulin sensitivity remain unclear. A single bout of exercise intervention in both diabetic and non-diabetic individuals can acutely improve insulin sensitivity, but this effect dissipates within days (3, 4). Long-term exercise interventions cannot effectively improve insulin activity without weight improvement (5, 6). These findings indicate that the effect of physical activity on insulin sensitivity is partly mediated by the reduction of body weight and/or body fat mass. One of the main effects of physical activity on body mass distribution is the prevention of subcutaneous fat mass from transferring into the abdominal cavity and leading to a major deposition of adipose tissue in the subcutaneous region (7, 8).

The lower-body region is one of the major areas for the accumulation of subcutaneous adipose tissue. Different adipose depositions have been recognized to cause different metabolic consequences (9, 10). Those who accumulate fat tissue in the trunk region (android obese) are more likely to develop diabetes and cardiovascular diseases (CVDs) than those with major lower-body-fat mass (LFM) deposition (gynoid obese) (10). One of the reasons for the higher prevalence of CVDs in men than in women is that men tend to develop android obesity, whereas women tend to develop gynoid obesity (11). LFM has been reported to play a protective role for CVDs due to its association with a favorable serum lipid profile and increased insulin sensitivity (11). However, the mediator of the interaction between body fat mass distribution and insulin sensitivity, as well as lipid metabolism, warrants further investigation.

Adiponectin is a peptide expressed specifically and abundantly in adipose tissue (12, 13) and has been suggested to be an important regulator of insulin action, thereby possibly linking adiposity with insulin sensitivity (14). Circulating adiponectin levels are reduced in individuals with obesity (15) and diabetes (16). A longitudinal study in Pima Indians presented that a high concentration of plasma adiponectin strongly predicts a lower incidence rate of type 2 diabetes independent of obesity (17). Furthermore, low adiponectin concentrations have been associated with a higher risk of type 2 diabetes (17, 18) and a more atherogenic lipid profile (19).

To date, the relationships among adiponectin, physical activity, and body fat mass distribution are equivocal. A study indicated that moderate physical activity training might improve adiponectin levels in middle-aged adults predisposed to metabolic syndrome (20). Another study including eight healthy subjects showed that circulating adiponectin concentration was increased by physical exercise training when body fat content was reduced but did not change when the body composition was unaltered (21). A study involving 40 obese young women demonstrated that no changes were observed in adiponectin levels after a nine-week intervention (22). In the present research, we conducted a cross-sectional study involving 101 sedentary and 100 endurance-trained healthy Japanese young women to investigate the circulating adiponectin levels and their relationships with regional adiposity, insulin sensitivity, serum lipid, and inflammatory markers. We aimed to explore the potential links between adiponectin with regional adiposity and various metabolic parameters in women with different physical activity statuses. Moreover, we tested the association of adiponectin with the insulin-sensitizing effects of physical activity and LFM.

2 Materials and methods

2.1 Study participants

The study population comprised 201 young women (aged 18–23 years) who are students of Mukogawa Women's University (MWU) in Nishinomiya, Japan. The study was approved by the MWU ethics committee, and written informed consent was obtained from each participant. The selection and recruitment procedures were described previously (23). The subjects in this study were categorized into two groups according to their physical activity habits. The 101 sedentary untrained students recruited from the Department of Food Sciences and Nutrition were not engaged in any regular sport activity. The 100 endurance-trained athletes were recruited from members of a volleyball club (28 students), a basketball club (46 students), and a

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track club (26 students). They have been training five hours per day and 5–7 days a week for two years or longer and participate regularly in competitive events in their respective sports specialties. All of them had similar anthropometric indices. All but six women were nonsmokers, and none had recently been on a diet or consumed alcohol daily. Neither did any of them receive medications.

2.2 Anthropometric and regional fat mass distribution

Body mass index (BMI) was calculated as weight (kg)/[height (m)]^2. A dual-energy X-ray absorptiometry with a scanner (Hologic QDR-2000, Waltham, MA) was applied to measure regional fat mass distribution. A scanned image of the whole body was divided into six subdivisions: head, trunk, left and right arms, and left and right limbs. The dividing borders between these subregions were differentiated by a line underneath the chin, a line between the humerus head and the glenoid fossa, and a line at the femoral neck (Figure 1). Trunk fat mass (TFM), also known as android fat mass, has been documented to be strongly and positively related to visceral adiposity measured with magnetic resonance imaging (24). The following parameters were introduced to describe regional fat deposition: i) total body fat mass ratio (% total fat), illustrated as a percentage of total fat tissue weight/body weight; ii) LFM ratio (L/Tr ratio), illustrated as LFM/TFM; and iii) arm fat ratio (A/Tr), illustrated as arm fat mass/TFM.

2.3 Glucose, insulin, and insulin resistance

Plasma glucose was measured through the hexokinase method [interassay coefficiency of variation (CV) < 2%]. Insulin was measured by an enzyme-linked immunosorbent assay (ELISA) with narrow specificity, excluding des-31, des-32, and intact proinsulin (Abbott Japan, Tokyo, Japan, interassay CV = 3.3%). The quantitative insulin-sensitivity check index (QUICKI) was used as a surrogate index for insulin sensitivity. QUICKI has an excellent linear correlation with the glucose clamp index of insulin sensitivity and is regarded as one of the most accurate surrogate indexes to determine human insulin sensitivity (25). QUICKI was calculated using the following formula: $QUICKI = 1/[\log(I_0) + \log(G_0)]$, where I_0 is the fasting insulin (microunits per milliliter) and G_0 is the fasting glucose (milligrams per deciliter).

2.4 Lipids, lipoprotein, and apolipoprotein

Serum lipids [triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C)] were measured using an autoanalyzer (AU5232, Olympus, Tokyo, Japan). Apolipoprotein A-1 (ApoA1) and apolipoprotein B-100 (ApoB) were measured with respective commercial kits using an Olympus autoanalyzer (AU600, Mitsubishi Chemicals, Tokyo, Japan). Low-density lipoprotein cholesterol (LDL-C) was determined using the Friedewald formula. The interassay CV were as follows: 5.0% for TG, 1.1% for TC, 3% for HDL-C, 5.0% for ApoA1, and 2.0% for ApoB.

2.5 Adipokines

Adiponectin was assayed by a sandwich ELISA employing an adiponectin-specific antibody. The intra- and inter-assay CV were 3.3% and 7.5%, respectively (Otsuka Pharmaceutical Co., Ltd., Tokushima City, Japan). Leptin was assessed by a radioimmunoassay kit purchased from LINCO Research (St. Charles, MO, interassay CV = 4.9%).

2.6 Inflammatory and acute response markers

White blood cell counts (WBC) were measured by an XE-2100 automatic blood routine analyzer (Sysmex Corporation, Kobe, Japan). Serum highly sensitive C-reactive protein (hsCRP) concentration was measured by an immunoturbidometric assay with reagents and calibrators purchased from Dade Behring Marbura GmbH

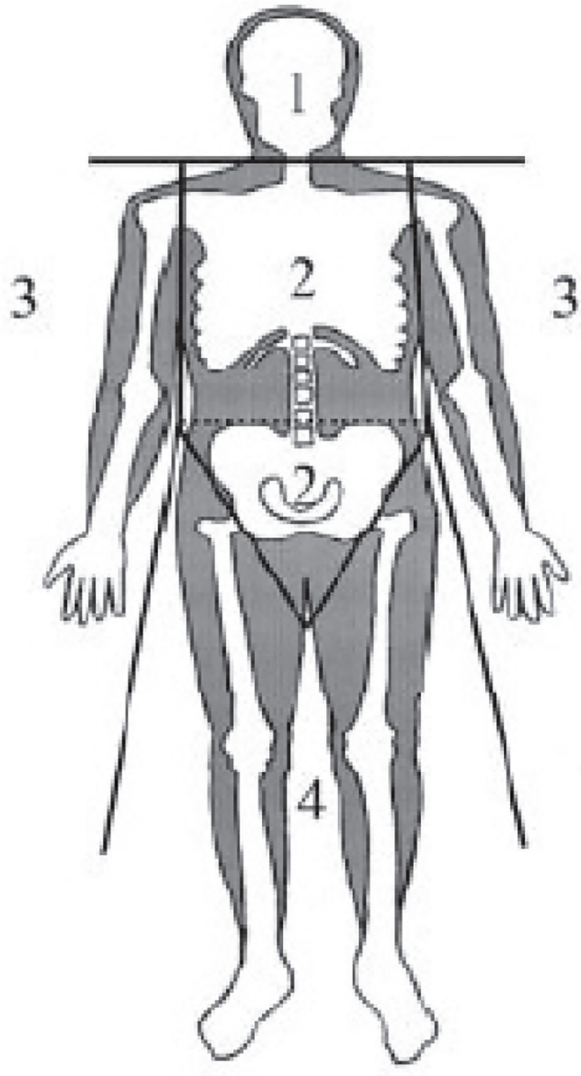

FIGURE 1
Standard regions of a dual-energy X-ray absorptiometry scan: 1, head; 2, trunk; 3, arms; 4, lower body.

(Marburg, Germany; inter-assay CV < 5.0%). Tumor necrosis factor-α (TNF-α) was measured by immunoassays (R&D Systems, Inc., Minneapolis, MN, interassay CV = 6.0%). Tissue plasminogen activator inhibitor-1 (PAI-1) was measured by an ELISA method (Mitsubishi Chemicals, interassay CV = 8.1%).

2.7 Statistical analysis

Data were expressed as mean ± SD. The normality of data distribution was examined using the Kolmogorov-Smirnov test. Comparison of demographic and metabolic variables was carried out by unpaired t-test and Mann-Whitney U test when the data were distributed non-normally. Correlations were conducted by univariate linear regression. Partial correlation analysis was applied to assess the relationship between two variables when confounding factors need to be adjusted. Multiple regression analysis was used to determine whether the association between the dependent and independent variables of interest remained significant after adjusting for other potentially confounding independent variables. The stepwise regression model was used to estimate the relative contribution of the independent variables and the variability of the dependent variable. Data were considered statistically significant when the p-value ≤ 0.05. All statistical calculations were performed using SPSS 27.0 (Chicago, IL).

3 Results

3.1 Anthropometric, regional adiposity, and metabolic characteristics

The two groups were matched by age. For body fat mass distribution, compared with the endurance-trained women, the sedentary subjects had higher total fat mass (+1.8 kg, p = 0.002), % total fat (+6.6%, p < 0.001), TFM (+1.0 kg, p = 0.003), % TFM (+3.1%, p < 0.001), arm FM (+0.21 kg, p = 0.006), and LFM (+0.48 kg, p = 0.018). Meanwhile, the BMI value was slightly lower in the sedentary women (–0.9 kg/m^2, p = 0.003). The A/Tr and L/Tr ratios were similar between the two groups. Systolic blood pressure (SBP) was slightly higher in the endurance-trained subjects (+3 mmHg, p = 0.008), while diastolic blood pressure (DBP) was similar between the two groups. For adipokines, a higher leptin concentration was observed in the sedentary group (+2.86 ng/ml, p < 0.001), although the adiponectin level was comparable between groups (10.77 ± 3.70 μg/ml in sedentary *vs.* 10.96 ± 4.12 μg/ml in trained, p = 0.743). For insulin sensitivity, QUICKI was lower in the sedentary subjects (–0.02U, p = 0.002). For the lipid profiles, LDL-C, ApoB, and ApoB/ApoA1 were higher in the sedentary subjects. For inflammatory factors, WBC, TNF-α, and hsCRP were similar between groups (Table 1).

TABLE 1 Anthropometric, regional adiposity, and metabolic characteristics (X ± SD).

	Sedentary	Endurance-Trained	P-Value
n	101	100	*NA*
Age (years)	20.3 ± 1.2	19.3 ± 1.2	0.278
BMI (kg/m^2)	20.61 ± 2.17	21.51 ± 2.00	**0.003**
Total Fat Mass (kg)	15.32 ± 4.39	13.51 ± 3.90	**0.002**
% Total Fat	29.4 ± 5.2	22.8 ± 4.7	**<0.001**
TFM (kg)	7.41 ± 2.58	6.41 ± 2.10	**0.003**
%TFM	14.0 ± 3.3	10.9 ± 3.0	**<0.001**
AFM (kg)	1.37 ± 0.56	1.16 ± 0.54	**0.006**
A/Tr (%)	19.0 ± 5.0	18.0 ± 6.0	0.271
LFM (kg)	5.91 ± 1.44	5.43 ± 1.42	**0.018**
L/Tr (%)	83.5 ± 15.3	87.6 ± 14.7	0.052
SBP (mmHg)	103.3 ± 7.3	106.3 ± 8.5	**0.008**
DBP (mmHg)	57.1 ± 4.9	56.4 ± 6.1	0.399
Adiponectin (μg/ml)	10.77 ± 3.7	10.96 ± 4.12	0.743
Leptin (ng/ml)	9.36 ± 3.99	6.50 ± 2.63	**<0.001**
FPG (mmol/L)	4.76 ± 0.38	4.78 ± 0.39	0.441
Fasting insulin (μU/ml)	7.48 ± 4.97	5.15 ± 2.73	**<0.001**
QUICKI	0.37 ± 0.04	0.39 ± 0.04	**0.002**

(Continued)

TABLE 1 Continued

	Sedentary	Endurance-Trained	P-Value
n	101	100	*NA*
TG (mmol/L)	0.66 ± 0.27	0.62 ± 0.26	0.324
TC (mmol/L)	4.69 ± 0.68	4.54 ± 0.66	0.105
HDL-C (mmol/L)	1.96 ± 0.35	2.00 ± 0.36	0.353
LDL-C (mmol/L)	2.43 ± 0.59	2.25 ± 0.52	**0.020**
ApoA1 (mg/dl)	164.23 ± 20.84	169.97 ± 21.93	0.058
ApoB (mg/dl)	73.41 ± 14.36	68.97 ± 12.96	**0.023**
ApoB/ApoA1	0.46 ± 0.12	0.41 ± 0.09	**0.004**
PAI-1 (ng/ml)	17.64 ± 8.86	16.74 ± 7.40	0.437
WBC (/μl)	6122 ± 1698	5728 ± 1469	0.081
TNF-α (pg/ml)	0.58 ± 0.64	0.50 ± 0.37	0.299
Log (hsCRP)	1.00 ± 0.48	1.02 ± 0.47	0.750

Numbers in bold: with statistical significance.

3.2 Univariate correlations

3.2.1 Association of adiponectin with body fat mass distribution

A simple correlation analysis (Table 2) revealed that adiponectin was reversely associated with BMI (r = –0.211, p = 0.034), TFM (r = –0.221, p = 0.027), and % TFM (r = –0.197, p = 0.048) and positively associated with L/Tr (r = 0.360, p < 0.001, Figure 2) in the sedentary individuals. For the endurance-trained women, only the L/Tr ratio was found to have a positive association with adiponectin (r = 0.296, p = 0.003, Figure 2). Notably, adiponectin was positively associated with total fat mass (r = 0.315, p < 0.001), % total fat (r = 0.241, p = 0.016), LFM (r = 0.292, p = 0.003), and L/Tr ratio (r = 0.291, p = 0.003) after adjustment for TFM in the sedentary group. For the endurance-trained women, plasma adiponectin was positively associated with total fat mass (r = 0.400, p<0.001), LFM (r = 0.393, p < 0.001), and L/Tr ratio (r = 0.331, p = 0.001) after adjustment for TFM (Table 2).

3.2.2 Association of adiponectin with serum lipids

In the sedentary group, adiponectin was positively associated with HDL-C (r = 0.431, p < 0.001) and ApoA1(r = 0.373, p < 0.001) and reversely associated with TG (r = –0.297, p = 0.003) and ApoB/ApoA1 (r = –0.253, p = 0.011). After adjustment for TFM, positive associations with HDL-C (r = 0.420, p < 0.001), ApoA1 (r = 0.363, p < 0.001), and TC (r = 0.271, p = 0.007) were observed, as well as a reverse association with TG (r = –0.258, p = 0.01). In the endurance-trained group, positive associations of adiponectin with HDL-C (r = 0.241, p = 0.016, Figure 3) and ApoA1 (r = 0.223, p = 0.026) were observed. After adjustment for TFM, the positive associations remained significant (for HDL-C, r = 0.237, p = 0.018 and for ApoA1, r =0.218, p = 0.03). However, no significant association of adiponectin with LDL-C was observed in both groups (Table 2).

3.2.3 Association of adiponectin with inflammatory markers

A reverse association of adiponectin with hsCRP (r = –0.196, p = 0.049) was found in the sedentary group but was not significant after the adjustment for TFM. In the endurance-trained group, a reverse association of adiponectin with PAI-1 (r = –0.216, p = 0.031) was observed, which remained significant (r = –0.210, p = 0.037) even after the adjustment for TFM (Table 2).

3.2.4 Association of adiponectin with insulin resistance

No significant association was found between adiponectin and QUICKI in both groups before and after adjustment for TFM (Table 2).

3.2.5 Association of LFM with serum lipids in the whole study cohort

After adjustment for TFM, LFM was positively associated with HDL-C (r = 0.160, p = 0.024) and negatively associated with ApoB/ApoA1(r = –0.144, p = 0.042). A borderline negative association with TG (r = –0.136, p = 0.055) was also observed in the whole study cohort. However, the associations failed to achieve significance after further adjustment for both TFM and adiponectin (Table 3).

3.3 Multivariate correlations

We performed multivariate linear regression analysis to determine the key predictors of adiponectin level among the variables that showed significant univariate associations with adiponectin. For the sedentary individuals, the L/Tr ratio and HDL-C were the strongest positive correlation factors, whereas TG was the negative correlation factor of adiponectin. HDL-C with L/Tr ratio and TG can explain the 29.5% variance of adiponectin in this study. In the endurance-trained group, the strongest predictors for adiponectin

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TABLE 2 Correlations of adiponectin with regional adiposity and metabolic variables in the two groups before and after adjustment for TFM.

	Sedentary	Endurance-Trained	Sedentary	Endurance-Trained
	Not adjusted		Adjusted for TFM	
	r	r	r	r
BMI	-0.211*	0.064	–0.040	0.150
Total Fat Mass	-0.142	0.041	0.315‡	0.400‡
%Total fat	-0.093	0.035	0.241*	0.206*
TFM	-0.221*	-0.053	NA	NA
% TFM	-0.197*	-0.068	0.042	–0.056
AFM	0.073	0.082	0.166	0.185
A/Tr	0.144	0.166	0.132	0.176
LFM	-0.003	0.159	0.292†	0.393‡
L/Tr ratio	0.360‡	0.296†	0.291†	0.331‡
Leptin	-0.089	-0.056	0.025	–0.062
FPG	0.079	-0.085	0.070	–0.094
QUICKI	0.032	-0.024	–0.031	–0.036
TG	-0.297†	-0.089	–0.258†	–0.092
TC	0.209	0.177	0.271†	0.176
HDL-C	0.431‡	0.241*	0.420‡	0.237*
LDL-C	0.047	0.082	0.112	0.086
ApoA1	0.373‡	0.223*	0.363‡	0.218*
ApoB	-0.087	0.021	–0.007	0.024
ApoB/ApoA1	-0.253*	-0.122	–0.194	–0.116
PAI-1	-0.162	-0.216*	–0.088	–0.210*
WBC	-0.192	-0.070	–0.157	–0.068
TNF-α	0.011	-0.160	–0.004	–0.160
Log (hsCRP)	-0.196*	-0.127	–0.163	–0.156

*: $P < 0.05$; †: $P < 0.01$; ‡: $P < 0.001$; NA, not applicable.

were the L/Tr ratio and HDL-C. The L/Tr ratio and HDL-C had a positive correlation with adiponectin. The two variables may jointly explain 13.5% of the variance of adiponectin in the model (Table 4).

4 Discussion

In this study, we unveiled that the endurance-trained young women were more sensitive to insulin compared with the sedentary women, but the two groups have similar adiponectin concentrations. In addition, the association between adiponectin and QUICKI did not reach significance, suggesting that adiponectin may not be involved in mediating the exercise-related improvement of insulin sensitivity. LFM was associated with a favorable lipid profile against CVDs in the whole cohort. However, this relationship disappeared after plasma adiponectin was taken into account, implying the involvement of adiponectin in the cardioprotective role of LFM. The HDL-C and L/Tr ratio had the strongest positive associations with adiponectin in both groups. The TG in the sedentary group and PAL-1 in the endurance-trained group were important factors that negatively correlated with adiponectin. These results suggest that the associations among adiponectin, lipid, and inflammatory factors vary in women with different physical activity statuses.

The negative relationships of adiponectin with BMI and total fat mass had been well documented (26, 27). Consistent with previous reports, the present research revealed a negative association of adiponectin with BMI and TFM in the sedentary Japanese women. However, no significant differences were found in the endurance-trained women. The most notable finding was that the total body fat mass, LFM, and L/Tr ratio were positively associated with adiponectin in both groups after adjustment for TFM. The positive association of total body fat mass with adiponectin may be a reflection of the correlation of LFM to adiponectin since it represents a major adipose deposition after adjustment for TFM. The multiple regression analysis revealed that the L/Tr ratio was the strongest predictor of adiponectin in both groups. Unlike other studies that emphasized the importance

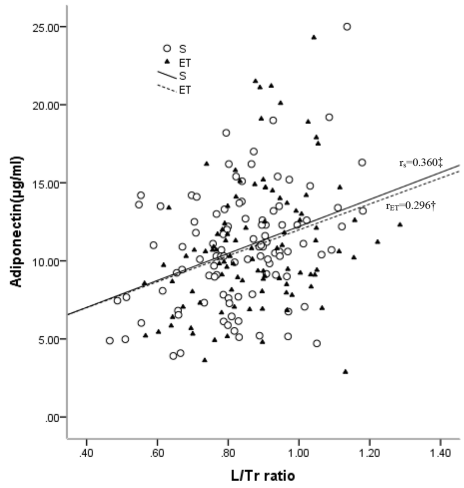

FIGURE 2
Relationships between plasma levels of adiponectin and L/Tr ratio in the sedentary (S) and endurance-trained (ET) groups. r_S: correlation coefficient of the sedentary group; r_{ET}: correlation coefficient of the endurance-trained group. *: P < 0.05; †: P < 0.01; ‡: P < 0.001 .

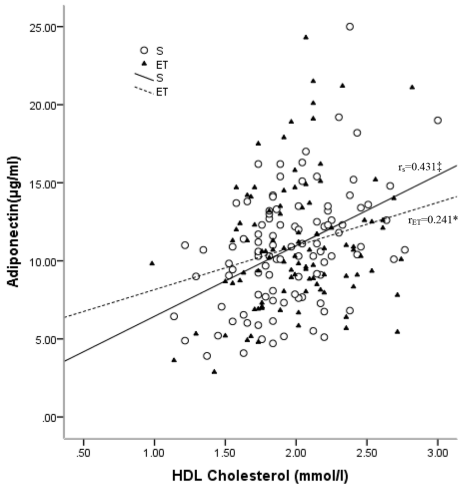

FIGURE 3
Relationships between plasma levels of adiponectin and HDL-C in the sedentary (S) and endurance-trained (ET) groups. R_S: correlation coefficient of the sedentary group; r_{ET}: correlation coefficient of the endurance-trained group. *: P < 0.05; †: P < 0.01; ‡: P < 0.001.

of abdominal fat mass, our observations suggested that LFM is an important determinant that is positively associated with adiponectin independent of TFM. One of the most important notions addressed in the current study was that LFM and TFM are likely to exert their impacts on circulating adiponectin levels in different ways. This hypothesis was supported by studies that found a lower adiponectin mRNA expression in the visceral adipose tissue compared with the subcutaneous adipose tissue, suggesting an antagonizing impact of intra-abdominal fat on adiponectin production (28). Another study in women with metabolic syndrome observed lower adiponectin mRNA expression levels in the visceral adipose tissue than the normal controls (29). A possible explanation for the difference in the production of adiponectin in different regional adiposities is that large visceral adipocytes with greater triglyceride storage produce less adiponectin than small adipocytes in the subcutaneous region (30). Given that large adipocytes are less insulin sensitive, the insulin sensitivity of adipocytes may be a determinant of adiponectin production (30).

In the current study, we found that LFM is associated with a favorable lipid profile against atherosclerosis. This observation is in line with our previous data (31) and other previous research (32), suggesting that the cardioprotective role of LFM is associated with an advantageous serum lipid-lipoprotein profile. However, these associations became non-significant after adiponectin was taken into account in the current study. These observations, together with the data implying that adiponectin gene mRNA expression is more abundant in the subcutaneous adipose tissue than in the visceral adipose tissue (28, 29), lead us to hypothesize that the antiatherogenic role of LFM may be mediated by adiponectin. Regarding the relationship between adiponectin and serum lipids, we found that plasma adiponectin is positively related to HDL-C and ApoA1 independent of TFM in both the sedentary and endurance-trained women. In addition, we found a negative association with TG exclusively in the sedentary subjects. These results suggest that adiponectin is associated with hepatic lipase (33) and exerts its lipid-modulating effect by antagonizing the activity of hepatic

TABLE 3 Partial correlations between LFM and serum lipids in the whole cohort.

	Adjustment for TFM		Adjustment for TFM and Adiponectin	
	r	P-Value	r	P-Value
TC	0.034	0.637	−0.043	0.549
TG	−0.136	0.055	−0.082	0.251
HDL-C	0.160	**0.024**	0.056	0.430
LDL-C	−0.031	0.66	−0.067	0.344
ApoA1	0.133	0.061	0.041	0.566
ApoB	−0.097	0.173	−0.103	0.146
ApoB/ApoA1	−0.144	**0.042**	−0.097	0.173

r, Partial correlation coefficient.
Numbers in bold: with statistical significance.

TABLE 4 Multiple-regression analysis for adiponectin as a dependent variable.

Independent Variables	B	SE (B)	Standard B	P-Value
Sedentary (r^2 = 0.295)				
HDL-C	4.74	1.185	0.452	**<0.001**
L/Tr ratio	6.56	2.2	0.273	**0.004**
TG	−2.977	1.366	−0.221	**0.032**
Endurance-trained (r^2 = 0.135)				
L/Tr ratio	7.817	2.653	0.279	**0.004**
HDL-C	2.54	1.098	0.219	**0.023**

B, regression coefficient; SE(B), standard error of regression coefficient; Standard B, standard regression coefficient.
Numbers in bold: with statistical significance.

lipase, which hydrolyzes triglyceride and phospholipids in HDL particles (34). Moreover, adiponectin can reduce hepatic lipid accumulation by stimulating fat oxidation induced by AMP-activated protein kinase activation (35). A reduction of hepatic lipid content may, in turn, improve lipid catabolism in the liver (36). In our study, the endurance-trained subjects displayed lower LDL-C and ApoB levels than the sedentary women. This finding may be partially due to the fact that the endurance-trained women were more insulin-sensitive, resulting in an enhanced catabolic rate of triglyceride. Moreover, the endurance-trained women have less TFM deposition than the sedentary women, leading to a lesser supply of non-esterified fatty acids for synthesizing triglyceride in the liver. Since both groups had similar circulating plasma adiponectin concentrations, it is plausible that adiponectin plays different roles in lipid regulation in young women with different physical activities.

Concurrent with our previous report on a young, healthy Japanese male population (37) and another study carried out in Pima Indian children (38), a significant association between adiponectin and insulin resistance was absent in the present study. This may be due to the narrow range of the QUICKI index and the relatively low BMI levels of our study subjects. The relationship between adiponectin and insulin resistance has been shown to be adiposity-dependent. In a cross-sectional study comprising 1,196 adolescents, adiponectin was found to have a negative association with fasting insulin levels only in overweight and obese subjects, but this association was absent in lean adolescents (39). In addition, serum adiponectin levels have been shown to decrease parallel to weight gain, as well as the progression of insulin resistance, in rhesus monkeys (40). These findings suggest that adiponectin may contribute primarily to insulin action changes associated with adiposity change. Therefore, we predicted that the failure to demonstrate the independent relationship between adiponectin and insulin resistance assessed by QUICKI in young, healthy women suggests that adiponectin may be associated primarily with adiposity and then modified by insulin resistance.

This study has several potential limitations that should be further investigated. First, the study design was cross-sectional and had an observational nature, which does not imply causality. Second, the levels of high-molecular-weight isoforms of adiponectin were not assayed in this sample cohort; thus, the total adiponectin level may only be a surrogate of the analysis. Finally, the cohort was relatively homogenous with a small range of insulin resistance index; thus, the relationship between adiponectin and insulin resistance may be underestimated. Although confounders such as obesity, age, sex, cigarette smoking, alcohol drinking, and drug administration were controlled, whether the results can be extended to more insulin-resistant subjects, such as an obese population, remains unknown.

5 Conclusions

Body fat distribution, especially the ratio of LFM to TFM, joined with HDL-C, are two important determinants of adiponectin in both sedentary and endurance-trained healthy young women. No significant difference regarding circulating adiponectin levels was observed between the two groups, which may partially be due to them having a similar HDL-C and L/Tr ratio. In addition, TG in the sedentary women and PAI-1 in the endurance-trained women are negatively associated with adiponectin. These results suggest that adiponectin plays different roles in lipid modulation and anti-inflammation in women with different physical activity statuses. Furthermore, LFM is associated with a favorable lipid profile in the whole study cohort, which became absent when adiponectin was taken into account, suggesting that adiponectin may be involved in this association.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethnic committee of Mukogawa Women's

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

Author contributions

YG - Conceptualization, methodology, writing - original draft. FZ - Investigation, data curation. JZ - Investigation, data curation. XN - Investigation and resources, data curation. LS - Investigation, data curation. YX - Investigation, funding acquisition, writing - review and editing. JH - Investigation, data curation. TK - Investigation, funding acquisition, writing - review and editing. BW - Supervision, writing - review and editing, funding acquisition. BW supervised the study, had full access to all data in the study, and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Glossary

% total fat	percentage of body fat mass (total body fat mass divided by body weight)
% TFM	percentage of trunk fat mass (trunk fat mass divided by body weight)
AFM	arm fat mass
A/Tr	arm fat mass divided by trunk fat mass
ApoA1	apolipoprotein A-1
ApoB	apolipoprotein B-100
BMI	body mass index
CRP	C-reactive protein
CV	coefficient of variation
CVD	cardiovascular disease
DBP	diastolic blood pressure
DXA	dual X-ray absorptiometry
ELISA	enzyme-link immunosorbent assay
ET	endurance-trained
FM	fat mass
FPG	fasting plasma glucose
HDL-C	high-density lipoprotein cholesterol
hsCRP	highly sensitive C-reactive protein
L/Tr	lower-body-fat mass divided by trunk fat mass
LDL-C	low-density lipoprotein cholesterol
LFM	lower-body-fat mass
QUICKI	quantitative insulin-sensitivity check index
MRI	magnetic resonance imaging
PAI-1	plasminogen activator inhibitor-1
S	sedentary
SBP	systolic blood pressure
TC	total cholesterol
TFM	trunk fat mass
TG	triglycerides
TNF-α	tumor necrosis factor-α
WBC	white blood cell count

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Short-term effect of polyethylene glycol loxenatide on weight loss in overweight or obese patients with type 2 diabetes: An open-label, parallel-arm, randomized, metformin-controlled trial

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Objective: Polyethylene glycol loxenatide (PEG-Loxe) is a novel, once-weekly glucagon-like peptide 1 receptor agonist that is approved in doses of 0.1 mg and 0.2 mg for the treatment of type 2 diabetes mellitus (T2DM). However, no clinical trials have been designed to determine the effect of 0.3 mg PEG-Loxe on weight loss in overweight or obese patients with T2DM. This trial aimed to evaluate the short-term effect of 0.3 mg PEG-Loxe, injected subcutaneously once weekly, for weight management in overweight or obese patients with T2DM.

Methods: This 16-week, open-label, parallel-arm, randomized, metformin-controlled trial was conducted at Shandong Provincial Hospital in Shandong, China. Patients with T2DM, who were overweight or obese (body mass index ≥ 25.0 kg/m^2) and had been treated with lifestyle interventions or a combination with oral antidiabetic drug monotherapy were randomized (2:1) to receive 0.3 mg PEG-Loxe or 1500 mg metformin. The primary endpoint was a change in body weight from baseline to week 16.

Results: Overall, 156 patients were randomized and exposed to treatment. Weight loss was 7.52 kg (8.37%) with PEG-Loxe and 2.96 kg (3.00%) with metformin, with a between-group difference of 4.55 kg (95% CI, 3.43 to 5.67) ($P < 0.001$). A significantly higher proportion of patients lost ≥5% (61.5% vs. 25.0%) or 10% (26.9% vs. 5.8%) body weight in the PEG-Loxe group than in the metformin group ($P < 0.01$). Additionally, PEG-Loxe resulted in marked improvements in several cardiovascular risk factors compared to metformin, including body mass index, waist circumference, visceral fat area, blood pressure, and lipid profile. PEG-Loxe and metformin displayed almost equal potency for glycemic control. The incidence of adverse events was 46.2% (48/104) and 44.2% (23/52) in the PEG-Loxe and metformin groups, respectively.

Conclusion: In overweight or obese patients with T2DM, a once-weekly subcutaneous administration of PEG-Loxe for 16 weeks, in addition to lifestyle interventions or oral antidiabetic drug therapy, resulted in significantly greater weight loss compared to metformin. Additional trials are necessary to establish whether these effects can be maintained in the long term.

Clinical trial registration: www.chictr.org.cn, identifier ChiCTR2200057800.

KEYWORDS

polyethylene glycol loxenatide, weight loss, overweight, obesity, type 2 diabetes, glucagon-like peptide 1 receptor agonist, metformin

1 Introduction

Type 2 diabetes mellitus (T2DM) is commonly associated with being overweight or obese. In China, more than half of patients with T2DM are overweight or obese (1). These conditions are associated with an increased risk of poor glycemic control, hypertension, dyslipidemia, and cardiovascular disease in patients with T2DM (2, 3).

Weight management is a vital aspect of treatment for patients with T2DM. However, weight gain is a side effect of some antidiabetic drugs, including thiazolidinedione, sulfonylurea, glinide, and insulin (4–6). Therefore, for overweight or obese patients with T2DM, antidiabetic drugs causing weight loss would be the preferred strategy for managing diabetes. Strong evidence shows that metformin, sodium-glucose cotransporter 2 inhibitors (SGLT2i), and glucagon-like peptide 1 receptor agonists (GLP-1RAs) can induce weight loss while improving glycemic control (7, 8).

GLP-1RAs are a relatively new class of drugs used to treat T2DM in the general population. The primary function of GLP-1RA is to improve glucose metabolism by increasing pancreatic β-cell insulin secretion and reducing α-cell glucagon secretion. Another well-known effect of GLP-1RA is weight loss through appetite suppression and reduced food intake (9). In addition to their roles in glycemic control and weight loss, several GLP-1RAs were shown to reduce the risk of major adverse cardiovascular events in patients with T2DM (10–13).

Polyethylene glycol loxenatide (PEG-Loxe) is a novel GLP-1RA derived from exendin-4, with 53% homology to human GLP-1 and an anti-PEG-Loxe antibody positive rate of < 2% (14–16). PEG-Loxe is approved in once-weekly doses of 0.1 mg and 0.2 mg for the treatment of T2DM and has been proven to be efficacious and well tolerated (15, 16). As a secondary endpoint, weight loss of <1 kg was observed in non-obese patients with T2DM administered PEG-Loxe doses in phase 3 trials (15, 16). In a phase 1 trial, administration of 0.3 mg PEG-Loxe resulted in a weight change of -1.9 kg in non-obese patients with T2DM (17). However, no clinical trials have been designed to determine the effect of this dose on weight loss in overweight or obese patients with T2DM. Therefore, this trial aimed to evaluate the short-term effect of PEG-Loxe dose of 0.3 mg, injected subcutaneously, once weekly for weight management in overweight or obese patients with T2DM.

2 Materials and methods

2.1 Trial design and participants

This 16-week, open-label, parallel-arm, randomized, metformin-controlled trial was conducted between March 2022 and October 2022 at the Department of Endocrinology, Shandong Provincial Hospital, Shandong, China. The Ethics Committee of Shandong Provincial Hospital approved the trial protocol (No. 2022-046/February 2022), which complied with the Declaration of Helsinki. Written informed consent was obtained from all the participants. The trial was registered in the Chinese Clinical Trial Registry (ChiCTR2200057800).

Key inclusion criteria included T2DM diagnosis (according to the 1999 World Health Organization criteria) (18), 18–65 years of age, body mass index (BMI) of ≥25.0 kg/m2, hemoglobin A1c (HbA1c) of 7.0–10.0%, and treated with lifestyle interventions or in combination with a stable dose of one oral hypoglycemic drug (thiazolidinedione, sulfonylurea, glinide, or a-glucosidase inhibitor) for at least 3 months. Key exclusion criteria included type 1 diabetes, gastrointestinal disorders associated with long-term nausea and vomiting, a history of acute or chronic pancreatitis, or had been treated with any GLP-1 RA or dipeptidyl peptidase-4 inhibitor within the last 3 months. Detailed inclusion and exclusion criteria are presented in Supplementary Figure S1.

2.2 Randomization and masking

Eligible patients were randomly assigned using an Interactive Web Response System in a 2:1 ratio to receive 0.3 mg PEG-Loxe (Hansoh Pharma) or 1500 mg metformin (Merck). Randomization was stratified according to the following two variables:

a. BMI: 25.0–29.9 kg/m^2 (overweight) or ≥30.0 kg/m^2 (obese); and
b. Pre-trial treatment: lifestyle interventions or OAD therapy.

Moreover, the clinical trial statistician was blinded to the two groups during data analysis.

2.3 Procedures

PEG-Loxe or metformin was added to the current treatment regimen of each patient: OAD therapy or lifestyle interventions. PEG-Loxe was injected subcutaneously once weekly. This treatment followed a fixed-dose-escalation regimen: an initial dose of 0.1 mg for 4 weeks, followed by 0.2 mg for 4 weeks, then a maintenance dose of 0.3 mg for 8 weeks. Similarly, a metformin maintenance dose of 1500 mg was administered in 500 mg weekly increments from 500 mg to 1500 mg.

In cases where the baseline HbA1c level was <7.5%, patients taking sulfonylurea or glinide were asked to decrease their dose to minimize the risk of hypoglycemia (10). Dose adjustment was performed according to the methods of Elhadd et al. and Kendall et al. (19, 20).

Patient visits were at 4, 8, and 16 weeks, and they included physical examination and data collection. The following information was obtained: demographic data, medical history, vital signs, visceral fat area (VFA), and laboratory test results. Laboratory tests included those for HbA1c, fasting plasma glucose (FPG), C-peptide, lipid profile, and liver function. Additionally, any adverse events (AEs) were recorded. For weight measurement, patients were instructed to remain in the fasting condition, wear light clothing, and take off their shoes. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse rate were measured using a blood pressure monitor (HEM-7312; OMRON, Kyoto, Japan). VFA was assessed using bioelectric impedance analysis (InBody 720, Seoul, South Korea). Fasting blood samples were collected in the morning and analyzed at the Clinical Laboratory of Shandong Provincial Hospital. β-cell function (HOMA-B) and insulin sensitivity (HOMA-S) were estimated using FPG and fasting C-peptide in an updated Homeostasis Model Assessment (HOMA2) obtained from the University of Oxford database (https://www.dtu.ox.ac.uk/homacalculator/). Hypoglycemia was classified as level 1, 2, or 3 based on the definitions of the American Diabetes Association guidelines (16) (Supplement).

2.4 Endpoints

Endpoints were collected at week 16, and the primary endpoint was a change in body weight. The secondary endpoints included the proportion of patients with ≥5% and ≥10% weight loss percentages; and changes in BMI, waist circumference (WC), VFA, HbA1c, FPG, C-peptide, HOMA-B, HOMA-S, total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), SBP, and DBP. The safety endpoints included AEs, serious AEs (SAEs), hypoglycemic events, levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and pulse rate.

2.5 Statistical analysis

The planned sample size of 150 patients was randomized 2:1 to receive either PEG-Loxe or metformin. This sample size was expected to provide a power of 80% to detect a difference of ≥2 kg weight loss between the PEG-Loxe and metformin groups, with a standard deviation (SD) of 3.66 (17, 21), α level of 0.05, and 20% withdrawal rate.

The normal distribution of variables was evaluated using the Kolmogorov-Smirnov test and by assessment of residual distribution. Baseline variables were analyzed using independent t test, Mann-Whitney U test, and χ^2 test.

The efficacy analyses were evaluated using the full analysis set, defined as patients exposed to ≥1 treatment dose and had a baseline assessment. Safety data were assessed using the safety analysis set, defined as patients exposed to ≥1 treatment dose. The primary endpoint (change in body weight) was analyzed using a mixed model for repeated measurements (MMRM), which included group, time, and the corresponding interactions as fixed effects, and baseline weight and sex as covariates. MMRM was used to analyze BMI, WC, VFA, HbA1c, FPG, C-peptide, HOMA-B, HOMA-S, TC, TG, LDL-C, HDL-C, SBP, DBP, ALT, AST, and pulse rate. Categorical variables were evaluated using the χ^2 test or Fisher exact test. Missing data were imputed using a multiple linear imputation analysis according to the rules of Rubin (22). Sensitivity analyses were performed on the per-protocol set, defined as patients who completed the trial without major protocol violations.

Results are shown as adjusted mean and 95% CI, if not indicated otherwise. A P value < 0.05 (two-tailed) was considered statistically significant. All statistical analyses were performed using Statistical Analysis System (SAS) version 9.4 (RRID: SCR_008567).

3 Results

Between March 2022 and June 2022, 212 patients were screened, of which 156 were enrolled and randomized to receive PEG-Loxe (n = 104) or metformin (n = 52). Twelve (11.5%) patients in the PEG-Loxe group and five (9.6%) patients in the metformin group withdrew from the study. The main reasons for withdrawal were AEs and failure to follow-up (Figure 1).

The baseline characteristics of the patients are presented in Table 1. At baseline, the only significant between-group difference was observed for VFA (greater in the PEG-Loxe group, P < 0.001).

3.1 Body weight

PEG-Loxe treatment resulted in significant weight loss compared to metformin treatment during the trial period. After 16 weeks, the primary endpoints (least-square mean (LSM) weight loss) were 7.52 kg (8.37%) and 2.96 kg (3.00%) for the PEG-Loxe and metformin groups, respectively, with a between-group mean difference of 4.55 kg (95% CI: 3.43, 5.67; P < 0.001) (Figures 2A, B; Table 2). Sensitivity analyses showed similar findings (Supplementary Table S1).

After 16 weeks, the proportions of patients with a weight loss of ≥5% were 61.5% and 25.0% in the PEG-Loxe and metformin groups (P < 0.001), respectively, and those of patients with a weight loss of

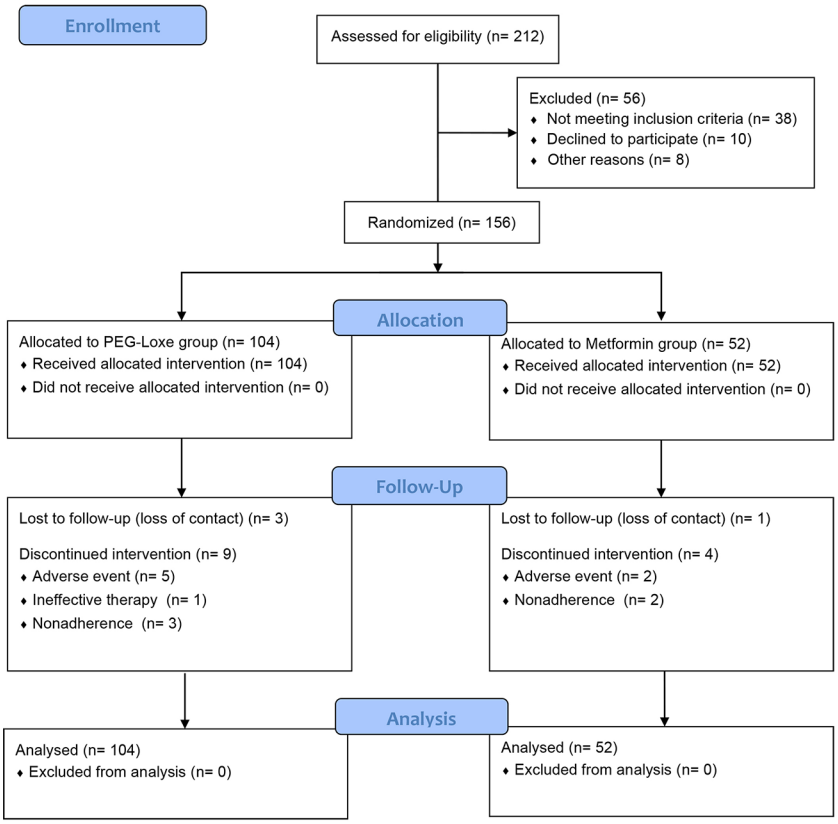

FIGURE 1
Consort flow diagram.

≥10% were 26.9% and 5.8% in the PEG-Loxe and metformin groups (P =0.001), respectively (Figures 2C, D).

3.2 BMI, WC, and VFA

The LSM (95% CI) change in BMI from baseline to week 16 was greater in the PEG-Loxe group [-2.55 (-2.74, -2.37) kg/m^2] than in the metformin group [-0.92 (-1.17, -0.67) kg/m^2] (P < 0.001). The LSM (95% CI) change in WC from baseline to week 16 was greater in the PEG-Loxe treated patients [-12.26 (-12.58, -11.55) cm] than in metformin-treated patients [-5.67 (-6.60, -4.75) cm] (P < 0.001). After 16 weeks, VFA reduction was significantly higher in the PEG-Loxe group [-26.02 (-27.60, -24.44) cm^2] than in the metformin group [-12.39 (-14.45, -10.32) cm^2] (P < 0.001) (Table 2).

3.3 Glucose control

The LSM changes in HbA1c after week 16 were similar between the two groups (PEG-Loxe, -1.22% [95% CI: -1.38 to -1.06]; metformin, -1.17% [95% CI: -1.39, -0.96]; P =0.69). No significant differences were observed in the mean FPG reduction relative to the baseline between the groups at week 16 (PEG-Loxe, -1.46 mmol/L [95% CI: -1.57, -1.34]; metformin, -1.49 mmol/L [95% CI: -1.65, -1.34]; P =0.70). HOMA2-B levels increased in both groups; the change in the PEG-Loxe group was greater than that in the metformin group (P =0.003). In addition, HOMA2-S increased in both groups, with greater changes observed in the metformin group than the PEG-Loxe group (P < 0.001) (Table 2).

3.4 Lipid profile and blood pressure

TC, TG, and LDL-C levels were improved with PEG-Loxe treatment compared to metformin treatment (P < 0.001). SBP was reduced by 3.18 mmHg and and 0.28 mmHg with PEG-Loxe and metformin, respectively (P < 0.001). DBP was reduced by 1.34 mmHg and 0.19 mmHg with PEG-Loxe and metformin, respectively (P < 0.001) (Table 2). Supplementary Table S2 also shows these efficacy variables at weeks 4 and 8.

3.5 Safety evaluation

After 16 weeks of treatment, the incidence of AEs was 46.2% (48/104) and 44.2% (23/52) in the PEG-Loxe and metformin groups, respectively (P =0.82). The incidence of SAEs was 2.9% (3/104) and 1.9% (1/52) in the PEG-Loxe and metformin groups, respectively. No deaths occurred during the study period. The most common AEs during the 16-week treatment were gastrointestinal disorders, with a greater incidence in the PEG-Loxe group (24.0%) than in the metformin group (17.3%) (Table 3). Gastrointestinal disorders were mostly mild to moderate and occurred primarily during the first four

TABLE 1 Baseline characteristics of patients.

	PEG-Loxe(n=104)	Metformin(n=52)	*P* value
Age, y	40.3 (10.0)	42.2 (9.5)	0.25
Women, N (%)	37 (35.6)	20 (38.5)	0.72
Duration, y	2.1 (1.7)	2.1 (1.5)	0.92
Body weight, kg	87.6 (13.9)	87.9 (13.7)	0.90
BMI, kg/m^2	30.0 (3.6)	30.1 (3.5)	0.92
WC, cm	102.1 (14.6)	104.6 (16.9)	0.34
VFA, cm^2 [median (IQR)]	127.0 (114.0–146.5)	113.5 (100.0–134.8)	<0.001
HbA1c, %	8.79 (0.83)	8.68 (0.95)	0.46
FPG, mmol/L	8.56 (0.87)	8.46 (0.96)	0.52
C-peptide, nmol/L [median (IQR)]	2.40 (1.65–2.90)	2.00 (1.40–2.88)	0.43
HOMA2-%B	122.1 (40.8)	119.3 (44.9)	0.70
HOMA2-%S [median (IQR)]	16.3 (13.5–23.8)	18.8 (12.9–27.7)	0.47
TC, mmol/L	5.05 (0.71)	5.36 (1.13)	0.09
TG, mmol/L	1.88 (0.53)	1.79 (0.52)	0.32
LDL-C, mmol/L	3.50 (0.66)	3.64 (0.80)	0.26
HDL-C, mmol/L [median (IQR)]	1.40 (1.20–1.65)	1.55 (1.11–1.80)	0.90
SBP, mmHg [median (IQR)]	132.0 (136.0–138.8)	134.0 (128.0–136.0)	0.59
DBP, mmHg [median (IQR)]	78.0 (70.0–81.5)	78.0 (70.3–80.0)	0.53
AST, U/L	44.7 (19.3)	39.6 (16.9)	0.12
ALT, U/L	48.8 (17.1)	45.2 (16.6)	0.23
Pulse rate, bpm [median (IQR)]	76.0 (69.0–82.0)	76.5 (70.0–83.0)	0.51
Previously treated with:			
lifestyle interventions, %	83 (79.8)	43 (82.7)	0.67
OAD therapy, %	21 (20.2)	9 (17.3)	0.67

BMI, body mass index; WC, waist circumference; VFA, visceral fat area; HbA1c, glycated hemoglobin; FPG, fasting plasma glucose; HOMA2-%B, updated homeostatic model assessment for beta cell function; HOMA2-%S, updated homeostatic model assessment for insulin sensitivity; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; AST, aspartate aminotransferase; ALT, alanine aminotransferase; bpm, beats per minute; OAD, oral antidiabetic drug; IQR, interquartile range. Data are expressed as mean (SD), unless otherwise indicated.

weeks of treatment. In the PEG-Loxe group, the incidence of gastrointestinal disorders was 11.5%, 5.8%, and 6.7% with 0.1 mg, 0.2 mg, and 0.3 mg treatment, respectively. Acute pancreatitis was not reported during this trial. In addition, the incidence of hypoglycemic events was 2.9% (3/104) and 3.8% (2/52) in the PEG-Loxe and metformin groups, respectively. No level 3 hypoglycemia was reported. Eight patients, five (4.8%) in the PEG-Loxe group and three (5.8%) in the metformin group, discontinued treatment because of AEs.

At week 16, the change in ALT was -6.88 U/L (95% CI: -7.54, -6.22) with PEG-Loxe and -2.33 U/L (95% CI: -3.17, -1.48) with metformin ($P < 0.001$). The changes in AST were -6.01 U/L (95% CI: -6.59, -5.43) with PEG-Loxe and -1.74 U/L (95% CI: -2.51, -0.97) with metformin ($P < 0.001$). Slight increases in pulse rate were observed in both groups: 2.07 bpm in the PEG-Loxe group and 0.43 bpm in the metformin group ($P < 0.001$) (Table 2).

4 Discussion

This is the first trial specifically designed to examine the efficacy of PEG-Loxe for weight management and the first trial to investigate PEG-Loxe at a higher dose of 0.3 mg with a fixed-dose-escalation regimen in patients with T2DM. A few studies have reported that a weight-maintenance phase occurred after approximately 12–16 weeks of GLP-1RA treatment (23–26); therefore, the treatment period was designed for 16 weeks to observe the short-term weight loss effect of PEG-Loxe. In the present trial, compared with metformin treatment, the addition of PEG-Loxe with lifestyle interventions or OAD therapy resulted in significantly greater weight loss at week 16. This was accompanied by significant improvements in several cardiovascular risk factors in overweight or obese patients with T2DM.

In previous phase 1–3 clinical trials, which enrolled patients with BMIs of 25–27 kg/m^2, 0.1 mg, 0.2 mg, and 0.3 mg PEG-Loxe

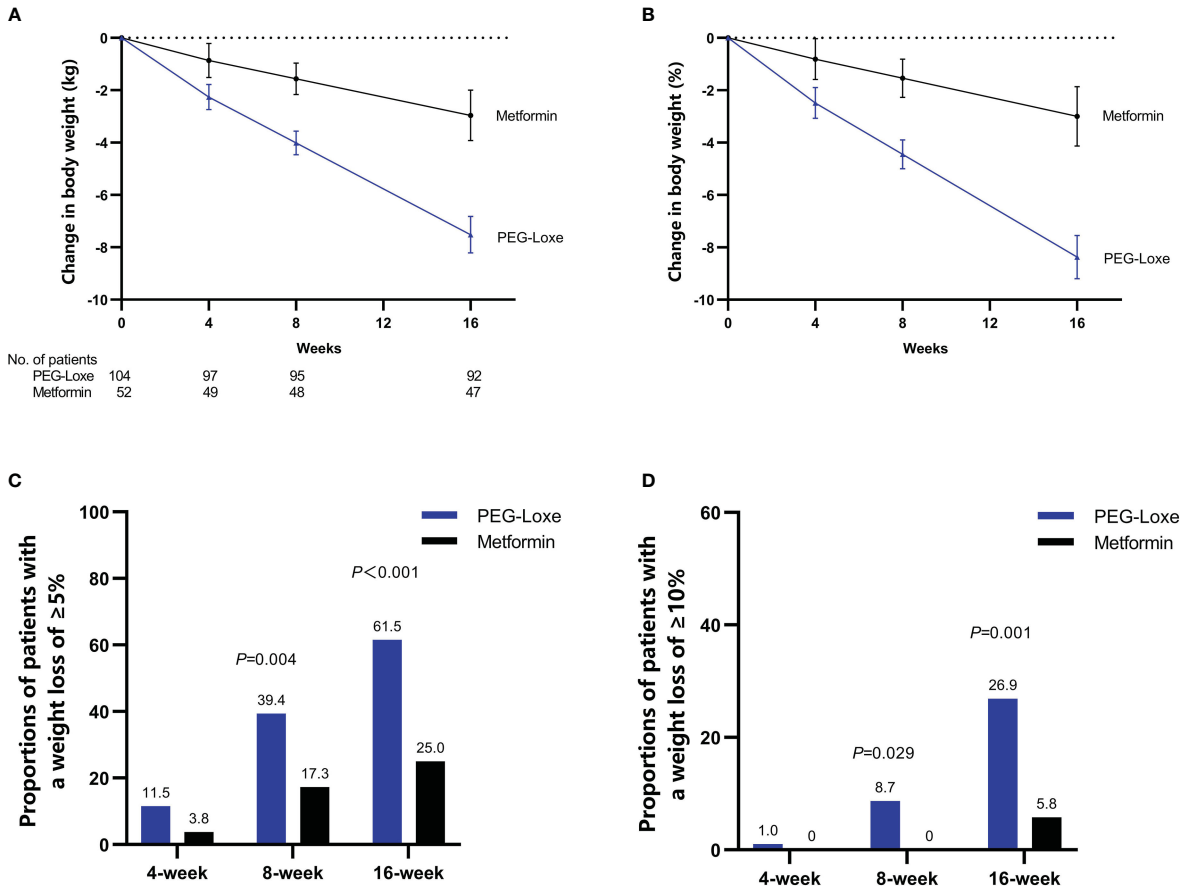

FIGURE 2
Efficacy endpoints during the 16-week treatment period: time course of absolute **(A)** and relative **(B)** changes in body weight; proportion of patients achieving ≥5% **(C)** or ≥10% **(D)** weight loss. Error bars indicate 95% CIs.

demonstrated a weak effect on weight loss (0.35–1.90 kg) after 8–24 weeks of treatment (15–17). In contrast, in the present trial, 0.3 mg PEG-Loxe achieved a significant weight reduction of 7.52 kg. Inconsistencies in these findings may be related to baseline characteristics of the patients. Patients with T2DM and obesity who initially had a higher BMI showed greater weight loss when they underwent GLP-1RA treatment (27). In comparison with previous trials, a higher baseline BMI (30 kg/m^2) in the present trial might have resulted in better weight loss.

Whether directly or indirectly, obesity contributes to the development of several cardiovascular risk factors and comorbidities, including dyslipidemia, hypertension, and cardiovascular disease (28, 29). Weight loss in the 5%–10% range or more is associated with improvements in these conditions (30). The Chinese Diabetes Society recommends short-term goals for weight management involving 5%–10% of weight loss in 3–6 months in overweight and obese patients with T2DM (31). In the present trial, the proportion of patients achieving clinically meaningful levels of weight loss (≥5% or 10%) was significantly higher with PEG-Loxe than with metformin after 16 weeks of treatment.

The glycemic control effects of 0.1 mg and 0.2 mg PEG-Loxe have been well established in patients with T2DM, as shown by HbA1c reduction ranging from 1.02–1.36% following 12 or 24 weeks of treatment (14–16). Furthermore, in a phase 1 trial, the change in HbA1c was 0.9% after 8 weeks of treatment with 0.3 mg PEG-Loxe (17). Using the same dose in the present trial, similar improvements in glycemic control were observed, with HbA1c reductions of 1.22% after 16 weeks of treatment. Moreover, in the present trial, PEG-Loxe and metformin displayed almost equal potency for glycemic control. The dose of metformin administered in this trial was 1500 mg/day, which is the conventional and most widely used dose in China. This dose conferred an HbA1c reduction of 1.17%, similar to a previous report wherein metformin reduced the HbA1c level by approximately 1.0–1.5% in patients with T2DM (31).

The safety profile of PEG-Loxe in the present trial was consistent with that in previous trials (15, 16). No new safety concerns have been identified. The main AEs associated with PEG-Loxe were gastrointestinal disorders (24.0%), which were predominantly mild to moderate and mainly occurred in the first 4 weeks (15, 16). To date, reports of a dose-escalation regimen for PEG-Loxe are not yet available. This present trial used a fixed-dose-escalation regimen to reduce possible gastrointestinal disorders. Consequently, 0.3 mg PEG-Loxe did not cause a significant increase in gastrointestinal disorders, and the incidences were comparable to those observed in previous trials using 0.1–0.2 mg PEG-Loxe (10.3–25.0%) (15, 16). In addition, GLP-1RAs do not increase the risk of hypoglycemia because of their glucose-dependent antidiabetic effects (32, 33). The incidence

TABLE 2 Primary and secondary endpoints at week 16.

	PEG-Loxe Group (n=104) Mean (95% CI)	Metformin Group (n=52) Mean (95% CI)	Between-Group Difference (95% CI)	*P* value
Body weight, kg	-7.52 (-8.21, -6.82)	-2.96 (-3.92, -2.00)	-4.55 (-5.67, -3.43)	<0.001
Body weight, %	-8.37 (-9.20, -7.55)	-3.00 (-4.13, -1.86)	-5.38 (-6.68, -4.07)	<0.001
BMI, kg/m^2	-2.55 (-2.74, -2.37)	-0.92 (-1.17, -0.67)	-1.63 (-1.92, -1.35)	<0.001
WC, cm	-12.26 (-12.98, -11.55)	-5.67 (-6.60, -4.75)	-6.59 (-7.58, -5.60)	<0.001
VFA, cm^2	-26.02 (-27.60, -24.44)	-12.39 (-14.45, -10.32)	-13.63 (-15.85, -11.42)	<0.001
HbA1c, %	-1.22 (-1.38, -1.06)	-1.17 (-1.39, -0.96)	-0.05 (-0.28, 0.19)	0.69
FPG, mmol/L	-1.46 (-1.57, -1.34)	-1.49 (-1.65, -1.34)	0.03 (-0.14, 0.21)	0.70
HOMA2-B, %	53.23 (47.38, 59.07)	40.36 (32.63, 48.08)	12.87 (4.40, 21.35)	0.003
HOMA2-S, %	0.41 (-0.52, 1.34)	4.88 (3.64, 6.12)	-4.47 (-5.84, -3.10)	<0.001
TC, mmol/L	-0.46 (-0.52, -0.39)	-0.19 (-0.27, -0.11)	-0.27 (-0.36, -0.18)	<0.001
TG, mmol/L	-0.39 (-0.44, -0.35)	-0.19 (-0.26, -0.13)	-0.20 (-0.27, -0.13)	<0.001
LDL-C, mmol/L	-0.43 (-0.49, -0.36)	-0.19 (-0.27, -0.11)	-0.23 (-0.33, -0.14)	<0.001
HDL-C, mmol/L	-0.16 (-0.21, -0.11)	-0.12 (-0.19, -0.06)	-0.04 (-0.10, 0.03)	0.25
SBP, mmHg	-3.18 (-3.78, -2.58)	-0.28 (-1.06, 0.51)	-2.90 (-3.77, -2.03)	<0.001
DBP, mmHg	-1.34 (-1.63, -1.06)	-0.19 (-0.57, 0.19)	-1.15 (-1.59, -0.72)	<0.001

BMI, body mass index; WC, waist circumference; VFA, visceral fat area; HbA1c, glycated hemoglobin; FPG, fasting plasma glucose; HOMA2-%B, updated homeostatic model assessment for b-cell function; HOMA2-%S, updated homeostatic model assessment for insulin sensitivity; TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure.

TABLE 3 Summary of safety.

	PEG-Loxe, No. (%) (n=104)	Metformin, No. (%) (n=52)	*P* value
Any AE	48 (46.2)	23 (44.2)	0.82
Any SAE			>0.99
Death	0 (0)	0 (0)	>0.99
Other	3 (2.9)	1 (1.9)	>0.99
AE by severity			
Severe	5 (4.8)	3 (5.8)	>0.99
Moderate	9 (8.7)	3 (5.8)	0.75
Mild	22 (21.2)	10 (19.2)	0.78
Discontinuation because of AEs	5 (4.8)	3 (5.8)	>0.99
AEs reported in ≥5% of patients by SOC/PT			
Gastrointestinal disorders	25 (24.0)	9 (17.3)	0.34
Nausea	13 (12.5)	4 (7.7)	0.43
Vomiting	6 (5.8)	1 (1.9)	0.43
Diarrhea	3 (2.9)	2 (3.8)	>0.99
Metabolism and nutritional disorders	8 (7.7)	4 (7.7)	>0.99
Decreased appetite	6 (5.8)	2 (3.8)	0.72
Infections and infestations	7 (6.7)	5 (9.6)	0.52
Upper respiratory tract infection	5 (4.8)	4 (7.7)	0.48
Hypoglycemia	3 (2.9)	2 (3.8)	>0.99
Level 1	3 (2.9)	1 (1.9)	>0.99
Level 2	0 (0)	1 (1.9)	0.33
Level 3	0 (0)	0 (0)	>0.99

AE, adverse event; SAE, serious adverse event; SOC, system organ class; PT, preferred term.

of hypoglycemic events in the present trial was comparatively low, and no level 3 hypoglycemia was reported. Additionally, GLP-1RAs are known to increase resting heart rate (34); in this trial, 0.3 mg PEG-Loxe increased the mean pulse rate by 2.07 bpm, which appears to be a class side effect of GLP-1RAs.

This study had several limitations. The open-label design of the present trial may have increased the risk of bias. Furthermore, the clinical trial duration was relatively short, and the full potential of 0.3 mg PEG-Loxe efficacy on weight loss may have been missed. Therefore, additional trials are warranted to assess whether these effects are maintained with long-term 0.3 mg PEG-Loxe treatment.

In conclusion, once-weekly subcutaneous PEG-Loxe administration resulted in significantly greater weight loss at 16 weeks, when compared with metformin administration, in overweight or obese patients with T2DM, with lifestyle interventions or OAD therapy. The effects of PEG-Loxe on body weight may provide a treatment option for overweight or obese patients with T2DM.

Data availability statement

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

Ethics statement

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

Author contributions

YD and XZ designed the trial. HC, QC, and XZ conducted the trial. HC and QC collected the data. XZ interpreted the data. YZ performed data analysis. HC, QC, and YD drafted the manuscript. XZ reviewed the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

Authors YD and YZ are employed in Hansoh Pharma.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1106868/full#supplementary-material

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Candidate loci shared among periodontal disease, diabetes and bone density

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Introduction: While periodontal disease (PD) has been associated with type 2 diabetes (T2D) and osteoporosis, the underlying genetic mechanisms for these associations remain largely unknown. The aim of this study is to apply cross-trait genetic analyses to investigate the potentially shared biology among PD, T2D, and bone mineral density (BMD) by assessing pairwise genetic correlations and searching for shared polymorphisms.

Methods: We applied cross-trait genetic analyses leveraging genome-wide association study (GWAS) summary statistics for: Periodontitis/loose teeth from the UKBB/GLIDE consortium (PerioLT, N=506594), T2D from the DIAGRAM consortium (N_{eff}=228825), and BMD from the GEFOS consortium (N=426824). Among all three, pair-wise genetic correlations were estimated with linkage disequilibrium (LD) score regression. Multi-trait meta-analysis of GWAS (MTAG) and colocalization analyses were performed to discover shared genome-wide significant variants (p_{MTAG} <5x10^{-8}). For replication, we conducted independent genetic analyses in the Women's Genome Health Study (WGHS), a prospective cohort study of middle-aged women of whom 14711 provided self-reported periodontal disease diagnosis, oral health measures, and periodontal risk factor data including incident T2D.

Results: Significant genetic correlations were identified between PerioLT/T2D (Rg=0.23; SE=0.04; p=7.4e^{-09}) and T2D/BMD (Rg=0.09; SE=0.02; p=9.8e^{-06}). Twenty-one independent pleiotropic variants were identified *via* MTAG (p_{MTAG}<5x10^{-8} across all traits). Of these variants, genetic signals for PerioLT and T2D colocalized at one candidate variant (rs17522122; $Prob_{H4}$ = 0.58), a 3'UTR variant of *AKAP6*. Colocalization between T2D/BMD and the original PerioLT GWAS p-values suggested 14 additional loci. In the independent WGHS sample, which includes responses to a validated oral health questionnaire for PD surveillance, the primary shared candidate (rs17522122) was associated with less frequent dental flossing [OR (95%CI)= 0.92 (0.87-0.98), p=0.007], a response that is correlated with worse PD status. Moreover, 4 additional candidate variants were indirectly supported by associations with less frequent dental flossing [rs75933965, 1.17(1.04-1.31), p=0.008], less frequent dental visits [rs77464186, 0.82(0.75-0.91), p=0.0002], less frequent

dental prophylaxis [rs67111375, 0.91(0.83-0.99), p=0.03; rs77464186, 0.80(0.72-0.89), p=3.8e^{-05}], or having bone loss around teeth [rs8047395, 1.09(1.03-1.15), p=0.005].

Discussion: This integrative approach identified one colocalized locus and 14 additional candidate loci that are shared between T2D and PD/oral health by comparing effects across PD, T2D and BMD. Future research is needed to independently validate our findings.

KEYWORDS

genetics, oral health, diabet mellitus, periodontal disease, bone densities, GWAS, survey and questionnaire

Introduction

Periodontal disease (PD) is a highly prevalent microbial induced chronic inflammatory disease with variable clinical expression. It has been estimated that 46% of US adults had periodontitis with 8.9% having severe periodontitis (1). PD is known to be associated with innate and adaptive immune responses (2), genetic susceptibility factors (3, 4), type 2 diabetes (T2D) (5), and osteoporosis (6). It has been shown that advanced PD progression correlated with poor glycemic control (7). Conversely, improvement in glycemic control among diabetic patients (8, 9) and reduction of healthcare expenditures (10, 11) were reported after periodontal treatment. Recent reviews of bone diseases among diabetic patients further shed light on the complex biology between diabetes, bone pathophysiology and microvasculature (12).

Associations between PD and T2D suggest overlapping etiology, but specific shared genetic mechanisms remain largely unknown, despite significant genetic correlation (13). Such mechanisms may be intrinsically difficult to discover due to complexity in the diversity of additional highly correlated comorbidities such as osteoporosis, hypercholesterolemia, and metabolic traits (14). Specifically, progress has been hampered in part by the lack of significant genetic risk variants and therefore implicated biology from the GWAS of PD, a situation that is in sharp contrast with the GWAS of T2D, which has abundant significant signals. Numerous candidate PD variants have been reported over the past decade, albeit with most studies reporting a sub-genome-wide level of statistical significance when using various PD definitions (15). Indeed, performing clinical periodontal examination in a large epidemiological study has been challenging and resource demanding (16). Thus, in the setting of GWAS that require large samples, PD has been defined with various alternative and possibly incommensurate approaches (e.g., various case definitions with different clinical measures or treatment histories) and/or self-reported responses (13). Even with sample sizes as large as 506594 (n_{cases}=36150), identification of genetic factors for PD has not been successful, likely in part due to heterogenous disease definitions (13). To standardize PD measures in epidemiological surveillance and research, the Centers for Disease Control and Prevention (CDC) and the American Academy of Periodontology (AAP) together created and validated a list of oral health questions (OHQs) for assessing periodontal status (17, 18). Participants who responded with OHQs indicating less-than-ideal oral health behaviors such as less frequent interdental cleaning were more likely to have severe PD (19). Lack of routine dental care was also shown to predict tooth loss (20). Selected CDC-AAP OHQs have recently been adopted in large genomic databases such as the UK Biobank (13), the Million Veterans Program (21) and the Women's Genome Health Study (WGHS) (22) whose oral health status in 2018 was obtained using established CDC-AAP OHQs.

In this study, to overcome difficulties in identifying significant genomic loci for PD, we explored potential shared genetics of the large scale GWAS summary statistics for periodontitis/loose teeth (PerioLT) from the UK Biobank/GLIDE (13), for T2D from the DIAGRAM consortium (23), and for bone mineral density (BMD) from the GEFOS consortium (24) using genetic correlation analysis (25) and multi-trait analysis of GWAS (MTAG) meta-analysis (26) followed by genetic colocalization analysis of candidate loci (27). The approach used here has potential to overcome challenges from the intrinsic heterogeneity in PD definitions by focusing statistical power on loci by PerioLT and either T2D or BMD, or both. If there are weak, sub-genome-wide significant ($p > 5x10^{-8}$) genetic signals in the published PerioLT GWAS that are nevertheless shared by other conditions, as had been suggested for PerioLT and T2D in the prior analysis (13), then MTAG provides a way to enhance these genetic signals by leveraging genome-wide genetic correlation with the other traits. In addition, to further characterize the candidate loci, we evaluated their associations with specific measures of periodontal status in the WGHS collected through OHQs.

Material and methods

GWAS summary statistics for periodontitis/loose teeth, type 2 diabetes, and bone mineral density

Summary statistics were publicly available from the GWAS consortia for the meta-analyses of case-control GWASs of periodontitis/loose teeth (abbreviated as PerioLT, downloaded from the UKBB/GLIDE, https://data.bris.ac.uk/data/dataset/7276c102292c49d4098a8c4396849218) (13), for the meta-analyses of case-control GWASs of type 2 diabetes (abbreviated as T2D, downloaded from the DIAGRAM consortium, https://diagram-consortium.org/downloads.html) (23), and for the meta-analyses of continuous heel bone mineral density (abbreviated as BMD,

downloaded from the GEFOS consortium, http://www.gefos.org/?q=content/data-release-2018) (24).

Study population of the Women's Genome Health Study

Participants in the WGHS were initially healthy, female healthcare professionals at least 45 years of age at baseline and represented participants in the Women's Health Study (WHS) who provided a blood sample at baseline. The WHS was conducted as a two-by-two randomized clinical trial in 1992-1994 investigating the effects of vitamin E and low dose aspirin in prevention of cancer and cardiovascular diseases with 10 years of follow-up (22, 28). Since the end of the trial, follow-up has continued in an observational mode. Additional information related to health and lifestyle were collected by questionnaires throughout the WHS trial and continuing observational follow-up. The WHS/WGHS was approved by the Institutional Review Board of Brigham and Women's Hospital, Boston Massachusetts and this report conforms to the STROBE guidelines (29).

Self-reported oral health questions in the WHS/WGHS

Information on the CDC-AAP validated OHQs (18) were collected in 2018 when WHS/WGHS participants were asked the questions that are provided in Box 1. These questions were administered to assess periodontal health. By December 2018, a total of 17955 questionnaires with information about updated oral/periodontal health were obtained. Among these, 14663 WGHS women of verified European ancestry had complete information for genotype, OHQs, and T2D diagnosis ascertained in December 2017.

WGHS/WHS covariates and the ascertainment of type 2 diabetes or osteoporosis

In the WGHS, covariates such as age, race, education, income, body-mass-index, histories of hypercholesterolemia or hypertension, and smoking behaviors were summarized from the baseline study entry questionnaire. Observational health outcomes such as cardiovascular disease, diabetes, and cancers were followed up yearly by questionnaires and validated by medical records as previously reported (28, 30, 31). In this analysis, we used the 2009 self-reported diagnosis of osteoporosis, which was also confirmed based on participants' report of having a bone density scan.

Genetic data in the WGHS

Genotyping and imputation in the WGHS have been detailed in previous reports (22). Genotyping was performed on the HumanHap300 Duo array or the combination of the HumanHap300 Duo and iSelect arrays (Illumina, San Diego, CA) with the Infinium II protocol. Imputation of genotypes for SNPs that were not directly measured by the arrays was performed using genotyped SNPs that passed a test of HWE (p-value > 10^{-6}) but were unrestricted by MAF, using the 1000G (phase 3, version 5) ALL panel (32) with MaCH (v.1.0.16) and Minimac. The majority of the WGHS participants have European ancestry verified with multidimensional scaling analysis using informative markers in PLINK. For this report, we used data

BOX 1 Self-reported Oral Health Questions (OHQs)

No.	Question
1	Do you think you might have gum disease?
	O Yes O No O Don't know
2	Overall, how would you rate the health of your teeth and gums?
	O Excellent O Very good O Good O Fair O Poor O Don't know
3	Have you EVER had treatment for gum disease such as scaling and root planing, sometimes called "deep cleaning?"
	O Yes O No O Don't know
4	Have you EVER been told by a dental professional that you lost bone around your teeth?
	O Yes O No O Don't know
5	Aside from brushing your teeth with a toothbrush, in the LAST 7 DAYS, on how many DAYS did you use dental floss or any other device to clean between your teeth?
	O 0 O 1 O 2 O 3 O 4 O 5 O 6 O 7
6	In the PAST 12 MONTHS, have you visited a dentist or dental hygienist?
	O Yes O No O Don't know
7	How often do you usually visit the dental office for routine check-ups or cleanings?
	O More than once per year O Once per year O Less than once per year O Don't know

from 14663 individuals of verified European ancestry who also had available information for type 2 diabetes and responses to the OHQs.

Genetic methods and statistical analyses

Genome-wide genetic correlation was estimated using LD score regression (LDSC, v.1.0.1, https://github.com/bulik/ldsc) (25) using the reference panel provided with the software. MTAG (v1.0.8, https://github.com/JonJala/mtag) was performed using default settings (26). Analysis was restricted to variants with MAF >0.01. Colocalization analysis was performed with the R function coloc (v.5.1.0) using default settings (27). Coloc evaluates the posterior probability of 4 alternative hypotheses within the local interval around an index variant: H1= causal variant for trait 1 only; H2= causal variant for trait 2 only; H3= two distinct causal variants; H4= one common causal variant; with the null hypothesis of H0 = no causal variant. Multi-clumping was done using PLINK (v1.9, www.cog-genomics.org/plink/1.9/) with the 1000 genomes European LD reference panel (v.3). In summarizing demographic characteristics, group means and proportions were compared by t-tests for continuous variables and by chi-square tests for categorical variables, respectively, using R statistical software. Statistical significance was judged by $p<5x10^{-8}$ for genome-wide analyses such as MTAG, by unadjusted two-sided $p<0.05$ for association analyses with clinical, i.e. non-genetic variables, or by $p<3x10^{-3}$ for genetic associations of the 15 candidate MTAG SNPs with OHQ responses in the WGHS to account multiple testing.

As described above, the CDC-AAP OHQs were designed for PD surveillance in settings where direct clinical periodontal evaluation is not feasible, such as the WGHS. In this report, we note that we are therefore testing genetic associations of candidate loci shared between T2D and PerioLT where the responses to the OHQs in the WGHS serve as a proxy for the latter.

Study flow diagram

A flow diagram is provided in Figure 1 to summarize the steps of analyses in this report.

Results

Genome-wide genetic correlations between periodontal disease, type 2 diabetes and bone mineral density

We estimated the genome-wide genetic correlations among GWASs of PerioLT (13), T2D (23), and BMD (24) using LDSC (Table 1) (25). PerioLT GWAS was significantly correlated with T2D [r_g(SE)= 0.23 (0.04); $p=7.4e^{-09}$], but there was no significant genetic correlation

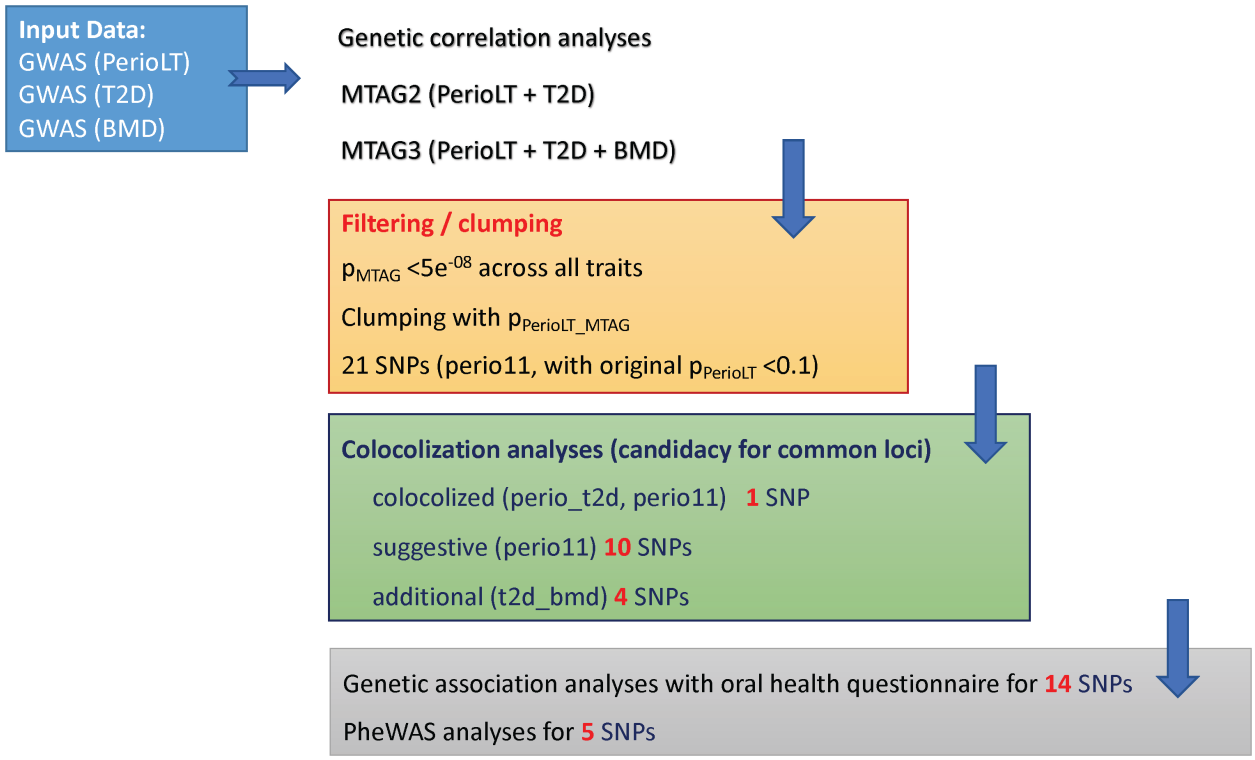

FIGURE 1
Study flow chart describing the steps in the analyses (1). Multi-trait meta-analysis of GWAS (MTAG) was conducted between PerioLT vs. T2D as well as among PerioLT/T2D/BMD (2). Filtering by the MTAG p-values $<5x10^{-8}$ for all three traits and subsequently clumping by the PerioLT MTAG p-values, we identified 21 candidate variants (3). Among them, colocalization analyses suggested 15 shared loci (4). Genetic association analyses for 14 candidate variants were done in the independent WGHS sample *via* a validated oral health questionnaire for periodontal disease surveillance. Phenome-wide association study (PheWAS) was conducted for 5 loci demonstrating independent significant associations with additional, i.e. non-periodontal, phenotypes.

TABLE 1 Genome-wide genetic correlations of GWASs of periodontitis/loose teeth (PerioLT), type 2 diabetes (T2D), and bone mineral density (BMD).

	PerioLT		T2D	
GWAS summary stats	r_g (SE)	Pv	r_g (SE)	Pv
Periodontitis/loose teeth	1	-	-	-
Type 2 diabetes	**0.23 (0.04)**	**$7.4e^{-09}$**	1	–
Bone mineral density	-0.02 (0.04)	0.57	**0.09 (0.02)**	**$9.8e^{-06}$**

r_g, genome-wide genetic correlation coefficient; Pv, p-value for r_g; SE, standard error; bolded values are statistically significant at p<0.05.

between PerioLT and BMD [r_g(SE)= -0.02 (0.04); p=0.57]. There was a significant genetic correlation between GWAS of T2D and BMD [r_g (SE)= 0.09 (0.02); p=$9.8e^{-06}$]. Such a finding has been suggested in an earlier report (12), but not evaluated on a genome-wide basis until the present investigation. The direction of the genetic correlation indicates that increased risk of T2D is associated with higher BMD. Our results suggest that a genetic relationship between PerioLT and BMD was not supported, despite the fact that many epidemiological reports suggested strong associations between PD and osteoporosis (6, 33). Moreover, in light of the significant genetic correlations between T2D/BMD in an opposite direction with that of PerioLT/BMD, it may be that alveolar bone loss due to PD is not genetically the same as those measured in heel BMD under the osteoporotic condition.

Candidate shared loci among T2D, BMD and PerioLT from cross-trait MTAG meta-analysis

Given the shared genetic correlation and strong GWAS signals of T2D and BMD, we conducted cross-trait MTAG GWAS meta-analyses between PerioLT/T2D or PerioLT/T2D/BMD to amplify the potentially true but weak genetic signals in the PerioLT GWAS (Table 2). The number of genome-wide statistically significant variants from MTAG for the PerioLT GWAS increased from 9 SNPs to 583 when analyzed with T2D, or to 615 SNPs when analyzed together with T2D/BMD. Among these, 548 and 244 SNPs had $p_{MTAG}<5x10^{-8}$ across all traits of PerioLT/T2D or PerioLT/T2D/BMD, respectively. We consolidated the $PerioLT_{MTAG}$ signals by multi-clumping the 548 and 244 PerioLT MTAG SNPs into the final 21 SNPs for subsequent colocalization analyses. Of note, out of the 21 SNPs, 11 of them had the original PerioLT GWAS p<0.1 (perio11 in Tables 2, 3).

Pairwise colocalization analyses of candidate SNP associations between PerioLT/T2D, T2D/BMD and BMD/PerioLT

At the candidate loci, we evaluated colocalization of genetic signals for pairs of the examined phenotypes. Table 3 shows the posterior probabilities of the hypothesis H4, which supports sharing of a causal variant between the phenotypes. Tables S1–S3 show the posterior probabilities of all alternative hypotheses (H1-H4). Of the 21 variants identified by MTAG (Table 2), one SNP (rs17522122) colocalized for PerioLT/T2D (H4 = 0.582), while none of the other colocalization alternatives for this variant involving PerioLT was significant (H1, H3 <0.05). The colocalization evidence for shared association at a second variant, rs3200401, was marginal for both PerioLT/T2D and PerioLT/BMD (H4 = 0.37 and 0.35, respectively). At an additional locus indexed by rs6711375, colocalization suggests there are independent associations for PerioLT and for T2D (Table S1; H3 = 0.905, H4 = 0.008). No additional candidate was supported for association with PerioLT by colocalization analysis, despite having PerioLT $p_{MTAG}<5x10^{-8}$. However, there was support for shared associations at between T2D and BMD at five loci (SNP=rs149290349, H4 = 0.977; rs8047395, 0.804; rs665268, 0.968; rs4376068, 0.955; rs76895963, 1.00), and support for independent associations for T2D and BMD at four loci (SNP=rs3200401, H3 = 0.948; rs1265758, 0.645; rs2010390, 0.985; rs75933965, 1.00), the first also a PerioLT candidate (above). We also retained rs77464186 for further analysis based on the elevated although not significant H3 probabilities

TABLE 2 MTAG cross-trait significant variants of among PerioLT, T2D, and BMD.

GWAS summary statistics*	N_{eff}	Original GWAS # SNPs $p<5e^{-08}$	MTAG PerioLT + T2D # SNPs $p_{MTAG}<5e^{-08}$	MTAG PerioLT + T2D + BMD # SNPs $p_{MTAG}<5e^{-08}$	Total Independent # SNPs
T2D (Mahajan)	228825	18911	13955	13528	
BMD (Morris)	426824	103257	–	79818	
PerioLT (Shungin)	506594	9	583	615	
# SNPs with p_{MTAG} $<5e^{-08}$ across all traits			548	244	
# SNPs after clumping with $p_{PerioLT\_MTAG}$			18	7 (4 overlapping)	21
List of perio11: from 21 SNPs with original GWAS $p_{PerioLT}$ < 0.1			9	5 (3 overlapping)	11

T2D, DIAGRAM type 2 diabetes meta-analysis (23); BMD, GEFOS heel bone mineral density analysis (24.); PerioLT, GWAS of periodontitis/loose teeth from the UKBB/GLIDE (13); N_{eff}, effective sample size from the downloaded dataset; Rg, genome-wide genetic correlation coefficient calculated from the LD Score Regression.

TABLE 3 Colocalization posterior probabilities and the original GWAS p-values of 21 SNPs identified from the MTAG analyses.

SNP	Candidacy for common loci	Source of MTAG	Prob H4 PerioLT/ T2D	Prob H4 T2D/ BMD	Prob H4 PerioLT/ BMD	Original GWAS Beta (SE); $p_{PerioLT}$	Original GWAS Beta (SE); p_{T2D}	Original GWAS Beta (SE); p_{BMD}
Colocalized								
rs17522122	perio11, perio_t2d	MTAG2	0.582	0.008	0.001	0.02 (0.0); $5e^{-5}$	0.04 (0.01); $4e^{-09}$	0.0 (0.0); 0.17
Suggestive								
rs3200401	perio11	MTAG2	0.368	0.052	0.345	0.02 (0.01); $8e^{-4}$	0.06 (0.01); $3e^{-14}$	-0.03 (0.0); $9e^{-23}$
rs149290349	perio11	MTAG2&3	0.017	0.977	0.029	-0.02 (0.01); 0.01	-0.13 (0.01); $3e^{-24}$	-0.03 (0.0); $1e^{-15}$
rs6711375	perio11	MTAG2	0.008	0.004	0.002	0.02 (0.0); $2e^{-4}$	0.04 (0.01); $3e^{-10}$	0.0 (0.0); 0.49
rs1265758*	perio11	MTAG2	0.015	0.004	0.015	-0.01 (0.0); $2e^{-3}$	-0.05 (0.01); $4e^{-13}$	0.0 (0.0); 0.03
rs2010390	perio11	MTAG3	0.132	0.015	0.091	0.01 (0.0); $3e^{-3}$	0.04 (0.01); $4e^{-10}$	0.03 (0.0); $1e^{-40}$
rs12255678	perio11	MTAG2&3	0.002	0	0.003	0.01 (0.0); 0.04	-0.15 (0.01); $1e^{-85}$	-0.02 (0.0); $2e^{-13}$
rs10770140	perio11	MTAG2	0.114	0.005	0	-0.01 (0.0); $6e^{-3}$	-0.07 (0.01); $3e^{-23}$	0.0 (0.0); 0.14
rs8047395	perio11	MTAG2&3	0.002	0.804	0.002	0.01 (0.0); 0.08	0.10 (0.01); $1e^{-52}$	-0.02 (0.0); $6e^{-16}$
rs665268	perio11	MTAG3	0.178	0.968	0.081	-0.01 (0.0); $3e^{-3}$	-0.05 (0.01); $1e^{-11}$	-0.01 (0.0); $1e^{-09}$
rs2546494	perio11	MTAG2	0.045	0.009	0	0.01 (0.0); $6e^{-4}$	0.04 (0.01); $6e^{-12}$	-0.0 (0.0); 0.12
Additional								
rs4376068	t2d_bmd	MTAG3	0.003	0.955	0.003	0.0 (0.0); 0.45	-0.11 (0.01); $1e^{-57}$	-0.01 (0.0); $5e^{-08}$
rs75933965	t2d_bmd	MTAG2	0.002	0	0.003	0.01 (0.01); 0.65	0.23 (0.01); $8e^{-75}$	-0.0 (0.0); 0.76
rs77464186	t2d_bmd	MTAG2	0.005	0.062	0	0.01 (0.01); 0.26	0.11 (0.01); $2e^{-33}$	0.01 (0.0); $6e^{-3}$
rs76895963	t2d_bmd	MTAG2&3	0.008	1	0.008	-0.01 (0.01); 0.64	0.48 (0.03); $5e^{-70}$	0.08 (0.01); $9e^{-25}$
Not suggestive								
rs4328980		MTAG2	0.002	0.001	0	-0.0 (0.0); 0.4	-0.08 (0.01); $1e^{-35}$	0.0 (0.0); 0.41
rs9348440		MTAG2	0.003	0.002	0	0.01 (0.01); 0.21	0.13 (0.01); $3e^{-44}$	0.0 (0.0); 0.22
rs13266634		MTAG2	0.003	0.003	0	-0.0 (0.0); 0.49	-0.11 (0.01); $1e^{-54}$	0.0 (0.0); 0.29
rs7020996		MTAG2	0.003	0.011	0	0.0 (0.0); 0.8	-0.15 (0.01); $4e^{-52}$	-0.0 (0.0); 0.12
rs11257655		MTAG2	0.009	0.008	0	0.01 (0.0); 0.11	0.09 (0.01); $4e^{-32}$	0.0 (0.0); 0.08
rs61875362		MTAG2	0.003	0.002	0	-0.0 (0.0); 0.4	-0.09 (0.01); $2e^{-41}$	-0.0 (0.0); 1

Prob, probability; H4, hypothesis of sharing 1 lead SNP; T2D, DIAGRAM type 2 diabetes meta-analysis (23); MTAG2&3, from both MTAG2 and MTAG3, with results of MTAG3 shown here; BMD, GEFOS heel bone mineral density analysis (24); PerioBL, GWAS of periodontitis/loose teeth from the UKBB/GLIDE (13); MTAG, multi-trait analysis of GWAS; MTAG2, MTAG of PerioLT and T2D; MTAG3, MTAG of PerioLT, BMD, and T2D; *, rs1265758 was not available in the WGHS data.
Candidacy for common loci annotation: perio_t2d, SNP identified with H4 probability of T2D/PerioLT colocalization >0.5; perio11, SNP identified from the MTAG analysis and with the original GWAS $p_{perioLT}$<0.1: t2d_bmd, additional SNP identified with H4 probability of T2D/BMD colocalization >0.7 or higher H3 probability of 2 different lead SNPs in T2D/BMD.

(0.163). Among the candidate SNPs, those that showed no evidence for association (P>0.1) in the original PerioLT GWAS are designated in the tables as "t2d_bmd" if there was colocalization between T2D and BMD.

Description of the remaining candidate shared loci and their MTAG results

Table 4 provides details of gene symbols, chromosome and position, allele frequency, type of variant, as well as the MTAG summary statistics of the 15 selected SNPs. The colocalized SNP between PerioLT and T2D, rs17522122, maps to the 3'-UTR of *AKAP6*. The A-kinase anchor proteins are a group of proteins highly expressed in various brain regions, cardiac and skeletal muscle and tongue, with biological functions involved in anchoring protein kinase A to the nuclear membrane or sarcoplasmic reticulum. The marginally colocalizing SNP for PerioLT, rs3200401, maps to a non-coding RNA near *MALAT1*, *MASCRNA* and *TALAM1*. Seven SNPs were derived from the three-trait MTAG analysis and had the following genomic contexts: rs149290349 (a missense variant of *ZFP36L2*), rs2010390 (an uncharacterized non-coding RNA), rs12255678 (an intron variant of *TCF7L2*), rs8047395 (an intron variant of *FTO*), rs665268 (a missense variant of *MLX*), rs4376068 (an intron variant of *IGF2BP2*), and rs76895963 (an intron variant of *CCND1, CCND2-AS1*). Among these, 4 SNPs (rs149290349,

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TABLE 4 MTAG association statistics for 15 selected variants.

SNP	Chr : Pos	A1	A2	Freq	Source of MTAG	Type	Candidate Genes	Beta (SE); $p_{PerioLT\_MTAG}$	Beta (SE); p_{T2D_MTAG}	Beta (SE); p_{BMD_MTAG}
Colocalized										
rs17522122	14:33302882	T	G	0.48	MTAG2	3'UTR	*AKAP6*	0.02 (0.0); $1e^{-08}$	0.04 (0.01); $2e^{-09}$	
Suggestive										
rs3200401	11:65271832	T	C	0.20	MTAG2	ncRNA	*MALAT1, MASCRNA, TALAM1*	0.03 (0.0); $9e^{-09}$	0.06 (0.01); $1e^{-13}$	
rs149290349	2:43451957	A	G	0.07	MTAG2&3	missense	*ZFP36L2*	-0.04 (0.01); $3e^{-10}$	-0.13 (0.01); $3e^{-21}$	-0.03 (0.0); $1e^{-12}$
rs6711375	2:161090873	A	G	0.68	MTAG2	SNV	–	0.02 (0.0); $3e^{-08}$	0.05 (0.01); $2e^{-10}$	
rs1265758	6:32323529	A	G	0.42	MTAG2	intron	*TSBP1, TSBP1-AS1*	-0.02 (0.0); $3e^{-08}$	-0.05 (0.01); $9e^{-13}$	
rs2010390	8:9047178	A	G	0.30	MTAG3	intron	*LOC101929128*	0.02 (0.0); $2e^{-08}$	0.04 (0.01); $9e^{-09}$	0.03 (0.0); $3e^{-44}$
rs12255678	10:114729482	T	G	0.75	MTAG2&3	intron	*TCF7L2*	0.02 (0.0); $6e^{-11}$	-0.15 (0.01); $8e^{-81}$	-0.02 (0.0); $6e^{-10}$
rs10770140	11:2193597	T	C	0.61	MTAG2	5' UTR	*TH, MIR4686*	-0.02 (0.0); $8e^{-10}$	-0.07 (0.01); $1e^{-22}$	
rs8047395	16:53798523	A	G	0.51	MTAG2&3	intron	*FTO*	0.03 (0.0); $2e^{-12}$	0.10 (0.01); $2e^{-51}$	-0.02 (0.0); $1e^{-17}$
rs665268	17:40722029	A	G	0.72	MTAG3	missense	*MLX*	-0.02 (0.0); $5e^{-08}$	-0.05 (0.01); $4e^{-11}$	-0.01 (0.0); $3e^{-08}$
rs2546494	17:46959525	A	G	0.51	MTAG2	intron	*LOC105371814*	0.02 (0.0); $3e^{-08}$	0.04 (0.01); $8e^{-12}$	
Additional										
rs4376068	3:185497635	A	C	0.69	MTAG3	intron	*IGF2BP2*	-0.02 (0.0); $2e^{-09}$	-0.11 (0.01); $2e^{-52}$	-0.01 (0.0); $6e^{-09}$
rs75933965	10:114749421	A	G	0.07	MTAG2	intron	*TCF7L2*	0.05 (0.01); $6e^{-13}$	0.23 (0.01); $7e^{-65}$	
rs77464186	11:72460398	A	C	0.84	MTAG2	intron	*ARAP1*	0.03 (0.01); $8e^{-09}$	0.11 (0.01); $1e^{-33}$	
rs76895963	12:4384844	T	G	0.98	MTAG2&3	intron	*CCND1, CCND2-AS1*	0.08 (0.01); $2e^{-09}$	0.48 (0.03); $1e^{-62}$	0.08 (0.01); $7e^{-19}$

MTAG, multi-trait analysis of GWAS; MTAG2, MTAG of PerioLT and T2D; MTAG3, MTAG of PerioLT, BMD, and T2D; MTAG2&3, from both MTAG2 and MTAG3, with results of MTAG3 shown here; Chr : Pos, chromosome and position (GRCh37/hg19); Freq, allele frequency of A1; SE, standard error; $p_{PerioLT\_MTAG}$, MTAG calculated p-values of the periodontitis/loose teeth from the UKBB/GLIDE (13); p_{T2D_MTAG}, MTAG calculated p-values of the type 2 diabetes (23); p_{BMD_MTAG}, MTAG calculated p-values of the GEFOS bone mineral density (24).

rs12255678, rs8047395, and rs76895963) were also found to be significant in the PerioLT/T2D two-trait MTAG analysis.

Characteristics of the Women's Genome Health Study participants with updated oral health measures

We provide characteristics of the WGHS participants who had verified European ancestry, had completed the oral health questionnaire (OHQ), and had information on their T2D status throughout the follow-up period in Supplementary Table S4. Distribution and risk factors for periodontal status from responses to OHQ, such as age at the time of the OHQ, baseline educational levels, smoking status, BMI, hypertension, hypercholesterolemia and updated osteoporosis status as are provided based on the confirmed T2D status. Women with T2D were more likely to self-report having fair/poor oral health, less likely to visit a dentist within the past year, less likely to have dental prophylaxis at least once per year, and more likely to floss two times or less per week. Genotype distributions of the shared candidate loci among the WGHS participants are provided in Supplementary Table S5. We were able to retrieve clinical dental records from a subset of women (Supplementary Table S6). Women who self-reported having fair/poor oral health, bone loss around teeth, or those with less dental prophylaxis (<1 per year), had fewer remaining natural teeth. Thus, in the WGHS, responses to the OHQ

demonstrated associations with worse PD status consistent with previous validation studies.

Genetic associations of the candidate shared PerioLT/T2D variants with responses to the oral health questions

Table 5 presents the genetic associations for 14 of the 15 selected variants with responses of OHQs among the WGHS participants. Data on one variant, rs1265758, were unavailable in the WGHS. The influence of each genetic variant on oral health measures was explored in multiple logistic regression models (Table 5 legend). Rs17522122, the top candidate variant that colocalized between PerioLT and T2D, was found to be inversely associated with less frequent dental flossing [OR (95%CI)= 0.92 (0.87-0.98), p=0.007]. Additional variants were found to be associated with the women's last visit to the dental office [rs77464186, OR(95%CI)= 0.82 (0.75-0.91), p=0.0002], frequency of dental prophylaxis [rs67111375, 0.91 (0.83-0.99), p=0.03; rs77464186, 0.80 (0.72-0.89), p=3.8e^{-05}], frequency of dental flossing [rs75933965, 1.17 (1.04-1.31), p=0.008], and a history of bone loss around teeth [rs8047395, 1.09 (1.03-1.15), p=0.005].

By further controlling for other traditional risk factors such as baseline BMI, education, hypertension and hypercholesterolemia, as well as the type 2 diabetes and osteoporosis status updated over follow-up, results of the genetic associations confirmed those shown in Table 4, though slightly attenuated (Supplementary Table S7). This observation suggests that the genetic effects are not functioning through these clinical conditions.

Lastly, we did not find significant genetic associations for these 14 variants with self-reported poor/fair oral health, a history of PD diagnosis, or a history of scaling and root planing (Supplementary Table S8).

Pleiotropy at candidate loci and tissues specific expression quantitative trait loci

We provide additional annotations for two variants associated with less frequent dental flossing (rs17522122 and rs75933965), two associated with less frequent dental prophylaxis (rs6711375, rs77464186), and the variant rs8037495 associated with bone loss around teeth (Supplementary Table S9). Multiple diverse phenome-wide association studies (PheWAS) results were identified for all five variants, implying a high level of pleiotropy. For example, GWAS associations with rs17522122 were enriched in phenotypes related to body fat. Rs6711375 and rs8047395 were identified with pleiotropic effects related to a variety of immune cell (leukocyte, lymphocyte, neutrophil and reticulocytes). The most pleiotropic associations highlighted were diabetes, body fat, body mass and body measures, and metabolic related traits. The variants associated with less frequent dental prophylaxis were also linked to expression quantitative trait loci, i.e., eQTLs, of several genes. Lastly, blood pressure, cancer,

TABLE 5 Oral health question (OHQs) with at least one significant genetic association.

	Dental Visit > 1 year ago		Prophylaxis < 1 per year		Floss <= 2 per week		Bone loss around teeth	
	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p	OR (95%CI)	p
Colocalized								
rs17522122_t	0.96 (0.89-1.04)	0.32	1 (0.92-1.09)	0.98	0.92 (0.87-0.98)	0.007	1.05 (0.99-1.11)	0.13
Suggestive								
rs3200401_t	1.03 (0.94-1.14)	0.49	1.07 (0.97-1.18)	0.19	1.07 (0.99-1.15)	0.09	0.98 (0.91-1.06)	0.68
rs149290349_a	0.87 (0.75-1.01)	0.08	0.9 (0.77-1.05)	0.20	1.04 (0.93-1.16)	0.47	0.96 (0.86-1.08)	0.50
rs6711375_a	0.94 (0.87-1.02)	0.15	0.91 (0.83-0.99)	0.03	1.02 (0.95-1.08)	0.62	1 (0.94-1.07)	0.88
rs2010390_a	1.01 (0.92-1.11)	0.84	1.07 (0.97-1.18)	0.19	0.97 (0.91-1.05)	0.48	1.04 (0.97-1.11)	0.31
rs12255678_t	1.02 (0.93-1.12)	0.64	1.02 (0.93-1.13)	0.61	1.03 (0.96-1.1)	0.43	0.99 (0.92-1.06)	0.77
rs10770140_t	1.03 (0.95-1.11)	0.52	1.08 (1-1.18)	0.06	1.02 (0.96-1.08)	0.54	0.97 (0.91-1.03)	0.25
rs8047395_a	1.04 (0.96-1.12)	0.35	1.02 (0.94-1.11)	0.59	0.98 (0.92-1.04)	0.55	1.09 (1.03-1.15)	0.005
rs665268_a	0.98 (0.9-1.07)	0.65	0.95 (0.86-1.04)	0.22	0.95 (0.89-1.01)	0.11	1.02 (0.95-1.08)	0.66
rs2546494_a	0.99 (0.91-1.06)	0.71	0.97 (0.9-1.05)	0.50	1.02 (0.96-1.08)	0.56	1 (0.94-1.06)	0.98
Additional								
rs4376068_a	0.96 (0.88-1.04)	0.28	0.99 (0.91-1.08)	0.84	0.97 (0.91-1.03)	0.35	1.02 (0.96-1.09)	0.53
rs75933965_a	1.02 (0.87-1.18)	0.83	0.97 (0.82-1.14)	0.69	1.17 (1.04-1.31)	0.008	0.97 (0.86-1.09)	0.63
rs77464186_a	0.82 (0.75-0.91)	0.0002	0.8 (0.72-0.89)	3.8e^{-05}	0.96 (0.88-1.04)	0.29	0.99 (0.92-1.08)	0.90
rs76895963_t	0.8 (0.61-1.08)	0.14	0.91 (0.67-1.28)	0.58	0.86 (0.69-1.1)	0.22	1.09 (0.85-1.4)	0.51

The associations to different oral health question responses were assessed with multivariate logistic regression using the genetic variables as the independent variables, adjusting for age at the time of OHQ responses, baseline information smoking (3 groups), and 10 genomic eigenvectors.
OR, odds ratio; CI, confidence interval.

cognitive function or physical activities GWAS results were previously reported with rs17522122. The *FTO* variant (rs8047395) was also mapped with tumors of central nervous system or other cancers, as well as bone mineral density.

Discussion

A possible explanation for relative lack of significant loci in the former UKBB/GLIDE PerioLT GWAS meta-analysis despite its large sample size may have been the potential heterogeneity in phenotype ascertainment across the contributing studies. To overcome this challenge, we boosted the PerioLT genetic signal with cross-trait MTAG meta-analysis, thereby identifying 21 genome-wide significant associations for PerioLT (after clumping), among which one was also nominally significant in the original GWAS and colocalized with the genetic signal for T2D (rs17522122). Testing for association with OHQs among the WGHS women, including adjustment for traditional risk factors and updated histories of diabetes and osteoporosis, supported some associations with less frequent dental care/prophylaxis (rs77464186) or less frequent flossing (rs17522122), or bone loss around teeth (rs8047359) despite the relatively small size of the sample. Rs6711375, which tagged a locus with potentially distinct signals for PerioLT and T2D (H3 = 0.91) and was marginally supported by association with less dental prophylaxis via OHQ, may have had a strong PerioLT MTAG signal due to inflation *via* residual LD to the strong T2D signal. Similarly, significantly replicating associations with dental phenotypes that did not colocalize with PerioLT are likely due to the highly pleiotropic nature of these candidate loci and their particularly significant associations with T2D and/or BMD, which may have inflated MTAG signals for PerioLT (26). For example, rs12255678 near *TCF7L2* and rs8047395 near *FTO* are the strongest associations in the genome for T2D and BMD, respectively. Therefore, we acknowledged such limitation from the MTAG results that these suggested shared PD candidate loci may only reflect very strong T2D or BMD associations.

That loci showing pleiotropy across a range of immune cell phenotypes and that T2D and BMD are associated with periodontal measures is consistent with current thinking about the origins of periodontal disease. Williams and colleagues report that cellular transcriptomic landscape of patients with periodontitis involved enhanced neutrophil and leukocyte infiltration due to the exaggerated stromal cell responsiveness (2). Meanwhile, bone changes are known among diabetic patients, perhaps with partial microvascular etiology (12), and mouse models link obesity and its metabolic dysregulation-associated inflammation to the size of preosteoclasts (myeloid-derived suppressor cells) populations (34). Both the genetic correlation of T2D/BMD and the colocalization of signals at some candidate loci are consistent with previously reported connections between these phenotypes from Mendelian randomization studies (35). Our result of lack of genetic correlation between PerioLT and BMD does not support a genetic relationship for the reported epidemiological associations between PD and osteoporosis (6, 33). We further suggest that the opposite direction of genetic correlation between PerioLT/BMD and T2D/BMD might imply different pathophysiology for alveolar bone loss than those measurements identified under the osteoporotic conditions.

A strength of our approach is the large sample size based on the consortium GWAS datasets as well as the WGHS/WHS genetic data for validations. Additionally, obtaining the validated CDC-AAP OHQs in the WGHS/WHS likely reduced heterogeneity in the periodontal phenotype that may have limited genetic signal in the original PerioLT GWAS. We do acknowledge that even as such OHQs have good sensitivity and specificity for periodontal disease surveillance (18), they may nevertheless be limited by the nature of self-reporting. Nevertheless, among the limited women's dental records retrieved in the WGHS/WHS, participants having less frequent dental prophylaxis, bone loss around teeth, or who self-reported fair/poor oral health had significantly fewer remaining teeth. Thus, the identified genetic associations between the reported shared PD/T2D candidate loci with responses to OHQs in the WGHS may be interpreted as genetic liability to surrogates for PD. Our integrated approach supports deployment of validated OHQs in future genomic studies so that important oral health information can be captured.

In summary, by exploring genetic analyses using GWAS summary statistics of PerioLT, T2D and BMD, we were able to identify one new candidate locus for PerioLT and several additional new suggestive loci. Among the WGHS women, significant genetic associations of these candidate variants with self-reported oral health measures remained even after accounting for other risk factors and the women's osteoporosis and diabetes status. Importantly, our observations may bear on significant association of periodontal disease or having a less than functional dentition with many systemic comorbidities such as T2D, cardiovascular disease (36), bone mineral density and hip fracture (37), as well as all-cause or disease-specific mortality (38). Future research must explore how the new loci are linked to underlying pathophysiology of periodontal diseases and its comorbidities.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board of Brigham and Women's Hospital, Boston Massachusetts. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Y-HY contributed to conception, design, data collection, data analysis, data interpretation and drafted the manuscript. JB and DC contributed to conception, design, data collection, data

interpretation and critically revised the manuscript. BS and PR contributed to conception, design and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1016373/full#supplementary-material

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Evolution of sodium-glucose co-transporter 2 inhibitors from a glucose-lowering drug to a pivotal therapeutic agent for cardio-renal-metabolic syndrome

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Cardio-renal-metabolic (CRM) syndrome, which involves type 2 diabetes mellitus (T2DM), chronic kidney disease (CKD), and heart failure (HF), is a serious healthcare issue globally, with high morbidity and mortality. The disorders that comprise CRM syndrome are independent can mutually affect and accelerate the exacerbation of each other, thereby substantially increasing the risk of mortality and impairing quality of life. To manage CRM syndrome by preventing vicious interactions among individual disorders, a holistic treatment approach that can simultaneously address multiple disorders underpinning CRM syndrome is of great importance. Sodium-glucose co-transporter 2 inhibitors (SGLT2i) lower blood glucose levels by inhibiting glucose reabsorption in the renal proximal tubule and were first indicated for the treatment of T2DM. Several cardiovascular outcome trials have demonstrated that SGLT2i not only lower blood glucose but also reduce the risk of hospitalization for HF and worsening renal function in patients with T2DM. Results have also suggested that the observed cardiorenal benefits of SGLT2i may be independent of their blood glucose-lowering effects. Several randomized controlled trials subsequently assessed the efficacy and safety of SGLT2i in patients without T2DM, and revealed considerable benefits of SGLT2i treatment against HF and CKD, regardless of the presence of T2DM. Thus, SGLT2i have become an essential therapeutic option to prevent the onset, slow the progression, and improve the prognosis of CRM syndrome. This review assesses the evolution of SGLT2i from a glucose-lowering drug to a therapeutic agent for CRM syndrome by evaluating epoch-making clinical studies, including randomized control trials and real-world studies.

KEYWORDS

SGLT2 inhibitor, chronic heart failure, chronic kidney disease, cardiorenal protection, cardio-renal-metabolic syndrome

Introduction

It is well known that metabolic, cardiovascular (CV), and renal diseases closely interact with each other, forming the so-called cardio-renal-metabolic (CRM) syndrome (1, 2), where each disease is not an independent complication but instead affects and exacerbates the other disorders. The risk of developing CV diseases, including heart failure (HF), in patients with type 2 diabetes mellitus (T2DM) is approximately double that observed in individuals without T2DM (3). A considerable proportion of patients with T2DM (~50%) develop chronic kidney disease (CKD), referred to as diabetic kidney disease, a leading cause of end-stage renal disease (ESRD) (4–6). Moreover, decreased renal function is associated with an increased risk of the onset of HF and CV death (7–10). In fact, the prognosis of patients with concomitant T2DM, HF, and CKD is extremely poor (11). Given the vicious interactions among these disorders, holistic interventions that can simultaneously target the multiple disorders in CRM syndrome are of great importance (12).

One class of glucose-lowering drugs, sodium-glucose co-transporter-2 inhibitors (SGLT2i) (13), has recently attracted much attention owing to its cardiorenal benefits. Several CV outcome trials (CVOTs) have demonstrated that SGLT2i reduce the primary and secondary events associated with CV and renal diseases in patients with T2DM (14–16). In addition, several randomized controlled trials (RCTs) have subsequently shown significant efficacy of SGLT2i treatment against HF and CKD regardless of the presence of T2DM (17–21). Owing to these startling results, SGLT2i have evolved from a glucose-lowering drug to an essential therapeutic option to prevent the onset, slow the progression, and improve the prognosis of CRM syndrome. This review describes the evolutionary journey of SGLT2i with reference to epoch-making studies, including RCTs and real-world observational studies.

Identification and development of SGLT2i

The development of SGLT2i started from the discovery of phlorizin in the bark of apple trees by Petersen in 1835 and the subsequent discovery of its effects on glucosuria and plasma glucose-lowering by von Mering in 1886 (22). In 1962, Alvarado and Crane showed that phlorizin competitively inhibits the cotransport of glucose and sodium in the proximal tubule of the kidney although the molecular entity of the transporter remained unknown (23). Two decades later, SGLT1, which transports D-glucose and D-galactose, was cloned from rabbit small intestine by Wright et al. at the University of California, Los Angeles (24). SGLT1 is predominantly expressed in S3 segments and assumed to be responsible for the reabsorption of 10% of the glucose filtered by the glomerulus (25). To elucidate the molecular entity of a similar glucose transporter in the proximal tubule responsible for the reabsorption of the remaining 90% of glucose, Kanai et al. at Osaka University screened a renal cDNA library using SGLT1 as a probe, which led to the identification of SGLT2 (26). In 1999, a Japanese pharmaceutical company developed T-1095, an undegradable SGLT2i. This is the first SGLT2i that was shown to enhance urinary glucose excretion and lower blood glucose levels in diabetic rats (27).

SGLT2i for the prevention of diabetic complications

It is well known that patients with diabetes frequently develop micro- and macrovascular complications, which worsen the prognosis and quality of life (QOL) of patients. More recently, a multinational cohort study demonstrated that HF and CKD are early and frequent complications in patients with T2DM (10), and their risk of developing these complications is double that of individuals without T2DM (11). For managing diabetic complications, glucose-lowering drugs are of great importance, together with diet and exercise therapy. Because patients with diabetes require long-term treatment, glucose-lowering drugs must not only be effective in terms of reducing hyperglycemia but must also be safe in terms of the risks of hypoglycemia and vascular diseases. Despite these requirements, some glucose-lowering drugs were reported to be potentially associated with an increased risk of CV events (28, 29). This led the United States Food and Drug Administration (FDA) to issue guidance for the development of new antidiabetic drugs in 2008, mandating the so-called "CV no harm study" to evaluate the CV risks of newly launched drugs in longer-term clinical trials with prespecified CV endpoints, usually a composite primary endpoint of CV death, non-fatal stroke, and non-fatal myocardial infarction (MI) (three-point major adverse CV events; 3P-MACE) or 4P-MACE (3P-MACE plus unstable angina). The European Medicines Agency (EMA) also stated the need for CV no harm studies, with safety outcomes consisting of 3P-MACE or 4P-MACE, as well as other events such as revascularization and/or worsening of HF.

In consideration of the FDA and EMA guidance, a meta-analysis of 21 phase 2b/3 clinical trials was performed to evaluate the risk of an increase in CVD in studies performed for the regulatory approval of dapagliflozin. This meta-analysis revealed numerically lower incidences of the composite of 4P-MACE as well as the individual components (30, 31). Moreover, the meta-analysis revealed that, dapagliflozin significantly reduced hospitalization for HF (HHF), with a hazard ratio (HR) of 0.361 (95% confidence interval [CI], 0.156 to 0.838). Subsequently, a prospective CVOT for dapagliflozin, the DECLARE-TIMI 58 trial (n = 17,160; median follow-up, 4.2 years), was conducted (16, 32). Large CVOTs also evaluated the risk of CVD for other SGLT2i, namely, the EMPAREG-OUTCOME trial (n = 4,687; median follow-up, 3.1 years) and the CANVAS program (n = 10,142; median follow-up, 2.4 years) (14–16). There are some differences in the characteristics of patients included in these trials. One is the baseline CV risk. EMPA-REG OUTCOME enrolled patients with established CVD whereas the other trials enrolled patients with either established CVD or risk factors for CVD, resulting in a different prevalence of CVD at baseline (100% in EMPA-REG OUTCOME, 65.6% in CANVAS, and 40.6% in DECLARE-TIMI 58). Another major difference is baseline renal function. While EMPA-REG OUTCOME and CANVAS excluded patients with eGFR of <30 and ≤30 mL/min/1.73 m^2, respectively, DECLARE-TIMI 58 excluded patients with a creatinine clearance of

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<60 mL/min. The percentage of patients with eGFR < 60 mL/min/1.73 m^2 in each trial was 25.9% in EMPA-REG OUTCOME, 20.1% in CANVAS, and 7.4% in DECLARE-TIMI 58 (14, 33, 34). Despite these differences in patient characteristics, the three trials consistently showed non-inferiority for 3P-MACE versus placebo from the safety perspective. Furthermore, SGLT2i demonstrated superiority for 3P-MACE versus placebo in EMPA-REG OUTCOME and CANVAS but not in DECLARE-TIMI 58. This apparent difference may be explained by the lower baseline risk of CVD in patients enrolled in DECLARE-TIMI 58. Indeed, a prespecified subgroup analysis showed that dapagliflozin significantly reduced the relative risk of MACE by 16% in patients with previous MI (35). Other evidence supporting this notion is that the incidence rate of 3P-MACE was the lowest in DECLARE-TIMI 58 among the three CVOTs, indicating lowest statistical power in that study (36). Furthermore, a meta-analysis of these three trials demonstrated that SGLT2i reduced 3P-MACE by 11% compared to placebo with no heterogeneity (HR 0.89, 95% CI 0.83 to 0.96, p = 0.0014; Q statistic = 1.20, p = 0.55, I^2 = 0%) (37). For the individual components, SGLT2i reduced the risk of MI with no heterogeneity (HR 0.89, 95% CI 0.89 to 9.98, p = 0.0177; Q statistic = 0.03, p = 0.98; I^2 = 0%) and CV death with a significant intertrial difference (HR 0.84, 95%CI 0.75 to 0.94, p = 00023; Q statistic = 9.95, p = 0.0069; I^2 = 79.9%). Furthermore, there were significant risk reductions for CV death and all-cause mortality in EMPA-REG OUTCOME but not in the other two trials. The difference in baseline CV disease risk may explain these differing outcomes. The results of a meta-analysis showing a numerically higher incidence and a greater efficacy of SGLT2i in patients with a history of ASCVD than in patients with multiple risk factors support this notion (37).

In DECLARE-TIMI 58, non-inferiority of dapagliflozin to placebo with respect to 3P-MACE was first assessed and superiority was sequentially tested with hierarchical closed testing procedure. The study verified non-inferiority of dapagliflozin, but not superiority, as described above. In addition to 3P-MACE, the effects of SGLT2i were rigidly tested on a composite of HHF and CV death as a co-primary endpoint. DECLARE-TIMI 58 first demonstrated a statistically significant risk reduction in HHF/CV death, and EMPA-REG OUTCOME and CANVAS also reported positive effects on this outcome (14–16, 38, 39). Consistent with the results of a meta-analysis of phase 2b/3 trials, the CVOTs demonstrated that SGLT2i significantly reduced the incidence of HHF compared with placebo, with relative risk reductions ranging from 27% to 35% (Table 1). Additionally, two large RCTs, the CREDENCE trial and the VERTIS CV trial, showed beneficial effects of SGLT2i on HHF in patients with T2DM and CKD or established CVD (40, 41). Moreover, no heterogeneity among patients with and without a history of HF was reported in these five trials (Table 1). These results demonstrate the effects of SGLT2i on primary and secondary prevention of HHF in a broad range of patients with DM.

The aforementioned large RCTs also evaluated the effects of SGLT2i on renal function. For example, dapagliflozin reduced the risk of renal events, defined as a composite of a sustained eGFR decrease by ≥40% to an eGFR of <60 mL/min/1.73 m^2, ESRD, and

TABLE 1 Hospitalization for heart failure among patients with type 2 diabetes mellitus with and without a history of heart failure.

Trials	Events per 1,000 patient-years		HR (95% CI)
	Treatment	Placebo	
Overall population			
EMPA-REG OUTCOME (14)	9.4	14.5	0.65 (0.50 to 0.85)
CANVAS program (15)	5.5	8.7	0.67 (0.52 to 0.87)
DECLARE-TIMI 58 (16)	6.2	8.5	0.73 (0.61 to 0.88)
CREDENCE (40)	15.7	25.3	0.61 (0.47 to 0.80)
VERTIS CV (41)	7.3	10.5	0.70 (0.54 to 0.90)
History of HF			
EMPA-REG OUTCOME	40.7	52.4	0.75 (0.48 to 1.19)
CANVAS Program	14.1	28.1	0.51 (0.33 to 0.78)
DECLARE-TIMI 58	27.7	37.2	0.73 (0.55 to 0.96)
CREDENCE	39.3	48.9	0.76 (0.48 to 1.22)
VERTIS CV	16.9	26.2	0.63 (0.44 to 0.90)
No history of HF			
EMPA-REG OUTCOME	6.4	10.8	0.59 (0.43 to 0.82)
CANVAS Program	4.3	5.7	0.79 (0.57 to 1.09)
DECLARE-TIMI 58	4.0	5.6	0.73 (0.58 to 0.92)
CREDENCE	11.7	21.5	0.54 (0.39 to 0.75)
VERTIS CV	4.7	6.0	0.79 (0.54 to 1.15)

HR, hazard ratio; CI, confidence interval; HF, heart failure.

renal death and it slowed the rate of eGFR decline (34). In addition, dapagliflozin was associated with less deterioration and more amelioration of albuminuria compared with placebo (42). These renoprotective effects were robustly examined in CREDENCE, which enrolled 4,401 patients with T2DM and CKD (40). Canagliflozin reduced the risk of a renal-specific composite of ESRD, doubling of the creatinine level, or death from renal causes, a secondary endpoint specified in a sequential hierarchical testing procedure. Furthermore, similar to HF, this renoprotective efficacy observed in large RCTs was consistent regardless of renal function at baseline, suggesting the prevention of onset and progression of CKD by SGLT2i (Table 2) (34).

In addition to these RCTs, real-world studies have investigated the cardiorenal benefits of SGLT2i in clinical settings (44–50). The CVD-REAL 2 trial, using data from six countries, showed a significant reduction in HHF, MI, stroke and all-cause mortality compared with other oral glucose-lowering drugs regardless of the history of CVD in patients with T2DM (44). A follow-up study of CVD-REAL 2 using data from 13 countries over a longer period yielded similar results when comparing SGLT2i with dipeptidyl peptidase-4 inhibitors (DPP-4i) (49). Several other real-world studies consistently reported a reduced risk of CV events and death with SGLT2i compared with other glucose-lowering drugs or DPP-4i (46, 47, 51).

In addition to these CV benefits, the renoprotective effects of SGLT2i have been demonstrated in real-world settings. The CVD-REAL 3 trial, a multinational observational cohort study, demonstrated a significant difference in the mean annual rate of change in eGFR between SGLT2i (0.46 mL/min/1.73 m^2 per year; 95% CI, 0.34 to 0.58) and other glucose-lowering drugs (−1.21 mL/min/1.73 m^2 per year; 95% CI, −1.35 to −1.06) (45). SGLT2i were also shown to slow the decline in eGFR in patients with T2DM and CKD in a Japanese real-world study (50). Moreover, SGLT2i can reduce the risk of ESRD (45). Although these real-world studies utilized propensity-score matching, the results should be interpreted carefully due to the possible involvement of unconsidered confounders. These lines of real-world evidence can contribute to

TABLE 2 Renal composite outcomes (worsening eGFR, end-stage renal disease, or renal death) according to renal function among patients with type 2 diabetes.

Trial	Events per 1,000 patient-years		HR (95% CI)
	Treatment	Placebo	
Overall population			
EMPA-REG OUTCOME (14)	6.3	11.5	0.54 (0.40 to 0.75)
CANVAS Program (15)	5.5	9.0	0.60 (0.47 to 0.77)
DECLARE-TIMI 58 (16)	3.7	7.0	0.53 (0.43 to 0.66)
CREDENCE (40)	27.0	40.4	0.66 (0.53 to 0.81)
VERTIS CV (43)	9.3	11.5	0.81 (0.63 to 1.04)
eGFR <60 mL/min/1.73 m^2			
EMPA-REG OUTCOME	NA	NA	0.66 (0.41 to 1.07)
CANVAS Program	11.4	15.1	0.74 (0.48 to 1.15)
DECLARE-TIMI 58	8.9	15.2	0.60 (0.35 to 1.02)
CREDENCE (45 to <60)	33.4	63.1	0.52 (0.38 to 0.72)
VERTIS CV	16.3	14.7	0.90 (0.59 to 1.38)
eGFR 60 to <90 mL/min/1.73 m^2			
EMPA-REG OUTCOME	NA	NA	0.61 (0.37 to 1.03)
CANVAS Program	4.6	7.4	0.58 (0.41 to 0.84)
DECLARE-TIMI 58	4.2	7.8	0.54 (0.40 to 0.73)
CREDENCE	14.9	18.5	0.81 (0.52 to 1.26)
VERTIS CV	10.5	7.0	0.66 (0.46 to 0.94)
eGFR ≥90 mL/min/1.73 m^2			
EMPA-REG OUTCOME	NA	NA	0.21 (0.09 to 0.53)
CANVAS Program	3.8	8.1	0.44 (0.25 to 0.78)
DECLARE-TIMI 58	2.5	4.9	0.50 (0.34 to 0.73)
CREDENCE	NA	NA	NA
VERTIS CV	9.3	9.6	1.04 (0.63 to 1.73)

eGFR, estimated glomerular filtration rate; HR, hazard ratio; CI, confidence interval. NA, Not available.

complement the results of RCTs and demonstrate the cardiorenal benefit of SGLT2i in patients with T2DM in clinical settings.

The real-world studies comparing SGLT2i and other glucose-lowering drugs suggest that the cardiorenal benefit seems to be independent of glucose-lowering effect. This idea was tested in a secondary analysis of clinical trials in which canagliflozin was compared with glimepiride (52). Despite the similar level of reductions in HbA1c, the decrease in systolic blood pressure was larger in the canagliflozin group than in the glimepiride group. Furthermore, the eGFR decline in the canagliflozin group was significantly slower than that in the glimepiride group. Another clinical trial enrolling Japanese patients with T2DM and CKD demonstrated that neither baseline HbA1c nor the changes in HbA1c significantly affected the decrease in the urinary albumin-to-creatinine ratio observed during 24 weeks of dapagliflozin treatment (53). Because SGLT2i lower blood glucose level by promoting the excretion of glucose into urine, their glucose-lowering effects become weaker as eGFR declines (54). Nonetheless, CVOTs reported that SGLT2i consistently reduced the risk of CV and renal outcomes even in patients with lower eGFR (≤45 mL/min/1.73m^2) (40, 55). Collectively, these findings suggest that SGLT2i confer a cardiorenal benefit independently of their glycemic effects.

The 2022 American Diabetes Association (ADA) standards of medical care in diabetes recommend a comprehensive approach to reduce the risk of diabetic complications, in which the following four factors are considered to be fundamental: management of glycemia, blood pressure, and lipids and the incorporation of specific therapies with efficacy on CV and renal outcomes (56). The importance of multifactorial interventions to target glycemia, blood pressure, and lipids is well established (57–59). Based on the evidence provided by RCTs, SGLT2i and glucagon-like peptide-1 receptor agonists (GLP-1 RA) are recommended to reduce the risk of adverse CV and renal events. The 2021 European Society of Cardiology (ESC) guidelines on CV disease prevention in clinical practice recommend SGLT2i and GLP-1 RA for reducing CV and/or cardiorenal outcomes in patients with T2DM (60). From the viewpoint of HF, SGLT2i are recommended for patients with stage A HF. The ADA consensus report for HF recommends that SGLT2i are an expected element of care in all individuals with diabetes and symptomatic HF and should be used in individuals with high CV risk, including those with stage B HF (61). However, DPP-4i and thiazolidines are not recommended for patients with diabetes with stages B, C, and D HF. For all patients with T2DM and stage ≥3 CKD, the use of SGLT2i is strongly recommended in the 2022 ADA standards of medical care in diabetes owing to their effects on slowing CKD progression and reducing HF risk (62). This recommendation is also based on the finding that the renoprotective effects of SGLT2i were not dependent on eGFR despite an attenuated glucose-lowering effect in patients with an eGFR of <45 mL/min/1.73 m^2 (Table 2).

SGLT2i for the treatment of HF

HF is a diabetic complication that must be managed; simultaneously, it is a serious issue for individuals without diabetes. The global prevalence of HF is estimated to be approximately 64 million, and it is the world's leading cause of hospitalization with a high risk of re-hospitalization (63, 64). As mentioned above, large CVOTs for SGLT2i have shown substantial risk reduction in HHF among patients with T2DM, including in patients with a history of HF (Table 2). The important remaining question is whether the beneficial effect of SGLT2i on HF can be extended to patients without T2DM.

The DAPA-HF trial was the first clinical trial to provide an answer to this question. The trial examined the efficacy and safety of dapagliflozin in addition to standard of care in patients with chronic HF and reduced ejection fraction (HFrEF) with or without DM (n = 4,744; median follow-up, 18.2 months) (17). Dapagliflozin achieved a 26% relative risk reduction for the primary outcome, defined as a composite of death from CV causes, HHF, and urgent HF visits, regardless of DM. Furthermore, this reduction was rapidly apparent, with a sustained statistically significant efficacy by 28 days after randomization (HR at 28 days 0.51, 95% CI 0.28 to 0.94) (65). Significant risk reduction was also observed for individual components of the composite outcome, with HRs of 0.82 (95% CI 0.69 to 0.98) for the risk of death from CV causes and 0.70 (95% CI 0.59 to 0.83) for the risk of HHF. Dapagliflozin was also associated with lower risk of death with a HR of 0.83 (95% CI 0.71 to 0.97) for death from any cause, and a HR of 0.79 (95% CI 0.63 to 0.99) for a composite of arrhythmia, resuscitated cardiac arrest and sudden death (66). In addition to these hard outcomes, DAPA-HF assessed QOL and showed a significant increase in the Kansas City cardiomyopathy questionnaire (KCCQ) score in the dapagliflozin group compared with the placebo group, indicating an improvement in patient-assessed symptoms. Although there were numerically fewer events in the dapagliflozin group than in the placebo group, there was no statistically significant difference in the renal composite outcome, defined as a ≥50% sustained decline in eGFR, ESRD, or renal death (HR 0.71, 95% CI 0.44 to 1.66). Consistently, the EMPEROR-Reduced trial, another large RCT of patients with HFrEF with or without DM, reported a 25% relative risk reduction in a composite of death from CV causes and HHF in patients treated with the SGLT2i empagliflozin (18). In addition, empagliflozin reduced the incidence of the composite renal outcome, defined as chronic dialysis, renal transplantation, a sustained reduction in eGFR of ≥40%, or sustained eGFR of <15 mL/min/1.73 m^2 among patients with a baseline eGFR of ≥30 mL/min/1.73 m^2 or <10 mL/min/1.73 m^2 among patients with a baseline eGFR of <20 mL/min/1.73 m^2. Empagliflozin also slowed the annual rate of decline in eGFR compared with placebo. A significantly greater improvement in the KCCQ score was reported in the empagliflozin group. Although EMPEROR-Reduced did not show a significant risk reduction for death from CV causes (HR 0.92, 95% CI 0.75 to 1.12) or death from any cause (HR 0.92, 95% CI 0.77 to 1.10), a meta-analysis of DAPA-HF and EMPEROR-Reduced showed significant risk reductions for both outcomes (67). These findings collectively show a cardiorenal benefit of SGLT2i in improving the prognosis and QOL of patients with HFrEF.

Approximately half of the patients with HF have LVEF of >40%, HF with preserved or mildly reduced ejection fraction (HFpEF or HFmrEF) (68, 69). The prognosis of this population is equivalently poor compared with HFrEF, with a 5-year mortality rate of approximately 40% (70). Several drugs with demonstrated benefits in HFrEF have been evaluated in HFmrEF and HFpEF, but none have shown conclusive efficacy (71–74). The EMPEROR-Preserved trial,

which enrolled 5,988 patients with HFmrEF and HFpEF regardless of DM, evaluated the efficacy and safety of empagliflozin, and reported for the first time a positive result of a 21% relative risk reduction in a composite of CV death and HHF (HR 0.79, 95% CI 0.69 to 0.90) (20). This was accompanied by sustained, statistically significant efficacy by 18 days after randomization (HR at 18 days 0.41, 95% CI 0.17 to 0.99) (75). In that trial, empagliflozin significantly slowed the slope of the eGFR decline and relieved the patient's symptoms and physical limitations, as measured by KCCQ (20, 76).

Although the results reported in EMPEROR-Preserved were epoch-making, the study left some uncertainties to be addressed. In particular, it was unclear whether these benefits are conserved in patients with a higher LVEF spectrum (>60%), in patients who start treatment during the subacute phase (i.e. during or soon after hospitalization), or in patients with a prior LVEF of ≤40% (HFrEF) that has since improved to >40% (HF with improved EF, HFimpEF). These gaps in evidence were addressed in the DELIVER trial, which evaluated the efficacy and safety of dapagliflozin in patients with HFmrEF and HFpEF (n = 6,263; median follow-up, 2.3 years) (21). In this trial, dapagliflozin significantly reduced the risk of the primary outcome events, CV death or worsening HF events, compared with placebo in the overall population (HR 0.82, 95% CI 0.73 to 0.92) and in a subpopulation of patients with an LVEF of <60% (HR 0.83, 95% CI 0.73 to 0.95). Consistent with the results of EMPEROR-Preserved, an early benefit of dapagliflozin was observed because the risk reduction for the primary outcome was statistically significant at 13 days after randomization and statistical significance was sustained from 15 days onward (77). An improvement in the KCCQ score was also reported, showing that dapagliflozin can ameliorate symptoms in patients with HFmrEF and HFpEF (21). Notably, DELIVER extended the findings of EMPEROR-Preserved trial based on the results of subgroup analyses, which demonstrated a consistent efficacy among patients with an LVEF of ≥60% (HR 0.78, 95% CI 0.62 to 0.98), HFimpEF (HR 0.74, 95% CI 0.56 to 0.94), and in the subacute phase (HR 0.78, 95% CI 0.6 to 1.03). Following evidence from a meta-analysis of DELIVER and EMPEROR-Preserved trials showing a consistent risk reduction for the composite of CV death and HHF (78), SGLT2i have been identified as the first drug that can be effective in patients with HFmrEF and HFpEF regardless of the presence of T2DM.

Four RCTs, DAPA-HF, DELIVER, EMPEROR-Reduced, and EMPEROR-Preserved, consistently showed the efficacy of SGLT2i in patients with HFrEF, HFmrEF, and HFpEF (Table 3). In addition, the SOLOIST-WHF trial assessed the efficacy and safety of sotagliflozin, a combined SGLT1 and SGLT2 inhibitor, in 1,222 patients with HF and T2DM, and demonstrated a significant risk reduction in a composite of CV death and worsening HF regardless of LVEF (>50% or ≤50%) (79) (Table 3). These results suggest that SGLT2i can be effective in patients with HF regardless of LVEF. This is a remarkable difference from the other drugs used for HF because the vast majority of them are only used to treat HFrEF owing to their attenuated effects in the higher LVEF spectrum (80). Two pooled meta-analyses of empagliflozin (pooled EMPEROR-Reduced and EMPEROR-Preserved, n = 9,718) and dapagliflozin (pooled DAPA-HF and DELIVER, n = 11,007) further support this notion (81, 82). Significant risk reductions for a composite of CV death and HHF were reported in both pooled analyses without heterogeneity across the full LVEF spectrum.

A meta-analysis of RCTs that enrolled >1,000 patients with HF across the full LVEF spectrum provided a combined view of the effects of SGLT2i on mortality (78). For CV death and all-cause death, the overall HR was 0.87 (95% CI, 0.79 to 0.95) and 0.92 (95% CI, 0.86 to 0.99), respectively, without heterogeneity among trials. For dapagliflozin, the pooled analysis was prespecified to assess its effect on CV death and all-cause mortality because of insufficient statistical power for evaluating these hard outcomes in individual trials (82). In the overall HF population, a significant benefit of dapagliflozin on mortality was observed irrespective of LVEF, with a HR of 0.86 (95%

TABLE 3 Randomized controlled trials of patients with heart failure.

	DAPA-HF (17)	DELIVER (21)	EMPEROR-Reduced (18)	EMPEROR-Preserved (20)	SOLOIST-WHF (79)
Drug	Dapagliflozin	Dapagliflozin	Empagliflozin	Empagliflozin	Sotagliflozin
No. of patients	4,744	6,263	3,730	5,988	1,222
LVEF (mean, %)	31.1	54.2	27.2	54.3	35
Median NT-proBNP (median, pg/mL)	1,437	1,011	1,910	994	1,800
eGFR (mean, mL/min/1.73 m^2)	65.8	61.0	62.2	60.6	49.7
T2DM (%)	45.1	44.8	49.8	49.1	100
Outcomes, HR (95% CI)					
HHF or CV death	0.74 (0.65 to 0.85)	0.82 (0.73 to 0.92)	0.75 (0.65 to 0.86)	0.79 (0.69 to 0.90)	0.67 (0.52 to 0.85)
HHF	0.7 (0.59 to 0.83)	0.77 (0.67 to 0.89)	0.69 (0.59 to 0.81)	0.71 (0.60 to 0.83)	0.64 (0.49 to 0.83)
CV death	0.82 (0.69 to 0.98)	0.88 (0.74 to 1.05)	0.92 (0.75 to 1.12)	0.91 (0.76 to 1.09)	0.84 (0.58 to 1.22)

LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro–B-type natriuretic peptide; eGFR, estimated glomerular filtration rate; T2DM, type 2 diabetes mellitus; HR, hazard ratio; CI, confidence interval; HHF, hospitalization for heart failure; CV, cardiovascular.

CI 0.76 to 0.97) for CV death and a HR of 0.90 (95% CI 0.82 to 0.99) for all-cause death. The meta-analysis of 21,947 patients, including outpatients and hospitalized patients, also showed that SGLT2i significantly reduced worsening HF events and improved symptoms. Collectively, SGLT2i demonstrated efficacy in reducing mortality, managing HF, and improving QOL, and prompted us to consider SGLT2i as a fundamental therapy for a broad range of patients with HF.

The 2021 ESC HF guidelines recommend dapagliflozin/empagliflozin as one of the four cornerstone drug therapies, alongside angiotensin receptor neprilysin inhibitors (ARNI)/angiotensin converting enzyme inhibitors (ACEi), β-blockers (BB), and mineralocorticoid receptor antagonists (MRA) for reducing HHF and death in all patients with HFrEF (Class I) (83). Moreover, the 2022 American Heart Association (AHA)/American College of Cardiology (ACC)/Heart Failure Society of America (HFSA) recommend guideline-directed medical therapy for Stage C or D HFrEF, consisting of four medication classes, including SGLT2i, ARNI/ACEi/angiotensin receptor blocker (ARB), BB, and MRA (Class I) (84). Additionally, the Japanese Circulation Society (JCS)/Japanese Heart Failure Society (JHFS) 2021 guideline focused update on diagnosis and treatment of acute and chronic heart failure recommends SGLT2i regardless of the presence of diabetes to further decrease the risk of exacerbation of HF or CV death in patients with symptomatic HFrEF combined with optimal basic treatment (ACEi/ARB, BB, and MRA) (85).

SGLT2i were included in the 2022 AHA/ACC/HFSA HF guidelines for the treatment of HFmrEF and HFpEF to reduce HHF and CV mortality (Class 2a) (84). This is a higher recommendation than those allocated to ARNI/ACEi/ARB, BBs, and MRAs for the treatment of HFmrEF and HFpEF. The results of DELIVER are expected to be reflected in future guidelines and provide further guidance for the use of SGLT2i in clinical practice independently of EF. A meta-analysis of five RCTs, including DAPA-HF, DELIVER, EMPEROR-Reduced, EMPEROR-Preserved and SOLOIST-WHF (78), will likewise help to reinforce the guideline recommendations for the use of SGLT2i in patients with HFrEF, HFmrEF, and HFpEF, as well as the clinical need to initiate guideline-directed medical therapy to improve the outcomes of patients with HF.

SGLT2i for the treatment of CKD

Although it was not a primary endpoint, protective effects of SGLT2i on renal function were demonstrated in three CVOTs, EMPA-REG OUTCOME, CANVAS, and DECLARE-TIMI 58, in which most enrolled patients had preserved renal function (14–16). The CREDENCE and DELIGHT trials further extended the renoprotective effects of SGLT2i to patients with T2DM and impaired renal function, in whom the glucose-lowering effects of SGLT2i are attenuated (40, 86). The proposed mechanism to explain these effects of SGLT2i on renal function, including reducing intrarenal hypoxia, may be relevant to patients with CKD without diabetes. The DAPA-CKD trial first evaluated the efficacy and safety of dapagliflozin in patients with CKD in the presence or absence of T2DM (n = 4,304; median follow-up, 2.4 years) (19). This trial was stopped early because of overwhelming efficacy. The primary outcome, assessed in terms of the time to the first event, was a composite of a sustained decline in eGFR of ≥50%, ESRD, and death from renal or CV causes. Dapagliflozin showed significant efficacy in reducing the risk of this composite by 39%, with a HR of 0.61 (95% CI 0.51 to 0.72), regardless of the presence of T2DM. Furthermore, there was no heterogeneity in the effects among the causes of CKD (87). Favorable effects of dapagliflozin were observed for each component of the composite outcome (19). In addition, the rate of eGFR decline was slower in the dapagliflozin group than in the placebo group (total eGFR slope difference 0.95 mL/min/1.73 m^2, 95% CI 0.63 to 1.27).

DAPA-CKD also assessed the effects of dapagliflozin on HF events and mortality. The HR for the CV composite of death from CV causes or HHF was 0.71 (95% CI 0.55 to 0.92) and the HR for death from any cause was 0.69 (95% CI 0.53 to 0.88) (19). These benefits were consistent with those observed in patients with HF as described above. With the evidence for T2DM, these findings further emphasize the beneficial effects of dapagliflozin in patients with CRM syndrome.

Results of the EMPA-KIDNEY trial (n = 6,609; median follow-up, 2.0 years) were recently published (88). This trial was also stopped early because of the clear efficacy of empagliflozin, as previously shown in CREDENCE and DAPA-CKD. Empagliflozin significantly reduced the risk of the primary composite outcome, progression of kidney disease (initiation of maintenance dialysis or receipt of a kidney transplant, a sustained decrease in the eGFR to <10 mL/min/1.73 m^2, a sustained decrease in eGFR by ≥40% from baseline, or death from renal causes) or CV death, by 28% (HR 0.72, 95%CI 0.64 to 0.82) regardless of diabetes status. The HRs for each component were 0.71 (95% CI 0.62 to 0.81) for progression of kidney disease and 0.84 (95% CI 0.60 to 1.19) for CV death. Furthermore, the rate of annual decline in eGFR was slower in the empagliflozin group than in the placebo group, with a between-group difference for the change from randomization to the final follow-up visit of 0.75 mL/min/1.73 m^2 per year (95% CI 0.54 to 0.96). Overall, EMPA-KIDNEY confirmed the efficacy of SGLT2i in patients with CKD regardless of T2DM.

The KDIGO 2022 clinical practice guideline for diabetes management in CKD (89) is a focused update of the 2020 guidelines with a relatively short interval to include some recently published evidence, particularly RCTs of SGLT2i (17–20, 79). In the focused update, SGLT2i are recommended as first-line therapy for patients with T2DM and CKD, regardless of the level of glycemia, to improve their renal and CV outcomes. Based on recently published RCTs, the 2022 guideline advocates initiating SGLT2i for patients with T2DM and CKD and an eGFR of ≥20 mL/min/1.73 m^2 instead of ≥30 mL/min/1.73 m^2, and continuing SGLT2i treatment for as long as tolerated, even if eGFR decreases to <20 mL/min/1.73 m^2, until kidney replacement therapy is initiated. SGLT2i are also recommended regardless of the patient's level of albuminuria. Therefore, SGLT2i are considered a foundation of pharmacologic therapy for T2DM and CKD.

Safety considerations

The safety profiles of SGLT2i as antidiabetic drugs were rigorously assessed in the large-scale CVOTs. Although the event

rates were low, there was a consistent increased risk of diabetic ketoacidosis (DKA) in the SGLT2i group than in the placebo group in a meta-analysis that included EMPA-REG OUTCOME, CANVAS, and DECLARE-TIMI 58 (37). On the other hand, an increased risk of amputations and fractures was only observed in one trial, resulting in moderate to high degree of heterogeneity. A more recent meta-analysis of 15 RCTs that enrolled patients with T2DM, CKD, and/or HF found no significant effect of SGLT2i on the incidence of amputation and fracture with no heterogeneity (90). This meta-analysis revealed a consistent increased risk of DKA as well as an increased risk of volume depletion. Although the meta-analysis included RCTs that enrolled patients with CKD, SGLT2i showed superiority but not inferiority in reducing the risk of acute kidney injury. These findings indicate the need for adequate patient education, including alerting patients to subjective symptoms of DKA and dehydration, such as lightheadedness, fatigue, abdominal pain, nausea, and vomiting, to confer a greater efficacy of SGLT2i treatment.

Discussion

In this review, we have summarized the results of RCTs and real-world data for SGLT2i treatment in patients with T2DM, HF, and CKD. Although the primary action of SGLT2i involves inhibition of SGLT2 expressed on proximal tubule cells, these drugs exhibit pleiotropic effects, which include reductions in body weight, blood pressure, intra-glomerular pressure, hyperuricemia, inflammation and oxidative stress, inhibition of the sympathetic nervous system, and improvements in erythropoiesis, cardiac energy metabolism and vascular function (91–93). The reduction in body weight is at least partly explained by a reduction in fat mass (94, 95). SGLT2i also reduce epicardial fat that releases pro-inflammatory mediators (96, 97). In addition, dapagliflozin was reported to inhibit the NLRP3 inflammasome, resulting in attenuation of fibrosis in diabetic mice (98). Further, the benefits on the heart may be mediated by improving myocardial energy efficiency. Ketone bodies were also elevated in patients treated with SGLT2i, which might contribute to improved cardiac function (99, 100). The increase in the erythropoietin level achieved by SGLT2i treatment appears to involve the suppression of hepcidin and ferritin and an increase of transferrin receptor protein 1 (101–103), which would correct anemia and improve clinical outcomes (104). Furthermore, inhibition of SGLT2 reduces the ATP-dependent tubular workload and oxygen requirements, alleviating hypoxia (105). Mitochondrial dysfunction has been implicated in both HF and CKD (106, 107), and several possible mechanisms were proposed by which SGLT2i preserve normal mitochondrial function (93). These effects probably contribute collectively to the cardiorenal protective effects of SGLT2i. Notably, an improvement in cardiac function leads to an improvement in renal function, and vice versa, owing to the mutual interactions between the heart and the kidney. Moreover, SGLT2i seem to prevent the onset of T2DM in patients with HF or CKD (108, 109), emphasizing the importance of using this drug in patients with CRM syndrome.

Although the efficacy of SGLT2i in CRM interactions is becoming increasingly clear, some issues remain unclear. Acute HF management with SGLT2i is one such example, and has received growing attention. A relatively small RCT was conducted and reported a significant clinical benefit of SGLT2i on the prognosis and symptoms in patients hospitalized for acute HF (110). A large-scale RCT is ongoing (NCT04363697) and is expected to provide rigorous evidence regarding this aspect. The efficacy and safety of SGLT2i in populations excluded from previous RCTs, such as the super-elderly (age >85 years) and/or highly frail individuals are also needed. Because of the considerable time and effort required to conduct such RCTs, it will be helpful to obtain evidence using real-world data to address the remaining issues and further solidify the CRM protective effects of SGLT2i.

Considering the emerging concept of CRM interactions, SGLT2i have shown better performance than initially expected. SGLT2i have not only been used as an antidiabetic agent but have also become a new treatment option for patients with HF (irrespective of LVEF or care settings) and/or CKD. Regardless of the presence of diabetes, patient management focusing on cardiorenal protection is important in terms of prognosis and QOL. SGLT2i can contribute to better treatment strategies for a huge number of patients suffering from CRM diseases.

Author contributions

All authors contributed to the conception of the review. HA and AN wrote the first draft, and NM and TY revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors HA, AN, and TY are employed by AstraZeneca K.K. Author NM was employed by AstraZeneca K. K.

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Prospecting of exosomal-miRNA signatures as prognostic marker for gestational diabetes mellitus and other adverse pregnancy outcomes

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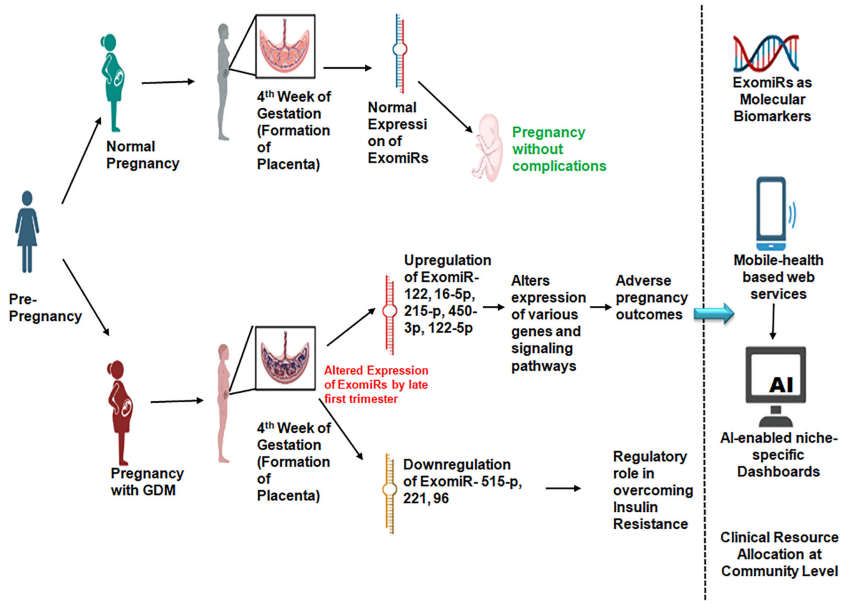
Exosomal microRNA (ExomiRs) serves as potential cargo molecules responsible for post-translation of gene expression and intracellular communication playing a vital role in acting as clinically relevant prognostic biomarkers for identifying pregnancy-associated complications in patients. ExomiRs are associated with Gestational Diabetes Mellitus (GDM) as potential targets for understanding the pathophysiology of beta-cell dysfunction. ExomiRs (ExomiR 122, ExomiR 16-5p, ExomiR 215-5p, ExomiR 450b-3p, ExomiR 122-5p) aid to act as biomarkers and regulate the progression of diabetes and its related complication. These ExomiRshave been reported to interfere with the regulation of various genes such as ZEB2, IRS1, IRS2, GLUT1, GLUT4, etc. and inhibition of several pathways like PI3K/AKT, Wnt, and mTOR signaling pathways leading to the modulation in the development of GDM affecting the clinical and pathological features of women. These ExomiRs have also been associated with other pregnancy-associated complications, including preeclampsia, hypothyroidism, pregnancy loss, and ectopic pregnancies. On the other hand, overexpression of certain ExomiRs such as Exomir-515-5p, ExomiR-221, and ExomiR-96 serve a regulatory role in overcoming insulin resistance. Taken together, the current review focuses on the prospective capabilities of ExomiRs for diagnosis and clinical prognosis of GDM women with respect to pregnancy outcomes.

KEYWORDS

clinical prognosis, exosomal microRNA, gestational diabetes mellitus, pregnancy outcomes, signaling pathways



GRAPHICAL ABSTRACT
Depicts the graphical abstract of the article demonstrating the role of ExomiRs as potential biomarkers for the prognosis of GDM and related pregnancy outcomes as well as how AI-enabled algorithms can help in clinical resource allocation at the community level. Additionally, the arrow pointing down from AI-enabled niche-specific dashboards towards the automated clinical resource allocation is missing.

Introduction

Exosomes are membranous vesicles produced in the endosomal compartment of most eukaryotic cells as a result of the lysosomal pathway and are usually 40-100 nm in size. These were discovered in 1983 (1) and were proposed to have no effect on neighboring cells and were considered either a cellular waste formed as a result of cell damage or a byproduct of cellular homeostasis until recently when they were found to act as complex cargo for delivering several proteins (2), lipids (3) and nucleic acids (2, 4) to the target cells (5), thereby playing a significant role in intercellular communication for serving pleiotropic cellular processes like signal transduction (6), immune responses (7) and antigen presentation (8). Thus, exosomes act as surrogate markers for different RNAs including microRNAs. These exosomes are shown to be released into the maternal circulatory system by the beginning of 6 weeks of pregnancy, i.e., the first trimester, and their concentration rapidly decrease within 48 hours postpartum (9, 10) thereby acting as an early predictor of Gestational Diabetes Mellitus (GDM) (11). GDM is a transient diabetic condition that women develop during their pregnancy tenure occurring mainly due to hormonal changes and metabolic exigencies of pregnancy accompanied by genetic and environmental factors.

Exosomal microRNA (ExomiR) are 21-25 nucleotide long (12) nano-sized, non-coding RNA molecules serving pivotal regulatory roles in the progression of various diseases including insulin resistance in pregnant women. These ExomiRs can act as biomedical tools for a better prognosis of GDM and other pregnancy-associated complications like preeclampsia, preterm births, neonatal sepsis, etc (13). These molecules not only regulate but also act as significant biomarkers for several diseases, thereby helping to better diagnose the diseases. GDM can be related to preterm birth cases of Assisted Reproductive Technology (ART) (14), which is generally used to overcome infertility problems.

Exosome biogenesis

Exosomes are vesicles formed by the process of endocytosis formed by the inward sprouting of the early endosome's limiting membrane from Multivesicular Bodies (MVBs). The invagination of the inner membrane within MVBs results in the formation of Intraluminal Vesicles (ILVs). Nucleic material, transmembrane, and peripheral proteins are integrated into ILVs during their formation and accumulate in the MVB lumen, which later has two distinct fates: diffusion with lysosomes for degradation, or diffusion with the cytoplasmic membrane, which releases the vesicles to the extracellular space *via* exocytosis as exosomes (5, 15).

Exosome biogenesis and secretion are thought to be aided by either the ceramide-dependent pathway or the Endosomal Sorting Complex Required for Transport (ESCRT)-dependent pathway. ESCRT, which recognizes ubiquitin-related proteins, is the most well-known pathway. These pathways may involve sphingomyelinases, which are composed of four protein complexes, including ESCRT-0, -I, -II, and -III and the associated ATPase Vps4 complex. Proteins like ubiquitinylated proteins and clathrin are recruited for internalization by the ESCRT-0 complex subunits. ESCRT-I and II initiate the sprouting process and facilitated e-ubiquitination of cargo proteins carried out by enzymes, before the ILVs are formed, which are grouped to create larger membranous vesicles, MVBs, in the intracellular compartment. The last stage of

membrane budding and partition is driven by the ESCRT-III complex (16).

The ceramide-dependent pathway serves as an alternative pathway for exosome formation. The ceramide-dependent pathway relies on the growth of glycolipoprotein micro domains (lipid rafts), where sphingomyelinases convert sphingomyelin into ceramide. The subsequent ceramide buildup causes micro domain fusion and starts the development of ILVs within MVBs (17, 18).

Packaging of miRNA into exosomes

The miRNAs are an important requirement for exosomal cell signaling. During the biogenesis of exosomes, there are miRNAs present in the cell which are passed into the exosomes *via* a loading process, which is yet to be identified. It has been demonstrated that argonaute proteins play a crucial role in exosomal loading, miRNA transport, and miRNA function. RISC may not even be present at all in exosomal miRNAs. Instead, they are recognized by particular proteins, such as hnRNPA2B1 and hnRNPA1, which recognize the particular miRNA-binding motifs. As a result, the miRNAs are then loaded into exosomes in a selective manner (19).

Although the underlying mechanisms of miRNA packaging are not fully understood, there are at least three putative methods for miRNA sorting into exosomes (Figure 1). Firstly, the pathway identified by Villarroya-Beltri et al. (19) helps in the packing of selective miRNAs into exosomes by using sumoylated heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), which can detect the GGAG pattern found in the 3′-end portion of the miRNA sequences. Additionally, other two members of hnRNP family, hnRNPA1 and hnRNPC, which may bind to exomiRs and are thus implicated in the sorting process, may be involved in miRNA sorting. The second approach is by Kosaka et al. (20), in which the overexpression of neural sphingomyelinase 2 (nSMase2) is involved resulting in an increase in exosomal miRNA levels. While inhibition of nSMase2 expression resulted in fewer exosomal miRNAs, the hnRNPA1 and hnRNPC protein families, which bind to exosomal miRNAs, might be factors responsible for facilitating miRNA sorting. The third and final approach by Koppers-Lalicet al. (21) deals with exosomes taken from either B cells or the urine that were the predominant source of the 3′ ends of uridylated endogenous miRNAs. This demonstrates that the 3′ ends of the miRNA sequence may be connected to a crucial sorting signal. The cytoplasmic lipid bilayers of the MVB limiting membrane are where the miRNAs with the greatest affinity to the raft-like area are accumulated. RNA-binding proteins transport miRNAs to be bound to this region. The specific binding motifs, like the GGAG pattern, may very well determine this transport. A spontaneous process of inward budding from the raft-like area takes place once the miRNA has attached to it, thereby producing ILVs and subsequently exosomes. The cytoplasmic leaflet of the membrane's ceramide molecules, as well as the lysophospholipid and glycosphingolipid molecules of the luminal leaflet, may be necessary for the budding process (22) and hence the ExomiRs are released into the maternal circulatory system.

Biodelivery of exomiRs

Fusion of the hydrophobic cytoplasmic leaflets of the exosome and plasma membrane is likely mediated by families of Soluble N-ethylmaleimide-sensitive factor Activating Protein Receptor (SNARE) and Rab proteins to produce a hemifusion stalk, thereby initiating the fusion of exosomes (23, 24). The exosome surface contains integrins, adhesion molecules, and lipid raft-like structures that facilitate contact, attachment, and fusion of the membrane with the target cell (25, 26). The formation of clathrin-coated vesicles during clathrin-mediated endocytosis, which is characterized by the participation of the triskelion scaffold (clathrin), occurs as a result of the sequential assembly of multiple transmembrane receptors and ligands (27). Most cell types exhibit this mechanism of the exosomal entrance, this involves internalized vesicles that uncoil and fuses with

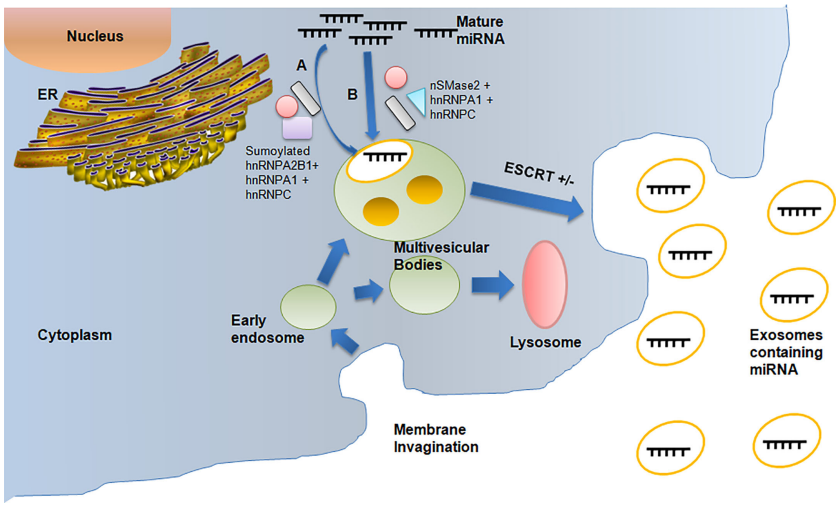


FIGURE 1
Depicts the packaging of miRNAs into exosomes during exosomal biogenesis resulting in the formation of ExomiRs, which helps in the biodelivery of miRNAs. **(A)** The miRNAs packaging during Exosome biogenesis occurs by sumoylated hnRNPA2B with the help of hnRNPA1 and hnRNPC. **(B)** Packagingof miRNA can also be done alternatively by nsMase2 with the help of hnRNPA1 and hnRNPC.

endosomes with Dynamin 2 forming the neck-like structure during invagination required for scission. Internalized vesicles then become uncoated and join endosomes. Clathrin-mediated endocytosis is one of the most conventional exosome uptake pathways. Thus the ExomiRs are transferred to the recipient cell. The cargo and exosome composition can also affect this tightly controlled process (27, 28).

One important endocytic method to move exosomes into the early endosome and affect their uptake is lipid raft-associated membrane invagination (29). By immobilizing exosomes on the cell surface at particular adherent locations, annexin AnxA2 increases lipid raft-mediated endocytosis (30), and flotillin, a component of lipid rafts, also favorably controls this process by associating with membrane micro domains enriched in cholesterol and sphingolipids. According to reports, the assembly of flotillin-1 and flotillin-2 causes membranes to experience curvature stress and creates caveola-like invaginations at the plasma membrane (28, 31).

ExomiRs as placental function marker

Complications in pregnancy are associated with significant difference in the level of circulating exosomes and hence the concentration of ExomiRs in maternal plasma, their composition, and bioactivity from that of normal pregnancies (32). These exosomes that are released into maternal bloodare responsible for placental development and maternal immune tolerance (33). The human placenta is a transient organ that provides the required oxygen and food to the fetus and removes the waste products from the fetal blood by the umbilical cord thus the proper development and functioning of the placenta is required for normal deliveries making it an essential part of the maternal-fetal communication system. Angiogenesis under hypoxic conditions perhaps is one of the keystone signaling pathways, responsible for the zygote to undergo the process of blastulation and gastrulation, thereby promoting the fetal growth through tissue differentiation which is mediated by upregulated expressions of hypoxia-induced vascular-endothelial growth factor-mediated downstream signaling pathways involving but not limited to the expression of MMPs and their downstream signaling intermediates (34).Various evidences support the hypothesis of the role of ExomiRsin the origin of pregnancy-related complications in the early stages of gestation. The total concentration of ExomiRs helps us to indicate the difference between normal and pregnant women. Additionally, the concentration of these ExomiRsis altered in women with pregnancy-associated complications.ExomiRscanmodulate the gene expression by post-transcriptional repression or messenger RNA degradation in a sequence-specific manner (35) leading to the onset of various complicated pregnancy outcomes in pregnant mothers. The upregulation and downregulation of various ExomiRs make them efficient biomarkers, helping in the prognosis of various pregnancy complications with most of the ExomiRs being upregulated during complicated pregnancies and only some being downregulated acting as non-invasive biomarker due to several epigenetic modifications indicating placental health. A maternal-fetal communication system based on ExomiRs may exist as evidenced by the rapid alteration in maternal blood ExomiR levels within 48 hours following delivery (Figure 2). According to a study, placental and maternal ExomiRs can both move to the maternal circulation with compartment-specific expression from the placenta and even into the fetal compartment (36).

The studies that are currently provided, however,suggest that exosome biology is altered during pregnancy-associated complications. To determine the precise function of exosomes in complicated pregnancies, it is necessary to apply particular and well-characterized isolation approaches. Exosomal secretion by trophoblastic cells in the placenta to the maternal peripheral circulation is thought to be responsible for the higher rates of delivery of these vesicles during gestation, which also

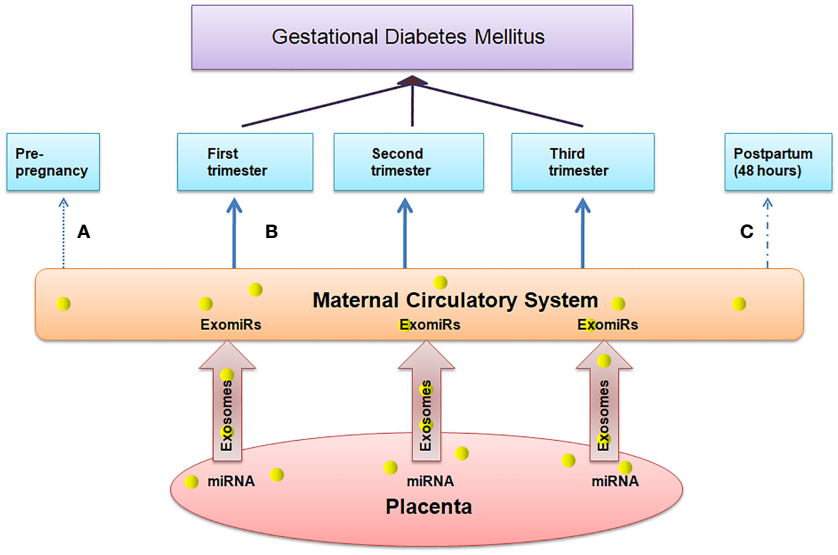


FIGURE 2
Depicts the association between the concentration of ExomiRs in maternal peripheral blood, which serves as a marker for placental health. **(A)** The concentration of ExomiRs in the maternal circulatory system is normal before pregnancy. **(B)** During complicated pregnancies like in GDM patients, the ExomiR concentrations, beginning from the first trimester, are observed to be altered, with most being upregulated and few being downregulated. **(C)** The ExomiR concentration is observed to be decreased after delivery and gets back to normal 48 hours postpartum.

happens in response to various pathological conditions. Exosomes that have been isolated from the maternal circulation during a typical pregnancy also show variations in their bioactivity as the gestational age increases. A major cause for the variation in the bioactivity of miRs in different trimesters of pregnancy is due to environmental factors like hypoxia, obesity, signaling pathways as well as epigenetic modifications. Compared to exosomes obtained from the second and third trimesters of pregnancy, those from the first trimester are shown to be more bioactive in stimulating endothelial cell migration (32). This phenomenon could be crucial in identifying the abnormal placentation in complex pregnancies since it may be linked to the cellular origin and/or exosomel composition and thus act as potential prognostic biomarker for adverse perinatal outcomes (33). Exosomal protein composition has also been seen to be alter in Preeclampsia (PE) affected women (32).

ExomiRs as an indicator of placental health in gestational diabetes mellitus

Several ExomiRs have been studied to interfere with the functioning of several genes and thus leading to insulin resistance in patients. This has been observed to be associated with several complications in patients like type 2 diabetes mellitus (T2DM) and Gestational Diabetes Mellitus (GDM) in pregnant women. ExomiRs also act as biomarkers that help in the early diagnosis of insulin resistance-related complications. Although the association between ExomiRs and GDM is yet to be uncovered, several genes are seen to be upregulated or downregulated making them efficient biomarkers for the prognosis of the disease. In a recent study, it was seen that screening patients for GDM in the second and the early third trimester helps us to indicate the specific pathophysiological placental features (7).

The upregulation of Exomir-122-5p in pregnant mothers with GDM shows their regulatory role in insulin resistance (37, 38), obesity (37) and regulation of glucose level (39, 40). This ExomiRis expected to prevent insulin from binding to the insulin receptor protein. GLUT-2 is anticipated to be inhibited by the ExomiR-122-5p which would result in reduced insulin production from pancreatic islet cells (41). Inhibition of the GLUT-2 receptor not only impairs glucose uptake by the cell but also causes the expression of other glucose transporters to be dysregulated resulting in dysglycemia during the course of pregnancy (42). The aggravated levels of ExomiR-122-5p found in GDM are expected to inhibit Adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK), which will thus prevent beta-oxidation and glucose transport (41). With its ability to activate insulin-sensitizing effects, AMPK is a phylogenetically conserved serine/threonine energy sensing kinase and is therefore a prime candidate for diabetes treatment. In addition to reducing hepatic glucose synthesis, it sends signals to enhance skeletal muscle glucose uptake and adipose (and other) tissue fatty acid oxidation (43). This inhibits the AMPK pathway and would also lead to hindering glucose uptake by the body cells thereby resulting in GDM. Moreover, the downregulation of beta-oxidation may also lead to adiposity in pregnant women (Figure 3). Exomir-122-5p also targets genes like Glucose-6-Phosphate Catalytic Subunit 3 (G6PC3) and Farnesyl-diphosphate farnesyltransferase 1 (FDFT1) essential for hydrolysis of glucose 6-phosphate in glycolysis and cholesterol biosynthesis respectively, impairing their proper functioning and leading to insulin resistance (44) and hence, GDM in patients.

ExomiR-16-5p regulates the PI3K/Akt, Wnt and mTOR signalling pathways, since these signalling pathways serve a key role in GDM (45–47). Upregulation ofExomiR-16-5p in GDM patients during the second trimester, IRS1 and IRS2 are negatively regulated, thereby showing the effect of ExomiR-16-5p on these genes. This impairs Wnt signalling pathway and may ultimately result in GDM by blocking the autophagic degradation of Dishevelled 2 (45, 48, 49) which modulates a glycogen synthase kinase allowing nuclear translocation of beta-catenin and subsequent activation of Wnt-

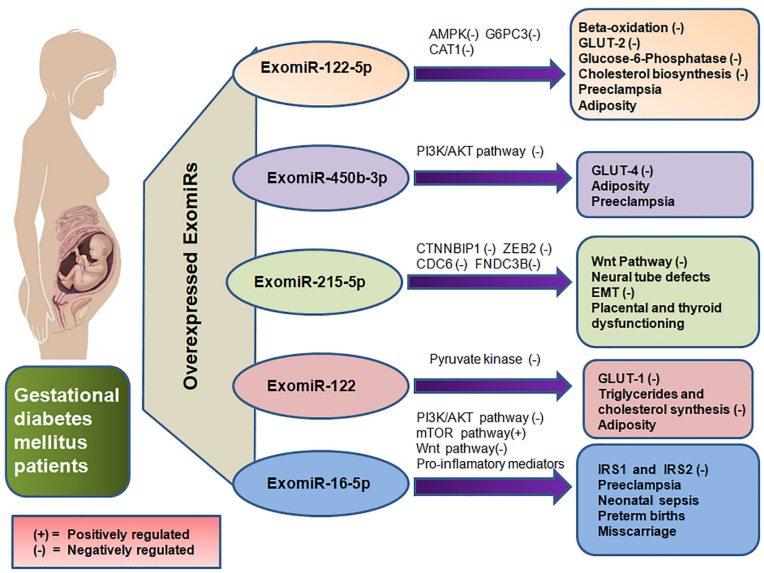


FIGURE 3
Depicts the overexpression of ExomiR in GDM patients, which results in their interference with the regulation of various genes and signaling pathways leading to several maternal and teratogenic outcomes thereby also acting as prognostic biomarkers for the GDM and its associated complications.

target gene. The placental mammalian target of rapamycin (mTOR) signal is activated by ExomiR-16-5p overexpression, which encourages mitochondrial function, protein synthesis and the transport of nutrients like amino acids, improving fetal nutrition utilisation. Exosomes from GDM patients are more enriched in proteins targeting the mTOR signalling pathway than exosomes from those with normal glucose tolerance (Figure 3). Exosomes from GDM patients may therefore control placental nutritional capacity by stimulating the mTOR signal in the placental environment (50). It has been proposed that altered signalling of protein kinase B/Akt (Akt) and mTOR in human placental endothelial cells may be the cause of insulin resistance in pregnant women with GDM and their neonates (45, 51).

ExomiR-215-5pdownregulates the Catenin Beta Interacting Protein 1 (CTNNBIP1) gene which encodes the CTNNBIP1 protein that is a negative regulator of the Wnt signaling pathway leading to GDM (52). It is also an important factor in the mechanism of ADSCs-Exo-mediated protection against podocyte injury by suppressing ZEB2 transcription leading to Diabetic Nephropathy (DN) (53). DN is considered a cause of chronic hyperglycemic conditions as a result of GDM leading to the damaging of multiple organs including kidneys (54). As ExomiR-251-5p acts as a downstream regulator of ZEB2 (Figure 3), it increases the proangiogenic effect leading to the ExomiR causing Neural Tube Defects (NTD). NTD-associated genes like ZEB2, with a two-fold or greater change in expression control diabetes exposure to theembryos (55).Thus ExomiR-215-5p not only serves a key role in GDM but also in neonatal diabetes.

PI3K/AKT signaling pathway is inhibited by ExomiR-450b-3p, which maintains insulin-induced protein Forkhead Box protein O1 (FOXO1) rejection and thereby impairing the GLUT-4 trafficking leading to impaired glucose tolerance (56). Additionally, the Akt signaling pathway phosphorylates FOXO1 transcription factors promoting adipogenesis as FOXO1 prevents the maturation and differentiation of adipocytes thereby playing a significant role in obesity (57).This leads to an increase in the Basal Metabolic Index (BMI) of the pregnant mother and hence increases the risk stratification of obesity-related pregnancy outcomes like GDM (Figure 3). Hyperlipidemia in the first trimester of pregnancy might lead to the development of GDM in the second trimester (58).

ExomiR-122 controls the expression of the GLUT-1 receptor by downregulating pyruvate kinase, thereby hampering the glycolytic fluxes and subsequently decreasing glucose metabolism (54). The major transporter for glucose transfer in the placenta, GLUT-1, is essentially expressed in the endothelial cells of the placental villi and syncytiotrophoblast (59). Syncytiotrophoblasts are primarily responsible for nutrient and gas exchange in the placenta. The downregulation of GLUT-1 by ExomiR-122 leads to a decrease in GLUT-1-mediated glucose transport activity (Figure 3) thereby leading to GDM (60).

Preeclampsia-associated exomiRs

GDM is most commonly linked to its pathophysiological outcome, Preeclampsia (PE),as a result of oxidative stress, pro-inflammatory factor release, and vascular-endothelial dysfunction. The occurrence of the hypertensive disorder, ie.,PE is positively correlated with blood glucose levels. The association between GDM and PE is not specific to obesity or primigravida but the association between the two increases with obesity and specifically gestational weight gain (61). In the early stages of pregnancy, new blood vessels develop for the supply of oxygen and nutrients to the fetus. These blood vessels do not work or develop properly in women with PE and this, in turn, leads to dysregulation of blood pressure in women with PE, which is generally determined in the second trimester of pregnancy.

ExomiR-122-5p has been observed to have a crucial role in metabolism of cholesterol by targeting Cationic amino acid transporter 1 (CAT1), which transports cationic amino acids (Figure 3) and can be linked to dyslipidemia in PE (62). ExomiR-122-5p elevated levels may be attributed to the abnormal glycosylation of the mucin-type O-glycosylated antigen, which is interconnected with the augmented maternal inflammatory responses seen in severe PE (63). Furthermore, recent research has linked the abnormal glycosylation of proteins in PE to the synthesis of new proteins that are involved in hepatic and renal dysfunction, implying that placenta-derived exosomes may be engaged in the end-organ abnormalities associated with severe forms of PE (64).

PI3K/Akt pathway overexpression by ExomiR-16-5p is involved in the osteogenic differentiation of cells thereby inhibiting the pro-apoptotic protein Cyt C, Apaf-1 and Bax. Nephronectin, an osteogenesis enhancer, is silenced which suppresses the early phases of osteoblast development in pregnant women. However, the bindingExomiR-16-5p to the 3'-UTR of nephronectin releases GalNT-7, which is another target also known to glycosylate proteins, including nephronectin to become active (65, 66). This increases the risk of PE (Figure 3) (67).

Overexpression of ExomiR-215-5p in pregnant women inhibits the proliferation and migration of trophoblasts during PE by limiting CDC6 (Figure 3). CDC6 gene codes for a protein, CDC6 that is required for the process of DNA replication. An examination of the cell cycle distribution of trophoblast cells reveals that the number of cells in the G1 phase visibly increases whereas the number of cells in the S-phase decreases significantly (68).ExomiR-215-5p also prevents the epithelial-mesenchymal transition (EMT) by impairing CDC6 *via* the epigenetic downregulation of E-cadherin expression (69).

Hyperlipidemia caused as a result of inhibition of PI3K/AKT signaling by ExomiR-450b-3p as a result of phosphorylation of FOXO1 transcription factor (57, 58) not only leads to adiposity-induced insulin resistance by impairing with GLUT-4 trafficking (56), but also the hypertensive disorder of PE as a result of obesity (Figure 3). Similarly, ExomiR-122-induced inhibition of GLUT-1 decreases glucose metabolism by downregulating pyruvate kinase (70) thereby also impairing the synthesis of triglycerides and cholesterol leading to obesity (71) and hence the onset of PE (60).

In PE, early insufficient trophoblast invasion causes improper spiral artery remodeling leading to placental ischemia and oxidative stress causing morbidity and mortality in mothers and infants and is considered a pregnancy-specific seizure disorder which is accompanied by the onset of proteinuria, and elevated blood pressure serving as recognition factors (72). Severe PE may lead the patient to undergo Cesarean delivery (C-section) (73).

ExomiRs-associated with other maternal and teratogenic outcomes

Several ExomiRs have been seen to have an association with adverse pregnancy outcomes with some of the placental origins, some are pregnancy state-specific and others are involved in a pathophysiological state of diabetes, which is associated with other severe pregnancy-related outcomes. Disruption of the tightly regulated endocrine system through sustained perturbation of hypothalamus-pituitary signaling cascade may lead to maternal complications including but not limited to GDM, preeclampsia, and hypothyroidism, which could either result in miscarriages, preterm births, pregnancy complications as well as increased pre-disposition of the offspring to neonatal sepsis (74).

During early adipogenesis, ExomiR-215-5p serves as a repressor of adipocyte differentiation *via* post-transcriptional regulation of Fibronectin type III Domain Containing 3B(FNDC3B) (52), which serves a ubiquitous role in the placenta. Decreased levels of ExomiR-215-5plead to ectopic pregnancy in the early stages of pregnancy accompanied by abdominal pain or vaginal bleeding (75). FNDC3B also serves a ubiquitous role in the thyroid leading to the onset of hypothyroidism. A study also shows that circulating ExomiR-215-5p in women in the second trimester of pregnancy was determined to be associated with the birth weight-at-gestational stage (76). Downregulation of NTD-associated genes like ZEB2 is caused as a result of ExomiR-251-5p upregulation (Figure 3), thereby regulating neural tube development hence altering embryonic expression leading to NTDs. These NTDs, in some cases, may progress from a wavy neural tube to spina bifida in various locations of the neural tube leading to exencephaly and craniorachischisis. This can be detected by the upregulation of ExomiRs in the mother's blood (55). ExomiR-16-5p enhances the secretion of proinflammatory cytokines in the human placenta by inhibiting the Apelin signaling pathway, where Apelin serves as a potent inhibitor of proinflammatory mediators thereby activating pro-labor hormones and cytokines including IL-1, IL-6, IL-8, and TNF-α. This leads to preterm births and C-sections (77). An elevation in the levels of proinflammatory cytokines likeIL-6 and IL-8 in the placenta also acts as a precursor for an increased risk of neonatal sepsis as a result of autophagy in the placenta (78). This can be well determined by the leukocyte count of the pregnant mother (79). Additionally, premature infants are more prone to Bronchopulmonary Dysplasia as a result of sepsis (80). Moreover, obesity induced by the overexpression of ExomiR-450b-5p and ExomiR-122 may also lead to cases of miscarriage among pregnant women (81).

Can epigenetic markers be prospected as theranostic target?

Not only ExomiRs aid to act as a potential causative agent for insulin resistance during pregnancy but there are certain ExomiRs that when overexpressed, lead to overcoming insulin resistance in patients by influencing glucose uptake.

Overexpression of ExomirR-221 targetsp21-activated kinase (PAK1) which regulates the proliferation and suppresses apoptosis of beta cells of islets of the pancreas thereby regulating insulin secretion (63, 82). ExomiR-96 when overexpressed in cells was found inversely correlated with the rise in blood glucose level in GDM conditions. It is also found to target PAK1 specifically like ExomiR-221 (82, 83). Therefore, these can possibly act as an effective tool to resist GDM-induced insulin resistance. These ExomiRs influence the cells' enhanced insulin selection leading to insulin secretion and also enhances the proliferative activity of cells. Their effect on cells' viability and apoptosis were partially reversed by PAK1 which meant that PAK1 was necessary for its protective impact on islet beta cells (65). Fasting hyperglycemia and severe glucose intolerance were also found to be present in PAK1-deficient individuals (83).

It was observed in Human Primary Trophoblasts (PHT) that overexpression of ExomiR-515-5p eventually significantly stimulates glucose uptake by cells. It regulates the functioning of Insulin like Growth Factor 1 Receptor (IGF1R) thereby stimulating glucose uptake (84). Proteins linked to glycolysis were differentially expressed in ExomiR-515-5p overexpressed PHT cells, according to a proteomics investigation (85). These data imply that in GDM patients, increased placental nutrition transfer may be a result of adipose tissue ExomiR515-5p mediated placental glucose uptake (85, 86).

Thus, these ExomiRs can not only be used as potential non-invasive biomarkers for the prognosis of GDM but also their ability to increase in glucose uptake makes them significant to be used as a clinical tool for reducing the risk of GDM and related pregnancy complications (87, 88).

Conclusion

The rise in the incidence of GDM, in turn gives rise to increasing number of maternal and fetal complexities with adverse consequences. To manage the burden of GDM, clinical interventions supplemented with bio-behavioral health-based interventions could significantly alleviate the clinical prognosis of GDM in the world. There have been numerous studies that characterized the expression of circulating miRNAs or ExomiRs from pregnant women, thus suggesting their role in pathogenesis of GDM, however, their potential molecular mechanisms are still unknown.

The main purpose of this review is to assess a panel of ExomiRs being prospected for early diagnosis of GDM in communities with multiple ethnicities, socio-cultural norms and lifestyle choices. This is very pertinent in Indian subcontinent where there are five distinct centers of origin having population with varying socio-cultural norms and lifestyle habits, which could perhaps create a variation in the existing panel of exomiRs which is being prospected. The identification of the race- and niche-specific ExomiRs as biomarkers can help in predicting and diagnosing GDM in the first trimester of pregnancy to avoid any pregnancy-associated complications through timely intervention at the community level.

The aggregation of hyperglycemic signals from the pregnant women at the community level to detect recurrent and emergent hotspots of GDM poses a major challenge for healthcare professionals. To this end, the deployment of a federated learning-based system to detect GDM using m-health platforms will not only facilitate optimal detection of GDM but also provide insights into the automated allocation of clinical resources, along with identification of the impending risk factors (precursors) contributing to the GDM epidemic, even in remote locations.

We believe that the use of AI-enabled dashboards (Figure 4) endowed with digital signals of hyperglycemia, as well as epigenetic/

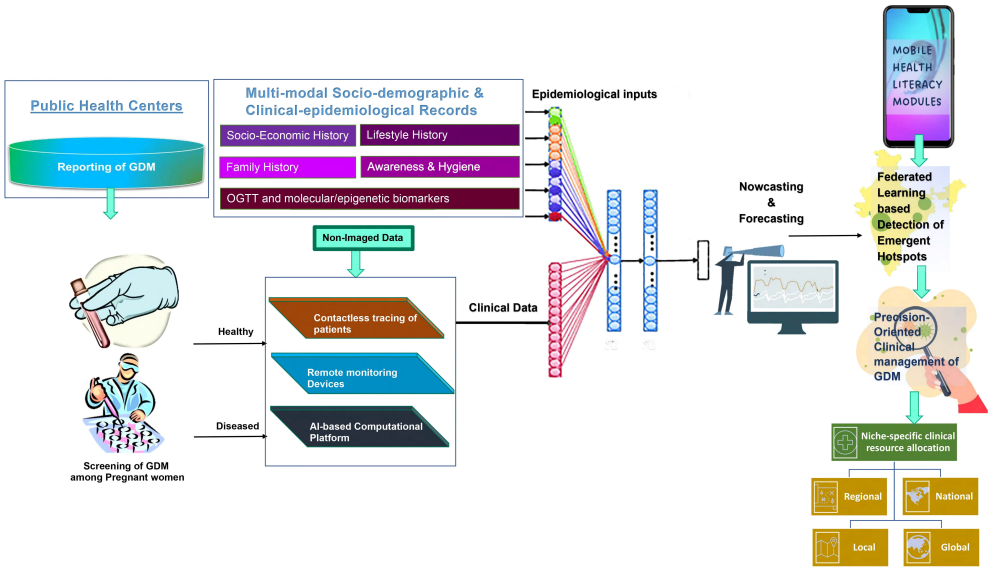


FIGURE 4
Shows the integration of digitized signals from the molecular markers, epidemiological data, as well as clinical data for the development of AI-enabled nowcasting and forecasting system, which when combined with the mobile-health-based health literacy modules, will help in the deployment of the federated learning-based detection of emergent hotspots of the Gestational diabetes (GDM)and its associated complications among pregnant women for the precision-oriented clinical management of the disease. This AI-enabled platform also forms the rationale for niche-specific allocation of the clinical resources at the community level.

molecular biomarkers, will facilitate precision-oriented large-scale screening of GDM in both rural, semi-urban, and urban milieus. This will facilitate the remote connection of the physicians' team with the patients along with the provision of health literacy modules to the vulnerable population. The heuristic capabilities of this iterative and interactive dashboard will be proactively used to develop nowcasting and forecasting strategies towards the development of niche-specific data-driven surveillance system, integrating all the stakeholders of the healthcare ecosystem for developing community empowering bottoms-up policies/programs to help the communities at local, regional, national, and global levels.

Author contributions

TM, RG, SK, ST, PR, and RJ designed the concept of the manuscript. TM and RG wrote the initial draft of the ms and made the concept figures with help from AU, PR, and RJ. SK, ST, PR, and RJ reviewed the paper extensively and provided their critical inputs in refining the concepts of the ms. RJ, TM, and RG were responsible for the overall concept and quality of the ms. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

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Causal associations between site-specific cancer and diabetes risk: A two-sample Mendelian randomization study

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Background: Both cancer and diabetes are complex chronic diseases that have high economic costs for society. The co-occurrence of these two diseases in people is already well known. The causal effects of diabetes on the development of several malignancies have been established, but the reverse causation of these two diseases (e.g., what type of cancer can cause T2D) has been less investigated.

Methods: Multiple Mendelian randomization (MR) methods, such as the inverse-variance weighted (IVW) method, weighted median method, MR-Egger, and MR pleiotropy residual sum and outlier test, were performed to evaluate the causal association of overall and eight site-specific cancers with diabetes risk using genome-wide association study summary data from different consortia, such as Finngen and UK biobank.

Results: A suggestive level of evidence was observed for the causal association between lymphoid leukaemia and diabetes by using the IVW method in MR analyses (P = 0.033), indicating that lymphoid leukaemia increased diabetes risk with an odds ratio of 1.008 (95% confidence interval, 1.001-1.014). Sensitivity analyses using MR-Egger and weighted median methods showed consistent direction of the association compared with the IVW method. Overall and seven other site-specific cancers under investigation (i.e., multiple myeloma, non-Hodgkin lymphoma, and cancer of bladder, brain, stomach, lung, and pancreas) were not causally associated with diabetes risk.

Conclusions: The causal relationship between lymphoid leukaemia and diabetes risk points to the necessity of diabetes prevention amongst leukaemia survivors as a strategy for ameliorating the associated disease burden.

KEYWORDS

site-specific cancer, diabetes, lymphoid leukaemia, Mendelian randomization, causality

Introduction

One of the twenty-first century's major threats to public health is the elevation of diabetes mellitus prevalence worldwide (1). An initial stage of insulin resistance and compensatory hyperinsulinemia which contribute to β-cell failure defines type 2 diabetes (T2D) (2). T2D is characterized by chronic hyperglycaemia, which damages end organs over time (2). The World Health Organization reports that out of six deaths, one is attributed to cancer, which makes cancer the second primary cause of mortality worldwide (3). Both cancer and diabetes are complex chronic diseases and have high economic costs for society. The co-occurrence of these two diseases in people has already been reported for more than 50 years (4). It is presumed that these two diseases may have similar developmental pathways, such as the malfunction of immunological regulation and cytokine activity (5). Common risk factors, such as obesity, genetic predisposition, and exposure to certain environmental factors, have been identified in the development of cancer and diabetes (5, 6). Given that abdominal adiposity has been found to promote a proinflammatory condition throughout the body, which increases the risk of cancer and diabetes, obesity has been proposed as one of the underlying reasons for these two diseases (6).

Epidemiological evidence has indicated that several malignancies are more likely to occur in people with T2D (7). For instance, diabetes significantly increases the relative risk of liver and pancreatic cancer (PC) (8, 9), but less evidence has been observed for other cancers. Because the development of some malignancies can precede and cause T2D, the potential reverse causation of these two diseases should also be considered. For instance, PC is likely to promote the development of T2D (10). According to a recent study from Korea, cancer can enhance the risk of developing diabetes among cancer survivors, independent of conventional diabetes risk factors (11). The diabetes risk was most significant in the first two years after cancer diagnosis, and elevated risk was continuously observed for as long as 10 years (11). Moreover, circulating cytokines aggravate hyperglycaemia in cancer patients by promoting insulin resistance and increasing hepatic gluconeogenesis (12). A standard tumour marker for PC is a higher level of CA19-9, and elevated serum CA19-9 levels have been related to the severity of inadequate glucose regulation (13, 14). It has been proposed that survivors of cancer treatment are at higher risk for endocrinopathies, such as diabetes and metabolic syndrome, for the rest of their lives (15). For example, recent work has revealed that diabetes is more likely to develop in people who survived childhood cancer (15). In addition, a long latency may exist between cancer treatment and the onset of different treatment-related conditions, emphasizing the necessity for lifelong awareness and monitoring (16).

Observational epidemiological research can be hampered by various potential biases caused by residual confounding (17). Moreover, the possible reverse causation of the exposure and outcome in these works makes it difficult to determine the direction of the correlations (17). The Mendelian randomization (MR) method, which uses genetic variants as instrumental variables, can infer the causal effects of exposure on outcomes. Because genetic variations are fixed at birth and normally cannot be modified by outcomes, MR analyses are less affected by reverse causality (18). Considering that the effects of cancer from different sites on diabetes risk may be different (19), the current study used the MR method to estimate the causal effects of overall and eight site-specific cancers on the risk of diabetes.

Abbreviations: SNP, single nucleotide polymorphism; GWAS, genome-wide association studies; MR, Mendelian Randomization; IVW, inverse-variance weighted; WM, weighted median; MR-PRESSO, Mendelian Randomization Pleiotropy RESidual Sum and Outlier; LD, linkage disequilibrium; ORs, odds ratios; CIs, confidence intervals; T2D, type 2 diabetes; PC, pancreatic cancer; UKB, UK Biobank; IVs, Instrumental variable; ILCCO, the international lung cancer consortium; GERA, the genetic epidemiology research on aging; DIAMANTE, the Diabetes Meta-analysis of Trans-ethnic Association Studies; TBI, total body irradiation; CID, chemotherapy-induced diabetes; CCSS, the childhood cancer survivor study.

Methods

Study design

MR examines the causal relationship between exposures and diseases using genetic variants (e.g., single nucleotide polymorphisms [SNPs]) as instrumental variables (IVs). In our analyses, the summary statistics of IVs were taken from genome-wide association study (GWAS) datasets of overall and site-specific cancers. Three requirements should be met for the selection of IVs. First, IVs are not directly associated with outcomes, and they only influence outcomes through exposure. Second, strong correlations exist between IVs and exposure. Third, IVs are not associated with the confounders (no horizontal pleiotropy exists). An MR framework was employed using GWAS summary data from different consortia to evaluate the causal association between overall and eight site-specific cancers and diabetes risk.

Data sources

Summary-level genetic data for overall and site-specific cancers were gathered from Finngen (20), the international lung cancer consortium (ILCCO) (21), the UK biobank (UKB) (22) and the genetic epidemiology research on aging (GERA) (23). Supplementary Table 1 provides more information on the data sources. GWAS datasets were used to extract the IVs for overall and lung cancer, in which the SNPs reached a genome-wide significance level ($P < 5 \times 10^{-8}$). We lowered the P value threshold for including SNPs as IVs to $P < 1 \times 10^{-5}$ if fewer than five IVs were selected (Supplementary Table 1). This threshold-lowering method has been previously adopted in MR studies (24). SNPs within 10,000 kb of each other were then clumped, with a linkage disequilibrium threshold of R2 > 0.001. The F-statistics of the IVs, an indicator of the ability of the IVs to predict the exposures (25), were estimated, and all exposures had F-statistics higher than 10 (Supplementary Table 2). The GWAS datasets for T2D, as the outcome, were from the Diabetes Meta-analysis of Trans-ethnic Association Studies (DIAMANTE) consortium (26).

Statistical analysis

The major method used to ascertain the relationships between different types of cancer and diabetes risk was the inverse-variance weighted (IVW) MR method. For sensitivity analyses, the weighted median (WM) method, MR-Egger, and MR pleiotropy residual sum and outlier (MR-PRESSO) test were also conducted. The potential heterogeneity was estimated by Cochrane's Q statistic, and the potential pleiotropy was assessed by the intercept of the MR-Egger test. Scatter plots were used to present the results of different MR methods. The estimate of the effect of SNPs after removing each SNP one by one was achieved by "leave-one-out" analysis. The causal effects of overall and site-specific cancer were represented using odds ratios (ORs) and 95% confidence intervals (CIs). The statistical significance of the MR analyses was adjusted using Bonferroni correction. The testing results that did not survive Bonferroni correction but had a $P < 0.05$ were defined as associations with suggestive level of evidence. R software was used for these analyses, in which the "TwoSampleMR" and "MR-PRESSO" R packages were employed.

Results

We first performed the MR analyses to examine the possible causal association of overall and eight site-specific cancers with diabetes using GWAS summary statistics from various consortia. Detailed information, as well as *P* threshold for IV selection for each GWAS summary dataset, is given in Supplementary Table 1. The results indicated that none of the tested associations survived Bonferroni correction with a *P* threshold of 0.05/9 = 0.006, but a suggestive level of evidence was observed for the causal association between lymphoid leukaemia and diabetes (IVW method, $P = 0.033$), indicating that lymphoid leukaemia increased diabetes risk, with an OR of 1.008 (95% CI, 1.001-1.014) (Figures 1, 2; Supplementary Figure 1, Supplementary Table 3). The F-statistic of the IVs used in these analyses ranged from 15.7 to 151.5, with a mean of 25.4, suggesting strong ability of the IVs to predict the exposures (Supplementary Table 2). For the observed causal association between lymphoid leukaemia and diabetes, sensitivity analyses using the MR-Egger and WM methods showed a consistent direction of the association compared with the IVW method. In addition, the leave-one-out sensitivity analysis revealed that the association of lymphoid leukaemia with diabetes became marginally significant after removing several SNPs, including rs147576549, rs17480734, rs59261129, rs61915331, and rs763477, with a *P* value ranging from 0.050 to 0.072 (Figure 3). Furthermore, no significant heterogeneity or horizontal pleiotropy was detected in the analysis of causality between lymphoid leukaemia and diabetes (Supplementary Tables 4, 5, respectively). MR-PRESSO consistently revealed no outlier IV in the analysis of lymphoid leukaemia, and the results were identical for the analyses of bladder cancer and PC after correcting for the identified outlier SNPs (Supplementary Table 6).

Discussion

Our study screened the possible causal association of a total of eight site-specific cancers with diabetes using MR methods based on GWAS summary datasets, and we found that lymphoid leukaemia was causally associated with diabetes risk. This observation is also reflected by the results of MR-Egger and WM MR analyses that showed a consistent direction of association. In addition, the MR-Egger intercept test and MR-PRESSO global test revealed that the causal association between lymphoid leukaemia and diabetes was not due to horizontal pleiotropy.

A class of deadly hematologic malignancies known as leukaemia is defined by malignant growth of white blood cells and their precursor cell (27). On the one hand, an increased leukaemia risk has been reported in patients with diabetes. For instance, a study in Sweden showed that patients with T2D had a noticeably higher incidence of leukaemia after hospitalization (28). Meta-analysis of 11 publications indicated that the OR of leukaemia for people with T2D was estimated to be 1.22 (29). On the other hand, leukaemia has been proposed as one of the childhood cancers that leads to higher risk of diabetes (30). Indeed, childhood cancer survivors were more likely to develop diabetes compared with their sibling controls according to one study from the childhood cancer survivor study (CCSS) group (31). Consistent results were observed in studies conducted in Scandinavia (32) and Canada (33).

Several mechanisms underlying the higher diabetes risk in patients with leukaemia have been proposed. Leukaemia cells can directly infiltrate the pancreas (34), and chemotherapeutic treatment using L-asparagine can also lead to β-cell malfunction, causing

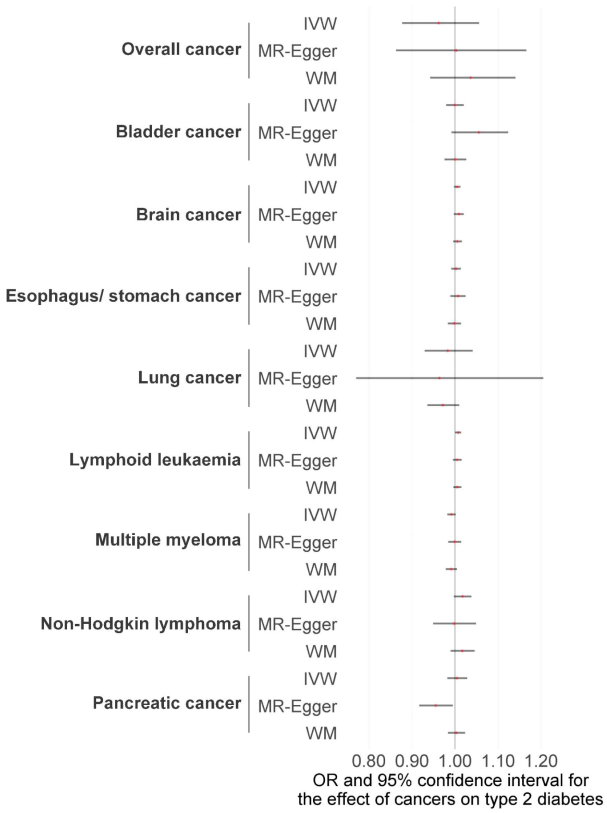

FIGURE 1
The potential causal relationships between site-specific cancer and diabetes risk were examined using various MR methods, including IVW, MR-Egger, and WM. IVW, inverse-variance weighted method; MR, Mendelian randomization; WM, weighted median method; OR, odds ratio.

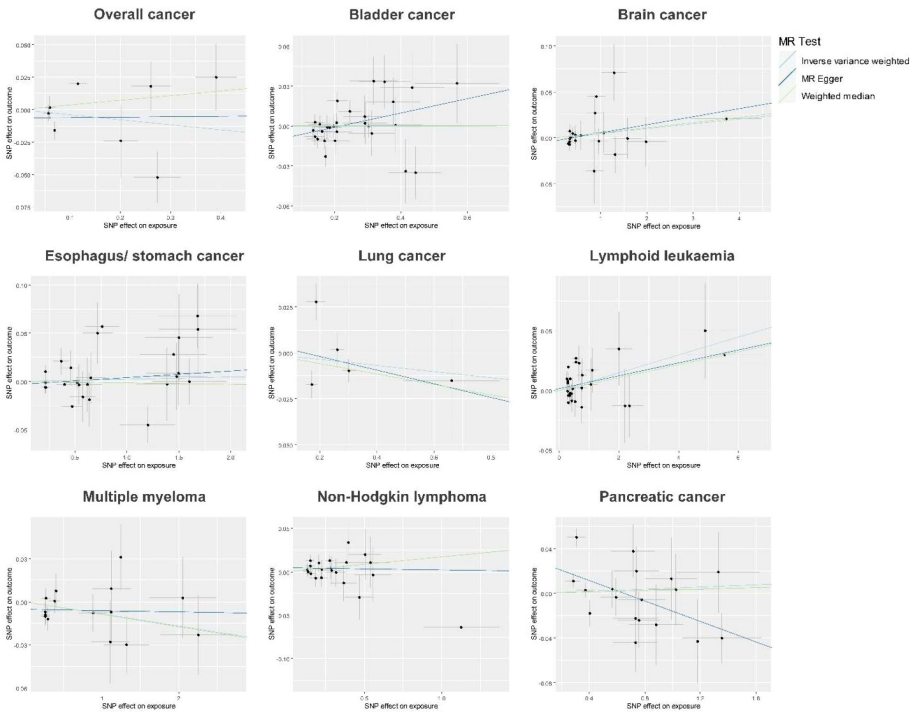

FIGURE 2
Scatter plots of the MR analyses showing the potential causal associations of site-specific cancer with diabetes. MR, Mendelian randomization; SNP, single nucleotide polymorphism.

hyperglycaemia in acute lymphocytic leukaemia (34), one of the most prevalent cancers among children (35). For chronic lymphocytic leukaemia, one case report indicated that a patient developed diabetes after being treated with fludarabine and cyclophosphamide therapy, which could potentially disrupt the local immune-regulatory balance (36). Corticosteroids are normally used as an integral part of combination chemotherapy in leukaemia treatment (37). However, some complications might arise during the usage of corticosteroids, of which two of the most common are hyperglycaemia and chemotherapy-induced diabetes (CID) (38). The development of diabetes after abdominal radiation is often linked to damage to the pancreas tail induced by the radiation, which leads to pancreatic insufficiency (39). For hematopoietic cell transplantation patients suffering from high-risk hematologic cancers, the precondition is normally achieved by total body irradiation (TBI) (40). The entire body is exposed to radiation during TBI, which affects the hypothalamic-pituitary axis and increases the risk of endocrinopathies (e.g., growth hormone deficiency) in cancer survivors (41). The risks of developing diabetes have been documented amongst children survivors exposed to TBI treatment, with a 12.6-fold risk ratio compared with their sibling controls (31). The major pathophysiologic mechanisms that contribute to the post-TBI development of diabetes have been proposed to be insulin resistance and hyperinsulinemia, rather than pancreatic insufficiency (16). It is also not uncommon for survivors of TBI exposure to present abnormality processes, such as altered adipokines and occurrence of inflammation (42).

CID contributes to poor clinical outcomes in leukaemia patients (43), and the underlying reasons could be multifactorial. One explanation is the increased susceptibility to infections in patients with CID undergoing intensive chemotherapy (44). Hyperglycaemia and hyperinsulinemia can further stimulate the neoplastic process, leading to unfavourable clinical outcomes in patients with leukaemia and CID (45). In patients suffering from acute myeloid leukaemia, researchers also reported an alteration in the glucose metabolism signature, which contributes to undesirable clinical outcomes (46). Thus, early commencement of CID screenings and relevant strategies to reduce its negative impact is advised because cancer survivors have an elevated chance of developing premature cardiovascular morbidity (47). Further research is warranted to elucidate the complex metabolic abnormality in cancer survivors, which could guide preventive and therapeutic endeavours to improve the quality of life of cancer survivors.

The association between cancer and diabetes can be site specific. For example, the risks of developing diabetes have been reported to be comparatively higher for survivors of PC compared with other types of cancers (48). A significant portion of patients recently diagnosed with PC present hyperglycaemia or T2D (49). In addition, T2D is alleviated after tumour removal, which reinforces the idea that T2D is related to PC (50). The risk of diabetes is elevated by PC because it promotes the secretion of insulin that leads to insulin resistance (51). Furthermore, pancreatic tissue destruction with an accompanying β-cell loss can also occur in patients with PC, which contributes to the development of diabetes (52). However, the causal effects of PC on T2D subtypes may be different. One MR analysis suggested that PC is causally associated with newly onset T2D but not long-standing T2D (53). The GWAS summary dataset of T2D used in our MR analysis did not separate subtypes of T2D, and the results indicated no causal association between PC and T2D. Similar to PC, six other site-specific cancers under investigation, including multiple myeloma, non-

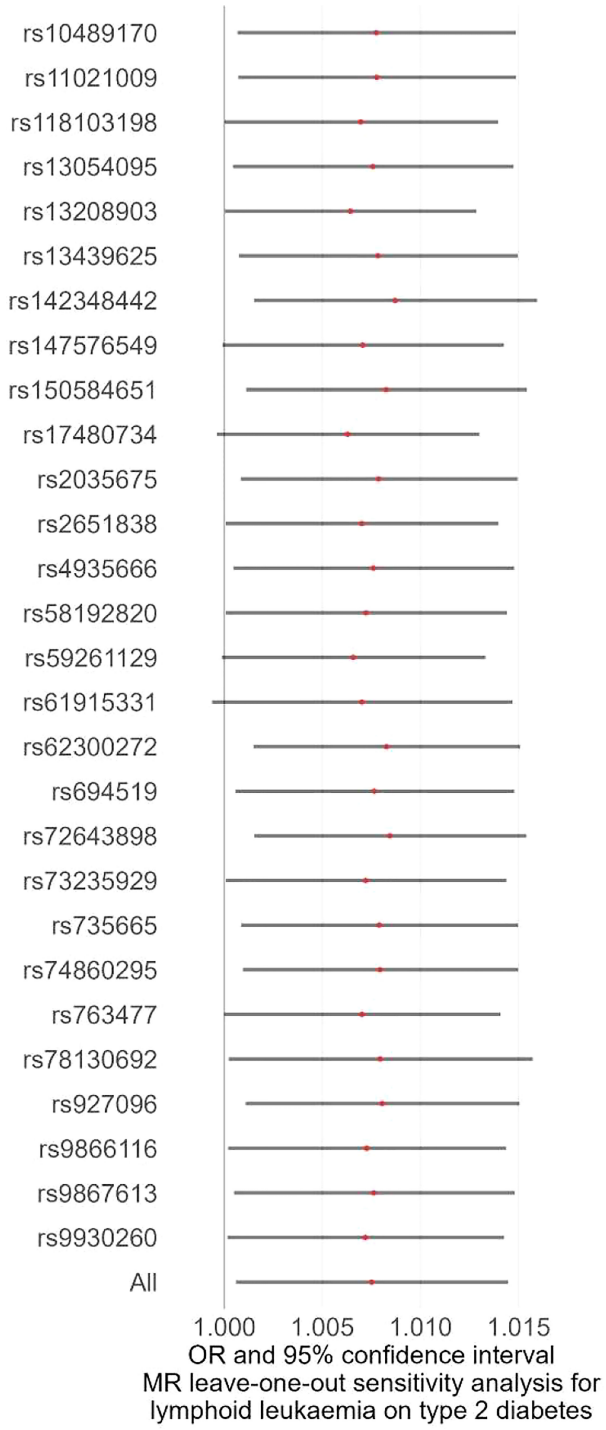

FIGURE 3
Leave-one-out analysis as a sensitivity analysis to examine the causal association between lymphoid leukaemia and diabetes. MR, Mendelian randomization; OR, odds ratio.

Hodgkin lymphoma and cancers of the bladder, brain, stomach, and lung, were also not causally associated with diabetes.

There were several areas of strength in this study. First, we employed an MR design to reduce the biases that can be introduced by reverse causality and residual confounding in conventional observational studies, which may lead to false-positive results. Second, numerous SNPs were used as IVs for overall and site-specific cancers, which was essential in facilitating the analysis of horizontal pleiotropy. Third, for sensitivity analyses aimed at estimating pleiotropy, several MR methods, such as MR-PRESSO and MR-Egger, were utilized. Lastly, the participants within the initial GWAS were mainly of European descent, which helped to reduce the bias attributable to population stratification. Despite the strengths, there were also several shortcomings in the present study, a key of which was the inability to completely exclude the possible effect of pleiotropy. Additionally, the interpretation of the results was limited to a certain ethnicity because the GWAS summary datasets were of European origin.

Conclusion

This comprehensive MR analysis has established a causal relationship between lymphoid leukaemia and diabetes risk, which points to the necessity of diabetes prevention amongst leukaemia survivors as a strategy for ameliorating the associated disease burden.

Data availability statement

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

Ethics statement

The GWAS used in the current work were approved by their relevant review board, and informed consent were collected from all participants.

Author contributions

RX and CG concepted the study. RX, TZ, CO, and XD performed the statistical analyses, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1110523/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Funnel plot of the MR analyses investigating the causal effects of site-specific cancer and diabetes. Abbreviations: MR: Mendelian randomization; SE: standard error; IVs: instrumental variables.

SUPPLEMENTARY TABLE 1
Information of GWAS summary datasets used in MR analyses.

SUPPLEMENTARY TABLE 2
The F-statistics of IVs.

SUPPLEMENTARY TABLE 3
MR analysis results.

SUPPLEMENTARY TABLE 4
Heterogeneity test results.

SUPPLEMENTARY TABLE 5
MR-Egger pleiotropy test results.

SUPPLEMENTARY TABLE 6
MR-PRESSO test results.

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Remnant cholesterol is independently asssociated with an increased risk of peripheral artery disease in type 2 diabetic patients

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Background: Remnant cholesterol (RC) has been correlated with a higher risk of atherosclerosis. It has been confirmed that in the general population, an elevated RC level is related to a 5-fold higher risk of peripheral arterial disease (PAD). Diabetes is one of the strongest risk factors for PAD development. However, the association between RC and PAD in the specific population of type 2 diabetes mellitus (T2DM) has not been investigated. Herein, the correlation was investigated between RC and PAD in T2DM patients.

Methods: In the retrospective study, the hematological parameter data of 246 T2DM patients without PAD (T2DM - WPAD) and 270 T2DM patients with PAD (T2DM - PAD) was collected. Differences in RC levels between the two groups were compared, and the association between RC and PAD severity was examined. Multifactorial regression was used to determine whether RC was a significant contributor to the development of T2DM - PAD. The diagnostic potential of RC was tested using receiver operating characteristic (ROC) curve.

Results: The RC levels in T2DM - PAD individuals were considerably greater than in T2DM - WPAD individuals ($P < 0.001$). RC had a positive correlation with disease severity. Further, multifactorial logistic regression analyses found that elevated RC levels were a major contributor to T2DM - PAD ($P < 0.001$). The area under the curve (AUC) of the RC for T2DM - PAD patients was 0.727. The cut-off value of RC was 0.64 mmol/L.

Conclusion: The RC levels were higher in T2DM - PAD patients, and were independently linked with its severity. Diabetic patients with RC levels > 0.64 mmol/L had an elevated risk of developing PAD.

KEYWORDS

type 2 diabetes, peripheral artery disease, remnant cholesterol, risk factor, lipid

Introduction

PAD is a chronic arterial occlusive disease of the lower limbs caused by atherosclerosis and is linked with substantial disability and death (1). T2DM is a main factor in the progression of atherosclerosis. The incidence of PAD rises in tandem with the occurrence of T2DM (2). In addition, diabetic people have a worse prognosis for PAD than non-diabetic ones (1). Thus, prompt diagnosis and treatment of PAD in diabetic subjects are necessary to reduce the danger of major adverse limb events (MALEs) (2).The ankle-brachial index (ABI) is currently recommended as the primary screening tool for PAD in diabetic patients and those with multiple risk factors (3). The ABI's limited sensitivity in detecting PAD in its earliest stage highlights the critical need to discover new markers that may detect PAD in diabetics at an earlier stage.

RC is the cholesterol in triglyceride-rich lipoproteins and consists of very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), and chylomicron remnants (4). RC-level assessment can be easily calculated using established formulas, which are easy-to-access, and may provide valuable data for clinical management (5). Evidence from large prospective cohort studies based on the general population suggests a causal relationship between high remnant cholesterol levels and cardiovascular disease(CVD), and it is well established that lowering these lipoproteins reduces atherosclerotic cardiovascular events in humans (6–8). Recent studies have confirmed the atherogenic potential of RC, however, many of these studies focused on elevated RC levels in coronary arterial disease (CAD) and cerebrovascular disease, demonstrating an association between elevated RC levels and the risk of ischemic heart disease, myocardial infarction, and ischemic stroke (8–10). Interestingly, a recent investigation showed that in the general population, an elevated RC level was associated with a five-fold higher risk of PAD, greater than for myocardial infarction and ischemic stroke (10). High RC levels are common in diabetic individuals and has been linked to atherosclerosis through lipid metabolism and insulin resistance (11). It's intriguing to speculate about whether or not RC also plays a part in the development of PAD in diabetics. Nevertheless, until now, there has been no study on whether there is a correlation between RC and PAD in T2DM population. The aim of the research was to examine whether higher RC levels were related to higher PAD risk among T2DM individuals.

Materials and methods

Study population

The cross-sectional research involved 514 gender-matched diabetic patients consecutively admitted to the Department of Endocrinology and Metabolism of the Liyuan Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China), from 1 March 2018 to 30 October 2022. T2DM patients with or without PAD were recruited. T2DM was defined as a fasting plasma glucose(FPG) level ≥ 7.0 mmol/L and/or 2-h plasma glucose(PG) ≥11.1 mmol/L during OGTT and/or HbA1c level ≥ 6.5%, based on the T2DM international criteria (ADA) (12). The inclusion criteria were patients aged 18-79 years with a confirmed diagnosis of T2DM. The excluding criteria were: a) coronary artery disease (CAD); b) history of stoke; c) diabetic retinopathy; d) acute complications of diabetes mellitus (such as diabetic ketoacidosis, hyperglycemia hyperosmotic state, and lactic acidosis); e) chronic kidney disease with an estimated glomerular filtration rate (eGFR) less than 60 mL/min; f) documented liver cirrhosis with Child–Pugh C dysfunction; g) history of active solid or hematological malignancy or autoimmune diseases; h) ABI > 1.4; i)RC < 0; j) suspected or confirmed pregnancy; k) undefined type of diabetes or clinical suspicion of non-type 2 diabetes mellitus; l) previous non-traumatic lower limb amputation; m) incomplete clinical data.

Each patient included in the study was evaluated for a history of PAD symptoms. The ABI was measured in patients with PAD-like symptoms. ABI was calculated according to the Transatlantic Inter-Society Consensus Document II (TASC-II) guidelines for the management of peripheral arterial disease (13). ABI was calculated as the ratio of ankle-to-brachial artery systolic pressure. ABI was computed by dividing the highest systolic pressure recorded in either the right or left brachial arteries or the anterior or posterior tibial arteries in each limb (14). The physician evaluated the patients' lower extremities using arterial Doppler-enhanced ultrasonography if they had symptoms in both legs. Patients with an ABI > 0.90 who were asymptomatic were not additionally evaluated for PAD.

Patients whose ABI < 0.9 underwent arterial Doppler-enhanced ultrasonography of the limb extremities. The common femoral artery, femoral artery bifurcation, popliteal artery, posterior tibial artery, and dorsalis pedis artery were examined. The evaluation and score of vascular pathology were as follows: a) Artery intima thickness: normal (< 1 mm), 0 point; moderately thickened (1 – 1.2 mm), 1 point; severely thickened (> 1.2 mm), 2 points. b) Hardening: normal, 0 point; mildly hardened (the intima was not thickened, the echo was increased, and with no plaque), 1 point; moderately to severely hardened (mildly hardened, accompanied with plaque or stenosis), 2 points. c) Plaque: normal (no plaque forming), 0 point; single plaque, 1 point; numerous plaques, 2 points; scattered plaques, 3 points. d) Stenosis: normal, 0 point; mild stenosis (narrowing by 30%–50%), 1 point; moderate or severe stenosis (narrowing by 50% – 75%), 2 points; occlusion (no blood flow), 3 points. The degree of PAD was categorized based on the total number of points: a) 0 point, normal; b) < 10 mild; c) 10 – 20 points, moderate; d) > 20 points, severe (15).

Demographic and clinical assessment

Demographic variables (age and gender), as well as laboratory results, such as blood count, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), were obtained from the electronic medical record system in Liyuan hospital. On the second hospital

morning, blood samples were collected from all patients' peripheries. Laboratory personnel unaware of the patient's diagnoses analyzed the blood samples.

RC levels were determined as TC (mmol/L) minus LDL-C (mmol/L) and HDL-C (mmol/L), as recommended by the dyslipidemia guidelines (16). The triglyceride glucose index (TyG index), neutrophil to lymphocyte ratio (NLR), monocyte to lymphocyte ratio (MLR), and platelet to HDL-C ratio (PHR) were calculated using the following formulas: TyG index = Ln [Triglyceride (TG, mg/dl) × FPG (mg/dl)/2]; NLR = neutrophil (10^9/L)/lymphocyte (10^9/L); MLR = monocyte (10^9/L)/lymphocyte (10^9/L); PHR = platelet (10^9/L)/HDL-C (mmol/L).

Statistical analysis

Statistical analyses were done using SPSS version 27.0 software (SPSS, Inc., Chicago, IL, United States). Graphs were created using Prism 9.0 (GraphPad Software). The normality of continuous variables was examined by the Shapiro-Wilk test. Continuous variables were described as means ± SDs and evaluated utilizing the Student's t-test (two groups) or the One-way ANOVA (three groups). Non-normally distributed continuous variables were described as medians (interquartile ranges) and assessed using the Mann-Whitney U test (two groups) or Kruskal-Wallis test (three groups). Categorical variables were described as the numbers and percentages of patients. Chi-square tests were performed to assess categorical variables. The link between RC and PAD phases was analyzed by utilizing spearman correlation and partial correlation analysis. The relationship between RC and other variables in PAD patients was analyzed by Spearman correlation analysis. Covariates were excluded from the correlation analysis. Univariate and multivariate logistic regression analysis were used to examine the association between RC and PAD. The optimal value for identifying the risk of PAD in this sample was calculated using ROC curve analysis. The optimal cutoff value was determined by maximizing the Yoden index. Statistical significance was defined as a two-sided P value < 0.05.

Results

Comparison of baseline clinical features and laboratory indicators between the PAD group and WPAD group

The demographic and clinical data of T2DM - PAD group and T2DM - WPAD group are summarized in Table 1. Among the 516 diabetic patients enrolled, 270 had PAD, and 246 did not (WPAD). Compared to WPAD patients, PAD patients had a higher prevalence of hypertension (P < 0.05), and showed significantly increased levels of age, diabetes duration, systolic blood pressure (SBP), urea, creatinine (Cr), C-reactive protein (CRP), RC, neutrophils, monocytes, NLR, MLR, and PHR (P < 0.05), and showed significantly decreased levels of diastolic blood pressure (DBP), alanine aminotransferase (ALT), eGFR, HDL-C, and lymphocytes (P < 0.05). The two groups did not differ for gender, history of smoking, drinking, and dyslipidemia, aspartate aminotransferase (AST), uric acid, FPG, glycosylated hemoglobin (HbA1c), TG, TC, LDL-C, non-HDL-C(N-HDL-C), TyG index, and platelets (P > 0.05). Significant differences in glucose-lowering measures and statin use were found between the two groups (both P < 0.05). The incidence of mild, moderate, and severe PAD was 50.7, 23.3, and 25.9% in PAD patients, respectively.

Clinical and laboratory features of T2DM - PAD patients: Subgroup analysis according to PAD severity

The three groups did not differ regarding gender, duration of diabetes, history of smoking, drinking, and dyslipidemia, SBP, DBP, and laboratory parameters such as ALT, AST, urea, Cr, FPG, HbA1c, TC, LDL-C, N-HDL-C, TyG index, neutrophils, lymphocytes, monocytes, NLR, and MLR (P > 0.05) (Table 2). As disease severity increased, history of hypertension, eGFR, and HDL-C presented a decreasing trend (P < 0.05), but TG, RC, platelets, and PHR showed an increasing trend (P < 0.05). Moderate PAD patients had the highest levels of age, uric acid, and CRP (P < 0.05). Significant differences were found between the three groups using only oral medication or only insulin (P < 0.05).

The violin - plot in Figure 1 found that the RC levels showed an increasing relationship with disease extent.

Correlation of RC and other lipid variables with severity levels of T2DM – PAD

The correlations between RC and other lipid variables were assessed by utilizing spearman correlation analysis (including TG, TC, LDL-C, HDL-C, and N-HDL-C) in PAD patients. Based on the data in Table 3, RC (r = 0.387, P < 0.001), TG (r = 0.151, P = 0.013), and HDL-C (r = -0.197, P < 0.001) were associated with the PAD severity levels. RC still maintained connections with PAD stages after adjusting for TG and/or HDL-C using partial correlation analysis.

Univariate and multivariate logistic regression analysis of RC for T2DM - PAD occurrence

As univariate logistic regression analysis showed (Table 4), age, duration of diabetes, history of hypertension, SBP, DBP, ALT, urea, Cr, eGFR, HDL-C, CRP, RC, NLR, MLR, and PHR were independently associated with PAD occurrence in T2DM patients (P < 0.05). After excluding the effects of confounding factors for multivariate logistic regression, age, duration of diabetes, HDL-C, RC, NLR, MLR, and PHR were still statistically significant. RC,

TABLE 1 Demographic and clinical data of diabetic subjects with and without PAD.

Variables	WPAD	PAD	*P* value
	(N=246)	(N=270)	
Gender (male, %)	135 (54.9%)	156 (57.8%)	0.507
Age (years)	**57 (50-62)**	**65 (59-71)**	**<0.001**
Diabetes duration (years)	**5 (1-10)**	**10 (5-18)**	**<0.001**
Smoking, n (%)	69 (28%)	73 (27%)	0.797
Alcohol, n (%)	60 (24.4%)	68 (25.2%)	0.839
Hypertension, n (%)	**120 (48.8%)**	**167 (61.9%)**	**0.003**
Dyslipidemia, n (%)	90 (36.6%)	92 (34.1%)	0.551
SBP (mmHg)	**127 (117-138)**	**132 (123-144)**	**<0.001**
DBP (mmHg)	**80 (72-86)**	**77 (70-85)**	**0.028**
ALT, U/L	**20.4 (14.7-30.7)**	**17 (12.1-23.1)**	**<0.001**
AST, U/L	20 (16-25.6)	18.5 (15.8-24.5)	0.140
Urea, mmol/L	**5.53 (4.49-6.38)**	**5.9 (4.53-7.2)**	**0.006**
Cr, UMOL/L	**62.4 (51.5-77.4)**	**70.7 (57.3-85.6)**	**<0.001**
eGFR (ml/min/1.73m^2)	**101.7 (91.3-110.9)**	**91.1 (72.2-103.7)**	**<0.001**
Uric Acid, μmol/L	309.8 (252.3-363.3)	319.4 (246.1-378.2)	0.461
CRP, mg/L	**1.2 (0.7-2.4)**	**2.1 (1.1-5.5)**	**<0.001**
FPG, mmol/L	9.98 (7.42-14.8)	9.99 (7.86-14.6)	0.796
HbA1c (%)	8.1 (6.7-9.9)	8.2 (7.2-9.8)	0.320
TG, mmol/L	1.55 (1.08-2.21)	1.66 (1.14-2.4)	0.275
TC, mmol/L	4.54 (3.92-5.32)	4.47 (3.64-5.41)	0.701
HDL-C, mmol/L	**1.14 (0.96-1.38)**	**1 (0.86-1.15)**	**<0.001**
LDL-C, mmol/L	2.82 (2.06-3.44)	2.52 (1.91-3.27)	0.053
N-HDL-C, mmol/L	3.38 (2.69-4.13)	3.36 (2.62-4.22)	0.899
RC, mmol/L	**0.55 (0.38-0.7)**	**0.75 (0.6-1.03)**	**<0.001**
TyG index	7.84 (7.25-8.48)	7.92 (7.35-8.49)	0.498
Neutrophil,10^9/L	**3.43 (2.83-4.46)**	**3.98 (3.11-5.11)**	**<0.001**
Lymphocyte, 10^9/L	**1.69 (1.41-2.03)**	**1.47 (1.13-1.85)**	**<0.001**
Monocyte, 10^9/L	**0.34 (0.27-0.41)**	**0.38 (0.3-0.49)**	**<0.001**
Platelet, 10^9/L	207 (178-243)	206 (171-258)	0.841
NLR	**2.05 (1.59-2.61)**	**2.66 (1.89-3.74)**	**<0.001**
MLR	**0.19 (0.16-0.25)**	**0.26 (0.19-0.35)**	**<0.001**
PHR	**179.26 (143.06-237.76)**	**204.08 (163.08-272.73)**	**<0.001**
Use antidiabetes agents			
Insulin, n (%)	**27 (11%) [a]**	**60 (22.2%) [b]**	**<0.001**
Oral drugs, n (%)	**143 (58.1%) [a]**	**117 (43.3%) [b]**	
Diet control only, n (%)	**41 (16.7%) [a]**	**21 (7.8%) [b]**	
Insulin + Drugs, n (%)	**35 (14.2%) [a]**	**72 (26.7%) [b]**	
Statins use, n (%)	**29 (11.8%)**	**55 (20.4%)**	**0.008**

(Continued)

TABLE 1 Continued

Variables	WPAD	PAD	*P* value
PAD			
Mild PAD, n (%)	/	137 (50.70%)	
Moderate PAD, n (%)	/	63 (23.30%)	
Severe PAD, n (%)	/	70 (25.90%)	

SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, creatinine; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; N-HDL-C, non-HDL-C; RC, **remnant cholesterol;** TyG index, triglyceride glucose index; NLR, neutrophil to lymphocyte ratio; MLR, monocyte to lymphocyte ratio; PHR, platelet/HDL-C ratio. P <0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance. a, b: after applying the chi-square test, different superscripts indicate statistically different categorical variables between the 2 groups.

TABLE 2 Demographic and clinical data of T2DM – PAD group according to PAD severity.

Variables	Mild PAD (N=137)	Moderate PAD (N=63)	Severe PAD (N=70)	*P* value
Gender (male, %)	82 (59.9%)	37 (58.7%)	37 (52.9%)	0.619
Age (years)	**64 (59-69)**	**68 (60-71)**	**67 (59-72)**	**0.043**
Diabetes duration (years)	10 (5-17)	10.5 (5-18)	10 (5-20)	0.447
Smoking, n (%)	39 (28.5%)	19 (30.2%)	15 (21.4%)	0.456
Alcohol, n (%)	43 (31.4%)	12 (19%)	13 (18.6%)	0.058
Hypertension, n (%)	**73 (53.3%)**	**43 (68.3%)**	**51 (72.9%)**	**0.011**
Dyslipidemia, n (%)	54 (39.4%)	15 (23.8%)	23 (32.9%)	0.093
SBP (mmHg)	130 (122-142)	132 (120-140)	135 (124-148)	0.093
DBP (mmHg)	77 (70-85)	76.5 (69-81)	78 (74-86)	0.230
ALT, U/L	17.9 (13.7-24.1)	16.2 (11.3-23.1)	15 (10.7-21.9)	0.115
AST, U/L	18.7 (16.3-24.1)	18.4 (15.6-25.5)	18.2 (15-25.5)	0.881
Urea, mmol/L	5.85 (4.51-6.8)	6.04 (4.89-7.41)	5.99 (4.45-7.89)	0.472
Cr, UMOL/L	70.1 (57.2-81.2)	71.25 (60.5-92.7)	71.3 (55.9-88.6)	0.253
eGFR (ml/min/1.73m^2)	**93.9 ± 19.71**	**90.1 ± 20.97**	**89.15 ± 20.32**	**0.042**
Uric Acid, μmol/L	**304.3 (233.9-353.1)**	**337.15 (259.1-391.5)**	**335.25 (261.7-395.8)**	**0.021**
CRP, mg/L	**1.7 (0.9-4.1)**	**2.65 (1.3-10.4)**	**2.55 (1.4-10.6)**	**<0.001**
Fasting glucose, mmol/L	10.52 (8.21-15.51)	9.95 (7.62-14.06)	8.74 (6.98-13.37)	0.060
HbA1c (%)	8.7 (7.2-10.2)	7.8 (6.9-9.6)	7.95 (7.2-9.1)	0.209
TG, mmol/L	**1.53 (1.09-2.09)**	**1.78 (1.14-2.71)**	**1.89 (1.22-2.9)**	**0.046**
TC, mmol/L	4.47 (3.63-5.44)	4.39 (3.49-5.19)	4.67 (3.82-5.25)	0.613
HDL-C, mmol/L	**1.01 (0.9-1.25)**	**0.97 (0.83-1.12)**	**0.94 (0.77-1.11)**	**0.005**
LDL-C, mmol/L	2.67 (1.97-3.55)	2.49 (1.86-3.23)	2.35 (1.84-3.03)	0.186
N-HDL-C, mmol/L	3.26 (2.51-4.27)	3.42 (2.62-4.06)	3.5 (2.72-4.23)	0.824
RC, mmol/L	**0.68 (0.54-0.87)**	**0.8 (0.67-1)**	**1.05 (0.75-1.45)**	**<0.001**
TyG index	7.90 ± 0.84	7.98 ± 0.84	7.99 ± 0.92	0.692
Neutrophil,10^9/L	3.73 (3.06-5.02)	3.96 (3.1-4.7)	4.305 (3.37-5.36)	0.142
Lymphocyte, 10^9/L	1.51 (1.2-1.87)	1.37 (1.06-1.78)	1.44 (1.14-1.85)	0.154
Monocyte, 10^9/L	0.38 (0.3-0.48)	0.37 (0.26-0.52)	0.41 (0.33-0.54)	0.258

(Continued)

TABLE 2 Continued

Variables	Mild PAD (N=137)	Moderate PAD (N=63)	Severe PAD (N=70)	*P* value
Platelet, 10^9/L	**200 (167-247)**	**207 (171-248)**	**227.5 (183-312)**	**0.032**
NLR	2.4 (1.81-3.56)	2.85 (2.06-3.93)	2.82 (2.19-3.79)	0.075
MLR	0.25 (0.19-0.34)	0.27 (0.19-0.38)	0.28 (0.21-0.37)	0.138
PHR	**185.53 (151.54-252.33)**	**211.42 (163.08-308.57)**	**247.45 (182.65-315.46)**	**<0.001**
Use antidiabetes agents				
Oral drugs	**68 (49.6%)** [a]	**27 (42.9%)** [a,b]	**20 (28.6%)** [b]	**0.013**
Insulin	**18 (13.1%)** [a]	**17 (27%)** [a,b]	**23 (32.9%)** [b]	
Insulin + drugs	37 (27%) [a]	15 (23.8%) [a]	22 (31.4%) [a]	
Diet control only	14 (10.2%) [a]	4 (6.3%) [a]	5 (7.1%) [a]	
Statins use	29 (21.2%)	11 (17.5%)	15 (21.4%)	0.806

SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cr, creatinine; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein; FPG, fasting plasma glucose; HbA1c, glycosylated hemoglobin; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; N-HDL-C, non-HDL-C; RC, **remnant cholesterol;** TyG index, triglyceride glucose index; NLR, neutrophil to lymphocyte ratio; MLR, monocyte to lymphocyte ratio; PHR, platelet/HDL-C ratio. P < 0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance. a, b: after applying the chi-square test, different superscripts indicate statistically different categorical variables between the 3 groups.

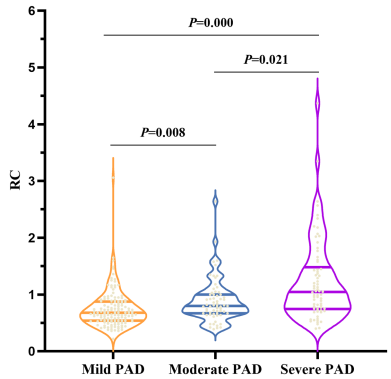

FIGURE 1
The RC levels according to PAD severity based on ultrasound results. RC, **remnant cholesterol.** In the violin plot, the three horizontal lines from top to bottom represent the upper quartile, the median, and the lower quartile in order. *P* < 0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance.

NLR, MLR, and PHR were considered independent risk factors for PAD occurrence in T2DM patients, while HDL-C was an independent protective factor.

Diagnostic performance of RC for T2DM - PAD

The ability of RC to identify T2DM - PAD patients was evaluated by the ROC curve. Figure 2 showed that RC exhibited a high predicting value for T2DM – PAD (AUC = 0.727). The optimum RC cut-off value for predicting the occurrence of PAD in the group was 0.64 mmol/L (Sensitivity 71.9%, Specificity 64.6%).

Correlation of RC with other parameters of T2DM - PAD patients

Correlations between RC and other indicators in PAD patients were assessed using Spearman correlation analysis. The RC had a significant and positive correlation with gender (r = 0.330), fasting glucose (r = 0.125), TG (r = 0.641), TC (r = 0.342), N-HDL-C (r = 0.379), TyG index (r = 0.485), and PHR (r = 0.123) (all *P* < 0.05) (Table 5).

Discussion

In this study, the relationship was first explored between RC and T2DM - PAD patients. The main conclusions were as follows: (1) RC levels had a positive association with the occurrence and severity of PAD, and RC was independently related to an increased risk of PAD in T2DM patients; (2) diabetic patients with RC levels > 0.64 mmol/L had an elevated risk of developing PAD.

Patients with T2DM and PAD have a cardiovascular mortality risk five times higher than patients with only one disease (17, 18). Hence, effective early screening and identification of T2DM - PAD individuals is crucial (19). Several potential biomarkers have been detected for PAD in diabetic patients, including HMGB 1, OPG, FGF 23, Omentin-1, Cyr61, and Sortilin (20–24). However, there are several limitations to obtaining these data in daily clinical practice. RC can be easily obtained using standard laboratory indices and may have substantial clinical use.

LDL-C is an established risk factor for atherosclerotic cardiovascular disease (ASCVD) (25). However, a high residual risk of CVD persists even in patients whose LDL-C levels meet therapeutic targets after statin therapy, as established by multiple recent meta-analyses (26, 27). RC may be an important contributor of this residual risk (28). In this study, RC levels were significantly higher in the PAD group than in the WPAD group, and LDL-C

TABLE 3 The correlation between stages of T2DM – PAD and the following lipid profiles.

Variables	Spearman Correlation Analysis		Partial Correlation Analysis	
	r	*P* value	r	*P* value
RC, mmol/L	**0.387**	**<0.001**	–	–
TG, mmol/L	**0.151**	**0.013**	**0.371**[a]	**<0.001**
TC, mmol/L	0.020	0.738	**0.416**[b]	**<0.001**
LDL-C, mmol/L	-0.111	0.069	**0.389**[c]	**<0.001**
HDL-C, mmol/L	**-0.197**	**<0.001**	**0.384**[d]	**<0.001**
N-HDL-C, mmol/L -	0.036 -	0.555 -	**0.410**[e] **0.388**[f]	**<0.001** **<0.001**

RC, remnant cholesterol; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; N-HDL-C, non-HDL-C. Associations between serum lipid profile and stages of PAD by Spearman correlation analysis and the association between RC and stages of DR by partial correlation analysis a: Adjusted for TG; b: Adjusted for TC; c: Adjusted for LDL-C; d: Adjusted for HDL-C; e: Adjusted for N-HDL; f: Adjusted for TG and HDL-C. P < 0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance.

levels were not significantly different (Table 1). The 2019 European Society of Cardiology guidelines recommend that the goal level of LDL be below 1.8 mmol/L with an LDL-C reduction of ≥ 50% from baseline (29). Unfortunately, LDL-C levels failed to meet the established criteria in both groups of patients. In the Supplementary Material, the two groups were divided respectively based on the use of statins or not. In the subgroups, LDL-C levels decreased significantly, whereas there was no statistical difference in RC levels. The results indicated that statins did not have a substantial effect on RC levels in T2DM patients with or without PAD (See Supplementary Tables 1, 2). Previous clinical studies have shown that statins reduce RC levels in patients with CAD (30, 31). A prospective cohort with a larger sample size is necessary to see whether statins reduce RC levels in patients with PAD. Comparing the PAD and WPAD groups of patients with LDL-C at the target level, a significant difference in RC levels was found. Elevated RC levels might explain the residual risk of PAD in DM patients with LDL-C level < 1.8 mmol/L (See Supplementary Table 3).

In this study, the severity of PAD was graded based on ultrasound measurements, which showed a positive correlation between RC levels and severity (Figure 1). Patients were also classified according to the severity of their clinical symptoms using the Fontaine classification (32); however, there was no link between the RC levels and the Fontaine classification. This finding provided more evidence that RC should be promoted in clinical settings alongside ultrasonography results for patient evaluation

TABLE 4 Univariate and binary logistic regression analysis results.

	Variable OR (95% CI)	*P* value	Variable OR (95% CI)	*P* value
Age	1.123 (1.096-1.150)	<0.001	**1.125 (1.086-1.167)**	**<0.001**
Diabetes duration	1.128 (1.095-1.162)	<0.001	**1.104 (1.063-1.147)**	**<0.001**
Hypertension	1.702 (1.199-2.417)	0.003		
SBP	1.019 (1.009-1.03)	<0.001		
DBP	0.981 (0.965-0.997)	0.019		
ALT	0.975 (0.962-0.988)	<0.001		
Urea	1.144 (1.045-1.252)	0.004		
Cr	1.01 (1.002-1.018)	0.011		
eGFR	0.97 (0.96-0.979)	<0.001		
HDL-C	0.178 (0.096-0.333)	<0.001	**0.141 (0.059-0.337)**	**<0.001**
CRP	1.059 (1.029-1.089)	<0.001		
RC	9.41 (5.1-17.363)	<0.001	**12.653 (6.112-26.197)**	**<0.001**
NLR	1.647 (1.394-1.945)	<0.001	**1.288 (1.032-1.608)**	**0.025**
MLR	1.795 (1.483-2.173)	<0.001	**1.568 (1.211-2.03)**	**<0.001**
PHR	1.004 (1.002-1.006)	<0.001	**1.006 (1.003-1.009)**	**<0.001**

SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; Cr, creatinine; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein; HDL-C, high-density lipoprotein cholesterol; RC, remnant cholesterol; NLR, neutrophil to lymphocyte ratio; MLR, monocyte to lymphocyte ratio; PHR, platelet/HDL-C ratio. P < 0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance.

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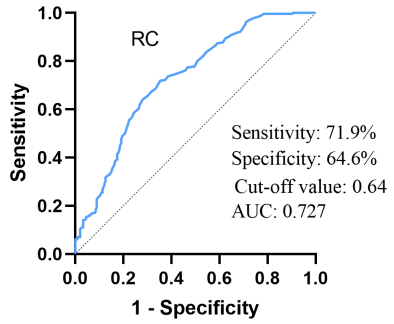

FIGURE 2
ROC curve analysis of the ability of RC to predict T2DM – PAD. RC, remnant cholesterol. AUC = 0.727, 95% CI:0.683–0.770, P = 0.000, cut-off: 0.64, sensitivity 71.9%, specificity 64.6%.

(See Supplementary Figure 1). After adjusting for other factors in the lipid profile using partial correlation analysis (all $P < 0.001$), a significant connection was found between RC and ultrasound grading. (Table 3)

The multifactorial regression, excluding the effects of confounding factors, showed that RC was independently associated with T2DM - PAD. This study also demonstrated that age and duration of diabetes were independent risk factors, consistent with previous studies (19). The roles of lipid metabolism and inflammation in atherosclerosis are well-established. It is generally accepted that NLR and MLR can be evaluated as inflammatory markers (33, 34). The platelet to HDL-C ratio as a novel inflammatory index has also garnered attention (35). The research also showed that HDL-C was a protective factor, and that NLR, MLR, and PHR were independent risk factors for PAD (Table 4). The ability of RC to predict T2DM - PAD was examined by using ROC curve, and the AUC was 0.727. The cut-off value was 0.64 mmol/L, indicating that diabetic patients with RC > 0.64 mmol/L had an elevated risk of developing PAD.

The TyG index, a surrogate for insulin resistance, is significantly related to the gold standard hyperinsulinemic-orthoglycemic clamp (36) and can be a reliable assessment of insulin resistance in patients. RC has been explored to be linked to insulin resistance (37). TyG index showed a correlation with RC (r = 0.485, $P < 0.001$) (Table 5), so it could be speculated that elevated RC levels in T2DM - PAD patients might be mediated by insulin resistance. In addition, one of the key mechanisms of pathogenesis for T2DM - PAD is the hypo-inflammatory response (38). It is worth noting that RC can also cause an inflammatory response, resulting in vascular endothelial damage (5). As shown in Table 5, CRP, NLR, MLR, and PHR levels were elevated in the T2DM - PAD individuals, but only PHR was significantly linked to RC (r = 0.123, $P = 0.044$). The correlation between inflammation and RC needs to be further verified by a large-scale investigation.

The fact that RC leads to atherosclerosis is the most likely cause of the link between raised RC levels and an increased risk of PAD (39). As with LDL particles, RC may enter the endothelium, where they are predominantly trapped because of their relatively large size (40), leading to the development of atherosclerosis as a result of cholesterol levels (39). Elevated RC levels are considered a risk factor for endothelial vasodilator dysfunction and can upregulate endothelial expression of endothelial-derived proatherogenic thrombogenic molecules *via* redox mechanisms (41, 42).It was reported that at high glucose concentrations, endothelial cells showed increased expression of low-density lipoprotein receptor 1 (LOX-1), thereby increasing vascular dysfunction (43). Interestingly, RC stimulated NAD(P)H oxidase-dependent superoxide formation and induction of cytokines in human umbilical vein endothelial cells (HUVECs) *via* activation of LOX-1, thereby exacerbating atherosclerosis (44). Furthermore, LOX-1-mediated uptake of RC plays important roles in atherogenesis by inducing LOX-1 expression and vascular smooth muscle cell migration, especially in the context of postprandial hyperlipidemia, diabetes, and metabolic syndrome (45). It could be hypothesized that in patients with DM and PAD, RC might also

TABLE 5 Correlation of RC with other potential risk factors in the T2DM-PAD patients.

Variables	Spearman Correlation Analysis	
	r	*P* value
Gender	**0.330**	**<0.001**
Age	-0.093	0.129
Diabetes duration	-0.027	0.660
Hypertension	-0.002	0.969
SBP	0.012	0.845
DBP	0.035	0.567
ALT	0.080	0.191
Urea	0.002	0.968
Cr	-0.083	0.175
eGFR	-0.019	0.754
HDL-C	-0.111	0.069
CRP	0.073	0.229
Fasting glucose	**0.125**	**0.040**
HbA1c (%)	0.061	0.315
TG	**0.614**	**<0.001**
TC	**0.342**	**<0.001**
HDL-C	-0.111	0.069
LDL-C	0.054	0.380
N-HDL-C	**0.379**	**<0.001**
TyG index	**0.485**	**<0.001**
NLR	-0.051	0.409
MLR	-0.041	0.500
PHR	**0.123**	**0.044**

SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferase; Cr, creatinine; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; CRP, C-reactive protein; RC, remnant cholesterol; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; N-HDL-C, non-HDL-C; TyG index, triglyceride glucose index; NLR, neutrophil to lymphocyte ratio; MLR, monocyte to lymphocyte ratio; PHR, platelet/HDL-C ratio. P < 0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance.

impact the etiology of PAD by inducing LOX-1 expression. Further studies are needed to determine the specific mechanism of action.

However, this current study also has some limitations. First, this was a retrospective cross-sectional study conducted in a single center, unable to determine the causal relationship between disease and RC. Second, the data were collected from clinical databases, and direct measurement of RC has not yet become a routine test for clinical lipid testing. Therefore, only get the calculated RC levels could be obtained. Calculated and measured RC are closely related (46, 47). Previous studies have shown that calculated RC underestimates the risk of myocardial infarction compared to directly measured RC (48). Nevertheless, calculated RC can be easily obtained from available lipid measurements at no additional cost, and therefore has a strong clinical utility. Third, although the non-fasting RC is critical in the development of atherosclerosis (49), only fasting RC levels were considered, possibly ignoring the possible results of non-fasting RC levels (6).Further prospective studies are required to analyze whether RC accelerates atherosclerosis progression.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by [2022] IEC CRYJ 0019. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

SJ and YiS conceived the study plan and contributed to the revision of the final manuscript. YiS collected, analyzed the data, and finished the manuscript writing. YZ and XB participated in data collection and literature search. WC, LW, MS, YaS, and LZ contributed to the manuscript writing and data interpretation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1111152/full#supplementary-material

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Serum glycoprotein non-metastatic melanoma protein B (GPNMB) level as a potential biomarker for diabetes mellitus-related cataract: A cross-sectional study

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Background: Diabetes mellitus (DM), a metabolic disease that has attracted significant research and clinical attention over the years, can affect the eye structure and induce cataract in patients diagnosed with DM. Recent studies have indicated the relationship between glycoprotein non-metastatic melanoma protein B (GPNMB) and DM and DM-related renal dysfunction. However, the role of circulating GPNMB in DM-associated cataract is still unknown. In this study, we explored the potential of serum GPNMB as a biomarker for DM and DM-associated cataract.

Methods: A total of 406 subjects were enrolled, including 60 and 346 subjects with and without DM, respectively. The presence of cataract was evaluated and serum GPNMB levels were measured using a commercial enzyme-linked immunosorbent assay kit.

Results: Serum GPNMB levels were higher in diabetic individuals and subjects with cataract than in those without DM or cataract. Subjects in the highest GPNMB tertile group were more likely to have metabolic disorder, cataract, and DM. Analysis performed in subjects with DM elucidated the correlation between serum GPNMB levels and cataract. Receiver operating characteristic (ROC) curve analysis also indicated that GPNMB could be used to diagnose DM and cataract. Multivariable logistic regression analysis illustrated that GPNMB levels were independently associated with DM and cataract. DM was also found to be an independent risk factor for cataract. Further surveys revealed the combination of

serum GPNMB levels and presence of DM was associated with a more precise identification of cataract than either factor alone.

Conclusions: Increased circulating GPNMB levels are associated with DM and cataract and can be used as a biomarker of DM-associated cataract.

KEYWORDS

diabetes mellitus, cataract, GPNMB, biomarker, serum

Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by high blood glucose levels. Owing to the rapidly increasing numbers of diabetic patients worldwide, it has become one of the most common and insidious chronic diseases with an estimated 4.2 million deaths among 20–79-year-old adults in 2019 (1, 2). In the Middle East and North Africa region, 16.2% of all-cause deaths are attributable to DM. In the South-East Asia and Western Pacific regions, the number of DM-related deaths in 2019 was 1,150,344 and 1,265,051, respectively (2). In addition, another study in 2021 showed that almost a half of all 20–79-year-old adults with DM were unaware of their diabetic status (3). Thus, advances in the study of biomarkers and mechanism are urgently needed for the prediction and therapy for DM.

The progression of DM are often accompanied by several complications such as acute kidney injury, cardiovascular diseases, heart failure, muscle infarction, and cognitive dysfunction (4–8). DM also affects ocular structures, contributes to the pathogenesis of cataract, and causes visual impairment in diabetic patients (9). According to the World Health Organization, cataract is an opacity of the lens with corrected visual acuity less than 0.7. Reports have regarded cataract as the leading cause of blindness globally and indicate that most cases of blindness cases from cataract occur in low- and middle-income countries (10–12). Globally, cost utility values for intraocular lens (IOL) implant surgery, one of the cost-effective treatments for cataract, remain considerable and cause a heavy public health burden (13). However, except for ocular examination, effective molecular biomarkers from blood indices are rarely discovered for the detection of cataract.

Glycoprotein non-metastatic melanoma protein B (GPNMB), also known as osteoactivin given its role in osteopetrosis in rats (14), is widely expressed in various types of cells such as macrophages (15), dendritic cells (16), osteoblasts (17), melanocytes (18), neurons (19) and hepatocytes (20). GPNMB contains an integrin-binding domain and extracellular heparin which contributes to binding to several types of cells such as vascular endothelial cells, keratinocytes, melanoma cells, fibroblasts and T cells (21–24). Study has identified the role of liver-secreted GPNMB in exacerbating obesity and insulin resistance by promoting lipogenesis (20). Cao C et al.'s (25) research has revealed the correlation of circulating levels of GPNMB with gestational DM. Moreover, the expression level of GPNMB is reportedly associated with type 1 DM-related renal function decline (26). Nevertheless, little is known about the role of GPNMB in diabetic cataract.

We investigated the role of GPNMB as a potential biomarker for DM-related cataract based on the data and samples collected from an ongoing cohort study—China Aging Longitudinal Study (CALS)—that enrolled a total of 26,000 healthy Chinese residents from seven geographic areas with the aim to investigate health and aging trends in China. The serum concentrations of GPNMB were measured, and some indicators of physical examination were analyzed.

Materials and methods

Study population

Subjects aged ≥25 years and without psychiatric disorders and alcohol or drug abuse were enrolled in CALS. From this existing cohort, we enrolled subjects from Long Tanhu Community in North China (426 participants) and excluded the following: subjects who required acute medical treatment or hospitalization within the first 3 months of GPNMB measurement (3 participants); those with severe diseases including cardiac, hepatic, or renal disease, and respiratory failure (10 participants); those unable to walk independently (2 participants); and those previously diagnosed with dementia (2 participants) and cancers (3 participants).

Finally, a total of 406 participants (155 men and 251 women; with DM=60, without DM=346) were included in this study. All subjects signed the informed consent form. Among these, 21.43% participants were diagnosed with cataract.

Clinical and biochemical measurements

Participants who had an opacity in the lens or were previously diagnosed with cataract were defined as the cataract group. Diabetes mellitus was defined by fasting serum glucose ≥126 mg/dL and/or glycosylated hemoglobin (HbA1c) ≥6.5% or those requiring treatment with anti-diabetes medication. All subjects were required to fast for 8 h before screening and filled in

questionnaires regarding medical history. Clinical characteristics such as height, weight, body mass index (BMI), and total body fat mass were obtained. Total body fat mass was detected with the body composition analyzer (TsingHua Tong Fang, BCA-2A). Body fat percentage (Fat%) was defined as total body fat mass divided by body weight. Fat mass index (FMI) was defined as total body fat mass divided by the height squared.

Concentrations of fasting blood glucose (GLU), total cholesterol (TC), total triglyceride (TG), high density lipoprotein-C (HDL-C), and low density lipoprotein-C (LDL-C) were assayed using enzymatic methods and detected by Hitachi Automatic Analyzer ((LABOSPECT 008 AS, Japan). Concentrations of insulin and folic acid (FOL) were assayed using a solid-phase enzyme-linked chemiluminescent immunoassay and detected by IMMULITE2000 Automatic Immune Analyzer (Siemens Healthcare Diagnostics, Inc.). HbA1c was measured by nitroblue tetrazolium method and detected by AU680 Automated Biochemical Instrument (Beckman Coulter, Inc.). Insulin resistance status was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR) according to the following formula: fasting serum insulin (μU/mL) × fasting serum glucose (mmol/L)/22.5 (27, 28).

Serum samples preparation and measurement

After 8 h of overnight fasting, blood samples were obtained *via* venipuncture from the median cubital vein, left in vacutainer tubes with coagulant at room temperature to clot for 15-30 min, centrifuged to collect the supernatant, and finally stored at -80°C until further analysis. Serum GPNMB concentrations were determined with an enzyme-linked immunosorbent assay kit (ELH-Osteoactivin, RayBiotech, Inc, Norcross, GA, USA). The assay had a sensitivity of 45 ng/mL to human GPNMB. The intra-assay and inter-assay coefficients of variation were <10% and <12%, respectively. The assay detection range was 49.15–12000 pg/mL. Serum samples were diluted 10 times before detection and measured according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Mac (version 26.0; IBM Corporation, Armonk, NY, USA) and R script 4.1.0. Continuous variables were presented as mean ± SD or median (interquartile range), while categorical variables were expressed as percentages. Differences between the two groups were analyzed by Wilcoxon rank sum test or Student's *t*-test. Comparison among three or more groups was performed using one-way analysis of variance (ANOVA) or Kruskal–Wallis H test. Categorical variables were compared using chi-squared test or Fisher's exact test. The association of study variables with DM or cataract was analyzed by univariable and multivariable logistic regression. The area under the receiver-operating characteristic (ROC) curve was calculated to test its predictive discrimination of DM and cataract. The optimal cut-off value was determined using the maximum sum of sensitivity and specificity based on the Youden index. Two-sided values of $P<0.05$ were considered to indicate statistically significant differences.

Results

The clinical and biochemical characteristics of subjects with or without DM are shown in Table 1. Subjects with DM were older and had higher BMI, FMI, Fat%, HOMA-IR, HbA1c, TG, FOL, INS, blood glucose and serum GPNMB levels than those without DM. Further, the DM group had a higher proportion of subjects diagnosed with cataract than the non-DM group. HDL-C levels were also lower in the DM than non-DM group. Circulating serum GPNMB levels were higher in subjects with DM than those without DM (Figure 1). We further plotted ROC curves based on GPNMB levels which showed a predictive ability of 0.734 to identify DM (asymptotic significance: <0.001) (Figure 2). The optimal cut-off value of GPNMB was 12,820.057 pg/mL (88.3% sensitivity and 53.2% specificity) to detect DM.

We then compared the variables in subjects with or without cataract (Table 2). Those with cataract were older and had higher FMI, Fat%, HOMA-IR, HbA1c, TC, TG, FOL, INS, blood glucose and serum GPNMB levels than those without cataract. A higher proportion of subjects with DM were detected in the cataract group than the cataract-free group. Furthermore, we performed analysis in subjects with DM and found that DM-bearing participants tended to be older and had a higher level of TC and serum GPNMB (Table 3). Circulating serum GPNMB levels were higher in subjects with cataract than those without cataract (Figure 3). The ability of serum GPNMB levels to predict the presence of cataract was 0.783, based on ROC curve analysis (asymptotic significance: <0.001) (Figure 4). The optimal cut-off value of GPNMB was 16,630.675 pg/mL (73.6% sensitivity and 73.0% specificity) to detect DM.

Table 4 shows the general characteristics of enrolled individuals grouped according to GPNMB tertiles. Lower age, Fat%, TC, TG, LDL-C, FOL levels, and more men were detected in tertile 1 than in tertiles 2 and 3. Nevertheless, the median levels of TC and LDL-C of individuals in tertile 2 were higher than those in tertile 3. Notably, a higher proportion of participants in tertile 3 were diagnosed as having DM and cataract than in tertiles 2 and 1.

As shown in Table 5, univariable logistic regression analysis revealed that age, BMI, FMI, Fat%, HOMA-IR, HbA1c, GLU, INS, TC, HDL-C, FOL, and log GPNMB were associated with DM. We included these variables in the multivariable logistic regression analysis. To avoid collinearity, we explored the linear correlation of log GPNMB with age, Fat%, GLU, TC, and FOL through Pearson's correlation coefficient analysis (Supplementary Figure 1) and finally excluded those five variables from the multivariable logistic regression analysis. It was indicated that HOMA-IR, HDL-C, and log GPNMB were independently associated with the presence of DM.

We also performed univariable and multivariable logistic regression analysis to investigate potential biomarkers for

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TABLE 1 Clinical and biochemical characteristics of study participants classified according to diabetes.

Characteristic	Overall	Diabetes (-)	Diabetes (+)	*P* value
Age (years), median (IQR)	50.000 (34.000, 63.750)	48.000 (33.000, 58.000)	66.500 (58.750, 76.000)	< 0.001
Male (%)	38.200	36.990	45.000	0.301
BMI (kg/m$^{2)}$, median (IQR)	23.700 (21.600, 26.040)	23.450 (21.500, 25.830)	24.690 (23.000, 27.230)	0.012
FMI (kg/m^2), median (IQR)	6.090 (5.038, 7.570)	5.980 (4.880, 7.370)	7.170 (5.920, 8.040)	< 0.001
Fat (%), mean ± SD	26.250 ± 8.2	26.270 ± 5.880	29.360 ± 5.820	0.001
HOMA-IR, median (IQR)	1.720 (1.200, 2.731)	1.640 (1.160, 2.410)	3.050 (1.790, 4.460)	< 0.001
HbA1c (%), median (IQR)	5.800 (5.600, 6.200)	5.800 (5.500, 6.000)	6.400 (5.820, 7.070)	< 0.001
GLU (mmol/l), median (IQR)	5.200 (5.000, 5.700)	5.200 (4.900, 5.600)	6.300 (5.570, 7.080)	< 0.001
TC (mmol/l), median (IQR)	4.740 (4.180, 5.363)	4.750 (4.200, 5.380)	4.620 (4.000, 5.170)	0.123
TG (mmol/l), median (IQR)	0.960 (0.640, 1.470)	0.920 (0.630, 1.400)	1.160 (0.890, 1.630)	0.007
HDL-C (mmol/l), median (IQR)	1.420 (1.190, 1.690)	1.450 (1.230, 1.710)	1.230 (1.060, 1.440)	< 0.001
LDL-C (mmol/l), median (IQR)	2.860 (2.335, 3.502)	2.860 (2.330, 3.510)	2.840 (2.340, 3.460)	0.405
FOL (ng/mL), median (IQR)	9.250 (6.473, 12.205)	8.760 (6.190, 12.010)	10.940 (8.920, 15.930)	< 0.001
INS (mU/L), median (IQR)	7.200 (5.300, 10.700)	6.800 (5.200, 9.700)	9.900 (6.120, 16.150)	< 0.001
Serum GPNMB conc (pg/mL), median (IQR)	13522 (6175, 19496)	12389 (5351, 18039)	20047 (15242, 24870)	< 0.001
Cataract (%)	21.400	17.630	43.330	< 0.001

Data are expressed as median (interquartile range), or %. BMI body mass index, FMI fat mass index, Fat% body fat percentage, HOMA-IR the homeostasis model assessment of insulin resistance, HbA1c glycated hemoglobin, GLU fasting blood-glucose, TC total cholesterol, TG triglyceride, HDL-C HDL cholesterol, LDL-C LDL cholesterol, FOL folic acid, INS insulin.
P values for student's t test or Wilcoxon rank sum test or Chi square test.

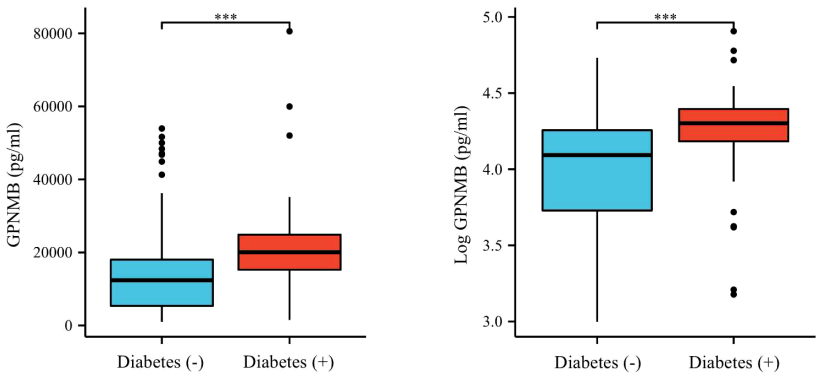

FIGURE 1
Plasma GPNMB levels depending on the existence of diabetes. ***P < 0.001 using a Wilcoxon rank sum test.

cataract (Table 6). Age, FMI, Fat%, HbA1c, GLU, TC, FOL, and log GPNMB were found to be associated with cataract by univariable logistic regression analysis. Multivariable logistic regression analysis showed that log GPNMB was an independent predictor of cataract. To gain further understanding of the relationship between serum GPNMB and DM-related cataract, we utilized ROC curve analysis based on serum GPNMB levels and the diabetic status (Figure 5). The area under the ROC curve was 0.789 with an asymptotic significance <0.001.

Discussion

To our best knowledge, our study is the first to demonstrate that serum GPNMB levels correlate with both DM and cataract. The results showed higher GPNMB levels in subjects with DM or cataract than in control subjects. In addition, serum GPNMB levels were independently correlated with both DM and cataract. ROC curve analysis also proved the potential of serum GPNMB levels as an independent biomarker for DM and cataract. Notably,

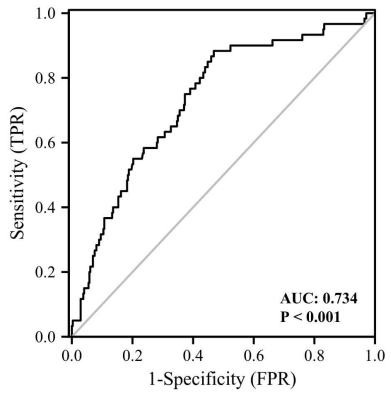

FIGURE 2
ROC curve analysis of the ability of plasma GPNMB to predict the presence of diabetes. AUC, area under curve.

the ability of GPNMB levels to diagnose cataract was improved with the presence of DM. Further research is required to validate these results.

GPNMB possesses an extracellular N-terminal signal peptide, a transmembrane domain, and a short cytoplasmic tail (21) and functions both as an inflammatory mediator and a circulating cytokine. Referring to its role in inflammatory response, a previous study detected the prevailing expression of GPNMB in macrophages, and showed that the expression level of GPNMB can be induced by interferon gamma (IFN-γ) and lipopolysaccharide and is elevated in inflammatory macrophages (15). In addition, overexpression of GPNMB in RAW264.7 cells, a macrophage cell line derived from mice, causes significant decrease of interleukin (IL)-6 and IL-12p40, inflammatory cytokines, as well as the inflammatory regulator nitric oxide (NO) (15). Thus, GPNMB is considered as a negative regulator of macrophage inflammatory capacity. Furthermore, macrophage inflammatory response is closely related to metabolic disorders (29). and DM is one of the most common metabolic diseases and can cause aberrant metabolism of blood glucose and induce the inflammatory response. By utilizing mouse models fed with high-fat diet, Prabata et al. (30) detected attenuated insulin and glucose tolerance in GPNMB-knock-out (KO) mice, and observed high levels of inflammatory cytokines produced by macrophages derived from GPNMB KO mice. In addition, the increase in inflammatory cytokines secreted by macrophages could be abrogated by added GPNMB extracellular domain. Prabata et al. (30) also found that GPNMB could bind to CD44 to prohibit nuclear factor kappa-B (NF-κB), thus abate the inflammatory response of macrophages.

GPNMB can also be cleaved into a soluble form that contains an ectodomain (ECD) and functions as a secreted cytokine (31). Gong et al. (20) revealed that GPNMB-ECD could interact with CD44 to trigger AKT signaling and further contributed to lipogenesis in adipocytes of withe adipose tissues. They further showed that

TABLE 2 Clinical and biochemical characteristics of study participants classified according to cataract.

Characteristic	Overall	Cataract (-), n=319	Cataract (+), n=87	*P* value
Age (years), median (IQR)	50.000 (34.000, 63.750)	45.000 (32.000, 55.000)	71.000 (65.000, 79.000)	< 0.001
Male (%)	38.200	39.810	32.180	0.241
BMI (kg/m^2), median (IQR)	23.700 (21.600, 26.040)	23.500 (21.400, 25.800)	24.300 (22.530, 26.600)	0.064
FMI (kg/m^2), median (IQR)	6.090 (5.038, 7.570)	5.940 (4.920, 7.360)	6.960 (5.880, 8.460)	0.001
Fat (%), mean ± SD	26.250 ± 8.2	26.120 ± 5.860	29.420 ± 5.730	< 0.001
HOMA-IR, median (IQR)	1.720 (1.200, 2.731)	1.620 (1.160, 2.460)	2.180 (1.460, 3.200)	< 0.001
HbA1c (%), median (IQR)	5.800 (5.600, 6.200)	5.800 (5.500, 6.100)	5.900 (5.700, 6.400)	0.001
GLU (mmol/l), median (IQR)	5.200 (5.000, 5.700)	5.200 (4.900, 5.600)	5.600 (5.200, 6.300)	< 0.001
TC (mmol/l), median (IQR)	4.740 (4.180, 5.363)	4.680 (4.180, 5.260)	4.970 (4.250, 5.620)	0.047
TG (mmol/l), median (IQR)	0.960 (0.640, 1.470)	0.890 (0.620, 1.400)	1.110 (0.840, 1.560)	0.002
HDL-C (mmol/l), median (IQR)	1.420 (1.190, 1.690)	1.420 (1.190, 1.680)	1.430 (1.210, 1.740)	0.451
LDL-C (mmol/l), median (IQR)	2.860 (2.335, 3.502)	2.830 (2.340, 3.390)	3.090 (2.310, 3.570)	0.252
FOL (ng/mL), median (IQR)	9.250 (6.473, 12.205)	8.580 (5.980, 11.660)	10.950 (8.540, 19.630)	< 0.001
INS (mU/L), median (IQR)	7.200 (5.300, 10.700)	6.800 (5.200, 10.250)	8.100 (5.900, 11.750)	0.011
Serum GPNMB conc. (pg/mL), median (IQR)	13522 (6175, 19496)	11778 (5230, 17232)	20658 (16163, 25958)	< 0.001
Diabetes (%)	14.800	10.660	29.890	< 0.001

Data are expressed as median (interquartile range), or %. BMI body mass index, FMI fat mass index, Fat% body fat percentage, HOMA-IR the homeostasis model assessment of insulin resistance, HbA1c glycated hemoglobin, GLU fasting blood-glucose, TC total cholesterol, TG triglyceride, HDL-C HDL cholesterol, LDL-C LDL cholesterol, FOL folic acid, INS insulin.
P values for student's t test or Wilcoxon rank sum test or Chi square test.

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TABLE 3 Clinical and biochemical characteristics of study participants with diabetes classified according to cataract.

Characteristic	Overall	Cataract (-), n=34	Cataract (+), n=26	*P* value
Age (years), median (IQR)	66.017 (53.942, 78.092)	60.235 (48.754, 71.716)	73.577 (65.502, 81.653)	< 0.001
Male (%)	45.000	21.700	23.300	0.228
BMI (kg/m^2), median (IQR)	24.690 (23.000, 27.230)	24.450 (21.750, 26.945)	24.690 (23.900, 27.400)	0.298
FMI (kg/m^2), median (IQR)	7.170 (5.915, 8.045)	6.870 (5.743, 8.028)	7.460 (6.440, 8.230)	0.346
Fat (%), mean ± SD	29.360 ± 5.820	29.032 ± 6.217	29.973 ± 5.144	0.619
HOMA-IR, median (IQR)	3.046 (1.787, 4.465)	2.555 (1.446, 4.415)	3.280 (1.870, 4.480)	0.486
HbA1c (%), median (IQR)	6.574 (5.528, 7.620)	6.485 (5.425, 7.545)	6.655 (5.603, 7.706)	0.606
GLU (mmol/l), median (IQR)	6.300 (5.575, 7.075)	6.000 (5.650, 6.800)	6.400 (5.200, 7.500)	0.783
TC (mmol/l), median (IQR)	4.583 (3.490, 5.678)	4.940 (4.200, 5.605)	4.510 (3.610, 4.710)	0.043
TG (mmol/l), median (IQR)	1.160 (0.888, 1.630)	1.220 (0.915, 1.780)	1.110 (0.890, 1.340)	0.323
HDL-C (mmol/l), median (IQR)	1.225 (1.058, 1.435)	1.320 (1.115, 1.480)	1.150 (1.050, 1.410)	0.447
LDL-C (mmol/l), median (IQR)	2.825 (1.912, 3.738)	3.051 (2.224, 3.878)	2.572 (1.618, 3.525)	0.055
FOL (ng/mL), median (IQR)	10.940 (8.920, 15.930)	10.410 (8.720, 12.122)	12.200 (9.530, 19.640)	0.154
INS (mU/L), median (IQR)	9.900 (6.125, 16.150)	9.550 (6.525, 15.875)	10.900 (6.125, 16.150)	0.707
Serum GPNMB conc. (pg/mL), median (IQR)	20050 (15240, 24870)	17270 (14110, 23060)	22710 (18870, 31080)	0.013

Data are expressed as median (interquartile range), or %. BMI body mass index, FMI fat mass index, Fat% body fat percentage, HOMA-IR the homeostasis model assessment of insulin resistance, HbA1c glycated hemoglobin, GLU fasting blood-glucose, TC total cholesterol, TG triglyceride, HDL-C HDL cholesterol, LDL-C LDL cholesterol, FOL folic acid, INS insulin.
P values for student's t test or Wilcoxon rank sum test or Chi square test.

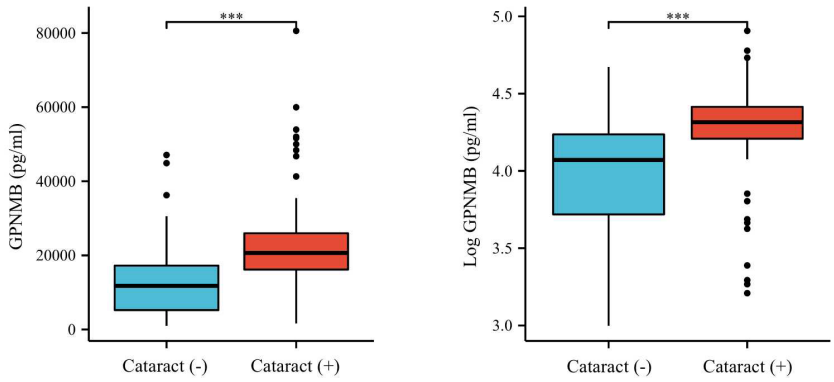

FIGURE 3
Plasma GPNMB levels depending on the existence of cataract. ***P < 0.001 using a Wilcoxon rank sum test.

GPNMB was capable of inducing obesity and insulin resistance in mice and this phenotype could be rescued by an anti-GPNMB antibody. Gong et al. (20) also performed a population-based study and ascertained that serum levels of GPNMB were correlated with human obesity. Research also determined the positive association between serum levels of GPNMB and disease presence and severity of Parkinson's Disease (32). Another study that concentrated on diabetic retinopathy—a microvascular complication characterized by aberrant angiogenesis—found that GPNMB knockdown attenuated retinal angiogenesis stimulated by high glucose both *in vivo* and *in vitro* (33).

In this study, elevated serum levels of GPNMB were determined in subjects with DM and cataract. Based on the results of previous research, it was quite possible that the increased circulating levels of GPNMB caused abnormal phosphorylation of AKT *via* binding to CD44 and then stimulated anomalous activation of AKT/PI3K signaling to promote lipogenesis and provoke diabetes. Moreover, activation of AKT signaling followed by downregulation of connexin 43 is required for transforming growth factor-beta 2 (TGF-β2)-induced epithelial mesenchymal transition (EMT) of HLE B-3 cells—a human lens epithelial cell line (34). It has been

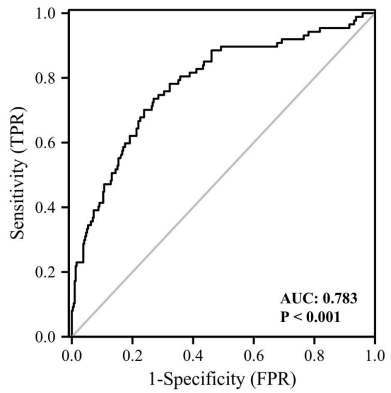

FIGURE 4
ROC curve analysis of the ability of plasma GPNMB to predict the presence of cataract. AUC, area under curve.

reported that lens epithelial cells that undergo EMT can result in posterior capsule opacification, which is the main cause and symptom of cataract (35). Yao et al. (36) demonstrated that integrin beta-1, a target protein of GPNMB, was essential for TGF-β2-mediated migration of lens epithelial cells. Thus, the raised serum levels of GPNMB may abate the expression of connexin 43 and stimulate the upregulation of integrin beta-1 to promote cataract formation. Notably, high glucose levels due to diabetes-induced insulin resistance also contributes to the pathogenesis of cataract (37). Accordingly, we concluded that the way in which GPNMB promoted DM might enhance the development of cataract. Further investigations are needed to reveal the underlying molecular mechanisms.

To minimize probable bias, we excluded subjects with alcohol and drug abuse, severe diseases, and those that underwent recent medical treatment. We aimed to only enroll subjects with a relatively steady metabolism, because DM and cataract are both metabolic diseases. Moreover, recruited individuals were all required to undergo 8h fasting before screening to maintain stable levels of serum cytokines. We reviewed the medical history of included individuals and performed ocular examination to avoid excluding those who had undergone cataract surgery before or were unaware of their cataract. Furthermore, we utilized different statistical methods to analyze the data, and found that the results were consistent. These

TABLE 4 General characteristics of patients by tertiles of plasma GPNMB level.

Characteristic	Tertiles of circulating GPNMB levels			*P* value
	Tertile 1 (<9650 pg/mL) n = 135	Tertile 2 (9650-17460 pg/mL) n = 135	Tertile 3 (>17460 pg/mL) n = 136	
Age (years), median (IQR)	42.000 (31.000, 53.500)	48.000 (34.000, 58.500)	63.500 (49.750, 73.500)	< 0.001
Male (%)	46.670	34.070	33.820	0.046
BMI (kg/m^2), median (IQR)	23.900 (21.500, 25.950)	23.450 (21.680, 25.660)	23.550 (21.670, 26.320)	0.860
FMI (kg/m^2), median (IQR)	5.900 (4.840, 7.360)	6.160 (5.170, 7.400)	6.540 (5.170, 7.890)	0.274
Fat%, mean ± SD	25.510 ± 6.050	27.040 ± 5.970	27.540 ± 5.640	0.022
HOMA-IR, median (IQR)	1.630 (1.210, 2.430)	1.720 (1.150, 2.430)	1.840 (1.200, 3.090)	0.274
HbA1c%, median (IQR)	5.800 (5.570, 6.100)	5.800 (5.600, 6.100)	5.900 (5.600, 6.300)	0.297
GLU (mmol/l), median (IQR)	5.200 (5.000, 5.600)	5.200 (4.900, 5.700)	5.300 (5.000, 6.000)	0.085
TC (mmol/l), median (IQR)	4.620 (4.170, 5.150)	4.900 (4.260, 5.500)	4.680 (4.170, 5.300)	0.047
TG (mmol/l), median (IQR)	0.850 (0.600, 1.340)	0.930 (0.640, 1.390)	1.040 (0.750, 1.520)	0.040
HDL-C (mmol/l), median (IQR)	1.380 (1.160, 1.600)	1.450 (1.230, 1.710)	1.420 (1.170, 1.710)	0.407
LDL-C (mmol/l), median (IQR)	2.760 (2.220, 3.340)	3.080 (2.500, 3.580)	2.820 (2.340, 3.460)	0.045
FOL (ng/mL), median (IQR)	7.940 (5.450, 10.950)	9.530 (6.870, 12.290)	10.300 (7.190, 15.320)	< 0.001
INS (mU/L), median (IQR)	6.950 (5.470, 10.100)	7.000 (5.350, 9.550)	7.550 (5.230, 11.750)	0.437
Cataract (%)	6.670	13.330	44.120	< 0.001
Diabetes (%)	4.440	12.590	27.210	< 0.001

Data are expressed as median (interquartile range), or %. BMI body mass index, FMI fat mass index, Fat% body fat percentage, HOMA-IR the homeostasis model assessment of insulin resistance, HbA1c glycated hemoglobin, GLU fasting blood-glucose, TC total cholesterol, TG triglyceride, HDL-C HDL cholesterol, LDL-C LDL cholesterol, FOL folic acid, INS insulin.
P values for ANOVA or Kruskal-Wallis H test or Chi square test.

TABLE 5 Univariable and multivariable logistic regression analyses for diabetes.

	Association with presence of diabetes			
	Single		Multiple	
	OR (95% CI)	P value	OR (95% CI)	P value
Age	1.076 (1.054-1.098)	<0.001	–	–
Gender	1.393 (0.801-2.424)	0.240	–	–
BMI	1.120 (1.031-1.216)	0.007	0.954 (0.743-1.225)	0.712
FMI	1.259 (1.102-1.438)	0.001	1.040 (0.739-1.464)	0.820
Fat (%)	1.092 (1.034-1.154)	0.002	–	–
HOMA-IR	1.322 (1.143-1.530)	<0.001	3.363 (1.101-10.268)	0.033
HbA1c	3.312 (1.966-5.581)	<0.001	1.207 (0.556-2.619)	0.634
GLU	2.484 (1.812-3.405)	<0.001	–	–
INS	1.052 (1.016-1.090)	0.005	0.753 (0.557-1.107)	0.064
TC	0.719 (0.518-0.998)	0.049	–	–
TG	1.195 (0.860-1.661)	0.289	–	–
HDL-C	0.186 (0.069-0.499)	0.001	0.171 (0.033-0.889)	0.036
LDL-C	0.805 (0.566-1.145)	0.227	–	–
FOL	1.082 (1.034-1.132)	0.001	–	–
Log GPNMB	14.871 (4.867-45.439)	<0.001	6.626 (1.316-33.370)	0.022

The symbol - indicates the cells without data.
Data are expressed as median (interquartile range), or %. BMI body mass index, FMI fat mass index, Fat% body fat percentage, HOMA-IR the homeostasis model assessment of insulin resistance, HbA1c glycated hemoglobin, GLU fasting blood-glucose, TC total cholesterol, TG triglyceride, HDL-C HDL cholesterol, LDL-C LDL cholesterol, FOL folic acid, INS insulin.
Log-transformation was used for GPNMB before statistical analysis. P values for univariable or multivariable logistic regression analysis.

TABLE 6 Univariable and multivariable logistic regression analyses for cataract.

	Association with presence of Cataract			
	Single		Multiple	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age	1.209 (1.158-1.261)	<0.001	–	–
Gender	0.717 (0.434-1.186)	0.195	–	–
BMI	1.040 (0.966-1.119)	0.299	–	–
FMI	1.190 (1.053-1.345)	0.005	1.074 (0.920-1.253)	0.364
Fat (%)	1.080 (1.000-1.167)	<0.001	–	–
HOMA-IR	1.104 (0.978-1.245)	0.109	–	–
HbA1c	1.643 (1.115-2.421)	0.012	1.569 (1.000-2.463)	0.050
GLU	1.495 (1.196-1.869)	<0.001	–	–
INS	1.019 (0.995-1.042)	0.117	–	–
TC	1.336 (1.040-1.716)	0.023	–	–
TG	1.189 (0.897-1.576)	0.229	–	–
HDL-C	1.488 (0.754-2.933)	0.252	–	–
LDL-C	1.215 (0.921-1.604)	0.169	–	–

(Continued)

TABLE 6 Continued

	Association with presence of Cataract			
	Single		Multiple	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
FOL	1.142 (1.095-1.192)	<0.001	–	–
Log GPNMB	36.785 (11.908-113.634)	<0.001	9.867 (2.837-34.324)	<0.001

The symbol - indicates the cells without data.
Data are expressed as median (interquartile range), or %. BMI body mass index, FMI fat mass index, Fat% body fat percentage, HOMA-IR the homeostasis model assessment of insulin resistance, HbA1c glycated hemoglobin, GLU fasting blood-glucose, TC total cholesterol, TG triglyceride, HDL-C HDL cholesterol, LDL-C LDL cholesterol, FOL folic acid, INS insulin.
Log-transformation was used for GPNMB before statistical analysis. P values for univariable or multivariable logistic regression analysis.

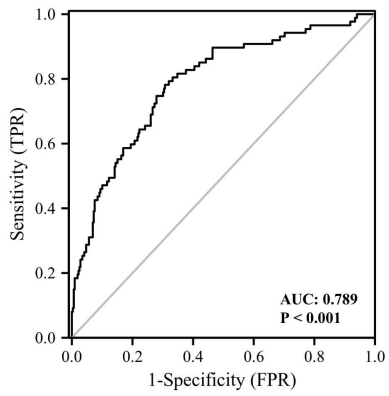

FIGURE 5
ROC curve analysis of the ability of plasma GPNMB and the presence of diabetes together to predict the presence of cataract. AUC, area under curve.

strategies added to the strengths of our study. A limitation of the study is the relatively small number of individuals in the subgroups. Further mechanistic studies are required to investigate the mechanism underlying GPNMB's effect on the pathogenesis of DM-related cataract.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee of Beijing Hospital (2019BJYYEC-054-02). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization: JC, J-PC, T-MZ. Methodology: JC, DH, Y-YL. Samples collection: DH, Y-YL, C-BL, L-TZ, G-QF, YW, L-QZ, JP, C-BL. Software Formal analysis: DH, Y-YL, CZ, X-FL. Data curation: JC, DH, Y-YL. Writing—original draft preparation: DH, Y-YL. Writing—review and editing: JC, J-PC, T-MZ, JP, L-QZ, TS. Supervision: JC, J-PC. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1110337/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The linear correlation of log GPNMB with different variables through Pearson's correlation coefficient analysis. *BMI* body mass index, *FMI* fat mass index, *Fat%* body fat percentage, *HOMA-IR* the homeostasis model assessment of insulin resistance, *HbA1c* glycated hemoglobin, *GLU* fasting blood-glucose, *TC* total cholesterol, *HDL-C* HDL cholesterol, *FOL* folic acid, *INS* insulin.

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Combination model of neutrophil to high-density lipoprotein ratio and system inflammation response index is more valuable for predicting peripheral arterial disease in type 2 diabetic patients: A cross-sectional study

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Background: Neutrophil/high-density lipoprotein (HDL) ratio (NHR), monocyte/HDL ratio (MHR), lymphocyte/HDL ratio (LHR), platelet/HDL ratio (PHR), systemic immune-inflammation index (SII), system inflammation response index (SIRI), and aggregate index of systemic inflammation (AISI) have been recently investigated as novel inflammatory markers. Herein, the correlation was investigated between these inflammatory biomarkers and peripheral arterial disease (PAD) in type 2 diabetes mellitus (T2DM) patients.

Methods: In this retrospective observational study, the hematological parameter data of 216 T2DM patients without PAD (T2DM-WPAD) and 218 T2DM patients with PAD (T2DM-PAD) at Fontaine stages II, III or IV stage had been collected. Differences in NHR, MHR, LHR, PHR, SII, SIRI, and AISI were analyzed, and receiver operating characteristic (ROC) curves were used to analyze the diagnostic potential of these parameters.

Results: The levels of NHR, MHR, PHR, SII, SIRI and AISI in T2DM-PAD patients were significantly higher than in T2DM-WPAD patients ($P < 0.001$). They were correlated with disease severity. Further, multifactorial logistic regression analyses showed that higher NHR, MHR, PHR, SII, SIRI, and AISI might be independent risk factors for T2DM-PAD ($P < 0.001$). The areas under the curve (AUCs) of the NHR, MHR, PHR, SII, SIRI, and AISI for T2DM-PAD patients was 0.703, 0.685, 0.606, 0.648, 0.711, and 0.670, respectively. The AUC of the NHR and SIRI combined model was 0.733.

Conclusion: The levels of NHR, MHR, PHR, SII, SIRI, and AISI were higher in T2DM-PAD patients, and they were independently linked with its clinical

severity. The combination model of NHR and SIRI was most valuable for predicting T2DM – PAD.

KEYWORDS

type 2 diabetes, peripheral artery disease, inflammation, lipid metabolism, biomarker

Introduction

Patients with T2DM and PAD have a cardiovascular mortality risk five times higher than patients with only one disease (1, 2). Therefore, early recognition and intervention of PAD in diabetic patients are necessary to lower the risk of major adverse limb events (MALEs) (3). The ankle-brachial index (ABI) is currently recommended as the primary screening tool for PAD in diabetic patients and those with multiple risk factors (4). Due to the low sensitivity of the ABI for early-stage PAD identification, finding new biomarkers that can identify PAD in diabetic individuals at an early stage is urgent.

Recent research has suggested that inflammation and lipid metabolism play a significant role in PAD pathogenesis (5, 6). Besides its capacity to transport cholesterol in the opposite direction, high-density lipoprotein cholesterol (HDL-C) has various protective properties, such as those related to infection, inflammation, antioxidants, and thrombosis (7). Obtaining the numbers of neutrophils, monocytes, lymphocytes, and platelets is inexpensive and easy *via* complete blood count, which are altered when an organism is inflamed. Furthermore, new hematological parameters related to HDL-C and complete blood cells, such as NHR (8), MHR (9), LHR (10), PHR (11), SII (12), SIRI (13, 14), and AISI (15) have been proposed as novel inflammatory biomarkers.

Relevant studies with single or two combined indicators in populations with diabetes or PAD are currently available (16–24). However, until now, no data has been found about how NHR, MHR, LHR, PHR, SII, SIRI, and AISI are linked to T2DM-PAD patients. Herein, the connection was explored between these inflammatory biomarkers and T2DM-PAD patients.

Materials and methods

Study population

Gender-matched individuals with T2DM were consecutively recruited from the Department of Endocrinology and Metabolism of the Liyuan Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China), from 1 June 2020 to 31 September 2022. The inclusion criteria are outlined in Table 1. Based on the T2DM international criteria (ADA) (25), T2DM was defined as a fasting plasma glucose level ≥ 7.0 mmol/L and/or 2-h plasma glucose ≥11.1 mmol/L during oral glucose tolerance tests (OGTT) and/or glycosylated hemoglobin (HbA1c) level ≥ 6.5%. Each patient included in the study was evaluated for a history of PAD symptoms or a verified diagnosis of PAD using the criteria established by the *Ad Hoc* Committee on Reporting Standards of the Society for Vascular Surgery and the International Society of Cardiovascular Surgery (26, 27). The ABI was measured in patients with PAD-like symptoms, and the physician evaluated the patient's lower extremities using arterial Doppler-enhanced ultrasonography if they had symptoms in both legs. Patients who had an ABI > 0.90 but showed no symptoms of PAD were not further tested for the disease.

The severity of PAD was assessed using the Fontaine classification, comprising four stages: I - asymptomatic; II - intermittent claudication; III - rest pain; and IV - ischemic ulcers or gangrene (28). Following the recommendations of the Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II), patients with ischemic rest pain, ulcers, or gangrene, attributable to objectively proven PAD, were considered affected by critical limb ischemia (CLI) (29). Based on the T2DM – PAD patients' clinical symptoms, the patients were divided into two groups, the first being those affected by stable PAD (Fontaine's II) and the second being those affected by CLI (Fontaine's III and IV).

Demographic and clinical assessment

Data from the electronic medical records of the relevant departments was analyzed. Factors such as age, gender, diagnoses, and lab results were recorded. On the second hospital morning, blood samples were collected from all patients' periphery. Laboratory personnel unaware of the patient's diagnoses analyzed the blood samples.

The NHR, MHR, LHR, PHR, SII, SIRI, AISI, and triglyceride glucose index (TyG index) were calculated using the following formulas: NHR = neutrophil/HDL ratio; MHR = monocyte/HDL; LHR = lymphocyte/HDL ratio; PHR = platelet/HDL ratio; SII = platelet × neutrophil-to-lymphocyte ratio, SIRI = monocyte × neutrophil-to-lymphocyte ratio; AISI = neutrophil × platelet× monocyte-to-lymphocyte ratio; TyG index = Ln [Triglyceride (TG, mg/dl) × Fasting plasma glucose (mg/dl)/2].

Statistical analysis

Statistical analyses were done using SPSS version 27.0 software (SPSS, Inc., Chicago, IL, United States). Graphs were created using Prism 9.0 (GraphPad Software). Continuous variables are described as means ± standard deviations (SDs) or medians (interquartile ranges), depending on the data distribution. Categorical variables

TABLE 1 Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
a) 18-79 years b) Confirmed diagnosis of type 2 diabetes mellitus at the time of admission c) Complete blood count parameters and lipid profile data are available	a) Undefined type of diabetes or clinical suspicion of non-type 2 diabetes mellitus b) Acute complications of diabetes mellitus (such as diabetic ketoacidosis, hyperglycemia hyperosmotic state, and lactic acidosis) c) Chronic kidney disease with eGFR below 60 mL/min (according to the CKD-EPI equation) d) Confirmed liver cirrhosis with Child–Pugh C functional impairment e) Leukocytosis (> 10×10^9 cells/L), leukopenia (< 4×10^9 cells/L), thrombocytosis (> 450×10^9 cells/L), or thrombocytopenia (< 100×10^9 cells/L) f) Autoimmune or chronic inflammatory pathology g) History or active solid or hematological malignancy h) Confirmed pancreatic insufficiency, chronic pancreatitis or previous pancreatic surgery i) ABI > 1.4 j) Taking immunosuppressive drugs, glucocorticoids or anticoagulants.

eGFR, estimated glomerular filtration rate; CKD-EPI equation: the Chronic Kidney Disease Epidemiology Collaboration equation; ABI, the ankle-brachial index.

are expressed as numbers and percentages of patients. Student's t-test and the Mann-Whitney U test were used to compare the two groups. One-way ANOVA (normally distributed variables) or the Kruskal-Wallis test (non-normally distributed variables) was used to analyze the differences between the three groups. For categorical variables, chi-square tests were performed. Spearman's correlation analysis was used to look at the connections between the different variables. Indicators with covariance were excluded from the correlation analysis. For example, NHR was covariant with neutrophils and HDL-C, and the Spearman correlation analysis was not conducted between the NHR and both variables. Binary logistic regression analysis was performed to explore the associations between NHR, MHR, PHR, LHR, SII, SIRI, and AISI and PAD. Due to the small variance of MHR as a continuous variable, the count unit was converted to $x10^8$/mmol during logistic regression, then included in the regression model. The optimum value for identifying PAD risk in this sample was calculated using ROC curve analysis. The optimal cut-off value was determined by maximizing the Yoden index. A bilateral $P< 0.05$ was defined as statistically significant.

Results

Comparison of baseline clinical characteristics and laboratory indicators between the PAD group and WPAD group

The demographic and clinical features of T2DM-PAD group and T2DM-WPAD group are summarized in Table 2. Among the 434 diabetic patients enrolled, 218 had PAD, and 216 did not (WPAD). Compared to WPAD patients, PAD patients had a higher prevalence of coronary artery disease (CAD) and hypertension, and showed significantly increased levels of systolic blood pressure (SBP), HDL, low-density lipoprotein (LDL), C-reactive protein (CRP), neutrophils, lymphocytes, monocytes, NHR, MHR, PHR, SII, SIRI, and AISI ($P<0.05$). The two groups did not differ for gender, history of smoking, drinking, and dyslipidemia, diastolic blood pressure (DBP), fasting glucose, TyG index, HbA1c, TG, total cholesterol (TC), platelets, and LHR ($P>0.05$). The prevalence of Fontaine stage II, and CLI were 62.8, 12.9, and 11.5% in PAD patients, respectively.

Clinical and laboratory features of T2DM - PAD patients: subgroup analysis using the fontaine classification

The two groups did not differ regarding gender, age, duration of diabetes, history of CAD, hypertension, and dyslipidemia, SBP, DBP and laboratory parameters such as lymphocytes, monocytes, platelets, and LHR ($P>0.05$) (Table 3). As disease severity increased, fasting glucose, HbA1c, TG, TC, HDL-C, LDL-C, and TyG index presented a decreasing trend ($P<0.05$), but CRP, neutrophils, monocytes, NHR, MHR, PHR, SII, SIRI, and AISI showed an increasing trend ($P<0.05$). Fontaine stage II patients had higher percentage of smokers (44.5%) and alcoholics (40.1%) ($P<0.05$).

The box - plot in Figure 1 indicated that CLI patients had higher levels of NHR, MHR, PHR, SII, SIRI, and AISI, and that all these indices showed an increasing relationship with disease extent.

Correlation of NHR, MHR, PHR, SII, SIRI, and AISI with other indicators of T2DM-PAD patients

Correlations between NHR, MHR, PHR, SII, SIRI, AISI and other indicators in PAD patients were assessed using Spearman correlation analysis. The NHR, MHR, PHR, SII, SIRI, and AISI were significantly correlated with Fontaine grading and CRP ($P<0.05$) (Table 4). However, these parameters had no significantly correlation with age, history of hypertension or dyslipidemia, SBP, DBP, fasting glucose, and TyG index. The PHR (r=-0.139) revealed a statistically weak connection with disease duration, whereas the NHR, MHR, SII, SIRI, and AISI were unrelated to disease duration. Additionally, a significant positive correlation was found between NHR and lymphocytes (r=0.151), monocytes (r=0.498), platelets (r=0.347), and TC (r=-0.207) (all $P<0.05$). The MHR was significantly linked with gender (r=-0.192), history of alcohol consumption (r=0.138), TC (r=-0.305), LDL-C (r=-0.192), neutrophils (r=0.455), lymphocytes (r=0.280), and platelets (r=0.204) (all $P<0.05$). The PHR was significantly associated with history of smoking (r=-0.136),

TABLE 2 Demographic and clinical data of diabetic subjects with and without PAD.

Variables	WPAD (N=216)	PAD (N=218)	*P* value
Gender (male, %)	113 (52.3%)	132 (60.6%)	0.084
Age (years)	**56 (50-61.5)**	**65 (59-71)**	**<0.001**
Diabetes duration (years)	**4 (1-10)**	**10 (5-18)**	**<0.001**
Smoking, n (%)	73 (33.8%)	83 (38.1%)	0.353
Alcohol, n (%)	62 (28.7%)	71 (32.6%)	0.383
CAD (%)	**33 (15.3%)**	**63 (28.9%)**	**<0.001**
Hypertension, n (%)	**102 (47.2%)**	**142 (65.1%)**	**<0.001**
Dyslipidemia, n (%)	93 (43.1%)	92 (42.2%)	0.857
SBP (mmHg)	**126 (115-137)**	**133 (123-143)**	**<0.001**
DBP (mmHg)	80 (72-87)	78 (72-85)	0.135
Fasting glucose (mmol/l)	10.75 (7.53-15)	10.07 (7.65-14.71)	0.687
HbA1c (%)	8.3 (6.75-10.4)	8.1 (7.2-9.7)	0.842
TG, mmol/L	1.55 (1.08-2.24)	1.67 (1.14-2.48)	0.264
TC, mmol/L	4.63 (3.97-5.45)	4.01 (3.46-5.4)	0.104
HDL-C, mmol/L	**1.15 (0.98-1.39)**	**1.01 (0.88-1.15)**	**<0.001**
LDL-C, mmol/L	**2.93 (2.19-3.46)**	**2.52 (1.86-3.43)**	**0.016**
CRP, mg/L	**1.1 (0.65-2.1)**	**1.9 (1-4.3)**	**<0.001**
TyG index	7.84 (7.26-8.5)	7.97 (7.35-8.53)	0.629
Neutrophil,10^9/L	**3.34 (2.83-4.13)**	**3.91 (3.2-4.86)**	**<0.001**
Lymphocyte, 10^9/L	**1.71 (1.45-2.03)**	**1.47 (1.15-1.81)**	**<0.001**
Monocyte, 10^9/L	**0.33 (0.27-0.4)**	**0.38 (0.3-0.48)**	**<0.001**
Platelet, 10^9/L	206.5 (176-239.5)	200 (168-249)	0.653
NHR, 10^9/mmol	**2.97 (2.62-3.72)**	**3.74 (3-5.26)**	**<0.001**
LHR, 10^9/mmol	1.48 (1.17-1.82)	1.45 (1.1-1.97)	0.908
MHR, 10^9/mmol	**0.29 (0.22-0.36)**	**0.37 (0.29-0.48)**	**<0.001**
PHR, 10^9/mmol	**172.05 (141.98-222.18)**	**195.06 (158.16-260.61)**	**<0.001**
SII,10^9/L	**409.56 (332.70-524.90)**	**534.59 (358.31-789.82)**	**<0.001**
SIRI,10^9/L	**0.63 (0.47-0.89)**	**1.00 (0.68-1.46)**	**<0.001**
AISI,10^{18}/L^2	**129.46 (94.53-193.60)**	**195.56 (127.62-316.40)**	**<0.001**
PAD			
Fontaine's II, n (%)		137 (62.8%)	/
CLI, n (%)		81 (37.2%)	/

CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; TyG index, triglyceride glucose index; NHR, neutrophil/HDL-C ratio; LHR, lymphocyte/HDL-C ratio; MHR, monocyte/HDL-C ratio; PHR, platelet/HDL-C ratio; SII, systemic immune-inflammation index; SIRI, system inflammation response index; AISI, aggregate index of systemic inflammation. P <0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance.

alcohol consumption (r=-0.179) and CAD (r=-0.271), HbA1c (r=0.143), TG (r=0.169), TC (r=-0.133), neutrophils (r=0.270), and monocytes (r=0.182) (all P<0.05). The SII was significantly associated with monocytes (r=0.205) and the SIRI was significantly correlated with gender (r=-0.238) smoking history (r=0.167), TG (r=-0.141), and platelets (r=0.246) (all P<0.05).

Univariate and multivariate logistic regression analysis of the influencing factors for T2DM-PAD occurrence

The univariate logistic regression analysis showed that age, duration of diabetes, SBP, LDL-C, CRP, NHR, MHR, PHR, SII,

TABLE 3 Subgroup analysis of the clinical characteristics based on the Fontaine classification in patients with PAD.

Variables	Fontaine's II (N=137)	CLI (N=81)	*P* value
Gender (male, %)	81 (59.1%)	51 (63%)	0.575
Age (years)	65 (59-69)	67 (60-72)	0.150
Diabetes duration (years)	10 (4.5-15)	11 (7-19)	0.066
Current smoker, n (%)	**61 (44.5%)**	**22 (27.2%)**	**0.011**
Alcohol, n (%)	**55 (40.1%)**	**16 (19.8%)**	**0.002**
CAD (%)	38 (27.7%)	25 (30.9%)	0.623
Hypertension, n (%)	89 (65%)	53 (52.8%)	0.944
Hyperlipidemia, n (%)	65 (47.4%)	27 (33.3%)	0.041
SBP (mmHg)	132 (120-143)	134 (125-144.5)	0.277
DBP (mmHg)	78 (70-85)	78 (73.5-86)	0.980
Fasting glucose (mmol/l)	**10.79 (8.47-15.61)**	**8.64 (6.54-11.795)**	**<0.001**
HbA1c (%)	**8.7 (7.2-10.25)**	**7.7 (7.15-8.85)**	**0.040**
TG, mmol/L	**1.81 (1.20-2.82)**	**1.45 (1.01-2.02)**	**0.004**
TC, mmol/L	**4.72 (3.895-5.52)**	**3.93 (3.285-5.12)**	**<0.001**
HDL-C, mmol/L	**1.05 (0.92-1.16)**	**0.96 (0.77-1.125)**	**0.003**
LDL-C, mmol/L	**2.59 (2.025-3.54)**	**2.32 (1.695-3.105)**	**0.019**
CRP, mg/L	**1.5 (0.9-3.35)**	**3 (1.5-11.1)**	**<0.001**
TyG index	**8.14 ± 0.92**	**7.65 ± 0.77**	**<0.001**
RBC ($\times10^9$/L)	**4.49 ± 0.49**	**3.99 ± 0.57**	**<0.001**
Neutrophil,10^9/L	**3.7 (3.15-4.5)**	**4.18 (3.33-5.345)**	**0.021**
Lymphocyte, 10^9/L	1.49 (1.125-1.855)	1.43 (1.16-1.76)	0.501
Monocyte, 10^9/L	0.37 (0.285-0.45)	0.4 (0.31-0.5)	0.075
Platelet, 10^9/L	200 (168-244.5)	212 (164.5-277)	0.349
NHR, 10^9/mmol	**3.6 (2.93-4.6)**	**4.74 (3.16-6.02)**	**<0.001**
LHR, 10^9/mmol	1.44 (1.09-1.86)	1.51 (1.13-2.2)	0.291
MHR, 10^9/mmol	**0.34 (0.28-0.45)**	**0.41 (0.29-0.57)**	**0.003**
PHR, 10^9/mmol	**192.11 (157.52-249)**	**200 (162.89-314.68)**	**0.032**
SII,10^9/L	**504.44 (355.53-694.09)**	**600.32 (361.22-1027.89)**	**0.018**
SIRI,10^9/L	**0.88 (0.64-1.31)**	**1.17 (0.77-1.73)**	**0.004**
AISI,10^{18}/L^2	**181.17 (121.82-276.4)**	**242.76 (141.15-429.44)**	**0.008**

CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; TyG index, triglyceride glucose index; NHR, neutrophil/HDL-C ratio; LHR, lymphocyte/HDL-C ratio; MHR, monocyte/HDL-C ratio; PHR, platelet/HDL-C ratio; SII, systemic immune-inflammation index; SIRI, system inflammation response index; AISI, aggregate index of systemic inflammation. P <0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance.

SIRI, and AISI were independently associated with PAD occurrence in T2DM patients (Table 5). After excluding the effects of confounding factors for binary logistic regression, age, duration of diabetes, NHR, MHR, PHR, SII, SIRI, and AISI were still statistically significant and considered independent risk factors for PAD occurrence in T2DM patients.

Diagnostic performance of different inflammatory indexes for T2DM-PAD

The ROC curve analysis was used to evaluate the ability of NHR, MHR, PHR, SII, SIRI, and AISI to identify T2DM-PAD patients. The results of the ROC curve analysis showed that each of these indicators

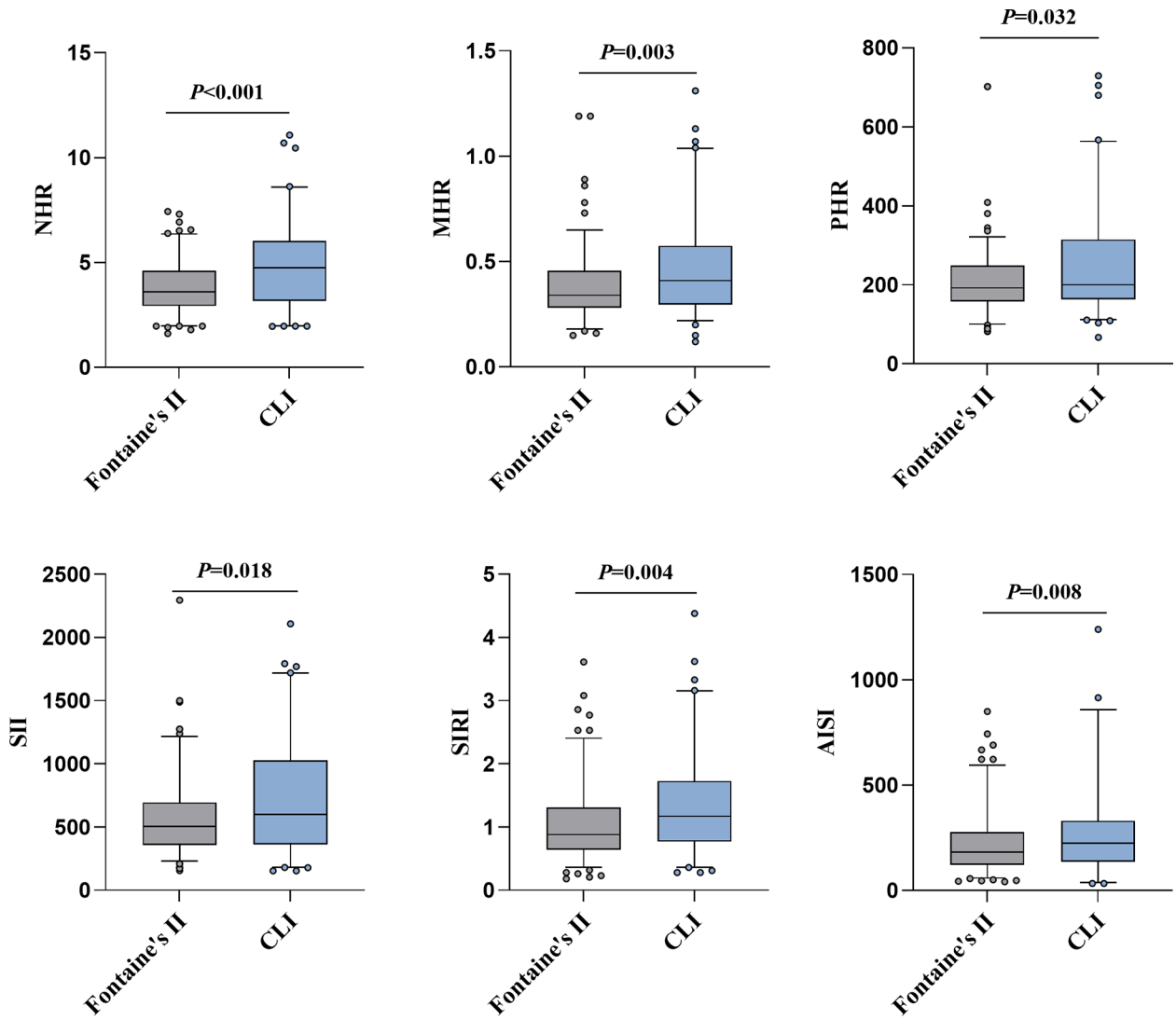

FIGURE 1
The NHR, MHR, PHR, SII, SIRI and AISI levels according to PAD severity. On the box plots, central lines represent the median, the length of the box represents the interquartile range and the lines extend to minimum and maximum values. Bold values indicate statistically significance.

exhibited a high discriminating value for T2DM-PAD. Both the SIRI (AUC 0.711, 95% CI: 0.663-0.760, P = 0.000, cut-off 0.95) and the NHR (AUC 0.703, 95% CI:0.655-0.751, P = 0.000, cut-off 3.44) had an AUC greater than 0.7 in the ROC analysis of T2DM-PAD. Additionally, the combination model of SIRI and NHR had an AUC of 0.733 (95% CI:0.686-0.779, P = 0.000, cut-off 0.57). Besides, the other indexes with AUCs greater than 0.6 were the MHR (AUC 0.685, 95% CI:0.636–0.735, P = 0.000, cut-off 0.33), AISI (AUC 0.670, 95% CI:0.619–0.721, P = 0.000, cut-off 218.94), SII (AUC 0.648, 95% CI:0.596–0.700, P = 0.000, cut-off 486.96) and PHR (AUC 0.606, 95% CI:0.553–0.658, P = 0.000, cut-off 166.47). The data are presented in Figure 2.

Discussion

Recently, it has been demonstrated that NHR, MHR, LHR, PHR, SII, SIRI, and AISI are novel inflammatory biomarkers and have significant clinical value due to easy access. Nevertheless, no evidence exists on the relationship between these inflammatory parameters and T2DM - PAD. To our knowledge, there is no published research on the association between SIRI, AISI, and diabetic patients. In this retrospective cross-sectional study, the association was first explored between seven novel serological indicators and T2DM-PAD patients. This study illustrated that NHR, MHR, PHR, SII, SIRI, and AISI were strongly associated with increased PAD prevalence in T2DM patients, and that all these indices were associated with disease severity. Additionally, the ROC curve analysis showed that NHR, SIRI, and their combination might predict the T2DM-PAD occurrence more effectively than other indexes.

The daily life of patients is often severely affected by PAD, imposing a substantial medical expense on individuals and society. Therefore, effective early screening and identification of T2DM - PAD patients is crucial (30). Evidence suggests that diabetes is one of the strongest risk factors for PAD development (31). Hence, only diabetic patients were chosen to be investigated in this study. Several potential

TABLE 4 Correlation of the inflammatory biomarkers with other parameters in the T2DM-PAD patients.

	NHR		MHR		PHR		SII		SIRI		AISI	
	r	p	r	p	r	p	r	p	r	p	r	p
Gender	-0.068	0.316	**-0.192**	**0.005**	0.093	0.169	-0.073	0.282	-0.238	**<0.001**	-0.119	0.078
Age	-0.024	0.772	-0.079	0.245	-0.133	0.050	-0.054	0.431	-0.023	0.740	-0.078	0.249
Diabetes duration	-0.200	0.773	-0.089	0.189	**-0.139**	**0.041**	-0.061	0.374	-0.041	0.547	-0.034	0.618
Fontaine classification	**0.275**	**<0.001**	**0.240**	**<0.001**	**0.188**	**0.005**	**0.186**	**0.006**	**0.227**	**<0.001**	**0.209**	**0.002**
Smoking	0.057	0.402	0.122	0.073	**-0.136**	**0.045**	-0.001	0.989	0.167	**0.013**	0.045	0.508
Alcohol	-0.027	0.697	**0.138**	**0.042**	**-0.179**	**0.008**	-0.089	0.188	0.116	0.089	0.020	0.770
CAD	-0.059	0.384	-0.083	0.223	**-0.271**	**0.001**	**-0.148**	**0.029**	-0.041	0.543	-0.122	0.072
Hypertension	0.051	0.454	0.038	0.577	-0.039	0.565	-0.066	0.331	0.023	0.738	-0.029	0.674
Hyperlipidemia	0.008	0.903	-0.034	0.532	-0.054	0.431	-0.039	0.566	-0.002	0.974	-0.031	0.649
SBP	0.082	0.226	-0.011	0.875	0.025	0.710	0.131	0.053	0.109	0.109	0.095	0.164
DBP	0.092	0.175	0.107	0.115	0.062	0.366	-0.004	0.949	0.031	0.650	0.024	0.725
Fasting glucose	-0.003	0.967	-0.078	0.253	0.024	0.723	0.046	0.503	-0.035	0.604	-0.009	0.896
HbA1c	0.031	0.646	-0.013	0.848	**0.143**	**0.034**	0.065	0.338	-0.044	0.516	0.031	0.647
TG	0.333	0.632	-0.042	0.541	0.169	**0.012**	-0.071	0.299	**-0.141**	**0.038**	-0.059	0.384
TC	**-0.207**	**0.002**	-0.305	**<0.001**	-0.133	**0.049**	0.032	0.633	-0.071	0.297	-0.016	0.814
HDL-C	–	–	–	–	–	–	-0.047	0.494	-0.043	0.530	-0.064	0.345
LDL-C	-0.065	0.341	-0.192	**0.004**	-0.072	0.293	0.094	0.165	0.000	0.998	0.035	0.604
CRP	**0.497**	**<0.001**	0.379	**<0.001**	0.378	**<0.001**	0.364	**<0.001**	**0.360**	**<0.001**	0.375	**<0.001**
TyG index	0.012	0.855	-0.068	0.319	0.124	0.069	-0.031	0.651	-0.115	0.090	-0.050	0.459
Neutrophil	–	–	0.455	**<0.001**	0.27	**<0.001**	–	–	–	–	–	–
Lymphocyte	**0.151**	**0.026**	0.280	**<0.001**	0.117	0.085	–	–	–	–	–	–
Monocyte	**0.498**	**<0.001**	–	–	**0.182**	**0.007**	**0.205**	**0.002**	–	–	–	–
Platelet	**0.347**	**<0.001**	0.204	**0.002**	–	–	–	–	**0.246**	**<0.001**	–	–

CAD, coronary artery disease; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycosylated hemoglobin; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; TyG index, triglyceride glucose index; NHR, neutrophil/HDL-C ratio; LHR, lymphocyte/HDL-C ratio; MHR, monocyte/HDL-C ratio; PHR, platelet/HDL-C ratio; SII, systemic immune-inflammation index; SIRI, system inflammation response index; AISI, aggregate index of systemic inflammation. P <0.05 (two-sided) was defined as statistically significant. Bold values indicate statistically significance.

biomarkers have been identified for PAD in diabetic patients, including HMGB 1, OPG, FGF 23, Omentin-1, Cyr61, and Sortilin (32–36). However, these biomarkers require complex and costly measurements and are not widely used in the clinic. The new inflammatory indicators presented in this work can be easily obtained using standard laboratory indices and may have substantial clinical use.

It is generally agreed that atherosclerosis is a chronic inflammatory disease of the arterial wall that stems from an insufficient inflammatory response and an imbalanced lipid metabolism. The role of inflammation and lipid metabolism in T2DM and PAD pathogenesis is of great interest (37, 38). Monocytes initiate and promote atherosclerosis progression by releasing pro-inflammatory cytokines, reactive oxygen species, and protein hydrolases (39). Neutrophils, the most abundant leukocyte subtype, exacerbate vessel wall inflammation *via* apoptosis of small muscle cells (40, 41). In contrast, lymphocytes can impair atherosclerosis progression (36). Platelets have a dual role in atherosclerosis: their adherence to the vascular wall promotes plaque formation (42), whereas their activation promotes inflammation and thrombosis (43). Elevated LDL and reduced HDL levels are key factors in atherosclerosis development and progression (44). The current results showed that PAD patients had significantly higher neutrophils, monocytes, and CRP and significantly fewer lymphocytes and HDL than WPAD patients, as well as a significant reduction in LDL, which could be hypothesized might be related to statin use. However, statin use was not an exclusion criterion, and the supplementary tables showed that the two groups of patients using statins had inflammatory indicators that were not significantly different (See Supplementary Tables 1, 2).

The MHR, NHR, PHR, and LHR have been investigated as novel inflammatory markers derived from peripheral blood cells and HDL-C in many systemic chronic inflammatory diseases. The SII, SIRI, and AISI are novel chronic low-grade inflammatory markers based on peripheral blood cells and platelets. The current results showed that NHR, MHR, PHR, SII, SIRI, and AISI were significantly higher in T2DM-PAD

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TABLE 5 Univariate and binary logistic regression analysis results.

	Variable OR (95% CI)	P	Variable OR (95% CI)	P
Age	1.15(1.11-1.18)	<0.001	1.12(1.09-1.16)	<0.001
Diabetes duration	1.13(1.09-1.67)	<0.001	1.1(1.06-1.15)	<0.001
Hypertension	0.48(0.33-0.7)	<0.001		
SBP	1.02(1.01-1.03)	<0.001		
LDL-C	0.83(0.69-1)	0.047		
CRP	1.09(1.04-1.14)	<0.001		
NHR	1.83(1.55-2.16)	<0.001	1.78(1.43-2.19)	<0.001
MHR	1.66 (1.41-1.95)	<0.001	1.45 (1.14-1.83)	<0.001
PHR	1.005(1.003-1.007)	<0.001	1.008(1.004-1.011)	<0.001
SII	1.001(1.002-1.003)	<0.001	1.002(1.001-1.003)	<0.001
SIRI	5.31 (3.27-8.62)	<0.001	3.84(2.15-6.84)	<0.001
AISI	1.005(1.003-1.007)	<0.001	1.005(1.003-1.007)	<0.001

SBP, systolic blood pressure; LDL-C, low-density lipoprotein cholesterol; CRP, C-reactive protein; NHR, neutrophil/HDL-C ratio; MHR, monocyte/HDL-C ratio; PHR, platelet/HDL-C ratio; SII, systemic immune-inflammation index; SIRI, system inflammation response index; AISI, aggregate index of systemic inflammation. P <0.05 (two-sided) was defined as statistically significant.

patients in WPAD patients and that they were significantly correlated with disease severity based on the Fontaine classification. Based on the ultrasound results of the lower limbs, we evaluated the degree of PAD disease and then categorized the patients with T2DM - PAD into three subgroups: mild, moderate, and severe. Individuals with severe PAD had higher concentrations of NHR, MHR, PHR, SII, SIRI, and AISI than those with mild PAD. Significant incremental increases were not reflected in the three sub-periods (See Supplementary Figure 1). These results were consistent with L. Santoro et al. that MHR was not associated with ultrasound grading in patients with only PAD (24), suggesting that these inflammatory indicators might be more appropriate for clinical application in combination with the Fontaine classification to assess disease severity. Spearman analysis showed that all indicators were positively associated with the Fontaine classification and CRP.

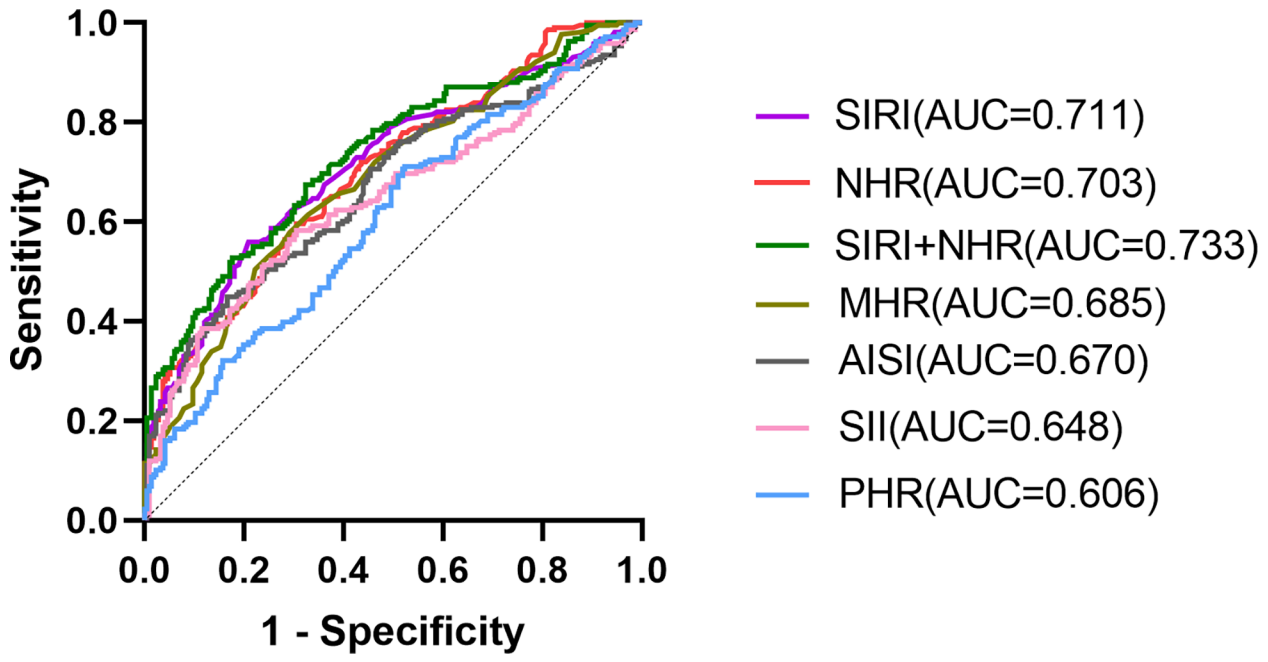

FIGURE 2
ROC curve analysis of the ability of these biomarkers to predict T2DM – PAD. SIRI (AUC 0.711, 95% CI:0.663–0.760, *P* = 0.000, cut-off 0.95, sensitivity 56%, specificity 79.2%); NHR (AUC 0.703, 95% CI:0.655–0.751, *P* = 0.000, cut-off 3.44, sensitivity 59.6%, specificity 69.4%); SIRI+NHR combined model (AUC 0.733, 95% CI:0.686-0.779, *P* = 0.000, cut-off 0.57, sensitivity 52.8%, specificity 82.4%); MHR(AUC 0.685, 95% CI:0.636–0.735, *P* = 0.000, cut-off 0.33, sensitivity 61.0%, specificity 70.4%);AISI(AUC 0.670, 95% CI:0.619–0.721, *P* = 0.000, cut-off 218.94, sensitivity45%, specificity83.3%);SII(AUC 0.648, 95% CI:0.596–0.700, *P* = 0.000, cut-off 486.96, sensitivity58.3%, specificity 69.4%);PHR (AUC 0.606, 95% CI:0.553–0.658, *P* = 0.000, cut-off 166.47, sensitivity71.1%, specificity 47.7%).

Meanwhile, NHR, MHR, SII, SIRI, and AISI were not correlated with the patients' age and disease duration, and PHR was weakly negatively correlated with the duration of diabetes. Although high glucose and insulin resistance enhance vascular inflammation (45), the data showed no significant correlation between these inflammatory markers and fasting glucose in T2DM-PAD patients or the total population, which might be due to sample size limits. The TyG index, a surrogate for insulin resistance, is significantly related to the gold standard hyperinsulinemic-orthoglycemic clamp (46) and can be a reliable assessment of insulin resistance in patients. Unfortunately, no correlation between TyG index and these indices was observed in the T2DM-PAD population. But in the total population, NHR (r=0.115), MHR (r=0.095), PHR (r=0.156) were found to be significantly correlated with TyG index (all P<0.05) (See Supplementary Table 3). Further large cohort studies are needed to analyze the relationship of these indicators with glycemic and insulin resistance. The univariate logistic regression showed that age, duration of diabetes, history of hypertension, SBP, CRP, NHR, MHR, PHR, SII, SIRI, and AISI were statistically significant. The multifactorial regression, excluding the effects of confounding factors, showed that NHR, MHR, PHR, SII, SIRI, and AISI were independently associated with T2DM-PAD. This study also demonstrated that age and duration of diabetes might be independent risk factors, consistent with previous studies (30). Combined with the Spearman correlation results, it was hypothesized that disease prediction by NHR, MHR, SII, SIRI, and AISI was not affected by age and duration of diabetes, increasing their clinical adjunctive value, but this hypothesis might require prospective cohorts to verify reliability. The ROC curve analysis showed that NHR, MHR, PHR, SII, SIRI, and AISI could predict T2DM-PAD well. The AUCs of MHR, AISI, SII, and PHR were over 0.6, and NHR and SIRI were over 0.7. The highest AUC (0.733) was detected when the NHR and SIRI were combined, indicating that it was better to use their combination for disease prediction in the clinic. When the combined model of NHR and SIRI value was greater than 0.57, it might suggest that the patient had a higher risk of developing PAD. This finding would serve as an easily available diagnostic aid for clinicians.

However, this current study also has some limitations. First, this was a retrospective cross-sectional study conducted in a single center, unable to determine the causal relationship between disease and indicators. Although these indices showed a good correlation with PAD, further studies are necessary to consider them as independent risk factors for the disease. Second, this research did not exclude patients on statins because neither group had significant differences in emerging inflammatory indicators on statins. Third, all patients' body mass index (BMI) data were not collected completely and BMI was not included in the analysis, so the possible effect of BMI as a confounding factor may had been overlooked. Fourth, the novel inflammatory indicators were not dynamically monitored. Therefore, whether their changes are related to PAD progression remains unknown. Further prospective studies are required to analyze whether the above indicators reduce atherosclerosis progression. SIRI effectively predicts MACE in patients undergoing percutaneous coronary intervention after acute coronary syndrome (14), and this predictive performance exceeds the neutrophil-lymphocyte ratio (NLR) and monocyte-lymphocyte ratio (MLR) (47). Prospective studies are needed to see if these indices have a similar effect in predicting MACE and MALE for PAD.

Data availability statement

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

Ethics Statement

The study protocol was approved by the ethics committee of the Liyuan Hospital, Tongji Medical College, Huazhong University ofScience and Technology. (Approval IRBID: [2022] IEC CRYJ 0018). All data used in this study were anonymized and the requirement forinformed consent was waived.

Author contributions

SJ and YiS conceived the study plan and contributed to the revision of the final manuscript. YiS collected, analyzed the data and finished the manuscript writing. YZ, YaS and LZ participated in data collection and literature search. WC, LW, MS, BX, RW, ZF, YY and FY contributed to the manuscript writing and data interpretation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

The reviewer Y-MW declared a shared affiliation with the authors to the handling editor at the time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1100453/full#supplementary-material

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The transcriptomic and epigenetic alterations in type 2 diabetes mellitus patients of Chinese Tibetan and Han populations

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Background: Due to the distinctive living environment, lifestyle, and diet, the Tibetan community in China has the lowest prevalence of T2DM and prediabetes among numerous ethnic groups, while Han community shows the highest statistic. In this study, we aim to conclude the clinical manifestations of both Tibetan and Han T2DM patients and their association with transcriptomic and epigenetic alterations.

Methods: A cross-sectional study including 120 T2DM patients from Han and Tibetan ethnic groups were conducted between 2019 to 2021 at the Hospital of Chengdu University of Traditional Chinese Medicine. The various clinical features and laboratory tests were recorded and analyzed between the two groups. The genome-wide methylation pattern and RNA expression were determined by Reduced Representation Bisulfite Sequencing (RBBS) and Poly (A) RNA sequencing (RNA-seq) from leucocytes of peripheral blood samples in 6 Han and 6 Tibetan patients. GO analysis and KEGG analysis were conducted in differentially expressed genes and those with differentially methylated regions.

Results: Compared to Han, Tibetan T2DM individuals intake more coarse grains, meat and yak butter, but less refined grains, vegetables and fruit. They also showed increased BMI, Hb, HbA1c, LDL, ALT, GGT and eGFR, and decreased level of BUN. Among the 12 patients in the exploratory cohort, we identified 5178 hypomethylated and 4787 hypermethylated regions involving 1613 genes in the Tibetan group. RNA-seq showed a total of 947 differentially expressed genes (DEGs) between the two groups, with 523 up-regulated and 424 down-regulated in Tibetan patients. By integrating DNA methylation and RNA expression data, we identified 112 DEGs with differentially methylated regions (overlapping genes) and 14 DEGs with promoter-related DMRs. The functional enrichment analysis demonstrated that the overlapping genes were primarily involved in metabolic pathways, PI3K-Akt signaling pathway, MAPK signaling pathway, pathways in cancer and Rap1 signaling pathway.

Conclusion: Our study demonstrates the clinical characteristics of T2DM differ subtly between various ethnic groups that may be related to epigenetic

modifications, thus providing evidence and ideas for additional research on the genetic pattern of T2DM.

KEYWORDS

DNA methylation, type 2 diabetes mellitus, Han, Tibetan, transcriptome

1 Introduction

The main features of type 2 diabetes mellitus (T2DM) include hyperinsulinemia, insulin resistance (IR) and islet cell damage, which can reach 50% at the time of diagnosis (1). With a high-energy diet, decreased physical activity, and an increase in obesity, the incidence of diabetes is rising globally, along with the rate of disability and mortality. People who have T2DM experience vascular and neurological consequences, as well as life, psychological, and financial stress. The diabetic population will predictably reach 147 million by 2045 (2). Most diabetes is a complex disease caused by a combination of multiple genes and environmental factors. Genetic factors are present in approximately 25% to 69% of people with T2DM worldwide (3) and over 560 genetic loci are identified to be relevant (4).

Epigenetics, including DNA methylation, histone modifications and microRNAs, lead to changes in gene function based on mitosis and meiosis without alteration in DNA sequence (5), in which DNA methylation has been recognized to be an important genetic factor contributing to T2DM (6). DNA methylation refers to the S-adenosyl methionine (SAM), as the methyl donor, transfers the activated methyl group to carbon 5 of the cytosine-phosphate-guanine (CpG) by the catalyzation of DNA methyltransferases (DNMTs). In general, gene expression is opposite to the level of methylation in the promoter region, which means low methylation levels result in up-regulation of gene expression, whereas high methylation results in down-regulation of expression (7, 8). As DNA methylation is reversible and can be interfered with, some chemicals can be used as targets to modify DNA methylation (9), providing a new perspective for T2DM treatment. Previous studies have shown that many genes are related to islet function, such as PDX1 (10), PPARGC1A (11), INS (12), GLP1R (13) and KCNQ1 (14), have been associated with the development of T2DM. Meanwhile, methylome-wide association studies (MWAS) for T2DM have identified differentially methylated sites (DMSs) in TXNIP (15), PHOSPHO1 (16), SREBF1 (17), ABCG1 (17), SOCS3 (18), and CPTA1 (19).

Environmental factors such as diet, exercise and obesity can also alter the epigenome. Tibetans are a distinct ethnic group in China that have historically lived at high altitudes. They primarily reside in the Tibetan Autonomous Region (TAR), as well as the provinces of Qinghai, Sichuan, Yunnan, and Gansu in China. Although highlanders had a lower incidence of diabetes, the number has quickly risen as a result of greater longevity and lifestyle changes (20, 21). According to nationwide research, the Han Chinese population had a 14.7% prevalence of diabetes and a 38.8% prevalence of prediabetes, whereas the Tibetan community had the lowest prevalence of both conditions at 4.3% and 31.3%, respectively (22). Lifestyle changes, particularly in calorie intake, are associated with the development of diabetes, possibly through epigenetic mechanisms (23, 24). This study aimed to demonstrate the differences in clinical characteristics between Tibetan and Han T2DM patients and to explore the transcriptomic and epigenetic alterations in the two groups.

2 Materials and methods

2.1 Study population

2.1.1 Cross-sectional cohort

A total of 60 Tibetan and 60 Han patients with T2DM were recruited at the Hospital of Chengdu University of Traditional Chinese Medicine from 2019 to 2021. All the patients were diagnosed with T2DM according to the 1999 WHO criteria (25). There was no kinship between the included study subjects and three consecutive generations for each patient are the same ethnic group. The exclusion criteria include 1) other types of diabetes; 2) having immune system diseases; 3) any types of tumors; 4) acute and chronic infections; 5) psychoneurological disorders; 6) recent use of drugs that affect lipid metabolism; 7) having liver or kidney failure or severe heart diseases; 8) disagreeing to participate in the study.

2.1.2 Subjects enrolled for exploratory cohort

Among the cross-sectional cohort, 6 Tibetan and 6 matched Han T2DM patients were selected for the exploratory cohort of RNA expression and DNA methylation. These two groups were selected by a matched pairs design based on shared characteristics including age, gender, weight, height and duration of T2DM to control lurking variables.

2.2 Clinical data collection

The general information for all patients included age, gender, body mass index (BMI), drinking and smoking history, family history of diabetes, the duration of T2DM, food intake, systolic blood pressure (SBP), diastolic blood pressure (DBP) and hemoglobin (Hb). In addition to HbA1c test, a standard 2-h OGTT test was performed by using a 75g glucose load to assess the patient's islet function and glycemic control. Plasma glucose and insulin level at 0 (fasting), 1, 2 and 3-hour postprandial blood glucose (PBG) were measured, and blood C-peptide was measured at 0 and 2-hour

postprandial. The biochemical analysis includes the total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), total bile acid (TBA), direct bilirubin (DBIL), blood creatinine (BCr), blood urea nitrogen (BUN), blood uric acid (BUA), were detected to estimate blood lipids, liver function, and kidney function. The Cockcroft-Gault equation was used to determine the estimated glomerular filtration rate (eGFR) (26).

2.3 Blood sample and DNA extraction

In the exploratory cohort, 3ml EDTA-treated peripheral blood sample of each participant was collected and stored in -80°C. Genomic DNA was extracted from peripheral blood using magnetic universal genomic DNA kit (TIANGEN Biotech (Beijing) co., Ltd). DNA concentration and quality were measured by Nanodrop.

2.4 Reduced representation bisulfite sequencing

1μg genomic DNA was digested using MspI enzyme for 16 hours at 37°C. After digestion, libraries were constructed as the Illumina Pair-End protocol with some modifications. Briefly, purified digested DNA was subsequently treated with a mix of T4 DNA polymerase, Klenow Fragment and T4 polynucleotide kinase to repair, blunt and phosphorylate ends. The DNA fragments were subsequently 3' adenylated using Klenow Fragment (3'-5' exo-) and following with ligation to adaptors synthesized with 5'-methylcytosine instead of cytosine using T4 DNA Ligase. the DNA was purified using QIAquick PCR purification kit (Qiagen) after reaction of each step. After purification, the library was subjected to 40°C for 30 min treatment in a thermo cycler with the lid heated at 57°C. After that, centrifuged the reaction mixture at 14,000 X g for 10 min and then transferred the supernatant into a new 0.2 ml PCR tube for the further bisulfite treatment, respectively. Bisulfite conversion treatment was performed using a ZYMO EZ DNA Methylation-Gold Kit (Zymo research, Irvine, CA, USA) according to the manufacturer's instructions. The final RRBS libraries were generated by PCR amplification using adapter compatible barcode primers, quantified by an Agilent 2100 Bioanalyzer (Agilent Technologies) and real-time PCR assay and then sequenced by Illumina Hiseq.

2.5 Methylation calculation and identification of DMRs

Low-quality reads that contained more than 5 'N's or had a low-quality value for over 50% of the sequence (Phred score< 5) were filtered. The sequencing reads of the samples were aligned to the human reference genome (hg19) using BSMAP (Version 2.74) (27). The methylated CpG (mCG) sites were identified following a previously described algorithm (28). The methylation levels for each sample were calculated using in-house Perl scripts. Differentially methylated regions (DMRs) were identified using metilene (Version 0.2-6) within a 500 bp sliding window at 250 bp steps with at least 10 CpGs covered by over 10× sequence reads, applying the thresholds of differential methylation $\beta \geq 15\%$, FDR for two-dimensional Kolmogorov-Smirnov-Test p-value< 0.05 (29). The enrichment analyses were conducted using WebGestalt (WEB-based Gene SeT Analysis Toolkit) (30).

2.6 RNA library construction and sequencing

Total RNA was extracted from cells using Trizol (Invitrogen) according to the manufacturer's protocol, and ribosomal RNA was removed using the Ribo-Zero™ kit (Epicentre, Madison, WI, USA). Fragmented RNA (the average length was approximately 200 bp) was subjected to first-strand and second-strand cDNA synthesis followed by adaptor ligation and enrichment with a low cycle according to instructions of NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NEB, USA). The purified library products were evaluated using the Agilent 2200 TapeStation and Qubit®2.0 (Life Technologies, USA). The libraries were paired-end sequenced (PE150, Sequencing reads were 150 bp) at Guangzhou MethylGene Co., Ltd. (Guangzhou, China) using the Illumina Xten platform.

2.7 Pre-processing of sequencing reads/ quality control

Raw fastq sequences were treated with Trimmomatic tools (v 0.36) using the following options: TRAILING: 20, MINLEN:235 and CROP:235, to remove trailing sequences below a Phred quality score of 20 and to achieve uniform sequence lengths for downstream clustering processes. Sequencing read quality was inspected using the FastQC software. Adapter removal and read trimming were performed using Trimmomatic. Sequencing reads were trimmed from the end (base quality less than Q20) and filtered by length (less than 25).

2.8 Quantification of gene expression level

Paired-end reads were aligned to the human reference genome (hg19) with HISAT2. HTSeq v0. 6.0 was used to count the numbers of reads mapped to each gene. The whole sample expression levels were presented as RPKM (expected number of Reads Per Kilobase of transcript sequence per Million base pairs sequenced), which is the recommended and most common method to estimate the level of gene expression.

2.9 Differential expression analysis

The statistically significant DE genes were obtained by an adjusted P-value threshold of<0.05 and |log2(fold change) | > 1 using the DEGseq software. Finally, a hierarchical clustering analysis was performed using the R language package gplots according to the RPKM values of differential genes in different groups. And colors

represent different clustering information, such as the similar expression pattern in the same group, including similar functions or participating in the same biological process.

2.10 GO terms and KEGG pathway enrichment analysis

All differentially expressed mRNAs were selected for GO and KEGG pathway analyses. GO was performed with KOBAS3.0 software, including cellular component (CC), molecular function (MF) and biological process (BP). GO provides label classification of gene function and gene product attributes (http://www.geneontology.org). GO analysis covers three domains: cellular component (CC), molecular function (MF) and biological process (BP). The differentially expressed mRNAs and the enrichment of different pathways were mapped using the KEGG pathways with KOBAS3.0 software (http://www.genome.jp/kegg).

2.11 Statistical analysis

The median and quartile were used for the statistical and data description of the normally distributed measures, and the number of cases (n) and percentages (%) were used for the statistical and data description of the categorical counts. Normality and homogeneity of all data were evaluated by Kolmogorov-Smirnov test. Student T-test or Mann-Whitney U test was applied to compare the differences of continuous variables. Pearson Chi-square test was employed to evaluate statistical differences of categorical variables. The Wilcoxon test was used to compare the continuous non-normally distributed variables between 6 Tibetans and 6 Hans in the exploratory cohort. Pearson correlation was used to identify the 14 overlapping genes and clinical characteristics with significant differences. All data were statistically analyzed by SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). Graphs were generated using Graphpad 7.0 software (GraphPad Software, Inc., San Diego, USA).

3 Results

3.1 The demographical and clinical characteristics between Tibetan and Han T2DM populations

A total of 120 participants were enrolled for the final analysis, including 60 Tibetans and 60 Hans. The patient flow chart is demonstrated in Figure 1. The basic and biochemical characteristics are shown in Table 1. Although no difference was observed in age, gender, and duration of T2DM, the BMI of Tibetans was significantly higher than Hans (26.08 vs 23.3, P = 0.017). Tibetans consume fewer refined grains (141.5 g/day vs 193.5 g/day, P< 0.001), vegetables and fruit (91 g/day vs 296.5 g/day, P< 0.001) than Han people, but they consume more coarse grains (171 g/day vs 63.5 g/day, P< 0.001), meat (181.5 g/day vs 100.5 g/day, P< 0.001), and yak butter (98.5 g/day vs 0 g/day, P< 0.001). Not surprisingly, the Hb level of Tibetans is higher than Hans (146.5 g/L vs 138.5 g/L, P< 0.001) due to the high-altitude, low-oxygen environment of Tibetan settlements. Despite similar BG, insulin, and C-peptide level, HbA1c of Tibetan T2DM patients was higher than Han patients (9.75% vs 8.65%, P = 0.001). Similarly, LDL level is significantly higher in Tibetan group compared to Han group (3.12 mmol/L vs 2.53 mmol/L, P = 0.002). Regarding the liver function, the blood tests also showed higher levels of ALT (30.5 IU/L vs 21.5 IU/L, P = 0.013), and GGT (38 IU/L vs 21 IU/L, P< 0.001). The level of BUN was lower (4.95 mmol/L vs 5.61 mmol/L, P = 0.002) and eGFR of Tibetan T2DM patients was statistically higher than Han patients (129.77 mL/min vs 96.5 mL/min, P< 0.001). There were no significant differences in other parameters of biochemical tests between the two groups.

A total of 12 patients with 6 in each group were selected by paired design for the exploratory cohort. The age ranged from 33 to 54 years

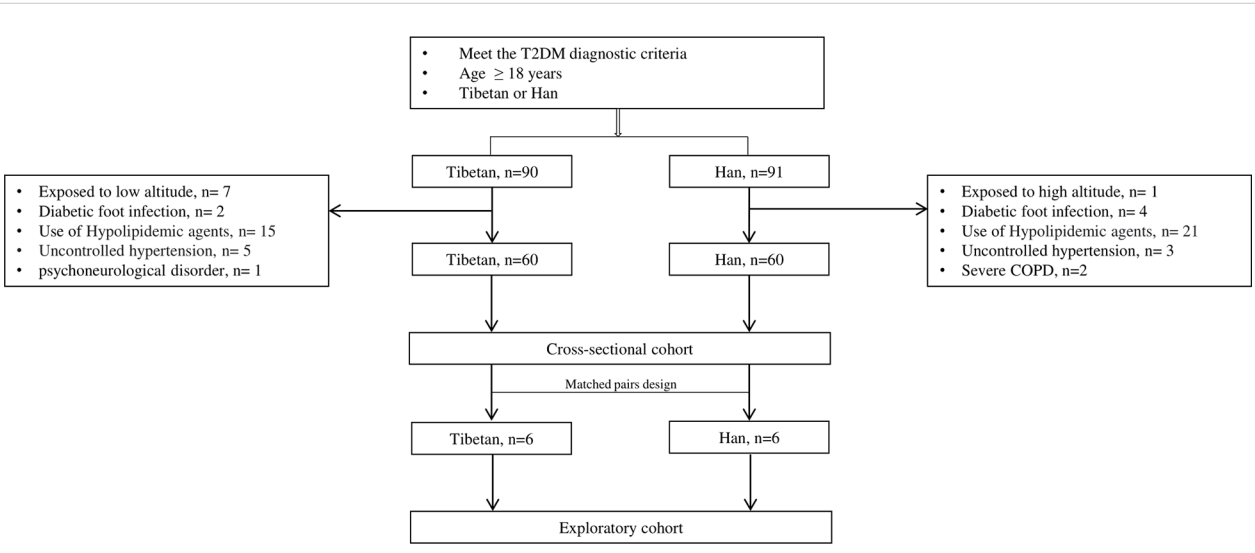

FIGURE 1
Flow chart of patient collection. COPD: chronic obstructive pulmonary disease.

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TABLE 1 Demographical and biochemical characteristics between Tibetan and Han T2DM patients in cross-sectional cohort.

	Tibetan (n=60)	Han (n=60)	P value
Age (years)	49 (42.25-60)	53 (45-63)	0.163
Male (female)	34 (56.7%)	31 (51.7%)	0.583
BMI (kg/m^2)	26.08 (23.63-28)	23.3 (21.88-25.19)	0.017*
Tabaco (n)	26 (43.3%)	22 (36.7%)	0.456
Alcohol (n)	31 (51.7%)	29 (48.3%)	0.715
Diabetes family history (n)	19 (31.7%)	22 (36.7%)	0.564
Duration of T2DM (years)	7 (3-11)	7 (3-12)	0.737
Food intake (g/day)			
Refined grains	141.5 (91.25-182.3)	193.5 (138.5-224.8)	<0.001*
Coarse grains	171 (46.5-80.75)	63.5 (46.5-80.75)	<0.001*
Meat	181.5 (142.3-219.8)	100.5 (74-121.8)	<0.001*
Vegetables and fruit	91 (71.25-110.8)	296.5 (231.3-377.5)	<0.001*
Yak butter	98.5 (65.75-126.8)	0 (0-7.25)	<0.001*
SBP (mmHg)	121.5 (110-133.25)	125 (117-143)	0.113
DBP (mmHg)	78 (70.25-85)	77.5 (70-85)	0.562
Hb (g/L)	146.5 (138.25-158)	138.5 (118.5-148.75)	<0.001*
HbA1c (%)	9.75 (8.23-11.8)	8.65 (7.13-10.63)	0.001*
FBG (mmol/L)	8.84 (7.37-8.84)	7.83 (5.99-9.42)	0.053
1-hr PBG (mmol/L)	15.26 (12.99-17.95)	15.86 (13.29-17.54)	0.836
2-hr PBG (mmol/L)	17.84 (15.55-20.75)	17.71 (14.97-20.98)	0.836
3-hr PBG (mmol/L)	16.99 (13.3-19.1)	16.58 (13.09-20.36)	0.774
0-hr Insulin (mIU/L)	7.64 (4.15-11.93)	7.06 (4.19-11.31)	0.661
1-hr Insulin (mIU/L)	21.46 (11.56-38.04)	25.35 (15.23-43.23)	0.183
2-hr Insulin (mIU/L)	25.08 (13.41-42.01)	29.71 (16.57-50.18)	0.062
3-hr Insulin (mIU/L)	18.99 (11.53-34.71)	23.89 (15.25-50.18)	0.317
0-hr C-peptide (nmol/L)	0.77 (0.59-1.05)	0.66 (0.51-0.97)	0.863
2-hr C-peptide (nmol/L)	1.4 (1.14-2.1)	1.71 (1.16-2.59)	0.171
TC (mmol/L)	4.55 (3.98-5.17)	3.96 (3.49-5.16)	0.170
TG (mmol/L)	1.28 (0.99-2.18)	1.45 (1.05-2.13)	0.601
HDL (mmol/L)	0.93 (0.85-1.09)	1.05 (0.85-1.34)	0.051
LDL (mmol/L)	3.12 (2.6-3.7)	2.53 (1.9-3.18)	0.002*
ALT (IU/L)	30.5 (17.75-39)	21.5 (16-28.75)	0.013*
AST (IU/L)	19.5 (15-25.25)	20.5 (17-23)	0.812
ALP (IU/L)	79 (64-104.25)	82 (64.25-100)	0.636
GGT (IU/L)	38 (23.75-59.5)	21 (16-30)	<0.001*
TBA (μmol/L)	3.2 (2.25-6.75)	4.55 (2.93-7.83)	0.051
DBIL (μmol/L)	12.65 (9.85-17.8)	11.95 (9.2-16.5)	0.486
BCr (μmol/L)	59.9 (55-66.8)	59.55 (49-69.78)	0.706

(Continued)

TABLE 1 Continued

	Tibetan (n=60)	Han (n=60)	P value
BUN (mmol/L)	4.95 (3.76-6.04)	5.61 (4.85-7.28)	0.002*
BUA (μmol/L)	299 (258-378)	308.5 (251.75-390)	0.894
eGFR (mL/min)	129.77 (97.81-155.2)	96.5 (77.87-117.1)	<0.001*

BMI, body mass index; SBP, Systolic blood pressure; DBP, diastolic blood pressure; Hb, hemoglobin; HbA1c, hemoglobin A1c; FBG, fasting blood glucose; PG, post-prandial blood glucose; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; TBA, total bile acid; DBIL, direct bilirubin; BCr, blood creatinine; BUN, blood urea nitrogen; BUA, blood uric acid; eGFR, estimated glomerular filtration rate. * P<0.05

old and the duration of T2DM ranged from 2 to 7.1 years. As shown in Supplementary Table S1, there were no significant differences in basic and biochemical parameters except HbA1c (9.9% vs 9%, P = 0.046), FBG (8.48 mmol/L vs 9.99 mmol/L, P = 0.028), 3-hr Insulin (12.11 mIU/L vs 21.07 mIU/L, P = 0.046), HDL (0.94 mmol/L vs 1.17 mmol/L, P = 0.046) and eGFR (143.7 mL/min vs 97 mL/min, P = 0.028).

3.2 Differentially methylated positions and regions

The whole-genome DNA methylation was detected by RRBS using peripheral blood samples from 6 Tibetan and 6 Han T2DM patients. After sulphite treatment, the conversion efficiency of all samples ranged from 98.82% to 99.27%. About 80% to 90% mCs were CG dinucleotides while about 10% to 20% were at CHG and CHH sites (G = A, C or T) (Supplementary Figure S1). Additionally, the methylation level of mC was around 80% to 100% while mCHG and mCHH were around 0% to 20%, with 20% as an interval (Supplementary Figure S2). We also explored the methylation levels in different genome regions. The level of methylation decreased in the 2kb upstream of transcription initiation but rose sharply in the exon region and reaches a maximum in the intron and 2kb downstream of genes (Supplementary Figure S3). The DMRs were mainly located in the intergenic region, accounting for 38.83%, followed by intron (32.15%) and exon regions (10.38%), respectively, in addition to 6.7% of DMRs within the gene promoter region (upstream 2kb) (Figure 2A). PCA found distinct clusters for study subjects (Figure 2B). The heatmap (Figure 2C) and volcano map (Figure 2D) have demonstrated the methylation difference between the two groups. Compared with Han group, we identified 5178 hypomethylated regions and 4787 hypermethylated regions in Tibetans (Table 2).

We performed GO functional analysis according to DMR-related genes, which were mostly enriched in protein binding (BP), nucleus (CC), cytoplasm (CC) and membrane (CC) (Figure 3A). KEGG analysis showed that DMR-related genes are mainly involved in metabolic pathway, pathways in cancer, cAMP signaling pathway, HTLV-I infection, cytokine-cytokine receptor interaction, calcium signaling pathway, alcoholism, regulation of actin cytoskeleton, hippo signaling pathway, Wnt signaling pathway, non-alcoholic fatty liver disease (NAFLD), insulin secretion, glycerophospholipid metabolism and type 2 diabetes mellitus (Figure 3B).

3.3 Transcriptome analysis

We conducted RNA-seq on peripheral blood samples from Han and Tibetan T2DM patients in order to investigate the relationship between DNA methylation and gene expression. Each sample produced about 8 giga bases (Gb) of filtered data. Additionally, using HISAT2 software, sequencing data were compared to the human reference genome with an average match rate of 90.2% per sample and an average unique mapping rate of 86.95% (Supplementary Table S2). Gene expression levels are calculated by RPKM as the number of reads per kilobase length from a given gene per million reads, and are calculated as $RPKM = \frac{Total\ exon\ reads}{Mapped\ reads \times exon\ length}$ (31).

A volcano map of significantly differentially expressed genes (DEGs) was created by differential gene expression analysis using the DESeq program, with 523 genes significantly up-regulated and 424 genes significantly down-regulated in the Tibetan group compared to the Han group (Figure 4A). The heat map revealed distinct gene expression patterns in the Tibetan and Han populations (Figure 4B).

Functional annotation showed that the most represented GO categories for DEGs were extracellular (CC), receptor-mediated endocytosis (BP), xenobiotic metabolic process (BP), negative regulation of endopeptidase activity (BP) and cellular response to hormone stimulus (BP) (Figure 5A), while KEGG enrichment analysis showed that the upregulated DEGs were mainly involved in steroid hormone biosynthesis, retinol metabolism, drug metabolism-cytochrome P450, PI3K-AkT signaling pathway, pentose and glucuronate interconversions, starch and sucrose metabolism, ascorbate and aldarate metabolism (Figure 5B).

3.4 Integrative analysis of transcriptome and DNA methylation

In general, gene expression is negatively correlated with DNA methylation. We divided each sample into four categories, including silence, low expression, medium expression, and high expression, according to the amount of gene expression and counted the methylation levels in the gene regions of each of the four categories of genes in a single sample. Our results showed DNA methylation was negatively correlated with gene expression in regions within 1k upstream of the gene, and genes with high methylation status were not expressed or were under-expressed (Figure 6).

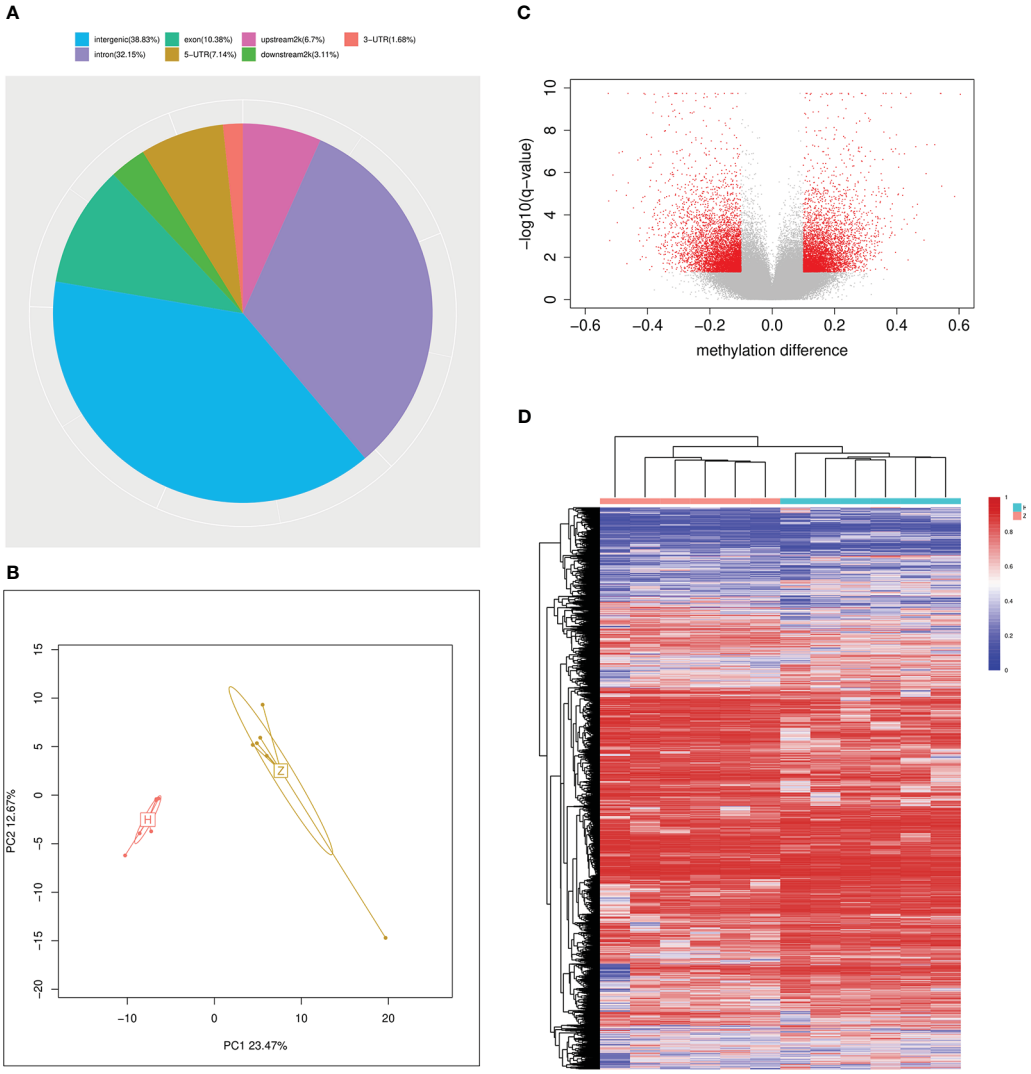

FIGURE 2
Summary of DMRs between Han and Tibetan T2D patients **(A)** The overall distribution of DMRs. **(B)** The principal component analysis plot using the differential methylated CpG sites between Han and Tibetans. **(C)** Volcano plot of methylation difference between Han and Tibetans. A total of 4787 CpG sites hypermethylated in Tibetans was represented by red point in the right side. A total of 5178 CpG sites hypomethylated in Tibetans was represented by red point in the left side. **(D)** Heatmap clustering analysis of DMRs of different gene functional regions. Highly methylated sites are shown in red and sparsely methylated sites are shown in blue. In addition, the pink clusters represent Tibetans and the blue clusters represent Han Chinese. H: Han, Z: Tibetan.

We observed 112 overlapping DEGs and DMR genes, of which 14 were promoter-related genes (Table 3). The GO enrichment analysis showed that the most significant enriched GO terms of overlapped genes are integral component of membrane (CC), plasma membrane (CC), homophilic cell adhesion *via* plasma membrane adhesion molecules (BP), calcium ion binding (BP) (Figure 7A).

According to KEGG enrichment analysis, these overlapping genes were primarily involved in metabolic pathway, including metabolism of xenobiotics by cytochrome P450, steroid hormone biosynthesis, retinol metabolism, ascorbate and aldarate metabolism, pentose and glucuronate interconversions, porphyrin and chlorophyll metabolism, drug metabolism, starch and sucrose metabolism, chemical carcinogenesis, PI3K-Akt signaling pathway, MAPK signaling pathway, pathways in cancer and Rap1 signaling pathway (Figure 7B). The relationship between overlapping genes and significant clinical characteristics was analyzed by Pearson correlation analysis. We found that the HbA1c was associated with the expression of *RHOD* (R = 0.697, P< 0.05), *LOC100134868* (R = -0.697, P< 0.01) and *LOC102723828* (R = -0.661, P< 0.05); FBG was negatively associated with *APOB* (R = -0.631, P< 0.05); HDL was positively associated with *PAX8-AS1* (R = 0.615, P< 0.05); and eGFR was related with *FOXA* (R = 0.794, P< 0.01) and *UMODL1-AS1* (R = 0.662, P< 0.05). In addition, insulin levels at three hours after 75g glucose load test showed positive association with the expression of *MIXL1*, *OXCT2*, *LAMA5-AS1*, *LOC100134868* and *LOC102723672* while negatively related to *AJAP1*, as shown in Supplementary table S3.

TABLE 2 The numbers and length of differentially methylated regions.

Type	Number of DMRs	Number of cytosine	Length of DMR region
HypoDMR	5178	49,492	1,087,603
HyperDMR	4787	45,966	1,000,093

DMRs, differentially methylated regions; Hypo, hypomethylated; Hyper, hypermethylated.

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TABLE 3 Overlapped genes of DEGs and promoter related DMR genes.

Gene	Location	log_2 fold change	P value	Description	Gene type	Methylation H-Z
AJAP1	Chr1	1.689	0.016	adherens junctions associated protein 1	Protein coding	0.206577
APOB	Chr2	2.284	0.018	apolipoprotein B	Protein coding	0.1933
COL1A1	Chr17	1.516	0.025	collagen type I alpha 1 chain	Protein coding	0.35821
FOXA1	Chr14	3.105	0.006	forkhead box A1	Protein coding	0.119007
MIXL1	Chr1	-4.042	0.000	Mix paired-like homeobox	Protein coding	-0.17532
MYCN	Chr2	1.648	0.021	MYCN proto-oncogene, bHLH transcription factor	Protein coding	0.10136
OXCT2	Chr1	-1.395	0.040	3-oxoacid CoA-transferase 2	Protein coding	-0.19075
RHOD	Chr11	1.386	0.030	ras homolog family member D	Protein coding	0.14237
LAMA5-AS1	Chr20	-2.823	0.039	LAMA5 antisense RNA 1	LncRNA	-0.13274
LOC100134868	Chr20	-2.221	0.001	uncharacterized LOC100134868	LncRNA	-0.19718
LOC102723672	Chr7	-1.591	0.015	uncharacterized LOC102723672	LncRNA	-0.19339
LOC102723828	Chr4	-3.703	0.006	None	LncRNA	-0.11715
PAX8-AS1	Chr2	-1.557	0.045	PAX8 antisense RNA 1	LncRNA	-0.12671
UMODL1-AS1	Chr21	2.228	0.033	UMODL1 antisense RNA 1	LncRNA	0.11357

Methylation H-Z: the methylation level of Han minus that of Tibetan T2DM patients. H: Han, Z: Tibetan.

4 Discussion

Our study reported the clinical characteristics of Han and Tibetan T2DM patients, indicating that the same disease has clinical differences between various ethnic groups and providing evidence for clinical individualization of T2DM treatment. We also revealed for the first time the differences in DNA methylation and RNA expression between Tibetan and Han T2DM patients, and synthesized the relationship between them, which provides a basis for further exploration of T2DM development mechanisms and identification of therapeutic targets.

Tibetans live in a high altitude, low oxygen, low temperature environment. Previous studies have shown that in a healthy population of Han Chinese and Tibetans living at the same altitude, the hemoglobin concentration of highland Han is higher than that of Tibetans (32). However, Han Chinese living at lower altitudes have lower hemoglobin concentrations (33), which is consistent with our results. The diet of Tibetans consists mainly of coarse grains, meat, yak butter and other high-fat, high-calorie, high-protein foods, thus have a higher BMI. However, no differences were shown in other lipid indicators in our cross-sectional cohort, except for higher LDL level in the Tibetan T2DM group, which indicates that Tibetans may have higher metabolism.

The Han and Tibetan populations also showed slight differences in liver function, with Tibetans having higher ALT and GGT levels,

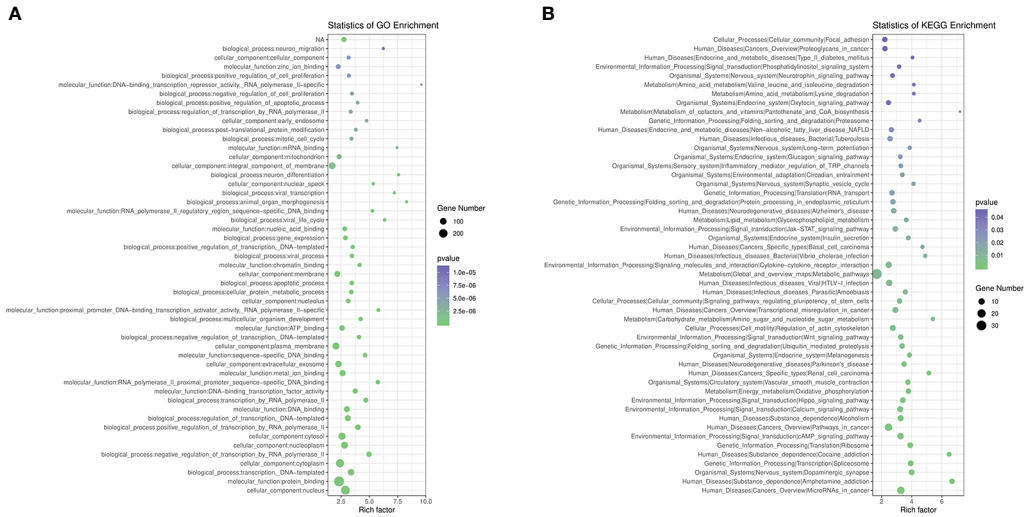

FIGURE 3
Pathway Analysis on DMR-related genes. **(A)**, GO analysis of DMR-related genes. **(B)**, KEGG analysis of DMR-related genes.

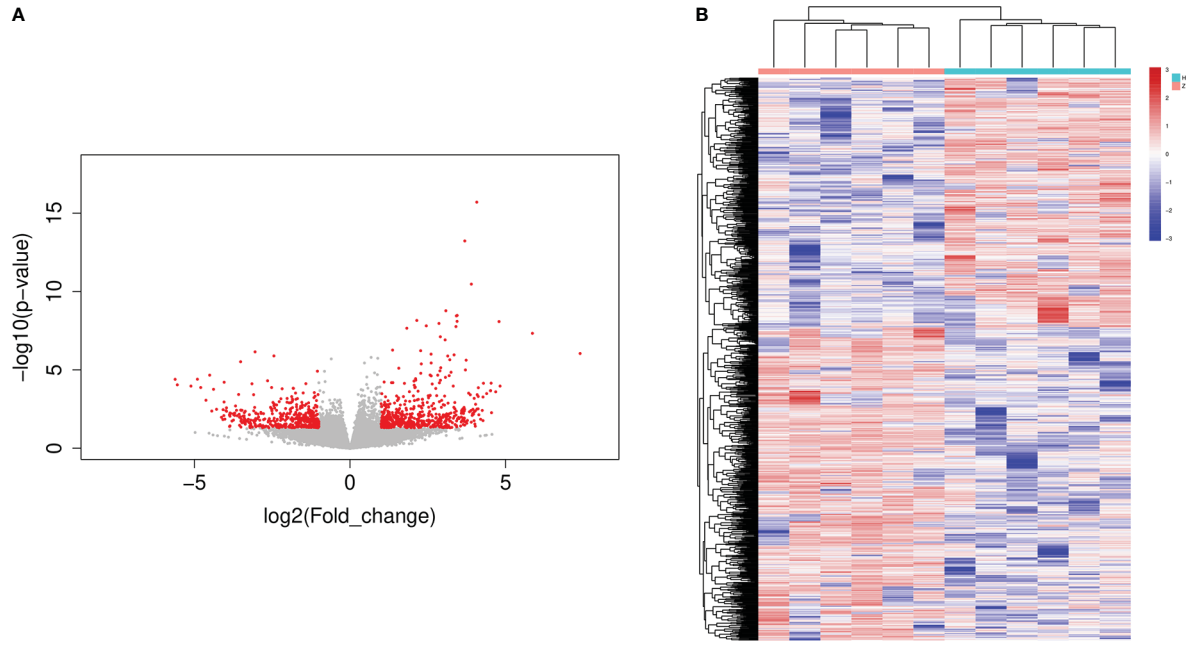

FIGURE 4
The volcano plot and heatmap of DEGs in Han and Tibetan T2D patients. **(A)** Volcano plot of DEGs. The x-axis represents the log2 fold change and the y-axis represents the log10 (P-value). The green dots represent downregulated genes and red dots represent upregulated genes. **(B)** Heat map of DEGs following clustering analysis. The vertical axis represents the sample, and the horizontal axis represents DEGs. Up: the number of up-regulated genes, down: the number of down-regulated genes, H: Han, Z: Tibetan.

but both in the normal range. Aminotransferases are considered indicators of hepatocyte health, and GGT also reflects biliary tract function. Elevated ALT is associated with age, obesity, elevated triglyceride levels, and low HDL cholesterol levels, but not with glycemic control (34). However, independent of common risk factors, ALT (35, 36) and GGT (37) are linked to an increase in the risk of T2DM. Although the eGFR level of Tibetan T2DM patients was higher and the BUN was lower than that of Han Chinese, both were at normal levels.

In the exploratory cohort, we further investigated the differences in DNA methylation and transcriptome between Han and Tibetan populations to interpret the differences in the development of T2DM between the two groups through a genetic perspective. The CpG island is a region of the DNA sequence rich in CpG sites, usually

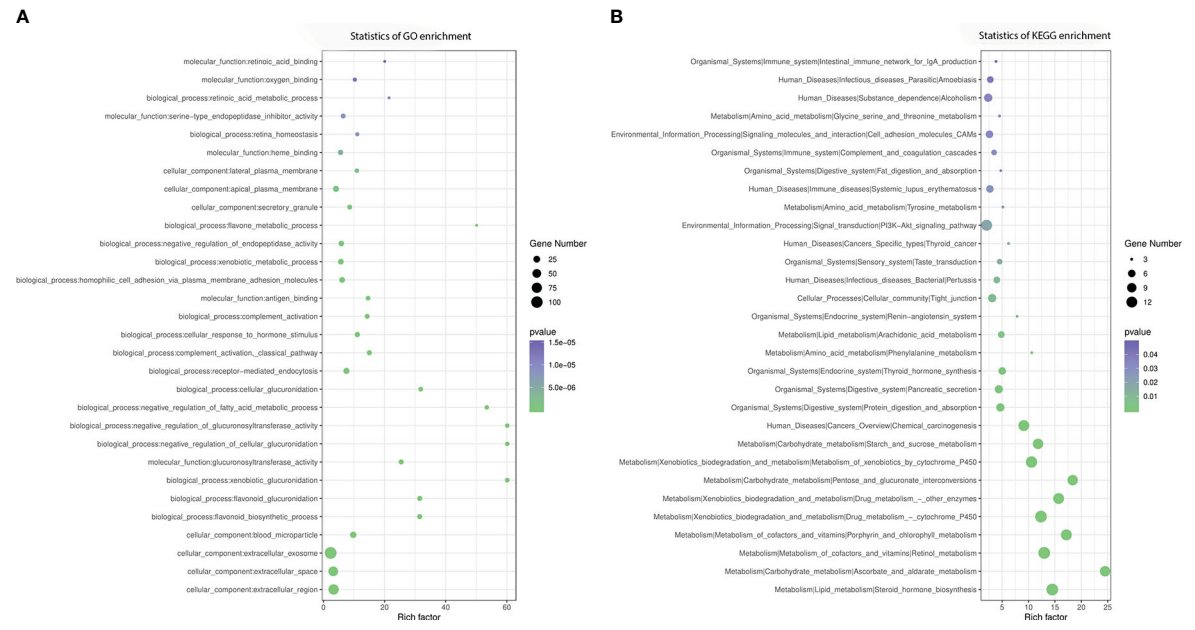

FIGURE 5
GO and KEGG enrichment analysis of DEGs. **(A)** GO analysis of differentially expressed genes, **(B)** KEGG analysis of differentially expressed genes.

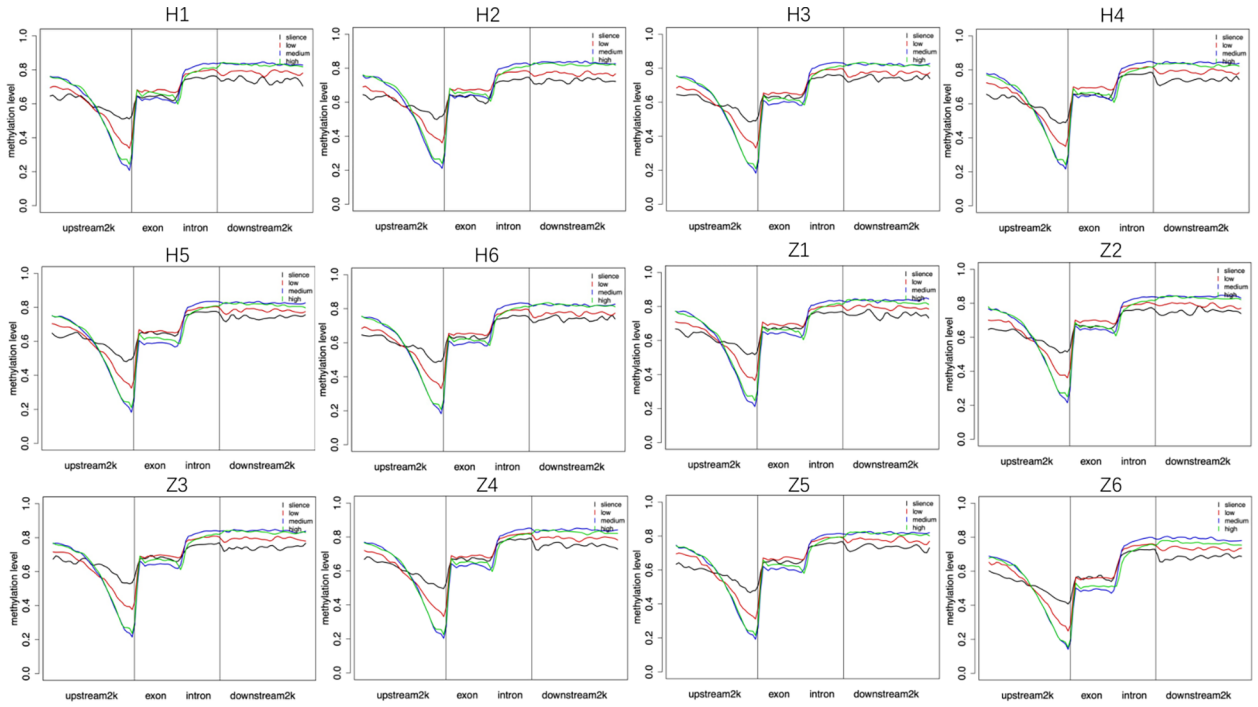

FIGURE 6
Relationship between DNA methylation and gene expression. Silence (RPKM=0), low: low expression (0<RPKM ≤ 1), medium: medium expression (1<RPKM ≤ 10), high: high expression (RPKM>10). H: Han, Z: Tibetan.

located in the promoters with an unmethylated state. When CpG islands are methylated, transcription factors become impaired in binding to promoters or bind to transcriptional repressors, altering the structure of chromatin. As a result, gene expression is altered without the changes of DNA sequence, affecting biological processes and leading to diseases (38, 39).

In our study, a total of 1613 genes with DMRs were found between Han Chinese and Tibetan T2DM patients. After GO and KEGG functional enrichment analysis, we identified signaling pathways that affect metabolism and other pathways that may play a key role in the development of T2DM, such as insulin secretion. Among them, cAMP signaling pathway, Wnt signaling pathway, and Hippo signaling pathway were more significant and relevant. cAMP is an intracellular mediator of insulin and adrenal glycogen catabolism in the liver (40). In mammals, cAMP activates cAMP-dependent protein kinase (PKA), which phosphorylates downstream protein targets and then regulates the function of ion channels, transcription factors and enzymes. Meanwhile, the cAMP signaling pathway regulates glucose homeostasis due to insulin secretion, glucose utilization, and glycogen synthesis and catabolism (41). The Wnt signaling plays an important role as an evolutionary pathway in regulating cellular homeostasis and energy homeostasis from the hypothalamus to the metabolic organs. The classical Wnt as well as non-classical Wnt pathways inhibit metabolism and lead to increased adipose tissue, resulting in metabolic stress and metabolic inflammation and obesity (42). The Hippo signaling pathway plays a role in pancreatic, hepatic, adipose and cardiac cells as well as in systemic metabolism, regulating glucolipid metabolism. Activation of the Hippo signaling pathway in hyperglycemic states induces proliferation and differentiation of pancreatic β-cells, increasing glucose uptake and utilization, thereby reducing insulin resistance, and improving insulin secretion (43).

In general, gene expression follows an opposite trend to the level of methylation in the promoter region. In the present study, we identified 947 differentially expressed genes, of which 112 overlapping genes had differential methylation levels, and a total of 14 genes with differentially methylated regions in the promoter region. Among the differentially expressed genes found to be differentially methylated in promoter regions, *APOB* encodes apolipoprotein B and is associated with LDL, celiac and LDL structural integrity, in lipid digestion, mobilization as well as transport (44). A study on the amount of non-insulin-dependent diabetic patients showed that *APOB* polymorphisms were effective in improving blood glucose and lipid levels of T2DM patients (45). *PAX8-AS1* is a non-coding RNA that is involved in the pathology of the disease despite its inability to encode protein synthesis. In a study examining non-coding RNA in leukocytes from patients with gestational diabetes mellitus (GDM), *PAX8-AS1* expression levels were significantly lower in GDM patients compared to healthy pregnant women and could be used as a diagnostic biomarker for GDM (46). The rest of key genes need to be further studied in the future.

Similar to previously described, KEGG analysis was mainly enriched in metabolic pathways that are related to IR or diabetes, including metabolism of xenobiotics by cytochrome P450 (47), steroid hormone biosynthesis (48), retinol metabolism (49), ascorbate and aldarate metabolism (50), pentose and glucuronate interconversions (51), starch and sucrose metabolism. Several canonical pathways outstood among the statistics, including PI3K-Akt pathway, MAPK pathways and Rap1 signaling pathway. Insulin secretion activates PI3K-Akt signaling pathway throughout the body

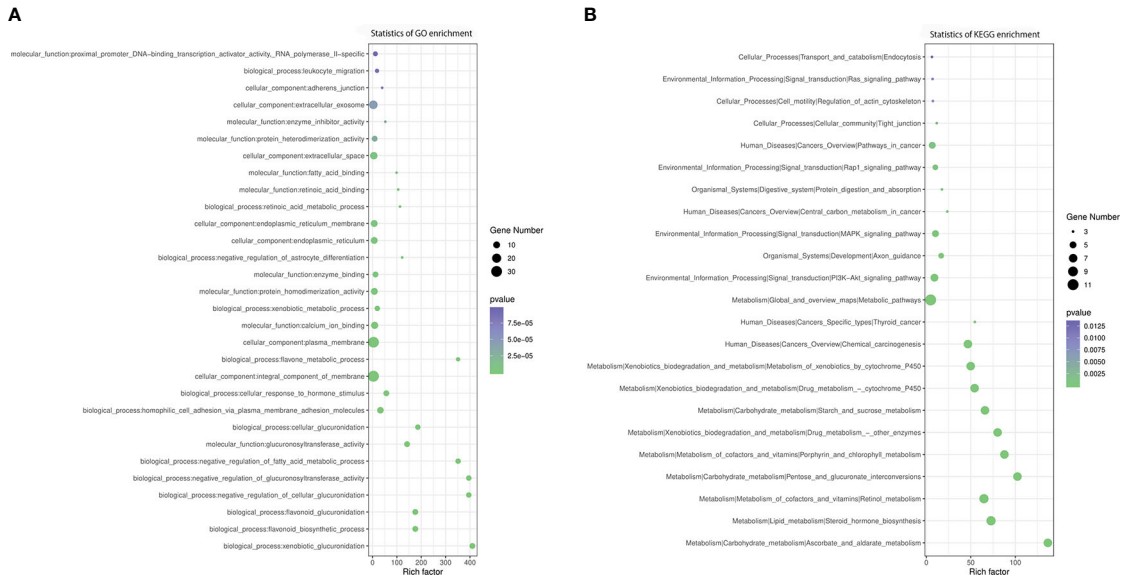

FIGURE 7
GO and KEGG enrichment analysis of DMR-related DEGs. **(A)** GO analysis of DMR-related DEGs. **(B)** KEGG analysis of DMR-related DEGs.

to increase glucose utilization, reduce glucose metabolism in the liver and muscle, and regulate the balance of lipid and glucose metabolism. However, impairment of this pathway leads to insulin resistance, which in turn worsens this pathway, leading to T2DM (52). In addition, insulin can also activate MAPK pathways but inappropriate MAPK signaling contributes to the development of metabolic syndrome and T2DM (53). An *in vitro* study showed that activated Rap1 is a key regulator of β-cell function, as evidenced by the promotion of glucose-stimulated insulin production, islet cell hypertrophy, and islet cell proliferation by activated Rap1A (54).

The overlapping genes primarily are associated with metabolism and insulin-related pathways, suggesting that the environment and lifestyle, such as diet, may play a role in altering DNA methylations levels, therefore affecting metabolism and insulin secretion and utilization in T2DM patients.

Conclusion

As the prevalence of T2DM varies in different ethnic groups in China, our study revealed the diverse clinical features of Tibetan and Han T2DM patients. The epigenetic and transcriptional patterns have provided a perspective on the mechanisms of T2DM in different ethnic groups, and the key genes are worthy to be further studied to reveal the importance of DNA methylation for the development of T2DM.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, accession number: PRJNA911064.

Ethics statement

The studies involving human participants were reviewed and approved by Hospital of Chengdu University of Traditional Chinese Medicine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JL and XW conceived of the presented idea, conducted the study of cross-sectional cohort. XW carried out the exploratory study of epigenome and transcriptome. QC and QW supervised the project. All the authors contributed to the final version of the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1122047/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The proportion of different types of methylated cytosines in 12 samples In the pie chart, red, green and blue indicate mCG, mCHH and mCHG, respectively. H: Han, Z: Tibetan

SUPPLEMENTARY FIGURE 2
Distribution of mC levels in mCG, mCH and mCHH. The figure showed the distribution of mC levels in 12 samples. The X axis showed the methylation level, and the Y axis showed the percentage of mC. Red, blue and green lines represent CG, CHG and CHH, respectively. H: Han, Z: Tibetan

SUPPLEMENTARY FIGURE 3
Distribution of DNA methylation levels in each gene elements. Red, blue and green lines represent CG, CHG and CHH, respectively. H: Han, Z: Tibetan

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Correlation of multiple lipid and lipoprotein ratios with nonalcoholic fatty liver disease in patients with newly diagnosed type 2 diabetic mellitus: A retrospective study

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Background and objective: The diagnostic value of lipid and lipoprotein ratios for NAFLD in newly diagnosed T2DM remains unclear. This study aimed to investigate the relationships between lipid and lipoprotein ratios and the risk of NAFLD in subjects with newly diagnosed T2DM.

Methods: A total of 371 newly diagnosed T2DM patients with NAFLD and 360 newly diagnosed T2DM without NAFLD were enrolled in the study. Demographics variables, clinical history and serum biochemical indicators of the subjects were collected. Six lipid and lipoprotein ratios, including triglycerides to high-density lipoprotein-cholesterol (TG/HDL-C) ratio, cholesterol to HDL-C (TC/HDL-C) ratio, free fatty acid to HDL-C (FFA/HDL-C) ratio, uric acid to HDL-C (UA/HDL-C) ratio, low-density lipoprotein-cholesterol to HDL-C (LDL-C/HDL-C) ratio, apolipoprotein B to apolipoprotein A1 (APOB/A1) ratio, were calculated. We compared the differences in lipid and lipoprotein ratios between NAFLD group and non-NAFLD group, and further analyzed the correlation and diagnostic value of these ratios with the risk of NAFLD in the newly diagnosed T2DM patients.

Results: The proportion of NAFLD in patients with newly diagnosed T2DM increased progressively over the range Q1 to Q4 of six lipid ratios, including the TG/HDL-C ratio, TC/HDL-C ratio, FFA/HDL-C ratio, UA/HDL-C ratio, LDL-C/HDL-C ratio, and APOB/A1 ratio. After adjusting for multiple confounders, TG/HDL-C, TC/HDL-C, UA/HDL-C, LDL-C/HDL-C and APOB/A1 were all strongly correlated with the risk of NAFLD in patients with newly diagnosed T2DM. In patients with newly-onset T2DM, the TG/HDL-C ratio was the most powerful indicator for the diagnosis of NAFLD among all six indicators, with an area under the curve (AUC) of 0.732 (95% CI 0.696–0.769). In addition, TG/HDL-C ratio>1.405, with a sensitivity of 73.8% and specificity of 60.1%, had a good diagnostic ability for NAFLD in patients with newly diagnosed T2DM.

Conclusions: The TG/HDL-C ratio may be an effective marker to help identify the risk of NAFLD in patients with newly diagnosed T2DM.

KEYWORDS

lipid ratios, lipoprotein ratios, TG/HDL-C ratio, NAFLD, newly diagnosed T2DM

Introduction

Non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) have a strong bidirectional association, and the prevalence of both is increasing simultaneously (1, 2). A recent meta-analysis reported the global prevalence of NAFLD in patients with T2DM was 55.5% (2). Moreover, the global prevalence of T2DM in patients with NAFLD and non-alcoholic steatohepatitis (NASH) patients was 22.51%, and 43.63%, respectively (3). There is now growing evidence that patients with T2DM combined with NAFLD tend to have poorer glycemic control than T2DM patients without NAFLD, and are at higher risk of developing NASH, cirrhosis or even hepatocellular carcinoma compared to NAFLD patients without T2DM (4). On the other hand, the incidence of chronic diabetic complications, such as cardiovascular disease (CVD), chronic kidney disease (CKD) and retinopathy, is also significantly higher in patients with T2DM combined with NAFLD than in those without combined NAFLD (4, 5).

Liver biopsy is the gold standard for the diagnosis of NAFLD and NASH cirrhosis. However, in clinical practice, the invasiveness, poor acceptability and high cost of liver biopsy make it difficult to use for widespread screening in the general population (6, 7). Conventional ultrasonography is commonly used for screening and diagnosis of NAFLD (7). However, due to the large number of patients with T2DM, routine liver ultrasound screening in all T2DM patients requires extremely expensive medical expenses. In addition, a large number of rural health centers or community hospitals lack ultrasound equipment and qualified ultrasonographers. Therefore, several previous studies have pinned hopes for early screening of patients with NAFLD on various serum markers (8–13). However, to date, no serum marker has become an accepted diagnostic indicator for NAFLD.

It is well known that serum biochemical indices of routine physical examination include liver enzymes and blood lipids. Previous studies have shown that liver enzyme levels are not useful for screening for NAFLD as their changes do not necessarily correspond to the degree of hepatic steatosis (14). Dyslipidemia, including increases in triglycerides (TG), cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C) and decreases in high-density lipoprotein-cholesterol (HDL-C), is strongly associated with NAFLD (11, 13, 15, 16). Several current data have indicated that lipid and lipoprotein ratios are more valuable than individual lipid values in predicting the risk of T2DM or NAFLD because they can reflect the interaction between lipid components (11–13, 15–17). Among them, the ratios of TG to HDL-C (TG/HDL-C) (12, 13), TC to HDL-C (TC/HDL-C) (11), uric acid (UA) to HDL (UA/HDL-C) (9), LDL-C to HDL-C (LDL-C/HDL-C) (16) and apolipoprotein B to apolipoprotein A1 (APOB/A1) (17) have been previously reported to be associated with the risk of NAFLD in different populations. Besides, TG/HDL-C and TC/HDL-C have been described as promising parameters for the diagnosis of prediabetes and T2DM (15, 18).

Currently, there are no studies on the relationship between the aforementioned lipid and lipoprotein ratios and NAFLD in a newly diagnosed T2DM population with no history of medication and no diabetic complications. Considering the high prevalence and risk of combined NAFLD in T2DM, there is a need for early identification of NAFLD in newly diagnosed diabetic patients for better early intervention. Therefore, this study sought to evaluate the value of the above-mentioned lipid-lipoprotein ratios for assessing the risk of NAFLD in patients with newly diagnosed T2DM.

Methods

Participants

This study was a retrospective study approved by the Ethics Committee of Tongji Hospital, Tongji University School of Medicine (K-2021-010). A total of 1021 patients who were first diagnosed with T2DM and not receiving anti-diabetic medication at the inpatient department of the Department of Endocrinology, Tongji Hospital, Tongji University, from June 2018 to December 2020 were enrolled.

The diagnosis of T2DM was based on the criteria of the World Health Organization (1999) (19). The diagnosis of NAFLD was made by abdominal ultrasound assessment of hepatic steatosis (20). The criteria were as follows: 1) diffusely enhanced liver echogenicity that was stronger than that of the kidneys or spleen; 2) attenuation of far-field echogenicity depth in the liver region; 3) vascular blurring on color Doppler ultrasound; 4) poorly displayed intrahepatic luminal structures. The exclusion criteria for this study were as follows: 1) subjects with a history of drinking, or alcohol consumption ≥140 g per week for men and ≥70 g per week for women; 2) subjects with a history of autoimmune hepatitis, drug-induced hepatic disease, viral hepatitis or other known diseases that may lead to fatty liver; 3) subjects treated with lipid-lowing agents or anti-diabetic medications; 4) subjects who did not receive liver ultrasound; 5) subjects with incomplete clinical information. Finally, 371 patients with newly diagnosed T2DM combined with NAFLD and 360 newly diagnosed T2DM patients without NAFLD were included in this study (Supplementary Figure 1).

Data collection

Basic clinical data and lifestyle information of the study population were collected, including age, sex, height, body weight, and smoking/alcohol consumption habits. Smoking/drinking habits depended on whether the individual currently smoked or drank excessively (140 g/week for men and 70 g/week for women). The levels of blood lipids, blood glucose, liver function and renal function were collected in this study, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), Gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), serum creatinine (Scr), UA, fasting blood-glucose (FBG), glycosylated hemoglobin (HbA1c), fasting insulin (FINS), TG, TC, free fatty acid (FFA), LDL-C, HDL-C, APOA1 and APOB. The lipid profiles, liver function, renal function and FBG were detected on an automatic biochemical analyzer (AU 5800, Beckman Coulter, USA). HbA1c was assessed by high-performance liquid chromatography (HLC-723G8, TOSOH CORPORATION, Japan). FINS was measured by an automatic electrochemiluminescence immunoassay analyzer (ADVIA centaur XP, Siemens, Germany).

Body mass index (BMI) was calculated as body weight (kg)/height^2 (m^2). Homeostasis model assessment-insulin resistance (HOMA-IR) reflects the state of insulin resistance (IR) in the body, and the equation is: HOMA-IR = fasting insulin (μU/dL) × fasting blood glucose (mg/dL)/22.5. TG/HDL-C, TC/HDL-C, FFA/HDL-C, LDL-C/HDL-C, UA/HDL-C, and AOB/A1 ratios were calculated as TG (mmol/L)/HDL-C (mmol/L), TC (mmol/L)/HDL-C (mmol/L), FFA (mmol/L)/HDL-C (mmol/L), LDL-C (mmol/L)/HDL-C (mmol/L), UA (μmol/L)/HDL-C (mmol/L), APOB (mmol/L)/APOA1 (mmol/L) respectively.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 software. Continuous variables with normal distribution were expressed as mean ± standard deviation (SD), and independent samples T-test was used to compare the non-NAFLD group with the NAFLD group. Continuous variables without a normal distribution were expressed as median (interquartile range), and compared between the non-NAFLD and NAFLD groups using the Kruskal-Wallis test. Categorical variables were shown as proportions, and compared using Chi-squared tests. We divided the TG/HDL-C ratio, TC/HDL-C ratio, FFA/HDL-C ratio, LDL-C/HDL-C ratio, UA/HDL-C and APOB/A1 ratio into four quartiles and converted them into conventional categorical variables, i.e. Q1 < 25%, Q2 25-50%, Q3 50-75% and Q4 ≥ 75%. Chi-square test was used to compare the proportion of NAFLD in patients with newly-onset T2DM in the above categorical variables. Continuous variables that did not conform to a normal distribution were log-transformed, and Pearson correlation analysis was conducted between the six lipid-lipoprotein ratios and each variable. After adjusting for potential confounders, a bivariate logistic regression analysis was performed in newly diagnosed T2DM patients to explore the association between several lipid ratios and NAFLD. Three models were used in this study, model 1 unadjusted; model 2 adjusted for age, sex, current smoking status, and BMI; and model 3 adjusted for age, sex, current smoking status, BMI, ALT, AST, GGT, ALP, Scr, FBG, HbA1c and FINS. The receiver operating characteristic (ROC) curve analysis was used to compare the relative diagnostic ability of the six lipids and lipoprotein ratios for new-onset T2DM combined with NAFLD. The indicator with the largest area under the ROC curve (AUC) was considered the best diagnostic marker.

Results

Clinical characteristics of the study subjects

A total of 731 newly diagnosed T2DM subjects were enrolled in the study, including 360 patients without NAFLD (non-NAFLD group), 371 patients with NAFLD (NAFLD group). That is, the overall proportion of NAFLD in patients with newly diagnosed T2DM was 50.8%. In non-NAFLD group, the mean age was 57.21 ± 16.83 years, with 58.9% (212/360) of males and 41.1% (148/360) of females. In NAFLD group, the mean age was 51.45 ± 15.86 years, of which 65.2% (242/371) were males and 34.8% (129/371) were females. Moreover, newly diagnosed T2DM subjects combined with NAFLD smoked more and had a higher BMI than subjects without NAFLD. As expected, patients with NAFLD had higher ALT, AST, GGT, ALP, UA, FBG, FINS, HOMA-IR, TG, TC, FFA, LDL-C and APOB than non-NAFLD group, while HDL-C and APOA1 were lower than non-NAFLD group. There was no significant difference in Scr and HbA1c between the two groups (Table 1).

The distribution of the ratios of TG/HDL-C, TC/HDL-C, FFA/HDL-C, LDL-C/HDL-C, UA/HDL-C, and APOB/A1 in the non-NAFLD and NAFLD groups, respectively, is shown in Supplementary Figure 2. In addition, the ratios of TG/HDL-C, TC/HDL-C, FFA/HDL-C, LDL-C/HDL-C, UA/HDL-C, APOB/A1 were significantly higher in new-onset T2DM patients with NAFLD than in patients without NAFLD (Table 1).

Associations of six lipid and lipoprotein-related indices with NAFLD in newly diagnosed T2DM

The proportion of NAFLD in newly diagnosed T2DM patients increased progressively across the Q1-Q4 range of six lipid-lipoprotein ratios, including TG/HDL-C (22.0, 49.4, 58.7 and 75.4%, respectively), TC/HDL-C (30.9, 44.6, 54.0 and 74.0%, respectively), FFA/HDL-C (34.4, 46.8, 57.6 and 66.3%, respectively), LDL-C/HDL-C (35.9, 44.3, 53.6 and 69.0%, respectively), UA/HDL-C (32.8, 45.8, 53.1 and 72.2%, respectively) and APOB/A1 (36.0, 41.8, 55.8 and 71.9%, respectively) (Figure 1). Compared to the lowest quartile (Q1) of the above six lipid-lipoprotein ratios, the proportion of NAFLD was significantly higher in the increasing quartiles (Q2-Q4) of the TG/HDL-C and TC/HDL-C ratios, and in the increasing quartile (Q3-Q4) of the FFA/HDL-C, LDL-C/HDL-C and UA/HDL-

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TABLE 1 Clinical characteristics of the study subjects in newly diagnosed T2DM with and without NAFLD.

	Non-NAFLD (N=360)	NAFLD (N=371)	*P*-Values
Age (years)	57.21 ± 16.83	51.45 ± 15.86	<0.001
Sex, male/female (n)	212/148	242/129	0.077
Current smoking (%)	79 (21.9)	113 (30.5)	0.009
BMI (kg/m^2)	23.65 ± 4.20	27.28 ± 4.82	<0.001
ALT (U/L)	19.30 (13.43-29.4)	30.30 (19.00-55.1)	<0.001
AST (U/L)	18.75 (14.90-24.85)	23.3 (17.4-38.30)	<0.001
GGT (U/L)	25.5 (17.45-38.85)	39.9 (26.60-66.05)	<0.001
ALP (U/L)	91.13 ± 29.98	98.52 ± 38.38	0.008
Scr (μmol/L)	71.45 (60.50-82.98)	74.7 (63.10-74.70)	0.202
UA (μmol/L)	306.06 ± 94.57	343.02 ± 100.63	<0.001
FBG (mmol/L)	9.72 ± 4.19	11.45 ± 5.50	<0.001
HbA1c (%)	10.60 ± 4.20	10.84 ± 2.59	0.280
FINS (μIU/mL)	9.18 (6.07-12.65)	11.72 (8.04-15.79)	<0.001
HOMA-IR	3.43 (2.14-5.62)	5.23 (3.36-7.85)	<0.001
TG (mmol/L)	1.27 (0.92-1.72)	1.85 (1.31-2.85)	<0.001
TC (mmol/L)	4.72 ± 1.15	5.25 ± 1.81	<0.001
FFA (mmol/L)	0.51 ± 0.23	0.58 ± 0.22	<0.001
LDL-C (mmol/L)	3.16 ± 0.91	3.35 ± 1.01	0.006
HDL-C (mmol/L)	1.07 ± 0.24	0.95 ± 0.22	<0.001
APOA1 (mmol/L)	1.16 ± 0.18	1.12 ± 0.18	0.006
APOB (mmol/L)	0.98 ± 0.23	1.09 ± 0.22	<0.001
TG/HDL-C	1.20 (0.84-1.80)	2.05 (1.37-3.23)	<0.001
TC/HDL-C	4.61 ± 1.19	5.57 ± 1.86	<0.001
FFA/HDL-C	0.51 ± 0.26	0.64 ± 0.32	<0.001
LDL-C/HDL-C	3.09 ± 0.92	3.62 ± 1.04	<0.001
UA/HDL-C	303.83 ± 131.20	386.35 ± 159.50	<0.001
APOB/APOA1	0.87 ± 0.22	1.00 ± 0.27	<0.001

Values are expressed as mean ± SD, median (quartile) or number (percentage). NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; Scr, serum creatinine; UA, uric acid; FBG, fasting blood-glucose; HbA1c, glycosylated hemoglobin; FINS, fasting insulin; HOMA-IR, homeostasis model assessment-insulin resistance; TG, triglycerides; TC, cholesterol; FFA, free fatty acid; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; APOA1, apolipoprotein A1; APOB, apolipoprotein B; TG/HDL-C, TG to HDL-C ratio; TC/HDL-C, TC to HDL-C ratio; FFA/HDL-C, FFA to HDL-C ratio; UA/HDL-C, UA to HDL-C ratio; LDL-C/HDL-C, LDL-C to HDL-C ratio; APOB/A1, APOB to APOA1 ratio.

C and APOB/A1 ratios (Figure 1). This increasing trend suggested that the greater the six lipid ratios in newly diagnosed T2DM patients, the higher the likelihood of NAFLD occurrence in those patients. Logistic regression analysis further demonstrated that the 6 lipid ratios in model 1 were positively correlated with NAFLD in new-onset T2DM patients without adjusting for other factors (Table 2). Pearson correlation analysis was shown in Supplementary Table 1, indicating that the 6 lipid ratios were strongly correlated with age, sex, BMI, hepatic function markers, renal function indicators, blood glucose indicators and blood lipid indicators, respectively. Therefore, we next corrected for these factors in models 2 and 3 of the logistic regression analysis (Table 2). After adjusting for age, sex, current smoking status and BMI in model 2, the 6 lipid ratios remained significantly and positively associated with NAFLD in newly diagnosed T2DM patients (Table 2). Even after adjusting for age, sex, current smoking status, BMI, ALT, AST, GGT, ALP, Scr, FBG, HbA1c and FINS in model 3, 5 lipid ratios (TG/HDL-C, TC/HDL-C, LDL-C/HDL-C, UA/HDL-C, and APOB/A1) remained positively associated with the risk of NAFLD in patients with newly diagnosed T2DM (Table 2). It should be noted that in model 1-3, APOB/A1 ratio had the strongest correlation with NAFLD in patients with newly diagnosed T2DM [model1 odds ratio (OR)= 10.72, P<0.001; model2 OR=4.81, P=0.001 and model3 OR= 6.25; P=0.006, respectively] (Table 2).

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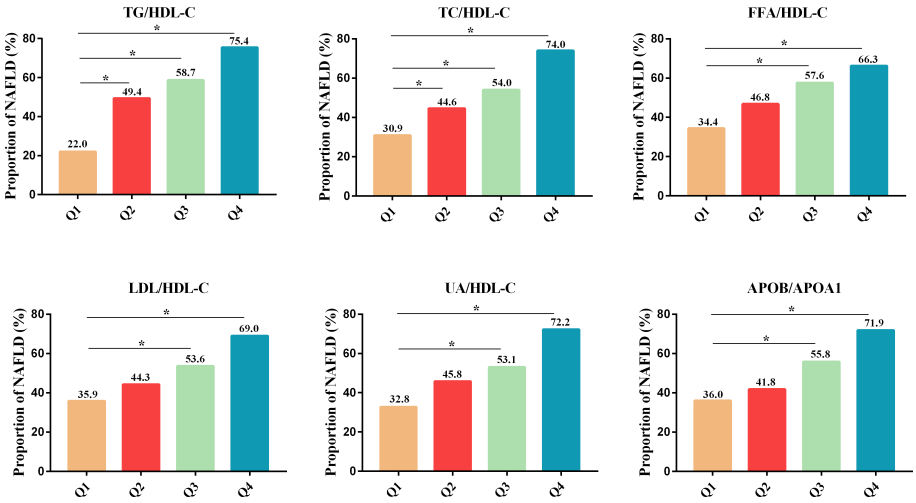

FIGURE 1
Proportion of NAFLD in patients with newly diagnosed T2DM across the quartiles of multiple lipid ratios (Q1-Q4). *P<0.001 vs. Q1. NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; TG/HDL-C, triglycerides to high-density lipoprotein-cholesterol ratio; TC/HDL-C, cholesterol to HDL-C ratio; FFA/HDL-C, free fatty acid to HDL-C ratio; UA/HDL-C, uric acid to HDL-C ratio; LDL-C/HDL-C, low-density lipoprotein-cholesterol to HDL-C ratio; APOB/A1, apolipoprotein B to apolipoprotein A1 ratio.

Diagnostic value of the six lipid-lipoprotein ratios for NAFLD in newly diagnosed T2DM patients

ROC curves were then constructed to compare the ability of the six lipid-lipoprotein ratios and their associated lipid metrics to discriminate NAFLD in newly diagnosed T2DM patients (Supplementary Figure 3). The area under the curve (AUC) of all lipid ratios was higher than that of individual lipid indicators, indicating that lipid ratios were superior to individual lipid values in the diagnosis of NAFLD in newly diagnosed T2DM patients (Supplementary Figure 3). Furthermore, the results of the ROC curve analysis corresponding to the six lipid ratios were shown in Figure 2 and Table 3, with the highest AUC for the TG/HDL-C ratio (AUC 0.732; 95% CI 0.696-0.769). Moreover, the sensitivity of the TG/HDL ratio (73.8%) was also the highest among all six indicators, with a specificity of 60.1% and a cut-off point of 1.405 (Table 3).

In addition, ROC curve analysis showed that all six metrics in model 3 had the highest ability to discriminate NAFLD in newly diagnosed T2DM patients among the three models (Supplementary Figure 4). Furthermore, after correction for age, gender, current smoking status, BMI, ALT, AST, GGT, ALP, Scr, FBG, HbA1c and FINS, the AUC of the TG/HDL-C ratio in model 3 remained the largest (AUC of 0.818; $P < 0.001$) (Figure 3). These results suggested that the TG/HDL ratio was the most promising diagnostic indicator of NAFLD in patients with new-onset T2DM after adjusting for patient age, sex, BMI, current smoking, or biochemical values.

Discussion

Early detection of NAFLD in patients with newly diagnosed T2DM is of great significance for the implementation of early intervention strategies. However, the invasiveness of liver biopsy or

TABLE 2 The association between the lipid ratios and the risk of NAFLD in patients with newly diagnosed T2DM.

	Model1		Model2		Model3	
	OR (95% CI)	*P*-Values	OR (95% CI)	*P*-Values	OR (95% CI)	*P*-Values
TG/HDL-C	1.62 (1.42-1.86)	<0.001	1.49 (1.25-1.77)	<0.001	1.45 (1.14-1.85)	0.002
TC/HDL-C	1.67 (1.46-1.90)	<0.001	1.41 (1.19-1.67)	<0.001	1.43 (1.11-1.83)	0.005
FFA/HDL-C	6.25 (3.40-11.49)	<0.001	2.35 (1.16-4.77)	0.018	1.41 (0.51-3.88)	0.508
LDL-C/HDL-C	1.74 (1.48-2.04)	<0.001	1.40 (1.13-1.73)	0.002	1.51 (1.09-2.08)	0.012
UA/HDL-C	1.00 (1.00-1.01)	<0.001	1.00 (1.00-1.00)	0.006	1.00 (1.00-1.01)	0.016
APOB/APOA1	10.72 (5.29-21.72)	<0.001	4.81 (1.93-12.01)	0.001	6.25 (1.71-22.79)	0.006

Model 1 is unadjusted.
Model 2 is adjusted for age, sex, current smoking, BMI.
Model 3 is adjusted for age, sex, current smoking, BMI, ALT, AST, GGT, ALP, Scr, FBG, HbA1c, FINS NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; Scr, serum creatinine; FBG, fasting blood-glucose; HbA1c, glycosylated hemoglobin; FINS, fasting insulin; TG/HDL-C, triglycerides to high-density lipoprotein-cholesterol ratio; TC/HDL-C, cholesterol to HDL-C ratio; FFA/HDL-C, free fatty acid to HDL-C ratio; UA/HDL-C, uric acid to HDL-C ratio; LDL-C/HDL-C, low-density lipoprotein-cholesterol to HDL-C ratio; APOB/A1, apolipoprotein B to apolipoprotein A1 ratio.

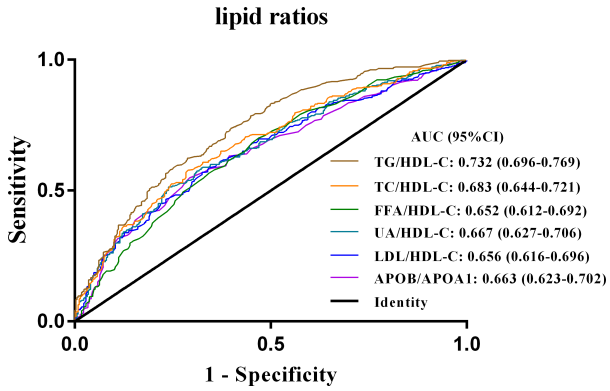

FIGURE 2
ROC curves of the six lipid ratios in patients with newly diagnosed T2DM combined with NAFLD. TG/HDL-C, triglycerides to high-density lipoprotein-cholesterol ratio; TC/HDL-C, cholesterol to HDL-C ratio; FFA/HDL-C, free fatty acid to HDL-C ratio; UA/HDL-C, uric acid to HDL-C ratio; LDL-C/HDL-C, low-density lipoprotein-cholesterol to HDL-C ratio; APOB/A1, apolipoprotein B to apolipoprotein A1 ratio; ROC curves, receiver operator characteristic curves; NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus.

the limitations of the expertise of ultrasound technicians and ultrasound instrumentation have made it difficult to use the above screening methods widely in the general population. Recent studies have found that lipid and lipoprotein disorders promote the development and progression of NAFLD (21, 22). Accumulating clinical evidence have indicated that dyslipidemia and lipoprotein disorders are associated with NAFLD in different populations (8–13, 16, 17), suggesting the possibility of lipid indices or lipid-lipoprotein ratios as diagnostic markers for NAFLD. In this study, we explored the efficacy of six lipid-lipoprotein ratio parameters (TG/HDL-C, TC/HDL-C, FFA/HDL-C, UA/HDL-C, LDL-C/HDL-C, APOB/A1) and their individual lipid indexes for the diagnosis of NAFLD in patients with newly diagnosed T2DM. Our study showed that all lipid-lipoprotein ratios were superior to individual lipid indices in the diagnosis of NAFLD in patients with newly-onset T2DM.

Previous studies on the correlation between lipid-lipoprotein ratios and NAFLD have been reported (9, 11, 16, 17, 23). Ren et al. (11) concluded that the TC/HDL-C ratio had a significant predictive value for NAFLD, and ROC analysis showed that the AUC (0.645) was greater than other serum lipids. In addition, Zhu et al. (9) suggested that the predictive value of UA/HDL-C was significantly higher than LDL-C/HDL-C, non-HDL-C/HDL-C and ALT/AST in a non-obese population, even when UA and LDL-C levels were within the normal range. In a 5-year longitudinal cohort study of 9767 non-obese subjects with normal lipids, Cox proportional hazard regression model confirmed that high LDL-C/HDL-C ratios significantly increased the risk of NAFLD in non-obese Chinese subjects with normal lipids (16). In addition, the APOB/A1 ratio was also associated with the prevalence of NAFLD in non-diabetic subjects (23), normal weight and overweight subjects (17). Although the correlation between lipid-lipoprotein ratio and NAFLD has been reported in non-obese, non-diabetic populations, the correlation between lipid- lipoprotein ratio and NAFLD in newly diagnosed T2DM patients has not been studied.

There is growing evidence revealed a strong association between TG/HDL-C and multiple metabolic disorders, including IR, diabetes, and cardiometabolic risk (13, 18, 24). For example, in a study investigating the relationship between lipid ratios and abnormal glucose tolerance, Guo et al. (18) found that serum TC, TG, TC/HDL-C, TG/HDL-C, and non-HDL-C were all strongly associated with prediabetes and T2DM. The AUC values of both TG and TG/HDL-C exceeded 0.70 in the diagnosis of prediabetes and T2DM. In addition, some studies have found a correlation between TG/HDL-C and NAFLD. For example, a retrospective study demonstrated that TG/HDL-C was independently associated with NAFLD in subjects undergoing health screening and could be used as a surrogate marker for NAFLD (12). In another retrospective cohort study of a Chinese non-obese population without dyslipidemia, there was an

TABLE 3 ROC curves of six lipid ratios for the diagnosis of NAFLD in patients with new-onset T2DM.

	AUC	95% CI	*P*-Values	Youden index	Cut-off point	Sensitivity	Specificity
TG/HDL-C	0.732	0.696-0.769	<0.001	0.339	1.405	0.738	0.601
TC/HDL-C	0.683	0.644-0.721	<0.001	0.289	5.175	0.575	0.714
FFA/HDL-C	0.652	0.612-0.692	<0.001	0.232	0.475	0.701	0.532
LDL-C/HDL-C	0.656	0.616-0.696	<0.001	0.245	3.485	0.569	0.675
UA/HDL-C	0.667	0.627-0.706	<0.001	0.275	360.2	0.514	0.761
APOB/APOA1	0.663	0.623-0.702	<0.001	0.280	0.945	0.573	0.767

NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; TG/HDL-C, triglycerides to high-density lipoprotein-cholesterol ratio; TC/HDL-C, cholesterol to HDL-C ratio; FFA/HDL-C, free fatty acid to HDL-C ratio; UA/HDL-C, uric acid to HDL-C (UA/HDL-C) ratio; LDL-C/HDL-C, low-density lipoprotein-cholesterol to HDL-C; APOB/A1, apolipoprotein B to apolipoprotein A1.

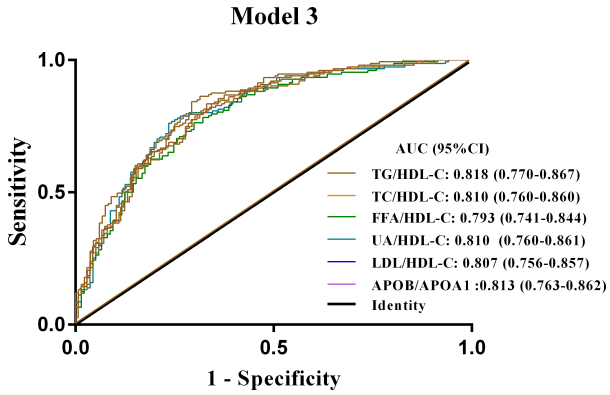

FIGURE 3
ROC curves for Model 3 of the six lipid ratios in patients with newly diagnosed T2DM combined with NAFLD. Model 3 is adjusted for age, sex, current smoking, BMI, ALT, AST, GGT, ALP, Scr, FBG, HbA1c and FINS. NAFLD, non-alcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; ROC curves, receiver operator characteristic curves; TG/HDL-C, triglycerides to high-density lipoprotein-cholesterol ratio; TC/HDL-C, cholesterol to HDL-C ratio; FFA/HDL-C, free fatty acid to HDL-C ratio; UA/HDL-C, uric acid to HDL-C ratio; LDL-C/HDL-C, low-density lipoprotein-cholesterol to HDL-C ratio; APOB/A1, apolipoprotein B to apolipoprotein A1 ratio.

independent correlation between TG/HDL-C and NAFLD (10). Although previous studies have identified correlations between TG/HDL-C and NAFLD in physical examination subjects and non-obese populations, no study has so far focused on the diagnostic value of TG/HDL-C for NAFLD in a newly diagnosed T2DM population. Our study suggests for the first time that TG/HDL-C may be a promising biomarker for early identification of NAFLD in newly diagnosed T2DM patients. We found that in patients with newly diagnosed T2DM, TG/HDL-C had an AUC of 0.732, a sensitivity of 73.8% and a specificity of 60.1% for identifying NAFLD, which was significantly higher than other five lipid- lipoprotein ratios.

Our study found that TG/HDL-C might have the potential to be used as a diagnostic indicator of NAFLD in newly diagnosed T2DM. The mechanism of the intrinsic association of TG/HDL-C with T2DM combined with NAFLD may be related to IR. Previous studies have revealed the strong correlation between TG/HDL-C and IR (25–28). And the onset of NAFLD and T2DM are also closely associated with IR (28–32). Excess fatty acids are produced due to increased lipolysis and enhanced fatty acid synthesis. These fatty acids enter the blood circulation and accumulate in peripheral tissues, such as the liver and adipose tissue, ultimately leading to IR (31). In addition, IR also enhances new lipogenesis in the liver and lipolysis in adipose tissue, thereby increasing the amount of fatty acids flowing to the liver (32). Lipids accumulate in the liver in the form of FFA-derived TG, which together with high levels of free cholesterol and lipid metabolites (e.g., unsaturated fatty acids, lipid peroxidation products, etc.), increase lipotoxicity (32, 33). Also, β-cell failure due to excess free fatty acids and lipid metabolites, as well as IR, are major pathogenic mechanisms of T2DM (33). The molecular mechanisms underlying the association between TG/HDL-C and the risk of NAFLD in newly diagnosed T2DM still deserve further exploration.

There are some limitations of our study. Firstly, it is uncertain whether the TG/HDL-C ratio remains a diagnostic indicator for NAFLD in patients with longer duration of T2DM. Follow-up studies of these patients will be able to clarify this issue. Secondly, all patients with T2DM recruited in this study were newly diagnosed and had not received oral lipid-lowering or hypoglycemic medications. The strict inclusion criteria resulted in a small sample size for inclusion. Thirdly, some newly diagnosed T2DM patients were not included in this study due to the lack of liver ultrasound imaging, which may lead to some degree of data bias.

Conclusion

In summary, this is the first study to assess the diagnostic value of multiple simple lipid parameter ratios for NAFLD in newly diagnosed T2DM patients. Our results found that the proportion of NAFLD in newly diagnosed T2DM patients elevated progressively with increasing ratios of six lipid parameters. Our study suggest that the TG/HDL-C ratio has the best diagnostic value for NAFLD in the newly diagnosed T2DM population, and may has the potential to be used as a screening marker for NAFLD in the newly diagnosed T2DM population in clinical practice and in large-scale screening.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Tongji Hospital, Tongji University School of Medicine (K-2021-010). Written informed

consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

RL and DK analyzed the patient data and drafted the manuscript. ZY and GZ contributed to data interpretation. KH, WX, PF coordinated the research. LZ and YZ contributed to data interpretation and critical revision of the manuscript. KZ and YX designed the study, revised and prepared the final version of the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1127134/full#supplementary-material

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An examination of causal associations and shared risk factors for diabetes and cardiovascular diseases in the East Asian population: A Mendelian randomization study

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Background: One of the major contributors to disability and mortality among diabetics is cardiovascular disease (CVD), with coronary artery disease (CAD) as the most prevalent type. However, previous studies have provided controversial evidence linking diabetes to other types of CVDs, such as atrial fibrillation (AF). In addition, the risk factors that predispose people to the risk of diabetes and its complications differ across ethnicities, but the disease risk profiles in the East Asian population have been less investigated.

Methods: The causal association between type 2 diabetes (T2D) and two types of CVDs (i.e., AF and CAD) in the East Asian population was first studied using Mendelian randomization (MR) analyses. Next, we examined the causal effect of 49 traits on T2D and CAD to identify their separate and shared risk factors in East Asians. A causal mediation analysis was performed to examine the role of T2D in mediating the relationship between the identified shared risk factors and CAD.

Results: T2D was causally associated with CAD, but not AF, in East Asians. A screening of the risk factors indicated that six and 11 traits were causally associated with T2D and CAD, respectively, with suggestive levels of evidence. Alkaline phosphatase (ALP) was the only trait associated with both T2D and CAD, as revealed by the univariable MR analyses. Moreover, the causal association between ALP and CAD no longer existed after adjusting T2D as a covariable in the causal mediation study.

Conclusion: Our study highlights the risk profiles in the East Asian population, which is important in formulating targeted therapies for T2D and CVDs in East Asians.

KEYWORDS

diabetes, cardiovascular diseases, coronary artery disease, atrial fibrillation, Mendelian randomization

Introduction

Up to 8.8% of the world's population suffers from diabetes, and International Diabetes Federation projections reveal that by 2040, the number of incidences will have risen to 642 million (1). One of the main contributors to disability among patients with diabetes is cardiovascular disease (CVD) (2, 3). The percentage of people with CVD is higher in diabetic patients than in adults without diabetes (4). CVD leads to the death of roughly 70% of type 2 diabetic patients at and above 65 years old (5). To elucidate, a systematic review that included 4,549,481 type 2 diabetes (T2D) patients showed an overall CVD prevalence of 32.2% (2). Coronary artery disease (CAD) (21.2%) was the most common kind of CVD reported (2). However, previous works have led to controversial conclusion about the association between diabetes and a particular type of CVD, such as atrial fibrillation (AF), the most prevalent type of arrhythmia in the world (6). For example, a study using a cohort of patients having new-onset AF did not establish the association between the symptoms of AF and diabetes (7).

Ethnic disparities in health conditions are well-recognized (8). For example, Asian Indians in the US are more likely to have diabetes, although they have lower chance to be obese (9). In addition, East Asians have more body fat and prone to visceral adiposity at a given body mass index (BMI), which promote the development of diabetes (10). The risk factors that contribute to the development of diabetes complications also differ across Asian and European populations (11). Thus, it is important to understand ethnic differences in disease risk profiles to formulate better treatment strategies.

Mendelian randomization (MR) is a method for inferring causation, which reduces the bias owing to reverse causality and residual confounding. In MR analyses, the genetic instruments are used as a proxy for exposures (12). In causal mediation analyses using a two-step MR design, the direct and indirect effects of exposure on the outcome can also be evaluated (13). Individual-level data was not applied in MR analyses because these analyses use summary statistics from genome-wide association studies (GWAS), which are normally produced using populations with large sample sizes (14). In addition, the availability of GWAS datasets makes it easier to screen disease risk factors at the phenome-wide level (15).

In the current study, we first investigated the potential causal association between T2D and two types of CVDs (i.e., AF and CAD) in the East Asian population. Next, we tested the causal effect of 49 traits on T2D and CAD to identify their separate and shared risk factors in East Asians. A causal mediation analysis was also performed to examine the role of T2D in mediating the relationship between identified shared risk factors and CAD.

Abbreviations: T2D, type 2 diabetes mellitus; MR, Mendelian randomization; GWAS, genome-wide association studies; IVs, instrumental variables; LD, linkage disequilibrium; FDR, False discovery rate; SNPs, single nucleotide polymorphisms; OR, odds ratio; CIs, confidence intervals; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; LPS, lipopolysaccharides; NO, nitric oxide; RBC, red blood cell; CVD, cardiovascular disease; CAD, coronary artery disease; AF, atrial fibrillation; ALP, alkaline phosphatase; DIAMANTE, the Diabetes Meta-analysis of Trans-ethnic Association Studies; IVW, the inverse-variance weighted method; TC, total cholesterol; APTT, activated partial thromboplastin time; DECODE, the Collaborative Analysis of Diagnostic criteria in Europe study.

Methods

The GWAS dataset for T2D was obtained from the Diabetes Meta-analysis of Trans-ethnic Association Studies (DIAMANTE) Consortium (16), in which GWAS was performed for the East Asian population. For other traits, the method for traits selection (Supplementary Figure 1) was similar to the one used in a recent paper (13). We only included the GWAS summary statistics datasets generated in the Biobank Japan study (17) to ensure that the MR analyses were conducted using genetic data from East Asians. Detailed information was included in Supplementary Table 1. The causal relationships between 49 traits (Supplementary Figure 1) and T2D/CAD were investigated by univariable MR analyses. For the identified trait (shared risk factor) that can lead to both T2D and CAD, we performed causal mediation analyses, where T2D was deemed as a potential mediator. A reciprocal link between mediator and exposure was not permitted in the mediation studies, so it was necessary to conduct a reverse univariable MR to infer whether these traits could be induced by T2D. The direct effect of trait (shared risk factor) on CAD was estimated using multivariable MR, in which T2D was adjusted as a covariable. The product of the beta coefficient of the effect of trait (shared risk factor) on T2D and the beta coefficient of the association between T2D and CAD (with trait adjusted as covariable) represented the indirect effects of trait (shared risk factor) on CAD.

In the univariable MR studies, the instrumental variables (IVs) used for exposure traits were selected according to various factors. First, the phenotypes should be highly associated with IVs ($P < 5\times10^{-8}$). Second, a linkage disequilibrium (LD) of $R^2 < 0.001$ and clumping with a 10-Mb window were used to ensure that the IVs were not related to each other. Third, each trait's IVs should have at least five variants as biallelic single-nucleotide polymorphisms (SNPs). In the univariable MR studies, the inverse-variance weighted (IVW) method, weighted median method, and MR-Egger were used, with the IVW approach being regarded as the primary method. Potential horizontal pleiotropy was examined using the MR-Egger intercept test. A 5% false-discovery rate (FDR) was used to correct multiple comparisons. The code for the MR studies was modified from a recent work (13), in which the R packages TwoSampleMR and MVMR, respectively, were applied to conducted the MR analyses.

Results

The results of the MR analysis using the IVW approach indicated a significant association between genetically predicted T2D and CAD ($P = 6.63\times10^{-5}$) (Figure 1 and Supplementary Figure 2). However, no causal association between T2D and AF was observed ($P = 0.97$) (Figure 1 and Supplementary Figure 2). The same relationship trajectory was apparent in the MR sensitivity analyses using the weighted median and MR-Egger methods

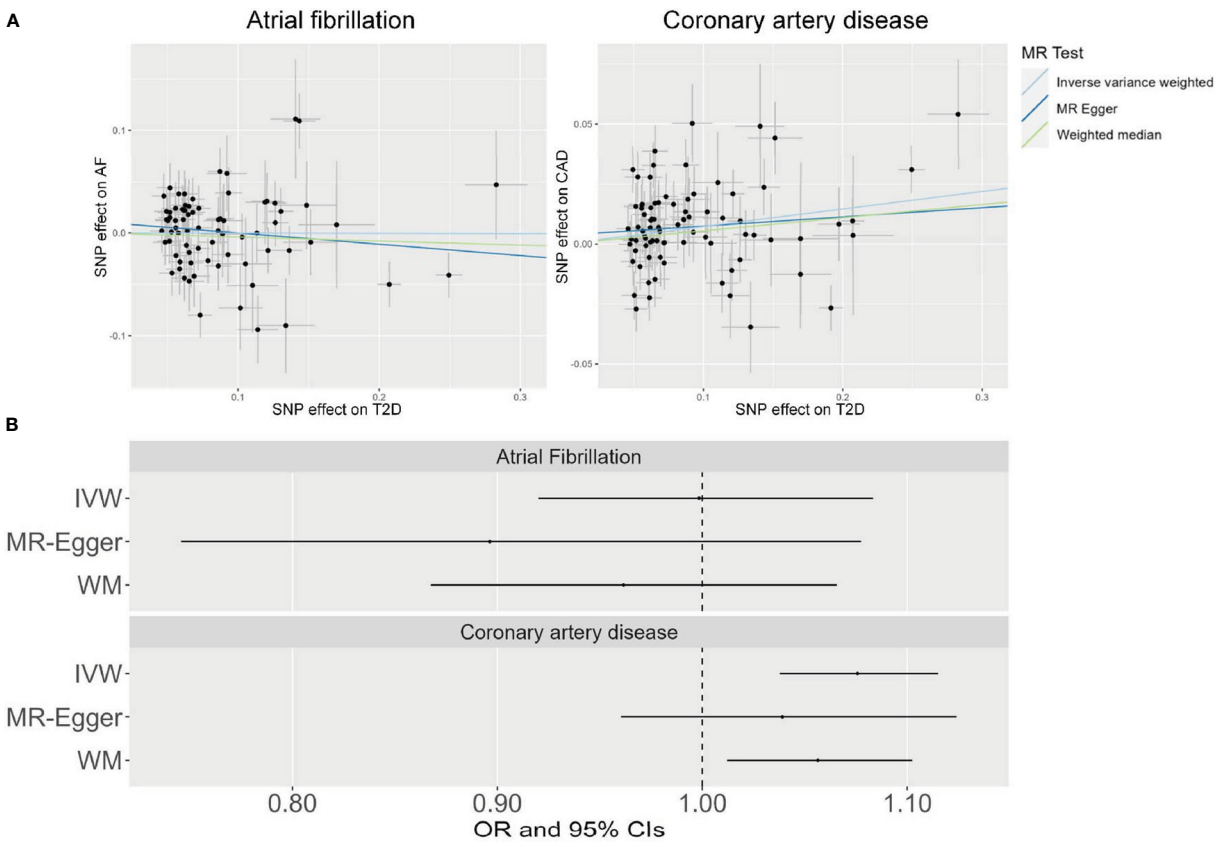

FIGURE 1
Scatter plots **(A)** and forest plots **(B)** showing the results of Mendelian randomization (MR) analyses studying the causal association between T2D and cardiovascular diseases in the East Asian population.

(Figure 1). Moreover, a leave-one-out sensitivity analysis suggested that not a single SNP was responsible for the causal effect of T2D on CAD (Supplementary Figure 3). The intercept term of the MR-Egger method was applied to examine the horizontal pleiotropy, which revealed that it was not significant ($P = 0.34$) in the studies.

After confirming the causal effect of T2D on CAD in the East Asian population, we next examine the separate and shared risk factors of these two diseases by including the GWAS summary datasets of 49 traits from the Biobank Japan study (Supplementary Table 1), in accordance with the criteria indicated in the flowchart (Supplementary Figure 1). Univariable MR analyses indicated that out of the 49 traits, six were associated with T2D at suggestive levels of evidence ($P < 0.05$) (Figure 2 and Supplementary Tables 2-3). Three of these six traits (i.e., hemoglobin A1c, blood sugar, and red blood cell count) survived 5% FDR correction for multiple comparisons (Supplementary Table 4). Reverse MR analyses suggested that alkaline phosphatase was the only trait that could not be altered by T2D (Figure 3 and Supplementary Tables 2-3). Eleven of 49 traits showed causal association with CAD at suggestive levels of evidence ($P < 0.05$) (Figure 4 and Supplementary Tables 2-3), and six of the 11 traits, namely, total cholesterol (TC), triglycerides, low-density-lipoprotein cholesterol

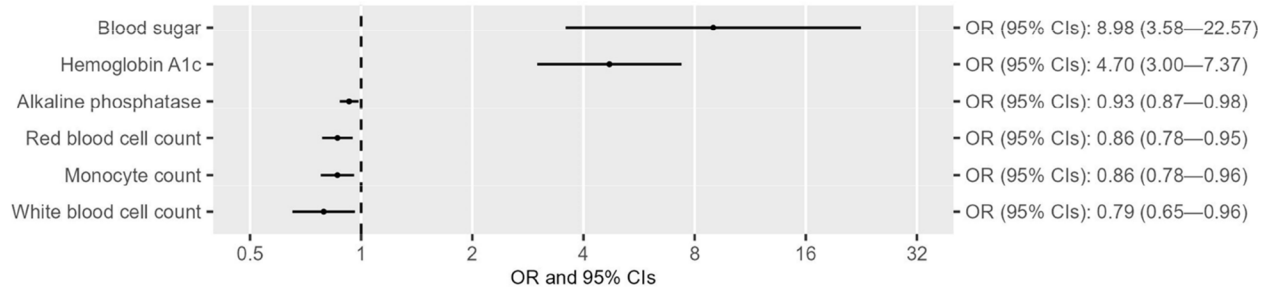

FIGURE 2
Forest plots showing the causal effect of traits on T2D with suggestive levels of evidence ($P < 0.05$) in the East Asian population.

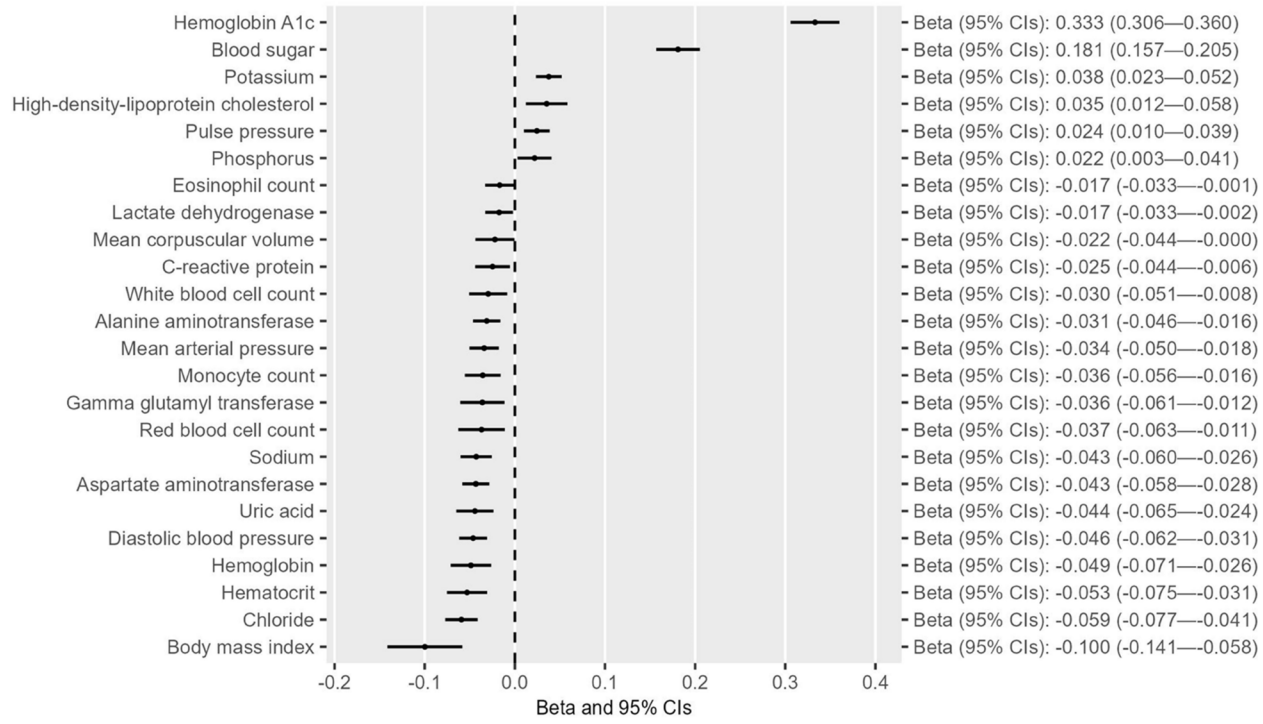

FIGURE 3
Forest plots showing the causal effect of T2D on traits with suggestive levels of evidence ($P < 0.05$) in the East Asian population.

(LDL), high-density lipoprotein cholesterol (HDL), alkaline phosphatase (ALP), and activated partial thromboplastin time (APTT), survived 5% FDR correction (Supplementary Table 4). Thus, the results revealed that ALP was causally associated with both T2D and CAD (Figure 5A), and the following causal mediation analysis based on two-step MR indicated that ALP was no longer associated with CAD after adjusting T2D as a covariable in the multivariable MR (Figure 5B).

Discussion

In the present study, the results of MR analyses suggested that T2D was causally associated with CAD, but not AF, in East Asians. The screening of the risk factors indicated that six and 11 traits were causally associated with T2D and CAD, respectively, with suggestive levels of evidence. ALP was the only trait associated with both T2D and CAD, as revealed by the univariable MR analyses. The causal

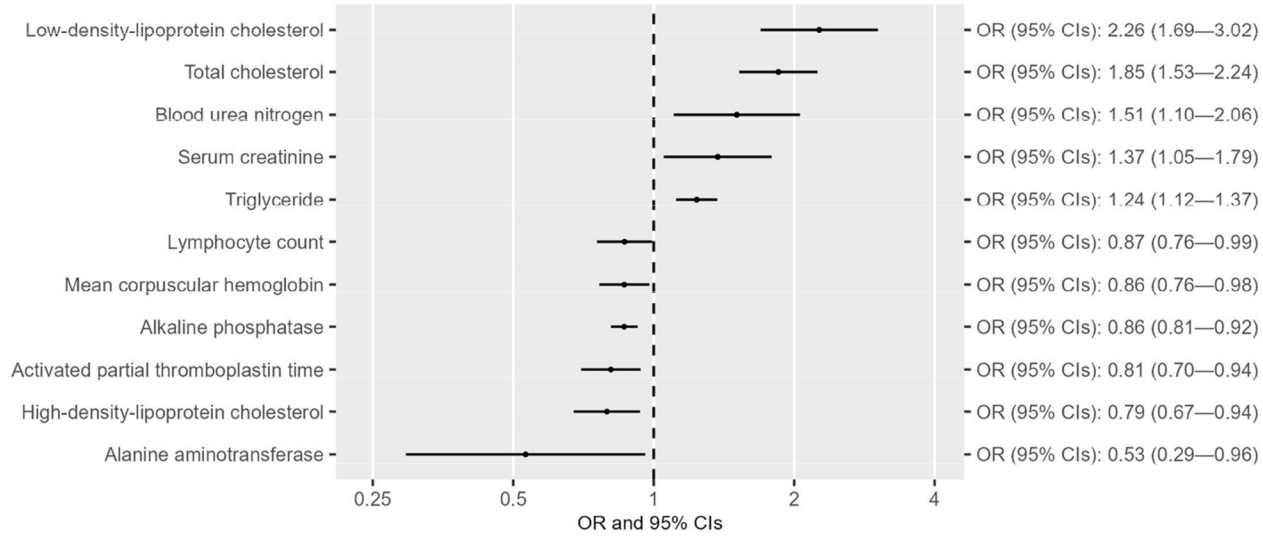

FIGURE 4
Forest plots showing the causal effect of traits on CAD with suggestive levels of evidence ($P < 0.05$) in the East Asian population.

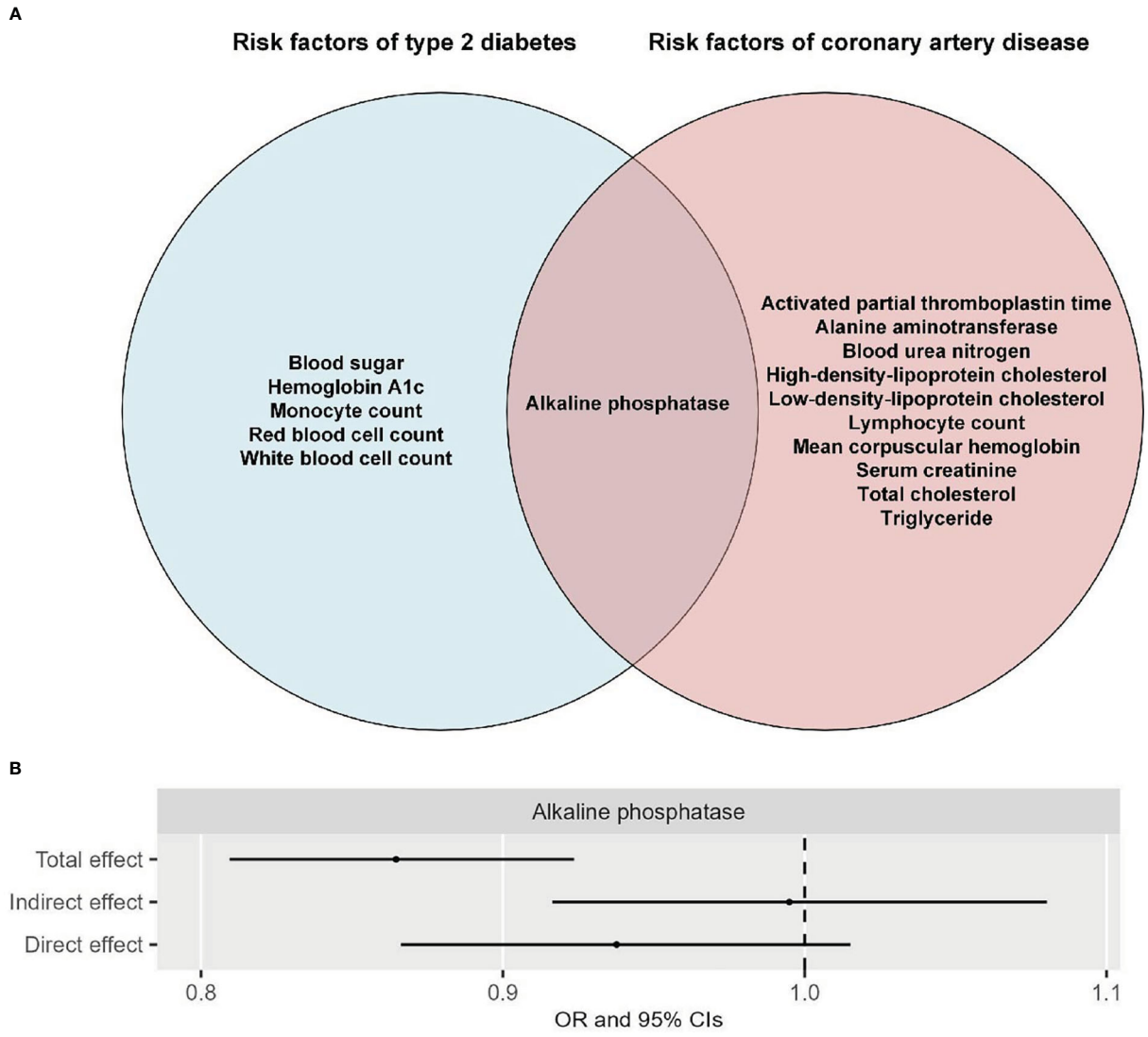

FIGURE 5
Shared and independent risks of T2D and CAD are presented in a Venn diagram **(A)**, and the total, indirect, and direct effects of alkaline phosphatase (ALP) on CAD are studied by causal mediation analyses **(B)** in the East Asian population.

association between ALP and CAD no longer existed after adjusting T2D as a covariable in the causal mediation study (direct effect).

T2D can approximately shorten life expectancy by a decade, and CVD is a major cause of death in T2D patients (18). However, the association of T2D and AF, as well as the exact pathophysiology of AF in diabetes patients, has not been fully established (19). The Framingham Heart Study correlated elevated glycemic levels with an increased risk of AF (20). Moreover, diabetes patients with AF had increased rates of overall and cardiovascular mortality, coupled with a decline in life quality compared with patients who only had AF but were not diabetic (21). However, a correlation between diabetes and non-paroxysmal AF was not observed (22). Diabetes cannot independently lead to AF after confounder adjustment, according to a survey in China (23). Thus, it is still unknown whether there is a causative association between diabetes and AF. Our MR study using genetic data from the East Asian population suggested that diabetes could not causally lead to AF.

CVD is a significant contributor to comorbidity and mortality among T2D patients, with CAD having the highest prevalence rate (2). Research has indicated that patients with diabetes have a higher susceptibility to CAD compared with non-diabetics (24). We consistently observed a causal association of diabetes with CAD in the East Asian population. Several reasons, such as insulin resistance, dyslipidemia, and hyperglycemia, have been postulated to explain the high vulnerability to CAD among patients with diabetes. These processes can be linked to abnormal functioning of the platelets, causing vascular smooth muscle dysfunction, and irregularity in the functioning of endothelial cells (25). Indeed, atherosclerotic plaques in diabetic patients are often lipid-laden, making them more prone to rupture compared with those of people without diabetes (26). In addition, critical to atherosclerosis is the process of inflammation, whose activation in T2D is often linked to insulin resistance and obesity (27). Hyperglycemia has also been linked with the promotion of epigenetic alterations that initiate the over-expression of genes

linked to vascular inflammation, thus establish a basis for atherosclerosis and endothelial dysfunction (28).

The Collaborative Analysis of Diagnostic criteria in Europe (DECODE) study indicated that the prevalence of diabetes was higher in urban Chinese and Japanese patients aged 30–69 years than in Europeans (29). Young patients have a higher chance to experience β-cell failure and long-lasting disease, making them have a higher risk for microvascular and macrovascular problems (10). For example, patients with T2D from East Asia are more likely than those from Europe to experience renal issues (10). One of the potential reasons for this interethnic disparity is that Asians, at a given BMI, usually have higher visceral adiposity compared to Caucasians, which is likely to be more harmful and can cause insulin resistance (30). For example, American Japanese patients have higher level of visceral adiposity than their Caucasian counterparts (31). For other race, the association between metabolic parameters and CVD can be different in Black and White population, and ethnicity is also responsible for the disparities in the metabolic syndrome associated CVD and T2D (32). Because of the ethnic differences in risk profiles, we screened and identified the independent and shared risk factors of diabetes and CAD using GWAS summary data generated from the East Asian population.

Red blood cell (RBC) changes are likely to happen in diabetes patients (33). For example, red blood cell parameters are correlated with glycemic control among adult patients with T2D in Eastern Ethiopia (34). Consistent with the literature, the causality interference by MR analyses in the present study suggested that RBC count was negatively associated with diabetes risk. Long-term hyperglycemia leads to the production of free oxygen radicals and the irreversible glycation of hemoglobin and RBC membrane proteins, resulting in a relative drop in RBC count (35). Thus, these processes make RBCs become less deformable and have a reduced chance of survival (36).

A moderate to very significant correlation between triglyceride levels and the risk of coronary heart disease has been observed (37). The measurement of TC is helpful in estimating CVD risk and making clinical decision for the start of statin therapy (38). Indeed, the risk of coronary heart disease increases by 24% for males and 20% for females for every 1 mmol/L increase in TC (39). For LDL cholesterol, angiographic trials confirm the significance of LDL cholesterol reduction in reducing the risk of CAD (40). Widespread epidemiological research indicates that low levels of HDL are a sign of increased cardiovascular risk (41). Consistent with these clinical observations, our MR analyses identified the causal association between lipid profile and CAD in the East Asian population. As a common coagulation screening test, the measurement of APTT can be used to estimate intrinsic coagulation pathway activity (42). The degree and severity of coronary stenosis can be estimated by using APTT in individuals undergoing coronary angiography; notably, the patients who had ST-Segment Elevation Myocardial Infarction (STEMI) had low APTT values (43). Moreover, a short APTT is correlated with higher thrombin production and an increased risk for thrombosis (44). Consistently, our study revealed that higher APTT was causally associated with decreased CAD risk in the East Asian population.

ALP is a plasma membrane-anchored enzyme that is widely present in nature (45). A correlation between baseline serum ALP levels and new-onset diabetes has been established in hypertensive individuals (46). In an Iranian population, the level of ALP and the risk of coronary heart disease were independently correlated (47). However, ALP was not associated with diabetes, according to the results of MR research, which only included persons of European ancestry (48). Our MR analysis in an East Asian population indicated that ALP was negatively associated with both diabetes and CAD, and the association between ALP and CAD was not significant after adjusting T2D in the multivariable MR. Mechanistically, ALP reduce the bioavailability of nitric oxide (NO), leading to an altered endothelial NO synthase activity (46). Besides serum ALP, intestinal alkaline phosphatase (IAP), as a membrane-bound glycoprotein mainly expressed in proximal small intestine, is also related to T2D (49). For instance, T2D can be observed in mice lacking IAP (50). Additionally, oral administration of IAP protects and even reverses high-fat-diet-induced T2D in wild-type mice by reducing metabolic endotoxemia and detoxifying lipopolysaccharides (LPS) (49).

This study has several limitations. First, a relatively high level of multiple comparison burden may exist when many traits are included in the analyses. To address this point, we also presented the results with suggestive levels of evidence. Second, as an inherent drawback, an MR study cannot completely rule out the potential horizontal pleiotropy. Thus, we used multiple MR methods as sensitivity analyses to enhance the credibility of our conclusion.

Conclusion

T2D is causally associated with CAD, but not AF, in the East Asian population. Multiple traits were identified as separate risk factors of T2D or CAD. A mediating effect of T2D on the association between ALP and CAD was observed. Our study highlights the risk profiles in the East Asian population, which is important for formulating targeted therapies for T2D and CVDs in East Asians.

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

YG and PS: conception and design, data analysis, and interpretation. JG, YL, YJ, XA, and XZ: collection and assembly of data, and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1132298/full#supplementary-material

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Identification and *in vitro* functional assessment of 10 CYP2C9 variants found in Chinese Han subjects

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Cytochrome P450 2C9 (CYP2C9) participates in about 15% of clinical drug metabolism, and its polymorphism is associated with individual drug metabolism differences, which may lead to the adverse drug reactions (ADRs). In this study, 1163 Chinese Han individuals were recruited to investigate their distribution pattern of *CYP2C9* gene and find out the variants that may affect their drug metabolic activities. We successfully developed a multiplex PCR amplicon sequencing method and used it for the genetic screening of *CYP2C9* in a large scale. Besides the wild type *CYP2C9*1*, totally 26 allelic variants of *CYP2C9* were detected, which included 16 previously reported alleles and 10 new non-synonymous variants that had not been listed on the PharmVar website. The characteristics of these newly detected CYP2C9 variants were then evaluated after co-expressing them with CYPOR in *S. cerevisiae* microsomes. Immunoblot analysis revealed that except for Pro163Ser, Glu326Lys, Gly431Arg and Ile488Phe, most of newly detected variants showed comparable protein expression levels to wild type in yeast cells. Two typical CYP2C9 probe drugs, losartan and glimepiride, were then used for the evaluation of metabolic activities of variants. As a result, 3 variants Thr301Met, Glu326Lys, and Gly431Arg almost lost their catalytic activities and most of other variants exhibited significantly elevated activities for drug metabolism. Our data not only enriches the knowledge of naturally occurring CYP2C9 variants in the Chinese Han population, but also provides the fundamental evidence for its potential clinical usage for personalized medicine in the clinic.

KEYWORDS

CYP2C9, allelic variant, genetic polymorphism, drug metabolism, Chinese Han population

1 Introduction

Cytochrome P450 (CYP) is one of the critical enzymes involved in the drug metabolism in human. It is responsible for the biotransformation of most foreign substances, including 70-80% of clinically used drugs (1). Variation in clinical response to drug treatment is very common among individuals, and this variation can be affected by many factors, including age, gender, hormone, disease status, genetic polymorphism, and so forth (2–4). It is reported that most CYP enzymes exhibit marked genetic polymorphism, including copy number variation, missense mutation, insertion, deletion, and most of these genetic variations can affect the protein expression level or drug metabolic activity of enzyme. Clinical evidence has confirmed the apparent correlation between genetic polymorphisms of CYP and adverse drug reactions (ADRs), especially for drugs with narrow therapeutic windows (5–7).

The cytochrome P450 2C (CYP2C) subfamily is one of the most important members of the P450 family, with strong correlations with DNA and protein sequences (>82%) (1). Among them, CYP2C9 is the most abundantly expressed in human body, accounting for about 20% of the total liver P450 protein (8). CYP2C9 enzyme is responsible for the metabolism of approximately 15% of drugs, such as the hypoglycemic agent glimepiride and tolbutamide, the anticoagulant warfarin, the antihypertensive drug losartan, the anticonvulsant phenytoin, as well as the non-steroidal anti-inflammatory drugs flurbiprofen and diclofenac (9). Similar to other CYP2C members, the distribution pattern of *CYP2C9* polymorphic alleles varies greatly among different populations, and most of allelic variants exhibited significantly changed drug metabolic activities compared with that of the wild type CYP2C9 protein (10–12). In order to carry out the individualized treatment for patients with different pharmacogenetic phenotypes and reduce the occurrence rate of related ADRs, the Pharmacological Clinical Pharmacogenetics Implementation Consortium (CPIC) recently issued three CYP2C9-related guidelines for warfarin (13), phenytoin (14), and non-steroidal anti-inflammatory drugs, respectively (15).

To date, 85 allelic variants of *CYP2C9* gene have been discovered and nominated by the Pharmacogene Variation (PharmVar) Consortium (https://www.pharmvar.org/gene/CYP2C9, accessed on Dec 2022). Like other CYP2C members, *CYP2C9* gene is highly polymorphic and exhibits different distribution patterns in different races and geographical regions. According to the previous reports, *CYP2C9*2* is the most prevalent defective allele in the Caucasian population (11.7%), while it is rarely identified in the Asian population (<0.1%) and African population (2.4%). In contrast, the main allelic variant of CYP2C9 in the Asian population is **3* (3.4% in East Asian and 11.3% in South Asian). *CYP2C9*5*, **6*, **8*, **9*, and **11* are nearly only restricted to African populations, and *CYP2C9*14* is almost uniquely found in South Asian individuals (16). Thus, clinical treatment decision on CYP2C9 meditated drugs in Caucasian populations may not have good generality and adaptability for other national populations. To better understand the specific polymorphic pattern of *CYP2C9* gene in the Chinese Han population, we previously conducted a large-scale genetic screening of *CYP2C9* in 2124 Chinese Han individuals and reported 21 new allelic variants in healthy subjects. Since then, four additional *CYP2C9* alleles *CYP2C9*58-*60* and **62* were also identified in the warfarin-sensitive Chinese patients (17–20). Both *in vitro* and *in vivo* studies on these newly uncovered CYP2C9 variants revealed that almost all of them exhibited significantly changed metabolic activities, although their allele frequencies are below 1% (21–23). These data indicated that some other rare *CYP2C9* alleles may still be undiscovered and need further investigation, considering that more than 1.4 billion Chinese Han populations lived in mainland China.

In this study, 1163 healthy Chinese individuals were used for the genetic polymorphism investigation on *CYP2C9* gene by a time- and labor-saving sequencing method. As a consequence, 10 new allelic variants were identified and functional evaluation experiments were also conducted to characterize their impacts on the enzyme's drug metabolic activity.

2 Materials and methods

2.1 Chemical materials

The FinePure Universal DNA Purification Kit was purchased from GENFINE Biotech (Beijing, China). The Taq plus master mix was obtained from Vazyme (Nanjing, China). PrimeSTAR Max DNA polymerase, restriction enzymes and DO Supplement-Ura were obtained from Takara Bio, Inc. (Otsu, Shiga, Japan). *Saccharomyces cerevisiae* strain YPH499 was obtained from ATCC (VA, USA). Yeast nitrogen base without amino acids, dextrose, galactose and losartan were purchased from Sigma-Aldrich (MO, USA). Baculosomes co-expressing human CYP2C9 and NADPH-cytochrome P450 oxidoreductase (OR) were purchased from BD Gentest (Woburn, MA, USA). The rabbit polyclonal anti-CYP2C9 antibody was obtained from Abcam (Cambridge, UK). The mouse monoclonal anti-OR antibody was from Santa Cruz Biotechnology (Dallas, Texas, USA). The Super Signal West Pico Trial Kit was obtained from Thermo Scientific (Rockford, IL, USA). Losartan was purchased from Sigma-Aldrich (St. Louis, MO, USA). Losartan carboxylic acid (E-3174), glimepiride and cyclohexyl hydroxymethyl glimepiride (M1) were obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). The NADPH-regenerating system was purchased from Promega (Madison, WI, USA). High-pressure liquid chromatography-grade solvents were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Other chemicals and solvents used were of analytical grade or the highest grade that was commercially available.

2.2 Genomic DNA extraction

All participants in this experiment were healthy Chinese Han individuals recruited in the Physical Examination Center of Beijing Hospital. The written informed consent form was signed when

blood collection and this study was approved by the Ethics Committee of Beijing Hospital. FinePure Universal DNA Purification Kit was used to extract DNA from white blood cells following manufacturer's recommend protocol, and genomic DNAs were diluted to the final concentration of approximately 40 ng/μL for PCR amplification.

2.3 Genotyping

To get a time-saving and cost-effective method for the genotyping of *CYP2C9* gene, a multiplex PCR amplicon sequencing method was developed in this study (Figure 1). The first round PCR reaction is used for the multiplex PCR amplification of all 9 exons of *CYP2C9* plus the exon-intron junction regions, and the second round of PCR reaction is aimed to obtain the amplicon library for the second-generation sequencing. Primers in the first round PCR reaction were designed by MFEPrimer (version 3.1) at the website of iGeneTech (https://mfeprimer3.igenetech.com/muld). Detailed primer information was listed in Table 1. A total amount of 40 ng genomic DNA was used as the input material for two rounds of PCR amplification. After purification with AMPure XP beads (Beckman, USA), barcoded library was quantified with Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyzer system (Agilent, USA) was used to measure the concentration and length of library fragments (from 270 to 420 bp). Qualified libraries were then sequenced on NovaSeq 6000 (Illumina, USA) with pair-end 150 sequencing strategy by iGeneTech Co (Beijing, China).

Raw reads were filtered to remove low quality reads using FastQC (Version 0.11.9) and clean data were mapped to the reference genome GRCh38 and annotated using Annovar software (24). The high-quality annotated data were then obtained after filtering with these parameters: Sequencing depth >50 and detected frequency is within the range of 0.4-0.6 (heterozygote) or 0.9-1.0 (homozygote). Then, detected mutation sites were aligned with PharmVar listed *CYP2C9* allele table to identify the allelic variants. For novel variants not included in the allele table, bi-directional sanger sequencing were used for sequence verification with our recently published primers (25).

2.4 Expression of CYP2C9 variants in the yeast cells

Full-length cDNA of the typical defective *CYP2C9* allele (*CYP2C9*3*) was constructed using previously described overlap extension PCR amplification method (21). Similarly, cDNA of newly discovered variants were obtained with primer pairs listed in Table 2 using wild type *CYP2C9*1* cDNA as the PCR template. The resulting full-length cDNA fragments were double digested with EcoRI and XhoI, and ligated to EcoRI/XhoI digested pESC-OR vector to get the dual expression yeast vector pESC-OR-CYP2C9. Using previously described method, all newly detected CYP2C9 variants were highly expressed with co-expressed CYPOR enzyme in yeast cell microsomes (18). The quantification of expressed CYP2C9 proteins was performed according to our previously reported method (20).

2.5 Enzymatic activity analysis

Based on our previously described methods (18, 22, 26), the drug metabolic activities of the wild type, typical defective variant CYP2C9.3, and seven allelic CYP2C9 variants found in this study were assessed with 2 typical CYP2C9 substrates: losartan and glimepiride. Briefly, reaction mixture contained 2-3 pmol of P450 from yeast microsomes, 5 μL purified cytochrome b5, and 2 μL substrates stock solution (dissolved in methanol) in 0.1M K_3PO_4 buffer (pH 7.5). The ultimate concentrations of losartan and glimepiride were 0.5-50 μM and 0.1-20 μM per reaction,

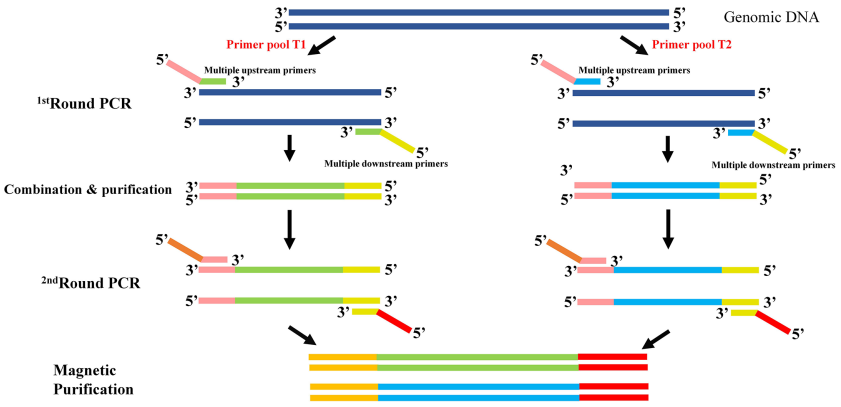

FIGURE 1
Schematic diagram of the multiplex PCR amplicon sequencing method for *CYP2C9* genotyping. Two rounds of PCR amplification were included in this method. The first round of PCR is for the multiplex amplification of all 9 exons of *CYP2C9* gene with pooled primers T1 or T2. After the combination and purification with magnetic beads, products were used as the template for the second round of PCR reaction and sequencing library construction. Universal primers in round 1 are illustrated as pink and yellow bars, and sequencing primers in round 2 are shown in brown and red bars.

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TABLE 1 Primers used for the first round of multiplex PCR reaction.

*Primer	**Forward (5′-3′)	**Reverse (5′-3′)	Amplicon (bp)	Position	Region
T1P1	TTGGAGTGCAAGCTCATGGTT	GTGAATTTACTTACCTTTTGCAAGCC	259	chr10:94938636-94938895	Exon 1
T1P2	GCAAGCTCATGGTTGTCTTAACAAG	AGGTGAATTTACTTACCTTTTGCAAGC	254	chr10:94938643-94938897	Exon 1
T1P3	GAGTGCAAGCTCATGGTTGTCT	GTGAATTTACTTACCTTTTGCAAGCCA	256	chr10:94938639-94938895	Exon 1
T1P4	ATTTGAAGCCTGTGTGGCTGA	ATGCAGCACCACTATGGGTTT	188	chr10:94941735-94941923	Exon 2
T2P1	GGACAAAATAGTAACTTCGTTTGCTGT	CATCCCCAAGACAGATGCTGAA	253	chr10:94941809-94942062	Exon 2
T1P5	TTCCTCTTTCTTGCCTGGGATC	GTAGTCCAGTAAGGTCAGTGATATGG	244	chr10:94942138-94942382	Exon 3
T2P2	GGGAGGATGGAAAACAGAGACT	CTTCCTCTTGAACACGGTCCT	243	chr10:94942062-94942305	Exon 3
T1P6	ACCCTGTGATCCCACTTTCATC	TGCACTTCAGAGCTTGATCCAT	250	chr10:94947782-94948032	Exon 4
T1P7	CCTGCAATGTGATCTGCTCCAT	TGCACTTCAGAGCTTGATCCATG	215	chr10:94947817-94948032	Exon 4
T2P3	AAACTACTATTATCTGTTAACAAATACAGTGTT	CAAAAATCTTGGCCTTACCTGGATC	259	chr10:94947698-94947957	Exon 4
T2P4	TCTGTTAACAAATACAGTGTTTTATATCTAAAGT	TCTCAGGAAGCAAAAATCTTGGC	257	chr10:94947710-94947967	Exon 4
T2P5	CTGTTAACAAATACAGTGTTTTATATCTAAAGTT	AGGAAGCAAAAATCTTGGCCTTAC	252	chr10:94947711-94947963	Exon 4
T1P8	GATCTGCTCCATTATTTTCCATAAACGT	GTCTGGGCAAGACTGTAGTATTCAA	233	chr10:94947827-94948060	Exon 4
T1P9	TCAATGGACATGAACAACCCTCA	GCTTCTCAAGCATTACTGATTGACC	173	chr10:94949224-94949397	Exon 5
T1P10	GGTTAGAATTGATCCTCTGGTCAGA	GTTGTGAGTTCCCGGGAAGTAA	260	chr10:94948898-94949158	Exon 5
T2P6	TGGTATATGGTATGTATGCTTTTATTAAAATCTT	GCTTTTGTTTACATTTTACCTTCTCCATT	260	chr10:94949043-94949303	Exon 5
T2P7	TTGGTATATGGTATGTATGCTTTTATTAAAATCT	CTTTTGTTTACATTTTACCTTCTCCATTTTCAT	260	chr10:94949042-94949302	Exon 5
T1P11	AGTTGGTCTACAGCCTCTGCTA	CTGTCCCAGCTCCAAACAAGT	243	chr10:94971939-94972182	Exon 6
T1P12	GCACAACCCTGAGATATGCTCT	ACCATGCCAGGCCAAGATATC	230	chr10:94972191-94972421	Exon 6
T2P8	TGCTGGTAAATAATTTGTCAGATAATTGCA	GACACTAGCAACACCTTCCCAA	252	chr10:94972058-94972310	Exon 6
T1P13	GCCATTTTTCTCCTTTTCCATCAGTTT	GTTGCAGTGTAGGAGAAACAAACTTAC	260	chr10:94981137-94981397	Exon 7
T1P14	TGTGCCATTTTTCTCCTTTTCCA	GAGAAACAAACTTACCTTGGGAATGAGA	251	chr10:94981134-94981385	Exon 7
T2P9	TCCAGGAAGAGATTGAACGTGTG	TTGGGGACTTCGAAAACATGGAG	231	chr10:94981188-94981419	Exon 7
T2P10	ATGCAAGACAGGAGCCACATG	GAGTTATGCACTTCTCTCACCCG	241	chr10:94981235-94981476	Exon 7
T2P11	CCAGGAAGAGATTGAACGTGTGA	TTGGGGACTTCGAAAACATGGA	230	chr10:94981189-94981419	Exon 7

(Continued)

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TABLE 1 Continued

*Primer	**Forward (5′-3′)	**Reverse (5′-3′)	Amplicon (bp)	Position	Region
T1P15	CCACTGTTTCTTCAACCTTCATGG	CTTGTACCCTGAAACACAAATGGA	246	chr10:94985968-94986214	Exon 8
T2P12	TGGTACTGCCCTTCTTTGGAAC	GTCAAACATCTCTGGGTTGGGA	205	chr10:94985902-94986107	Exon 8
T2P13	CTTCATGCCTTTCTCAGCAGGTA	TGGATTAACTCCCCAAAGTCCAC	222	chr10:94986154-94986376	Exon 8
T1P16	ACCCATCCACCCATCTATCTCT	GGTTCTTTGGGTCAACCAGAGA	255	chr10:94988699-94988954	Exon 9
T1P17	CCTGTCTGAAGAAGAGCAGATGG	CTCTCCGTAATGGAGGTCGAATG	170	chr10:94989019-94989189	Exon 9
T2P14	CATGAGGAGTAACTGCTCTCTGTG	GACTGCACAGCAGCAGC	255	chr10:94988808-94989063	Exon 9

*Primers starting with T1 and T2 were mixed in the primer pool T1 and T2, respectively.
**All primers contained a universal part in the 5' direction and only the sequences for 3' specific part were listed in this table.

respectively. After a 5 min pre-incubation, an NADPH-regenerating system (1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, and 0.4 unit/mL glucose-6-phosphate dehydrogenase) was added to start the reaction at 37°C in a final volume of 200 µL and proceeded for 30 min (losartan) or 50 min (glimepiride). The incubation was terminated by adding an equal volume of the stop solution containing 150 µL acetonitrile and 50 µL internal standard midazolam (500 ng/mL). After vortexing, the incubated mixture was centrifuged at 12,000 × *g* for 5 min, and 200 µL aliquots were then removed and used for the following

TABLE 2 Primers used for the yeast expression vector construction.

Primer	*Sequence (5′-3′)	Amplicon	Variant	Note
2C9-UF	CCGA*GAATTC*ATGGATTCTCTTGTGGTCC	1499	wild type	EcoRI site listed in underline
2C9-UR	AACC*CTCGAG*TTAGACAGGAATGAAGCACAG			XhoI site listed in underline
2C9-L71R-F	ATTTTGGCCgGAAACCCA	1281	L71R	xx-R paired with UF
2C9-L71R-R	TGGGTTTCcGGCCAAAAT	236		xx-F paired with UR
2C9-P163S-F	AGGCCTCAtCCTGTGAT	1005	P163S	xx-R paired with UF
2C9-P163S-R	ATCACAGGaTGAGGCCT	511		xx-F paired with UR
2C9-T301M-F	GACAGAGAtGACAAGCA	590	T301M	xx-R paired with UF
2C9-T301M-R	TGCTTGTCaTCTCTGTC	926		xx-F paired with UR
2C9-E326K-F	CCAGGAAaAGATTGAACG	515	E326K	xx-R paired with UF
2C9-E326K-R	CGTTCAATCTtTTCCTGG	1002		xx-F paired with UR
2C9-C372R-F	CAGTGACCcGTGACATT	378	C372R	xx-R paired with UF
2C9-C372R-R	AATGTCACgGGTCACTG	1138		xx-F paired with UR
2C9-I389V-F	CCATATTAgTTTCCCTG	327	I389V	xx-R paired with UF
2C9-I389V-R	CAGGGAAAcTAATATGG	1189		xx-F paired with UR
2C9-H396Y-F	CTGTGCTAtATGACAAC	306	H396Y	xx-R paired with UF
2C9-H396Y-R	GTTGTCATaTAGCACAG	1210		xx-F paired with UR
2C9-N398H-F	GCTACATGACcACAAAG	302	N398H	xx-R paired with UF
2C9-N398H-R	CTTTGTgGTCATGTAGC	1214		xx-F paired with UR
2C9-G431R-F	TCTCAGCAaGAAAACGG	201	G431R	xx-R paired with UF
2C9-G431R-R	CCGTTTTCtTGCTGAGA	1315		xx-F paired with UR
2C9-I488F-R	AACCCTCGAGTTAGACAGGAAaGAAGC	1499	I488F	paired with UF

*The mutated site of each variant is illustrated as lower-case letter.

measurements. The incubations were performed in triplicate, and the mean values and S.D. from three experiments were provided for analysis.

Detection and quantification of the metabolites after incubation were performed on the ACQUITY UPLC I-Class/Xevo TQD IVD System (Waters, Milford, MA, USA). Aliquots of samples were placed into an ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm; 1.7 μM; Waters), and the column temperature was maintained at 40°C. The initial mobile phase comprised A (pure acetonitrile, >98%) and B (ultrapure water), and the flow rate was 0.4 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer equipped with positive electrospray ionization (ESI) by multiple reactions monitoring (MRM) of the transitions. The linearity gradient elution condition for losartan was as following: 0–0.5 min (30%A), 0.5–1.0 min (30-95%A), 1.0–2.0 min (95%A), 2.0–2.3 min (95–30%A); The linearity gradient elution condition for glimepiride was set as 0–0.3 min (10-25%A), 0.3–2.0 min (25-95%A), 2.0–2.5 min (95%A), 2.5–2.6 min (95–10%A). The running time for all detections was 3.0 min. MRM transitions were m/z 437.20 → 235.00, m/z 507.30 → 126.10, and m/z 325.98 → 291.07 for E-3174, hydroxyglimepiride and midazolam, respectively. Nitrogen was used as the desolvation gas (1000 L/h) and cone gas (50 L/h). The dwell time was 0.063 s for E-3174 and 0.108 s for hydroxyglimepiride, the capillary voltage was set as 3.00 kV and the desolvation temperature was maintained at 500°C.

The enzymatic kinetic parameters Km, Vmax, and clearance rate Clint (Vmax/Km) were calculated by GraphPad Prism (version 9; GraphPad Software, Inc., CA, USA). IBM SPSS software (version 25.0, Magneto, New York, USA) was then used to evaluate the catalytic activity difference between the wild type and expressed variants by independent-samples T test.

3 Results

3.1 Distribution pattern of *CYP2C9* alleles in the Chinese Han population

As illustrated in Figure 1, two rounds of PCR amplification were included in the newly developed multiplex PCR amplicon sequencing method. All 9 exons and exon-intron regions of *CYP2C9* gene could be efficiently and specifically amplified after the first round PCR with multiplex PCR primers listed in Table 1. The products were pooled and purified with magnetic kit. Then, the purified amplicons were used for the second round of PCR amplification with universal primers to obtain the library for the second-generation sequencing. Using this system, we efficiently identified 39 allelic variants of *CYP2C9* in 1163 individuals, which include 16 previously reported nonsynonymous variations, 13 synonymous variations and 10 new nonsynonymous variations (Table 3). Similar to other studies, the most common defective allele in Chinese Han population is *CYP2C9*3* with a allele frequency of 3.998% and 7.57% of studied subjects are heterozygote carrying **1/*3*. In addition, 16 previously reported alleles (*CYP2C9*2, *8, *13, *16, *29, *31, *34, *36, *37, *39, *45, *48, *53, *56, *60*, and **75*) were also detected in this study, and most of these allelic variants are heterozygous with the wild type with a total genotype frequency less than 4% which indicates that these alleles are rare in the Han Chinese populations (Table 4).

3.2 Identification of 10 new *CYP2C9* allelic variants

In this study, 10 non-synonymous *CYP2C9* variations (L71R, P163S, T301M, E326K, C372R, I389V, H396Y, N398H, G431R, and I488F) were newly identified, which have not yet been nominated by the Pharmacogene Variation (PharmVar) Consortium (https://www.pharmvar.org/gene/CYP2C9). Their sequencing electropherogram pictures are shown in Figure 2. As illustrated in Table 3, these newly detected variants are located at almost all exons which include exon 2, exon 4, exon 6 - exon 9. Individuals carrying these variants were all heterozygous with wild type *CYP2C9*1* and most of the variants could be detected in only one person, except for Asn398His which was found to be carried by two subjects. Specially, 7 of these 10 variants were reported for the first time and could be regarded as novel *CYP2C9* variants because they have not been registered by the dbSNP database or any other public databases currently.

3.3 Expression of newly detected CYP2C9 variants in yeast cells

In order to characterize the biological effects of newly detected CYP2C9 variants, the yeast expression system was used to efficiently co-express CYP2C9 enzyme and NADPH-cytochrome P450 oxidoreductase (CYPOR) according to the methods described previously (18). Immunoblot results indicated that most of newly detected CYP2C9 variants exhibited comparable protein expression level to that of wild type enzyme CYP2C9.1, except for variants Pro163Ser, Glu326Lys, Gly431Arg and Ile488Phe which showed obviously lower protein expression levels than the wild type (Figure 3).

3.4 Drug metabolic activity analysis of CYP2C9 variants

To better understand the impacts of newly detected CYP2C9 variants on drug metabolic activity of enzyme, two typical CYP2C9 mediated drugs losartan and glimepiride were included in this study. As a result, three variants (Thr301Met, Glu326Lys and Gly431Arg) showed no catalytic activities towards both drugs. Whereas 4 variants exhibited elevated activities for the metabolism of losartan (Table 5) and 6 variants exhibited increased intrinsic clearance rate for glimepiride, as compared with the wild type enzyme CYP2C9.1 (Table 6). These data indicated that most of newly detected CYP2C9 variants could significantly change the metabolic ability of enzyme (Figures 4, 5).

4 Discussion

In this study, we developed a timesaving and cost-effective genotyping method for *CYP2C9* gene. This method is based on the combination of MPCR (Multiplex polymerase chain reaction) and NGS (Next generation sequencing) techniques and has many advantages over traditional Sanger sequencing method. Firstly, this method is easy to be operated in a large scale with automatic protocol, leading to the lower cost, reduced man-made errors and improved accuracy than Sanger method; Secondly, newly developed method can present overall genetic information of *CYP2C9* gene in the target region, that can be used for the genotyping of previously reported alleles and for the discovery of novel variants with unreported mutations, simultaneously; Finally, short time, typically only 2-3 weeks, is needed for the genotyping of hundreds of the samples. In contrast, for traditional Sanger sequencing method, several months maybe needed for the sequencing and analyzing of all 9 exons of *CYP2C9* in a large scale. In brief, our method not only reduces the costs for genotyping, but also greatly improves the efficiency and accuracy of sequencing, favoring its application in large sample scale, multi-center or multi-targets genotyping projects.

Like other *CYP2C* members, *CYP2C9* gene shows marked differences in the allelic frequency in different biogeographic groups and races. These genetic polymorphisms are highly related to the adverse drug reactions (ADRs), especially for the drugs with narrow therapeutic window (27), such as the hypoglycemia caused by hypoglycemic drugs (28), the gastrointestinal bleeding caused by non-steroidal anti-inflammatory drugs (29, 30), and severe bleeding caused by anticoagulation therapy (31, 32), etc. Therefore, digging

TABLE 3 Allelic *CYP2C9* variants identified in 1163 Chinese Han individuals.

Allele	Gene position	Nucleotide change	rsID	Amino-acid effect	Region	n	Allele frequency(%)
**36*	1A>G	1A>G	rs114071557	SCM	exon 1	2	0.086
/	54A>T	54A>T	rs544425883	S18S	exon 1	3	0.129
**37*	146A>G	146A>G	rs564813580	D49G	exon 1	4	0.172
new	3219T>G	212T>G	rs538852786	L71R	exon2	1	0.043
/	3235G>A	228G>A	rs17847036	V76V	exon2	12	0.516
**13*	3276T>C	269T>C	rs72558187	L90P	exon2	8	0.344
**39*	3300G>T	293G>T	rs762239445	G98V	exon2	3	0.129
/	3547C>A	369C>A		I123I	exon3	1	0.043
**45*	3572C>T	394C>T	rs199523631	R132W	exon3	1	0.043
**2*	3608C>T	430C>T	rs1799853	R144C	exon3	5	0.215
**8*	3627G>A	449G>A	rs7900194	R150H	exon3	1	0.043
/	9098C>T	483C>T		A161A	exon4	2	0.086
new	9102C>T	487C>T		P163S	exon4	1	0.043
/	9155C>T	540C>T		S180S	exon4	1	0.043
**48*	9235T>C	620T>C	rs1326630788	I207T	exon4	1	0.043
/	9245C>T	630C>T	rs773479415	S210S	exon4	1	0.043
/	10521A>G	738A>G		E246E	exon5	3	0.129
/	10533A>G	750A>G		E250E	exon5	1	0.043
/	10545A>G	762A>G		S254S	exon5	30	1.290
**29*	33437C>A	835C>A	rs182132442	P279T	exon6	8	0.344
**16*	33497A>G	895A>G	rs72558192	T299A	exon6	2	0.086
new	33504C>T	902C>T	rs757970831	T301M	exon6	1	0.043
**53*	33551C>T	949C>T	rs1237225311	P317S	exon6	1	0.043
new	42515G>A	976G>A		E326K	exon7	1	0.043
**31*	42519T>C	980T>C	rs57505750	I327T	exon7	2	0.086
**34*	42543G>A	1004G>A	rs367826293	R335Q	exon7	1	0.043

(Continued)

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TABLE 3 Continued

Allele	Gene position	Nucleotide change	rsID	Amino-acid effect	Region	n	Allele frequency(%)
*3	42614A>C	1075A>C	rs1057910	I359L	exon7	93	3.998
new	42653T>C	1114T>C		C372R	exon7	1	0.043
/	42676T>C	1137T>C	rs141283168	Y379Y	exon7	15	0.645
*56	47360A>G	1159A>G	rs764211126	I387V	exon8	1	0.043
new	47366A>G	1165A>G		I389V	exon8	1	0.043
new	47382C>T	1186C>T		H396Y	exon8	1	0.043
new	47393A>C	1192A>C		N398H	exon8	2	0.086
/	47398A>G	1197A>G		K399K	exon8	1	0.043
*75	47454A>C	1253A>C	rs1254213342	N418T	exon8	1	0.043
new	50164G>A	1291G>A		G431R	exon8	1	0.043
*60	50273T>C	1400T>C	rs767284820	L467P	exon9	1	0.043
/	50277C>T	1404C>T		D468D	exon9	1	0.043
/	50298A>T	1425A>T	rs1057911	G475G	exon9	92	3.955
new	50335A>T	1462A>T	rs1442749761	I488F	exon9	1	0.043

TABLE 4 Genotype frequencies of CYP2C9 allelic variants in 1163 Chinese Han individuals.

Genotype	n	Frequency (%)
*1/*1	1020	87.70
*1/*2	5	0.43
*1/*3	88	7.57
*3/*3	2	0.17
*3/*13	1	0.09
*1/*8	1	0.09
*1/*13	7	0.60
*1/*16	2	0.17
*1/*29	8	0.69
*1/*31	2	0.17
*1/*34	1	0.09
*1/*36	2	0.17
*1/*37	4	0.34
*1/*39	3	0.26
*1/*45	1	0.09
*1/*48	1	0.09
*1/*53	1	0.09
*1/*56	1	0.09
*1/*60	1	0.09
*1/*75	1	0.09
*1/*84	1	0.09

out the "special subgroups" with abnormal drug metabolism in the population is one of the key factors for reducing the occurrence of ADRs in the clinic. For instance, warfarin is the most commonly used oral anticoagulant, but its therapeutic index is narrow and wildly variable among different patients. Genetic polymorphisms of *CYP2C9* and Vitamin K epoxide reductase complex subunit 1 *(VKORC1)* are one of the most concerned factors for the optimal warfarin dose determination in clinic (33). S-warfarin is mainly metabolized *via* CYP2C9 to 7-hydroxy warfarin. Typical missense variant *CYP2C9*3* caused a remarkable decrease in the S-warfarin clearance rate, leading to the increased risk of venous thromboembolism and bleeding in patients (13). Our recent studies revealed that a lot of rare *CYP2C9* alleles are carried by Chinese individuals and most of missense mutations in *CYP2C9* gene are highly related to the low dose of warfarin in Chinese population (18, 20, 34). In this study, we developed one time-saving and cost-effective genotyping method for *CYP2C9* and performed a genetic screening in 1163 Chinese individuals. Similar to our previous study, *CYP2C9*3* is the most prevalent defective alleles in Chinese population although the allele frequency detected in this study is slightly higher than previous report (17). Additionally, *CYP2C9*13* and *29 exhibited relatively higher frequencies than other allelic variants which is in agreement to our previous reports (17, 25). For the first time, we reported one Chinese individual carrying allele *CYP2C9*8* which was previously regarded as only limited to individuals of African ancestry (35). Specially, we detected 10 new allelic variants that have not been listed on the PharmVar consortium website (Table 3; Figure 2). These data indicated that *CYP2C9* was highly polymorphic in Chinese population and more attention should be paid to the distribution pattern and its potential clinical application in clinic, considering that more than 1.4 billion people lived in the mainland of China.

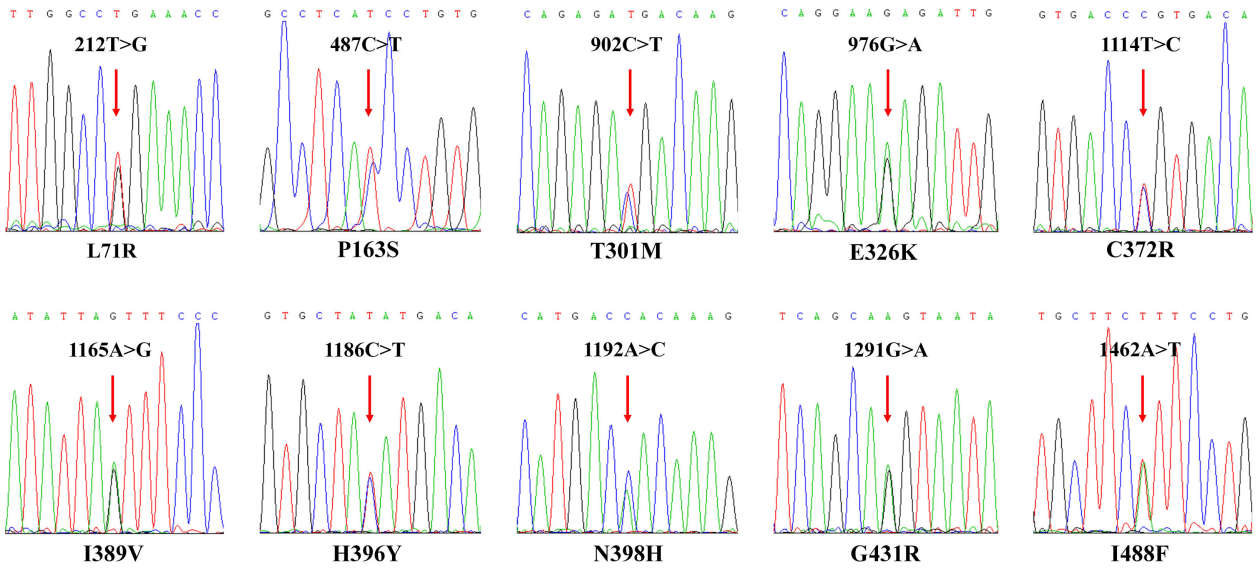

FIGURE 2
Variation verification of newly detected CYP2C9 variants. Sanger sequencing electropherogram pictures of newly detected CYP2C9 variants. The red arrow indicates that the variation sites detected in carriers and the amino acid substitutions are illustrated at the bottom of captured pictures.

Glimepiride is one of the most used oral sulfonylureas (SU) drugs in the clinical treatment of type 2 diabetes mellitus (T2DM). Hypoglycemia is the most common adverse effect related to SU therapy and severe hypoglycemia might significantly increase the cost of medication and decrease the quality of life for T2DM patients (36, 37). Since CYP2C9 is the major enzyme involved in SUs metabolism, the risk of hypoglycemia induced by SUs would be elevated in deleterious CYP2C9 variant allele carriers (38). According to a recent meta-analysis, CYP2C9 variant alleles have increased risk of hypoglycemia than wild-type *CYP2C9*1/*1* after the SUs treatment. The incidence of hypoglycemia would be increased by 80% in *CYP2C9*2* carrier (39). Previous studies have also reported that the AUC of tolbutamide was increased by 150% and 190% in *CYP2C9*1/*2* and **1/*3* carriers, respectively (40); Similarly, for glimepiride, the AUC was increased by 167% in CYP2C9*3 carriers in comparison to *CYP2C9*1/*1* individuals (41). In this study, the *in vitro* metabolic activity analysis results revealed that 3 newly detected CYP2C9 variants had no catalytic

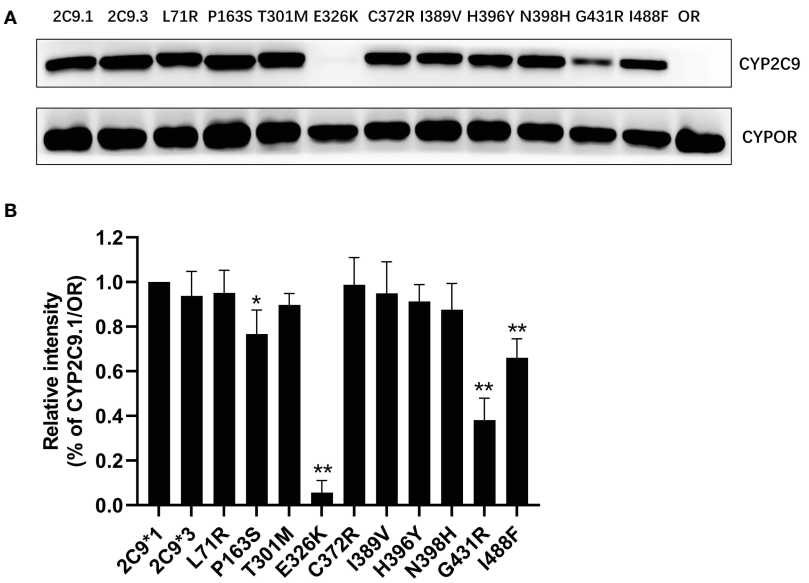

FIGURE 3
The immunoblotting results for expressed CYP2C9 variants in yeast microsomes. **(A)** Expressed CYP2C9 variants and CYPOR enzyme were detected by their corresponding antibodies after the SDS-PAGE gel separation. OR: microsome from yeast cells only expressing CYPOR enzyme. **(B)** Relative CYP2C9/OR intensities. Each bar represents the mean ± SD of three independently experiments. **P* < 0.05, ***P* < 0.01 *vs* CYP2C9.1/OR.

TABLE 5 Enzyme kinetic values of recombinant wild type and CYP2C9 variants towards losartan.

Variants	Vmax (pmol/min/pmol P450)	Km (μM)	Clearance (Vmax/Km)	Relative clearance(/CYP2C9.1)
CYP2C9.1	0.26 ± 0.02	2.53 ± 0.24	0.10 ± 0.0079	100.00%
CYP2C9.3	0.02 ± 0.00*	4.35 ± 0.27*	0.01 ± 0.00053*	5.24%*
L71R	0.32 ± 0.02*	2.47 ± 0.59	0.13 ± 0.025	128.16%*
P163S	0.30 ± 0.01*	2.60 ± 0.25	0.11 ± 0.0092	109.16%
C372R	0.31 ± 0.00*	1.72 ± 0.09*	0.18 ± 0.0068*	174.31%*
I389V	0.23 ± 0.02	2.16 ± 0.36	0.11 ± 0.012	102.94%
H396Y	0.42 ± 0.01*	1.80 ± 0.26*	0.23 ± 0.026*	223.68%*
N398H	0.30 ± 0.01*	2.28 ± 0.20	0.13 ± 0.0093*	125.38%*
I488F	0.32 ± 0.02*	2.81 ± 0.53	0.12 ± 0.016	111.27%

Data are presented as the mean ± S.D. of 3 different expression experiments. *P < 0.05 vs. wild-type CYP2C9.1.

activity for glimepiride metabolizing and carriers for these variants might exhibit significantly reduced drug metabolizing activity for SU. However, most of other newly detected allelic variants exhibited significantly increased enzyme activity for glimepiride metabolism which indicated that carriers with these variants might possess the higher drug metabolizing activity for SU than individuals with wild type *CYP2C9*1/*1*. (Figures 4, 5 and Table 6). These data indicated that different amino acid substitution at different sites of CYP2C9 protein had different effects on the drug metabolizing activity of enzyme.

Typical tertiary structure of a cytochrome P450 enzyme mainly consists of twelve α-helices (A-L) and four β-sheets (1–4) with the heme locating between the helices I and L. There are six substrate recognition sites (SRSs) in CYP2C9 enzyme which locate at the amino acids 96-117 (between the helices B and C), 198-205 (between the helices F and G), 233-240 (between the helices F and G), 286-304 (in the center of the helix I), 359-369 (at the N-terminus of β strand 1-4), and 470-477 (at the turn at the end of β sheet 4), respectively (42). In this study, 3 allelic variants, Thr301Met, Glu326Lys and Gly431Arg showed activity deficiency for both losartan and glimepiride. In the crystal structure of CYP2C9, Thr301 is involved in the SRS4, and Gly431 belongs to heme-binding motif residues. Therefore, the amino acid substitution at position 301 or 431 is estimated to affect the substrate recognition or heme propionate binding capacity for CYP2C9. Similar to our results, another allelic variant at position 301, Thr301Lys, also showed no enzymatic activity (43). These data indicated that Thr301 might be crucial for the drug metabolic activity of enzyme. Glu326 is located at the helix J of CYP2C9 and it has a strong binding strength with 5 amino acid residues within 5Å distance. Previous study revealed that variant Glu326Asp (CYP2C9*65) had deleterious effect in SIFT and Polyphen prediction (12). Combined with the data in this study, it is estimated that the replacement of Glu326 might influence the enzyme activity significantly. Different from these 3 defective variants, most of other newly detected variants exhibited significantly increased metabolizing activities towards both losartan and glimepiride *in vitro* (Figure 5; Tables 5, 6). These data indicated that carriers with these allelic variants might have higher metabolizing activity for CYP2C9 mediated drugs.

In summary, we developed a time-saving next generation sequencing based method for *CYP2C9* genotyping and performed a large-scale polymorphic screening of *CYP2C9* gene in Chinese Han population. Totally 16 previously reported allelic variants and 10 new non-synonymous variations were detected in this study. When

TABLE 6 Enzyme kinetic values of recombinant wild type and CYP2C9 variants towards glimepiride.

Variants	Vmax (pmol/min/pmol P450)	Km (μM)	Clearance (Vmax/Km)	Relative clearance(/CYP2C9.1)
CYP2C9.1	45.12 ± 6.04	2.25 ± 0.80	21.39 ± 5.77	100.00%
CYP2C9.3	12.70 ± 2.47*	29.30 ± 4.75	0.43 ± 0.014*	2.02%*
L71R	37.85 ± 1.44	1.69 ± 0.33	22.85 ± 4.042	108.45%
P163S	69.21 ± 2.49*	1.85 ± 0.45*	38.92 ± 9.57	182.72%*
C372R	72.28 ± 1.61*	1.60 ± 0.15	45.43 ± 4.65*	219.92%*
I389V	122.23 ± 7.17*	1.86 ± 0.34	66.89 ± 9.11*	319.66%*
H396Y	117.10 ± 5.56*	1.04 ± 0.11	113.68 ± 11.67*	546.32%*
N398H	89.29 ± 3.82*	1.13 ± 0.09	77.27 ± 2.73*	379.09%*
I488F	90.87 ± 5.32*	1.55 ± 0.23	59.18 ± 5.42*	284.93%*

Data are presented as the mean ± S.D. of 3 different expression experiments. *P < 0.05 vs. wild type CYP2C9.1.

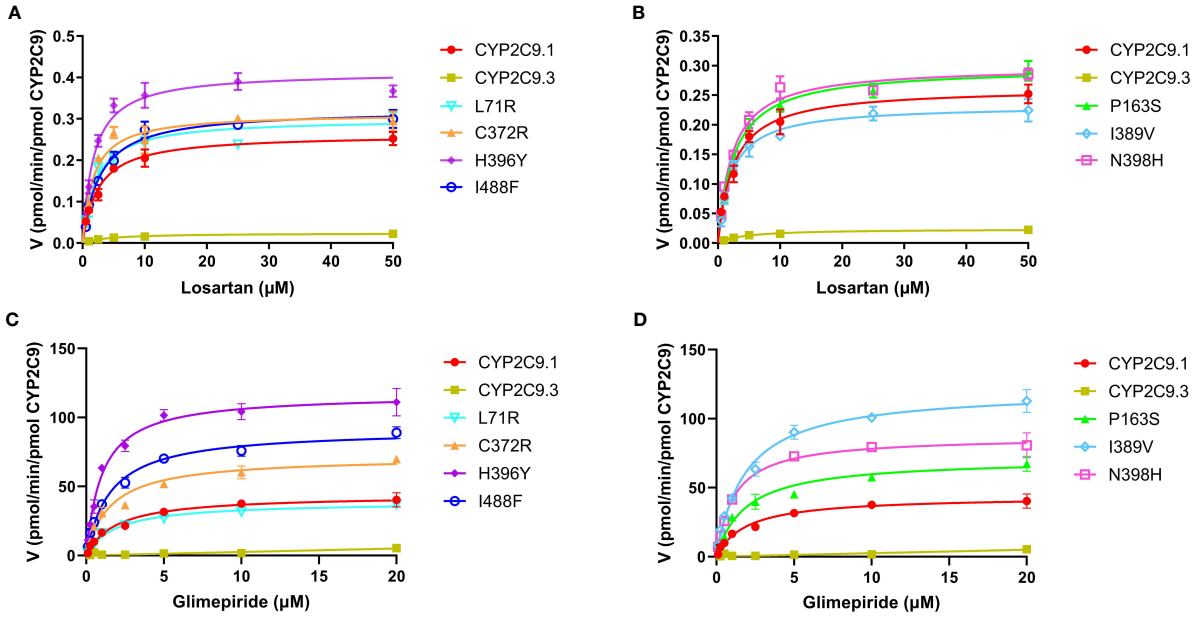

FIGURE 4
Michaelis-Menten curves of the enzymatic activities of expressed CYP2C9 variants toward losartan **(A, B)** and glimepiride **(C, D)**. Each point represents the mean ± S.D. of 3 separate experiments.

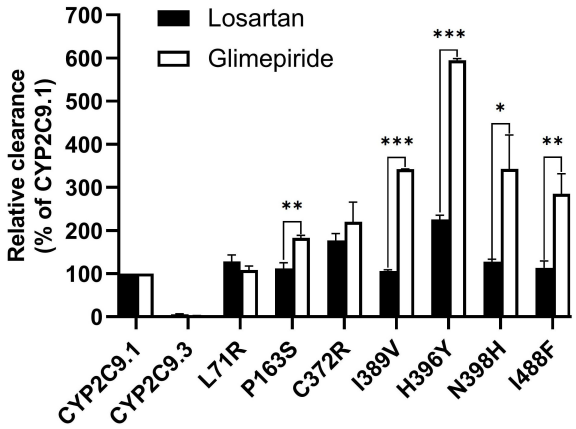

FIGURE 5
The relative clearance rates of losartan and glimepiride among wild type CYP2C9.1, typical defective variant CYP2C9.3 and 7 newly detected variants. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$.

expressed in yeast microsomes, most of newly detected variations showed similar protein expression level to wild type. Further drug metabolic activity analysis revealed that 3 variants were loss of function isoforms and most of other newly detected variants exhibited significantly increased metabolizing activities for both losartan and glimepiride. Our study greatly enriched the knowledge of genetic polymorphism of *CYP2C9* in Chinese Han population, and the clinical significance of newly detected CYP2C9 alleles still needs further investigation by enlarging the sample size and deep correlation analysis between genetic information and clinical features.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Genbank-BankIt2667312 Seq_C487T OQ376733, BankIt2667312 Seq_G976A OQ376734, BankIt2667312 Seq_T1114C OQ376735, BankIt2667312 Seq_A1165G OQ376736, BankIt2667312 Seq_C1186T OQ376737, BankIt2667312 Seq_A1192C OQ376738, BankIt2667312 Seq_G1291A OQ376739.

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Beijing Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

DD, JY, and HC contributed to conception and design of the study. QiZ, YQ, SW, FZ, LZ, QuZ, PG, YH, and HY performed the experiments. QiZ, YQ, SW, FZ, QuZ, QL, JC, HW, and DW performed the statistical analysis. QiZ wrote the first draft of the manuscript. YQ and DD wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Microvascular and macrovascular complications of type 2 diabetes mellitus: Exome wide association analyses

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Background: Type 2 diabetes mellitus (T2DM) is a chronic, metabolic disorder in which concomitant insulin resistance and β-cell impairment lead to hyperglycemia, influenced by genetic and environmental factors. T2DM is associated with long-term complications that have contributed to the burden of morbidity and mortality worldwide. The objective of this manuscript is to conduct an Exome-Wide Association Study (EWAS) on T2DM Emirati individuals to improve our understanding on diabetes-related complications to improve early diagnostic methods and treatment strategies.

Methods: This cross-sectional study recruited 310 Emirati participants that were stratified according to their medically diagnosed diabetes-related complications: diabetic retinopathy, diabetic neuropathy, diabetic nephropathy, and cardiovascular complications. The Illumina's Infinium Exome-24 array was used and 39,840 SNPs remained for analysis after quality control.

Findings: The analysis revealed the associations of various genes with each complication category: 1) diabetic retinopathy was associated to *SHANK3* gene in locus 22q13.33 (SNP rs9616915; p=5.18 x10^{-4}), *ZSCAN5A* gene in locus 19q13.43 (SNP rs7252603; p=7.55 x10^{-4}), and *DCP1B* gene in locus 12p13.33 (SNPs rs715146, rs1044950, rs113147414, rs34730825; p=7.62 x10^{-4}); 2) diabetic neuropathy was associated to *ADH4* gene in locus 4q23 (SNP rs4148883; p=1.23 x10^{-4}), *SLC11A1* gene in locus 2q35 (SNP rs17235409; p=1.85 x10^{-4}), and *MATN4* gene in locus 20q13.12 (SNP rs2072788; p=2.68 x10^{-4}); 3) diabetic nephropathy was associated to *PPP1R3A* gene in locus 7q31.1 (SNP rs1799999; p=1.91 x10^{-4}), *ZNF136* gene in locus 19p13.2 (SNP rs140861589; p=2.80 x10^{-4}), and *HSPA12B* gene in locus 20p13 (SNP rs6076550; p=2.86 x10^{-4}); and 4) cardiovascular complications was associated to *PCNT* gene in locus 21q22.3 (SNPs rs7279204, rs6518289, rs2839227, rs2839223; p=2.18 x10^{-4},3.04 x10^{-4},4.51 x10^{-4},5.22 x10^{-4}

respectively), *SEPT14* gene in locus 7p11.2 (SNP rs146350220; p=2.77 x10^{-4}), and *WDR73* gene in locus 15q25.2 (SNP rs72750868; p=4.47 x10^{-4}).

Interpretation: We have identified susceptibility loci associated with each category of T2DM-related complications in the Emirati population. Given that only 16% of the markers from the Illumina's Infinium Exome chip passed quality control assessment, this demonstrates that multiple variants were, either, monomorphic in the Arab population or were not genotyped due to the use of a Euro-centric EWAS array that limits the possibility of including targeted ethnic-specific SNPs. Our results suggest the alarming possibility that lack of representation in reference panels could inhibit discovery of functionally important loci associated to T2DM complications. Further effort must be conducted to improve the representation of diverse populations in genotyping and sequencing studies.

KEYWORDS

T2DM, diabetes, EWAS, retinopathy, nephropathy, neuropathy, macrovascular complications, microvascular complications

1 Introduction

Type 2 Diabetes Mellitus (T2DM) is a chronic, metabolic condition, characterized by elevated blood glucose levels (1). Although the pathogenesis of T2DM is complex, a number of factors that increase the risk for the disease have been identified, including modifiable risk factors (body mass index (BMI), physical inactivity, diet) and nonmodifiable risk factors (age, ethnicity, comorbid diseases, family history and genetic predisposition) (2). The clinical presentation and disease progression of patients with T2DM are heterogeneous, which may lead to a delay of diagnosis, multiple pathophysiological abnormalities, and varying susceptibility to complications. Complications from T2DM can be classified as microvascular complications, such as retinopathy, neuropathy and nephropathy, or macrovascular complications, including cardiovascular, cerebrovascular, and peripheral vascular disease (3). Although there is a strong inheritance of risk of developing T2DM, less is known about the heritability and genetic component of diabetes complications (4). Further studies must be conducted to elucidate the genetic variants associated to each diabetic complication to improve early diagnostic measures and therapeutic strategies.

Genome wide association studies (GWAS) has played a major role in identifying susceptibility loci associated with these various categories of diabetes-driven complications. More than 300 genetic loci have been associated with T2DM, which explain >19% of the phenotypic variance in risk for T2DM risk (5). Early family and twin studies have suggested a high concordance rate of the diabetic complications, with heritability estimated at 18 to 60% (6–10). GWAS studies have identified susceptible loci for diabetic retinopathy (*WDR72, NVL*, and *CCDC146*) (11–13), diabetic neuropathy (*XIRP2*, and *APOL1*) (13, 14), diabetic nephropathy (*GABRR1*, and *GYPA*) (7, 13), and cardiovascular complications (*PDE4DIP, NAT8, F5, LPA*, and *RPS6KA2*) (13, 15, 16). However, a number of the single nucleotide polymorphisms (SNPs) that failed to replicate in multiple populations demonstrate the strong influence of population specificity on genetic variation discrimination and contribution to the phenotype of interest. Therefore, discovery and replication investigations in populations of various ancestries are required to identify population-specific traits (17–19). This variability is the leading cause of clinical translation discrepancies due to the scarcity of genetic research specifically to the Middle East region, with multiple countries reporting a T2DM prevalence >20%, including Kuwait, Egypt and the United Arab Emirates (UAE) (20–22).

With the rising prevalence of diabetes-related complications, there is an urgency of conducting genetic studies to uncover new target pathways, and enhance our ability to use precision medicine for targeted therapeutic measures. By identifying new genotypes in an underrepresented region, in this case the UAE, this will yield to the discovery of novel genetic associations in diabetic-related complications. In this study, we aim to conduct an Exome wide association study (EWAS) to identify susceptibility loci associated with diabetic complication development within the Emirati population.

2 Methods

2.1 Ethics approval

An ethical request was submitted to the Dubai Health authority (DHA) whereby it was accepted under reference number DSREC-07/2020_19 and conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent before taking part in this research. All data was de-identified prior to use.

2.2 Study group and phenotype definitions

This prospective, cross-sectional study recruited a total of 338 T2DM patients from the Dubai Diabetes Center (DDC), during the period between October of 2020 and July of 2021. All the patients were diagnosed in accordance to the American Diabetes Association (ADA) diagnosis criteria of a HbA1c ≥ 6.5 and were receiving treatment for their condition. To limit misclassification and ascertainment bias, the patient recruitment process was randomized for a more accurate representation of diabetes within the region.

The blood samples were collected in a sterile 5ml sample tube supplemented with ethylenediaminetetraacetic acid from the cubital vein. Samples were transported in a sealed biohazard bag using a cool transport container to Khalifa University, Center for Biotechnology, in Abu Dhabi for genotypic and analysis. The questionnaire included details on the demographic information, clinical details including physical measurements and medical status, medications prescribed, and biochemical parameters. In this questionnaire, it was ensured that the following clinical data was recorded: date of T2DM diagnosis, presence or absence of a diabetes-related complications, type of complication, and HbA1c measurements attained from the DHA's Salama electronic medical record system. The patients with the presence of complications were stratified into four different phenotype-based categories: retinopathy, neuropathy, nephropathy, and cardiovascular complication. The group stratification was defined as follows:

1. Retinopathy complication: records of proliferative or non-proliferative retinopathy, or laser since the diagnosis of T2DM.
2. Neuropathy complication: records of foot ulcers, gangrene, amputation of the toe/foot/leg, pain in calf muscle while walking, shunting and angioplasty on artery in the leg since the diagnosis of T2DM.
3. Nephropathy complication: records of protein or albumin in the urine, albuminuria in the range of 30 – 299 mg/g, estimated Glomerular Filtration Rate (eGFR) <30 since the diagnosis of T2DM.
4. Cardiovascular complication: records of coronary artery bypass grafting or a cerebrovascular accident since the diagnosis of T2DM.

2.3 DNA extraction and genotyping

DNA extraction of 338 T2DM patients was conducted, as per the manufacturer's instructions using the Qiagen DNA extraction kit. DNA samples were genotyped with the Infinium Exome BeadChip (Illumina, USA) scanned with the iScan System microarray scanner (Illumina, USA). This BeadChip has a total of 244,883 fixed markers. The raw data was uploaded onto GenomeStudio 2.0 and converted into PLINK format. Quality control (QC) was done to check for discordant gender information, missing genotype data (<98%), outlying heterozygosity rate (±3), and related individuals (PI_HAT>0.5). This led to the removal of 28 individuals (1 individual had low genotype quality and 27 individuals were related) for not passing the QC. The SNPs were filtered using the following parameters: low minor allele frequency (<0.01), low genotyping rate (<95%), and deviation from Hardy-Weinberg Equilibrium ($p<10^{-6}$). The number of variants excluded for each filtering parameter was 202075 variants, 2946 variants, and 22 variants, respectively. A total of 310 individuals and 39,840 SNPs passed QC and remained for analysis.

2.4 Statistical analysis

Association analyses corresponding to the following four complication groups were conducted for descriptive statistics and genetic association (EWAS): retinopathy complications, neuropathy complications, nephropathy complications, and cardiovascular complications. For each category, the cases were those that were assigned to that category and the control group were all the remaining individuals that did not experience that particular complication. Statistical analysis of demographic characteristics and anthropometric measurements was conducted. Pearson χ^2 was used to measure the association of categorical variables. Independent sample t-test, presented as mean and standard deviation, or nonparametric Mann-Whitney U-test, presented as median and inter-quartile region, were used to study continuous variables. Statistical analysis was performed in R (version 3.4), SPSS (version 46.0) and PLINK (version 1.9).

For the genetic case-control comparisons, logistic regression, assuming additive allelic effects for genotypes SNPs, were conducted, while adjusting for age, sex, and BMI. Exome-wide association markers surpassed a conservative Bonferroni-corrected significance threshold of discovery $p<1.2\times10^{-6}$ (0.05/39,840), whereas markers that identified associations that reached a suggestive association threshold of $p<5\times10^{-4}$. A quantile-quantile (Q-Q) plot analysis was conducted to check whether the distribution of the inflation p-values deviated from the expected distribution under the null hypothesis of no genetic association and the impact of population stratification was evaluated by calculating the genomic control inflation factor [λGC]. A Manhattan plot was generated with -log10p-values. Q-Q plots and Manhattan plots were generated using the Locuszoom tool. Regional plots were generated by using LocusZoom.

3 Results

A cohort of 310 T2DM patients of which 153 were men and 157 were women aged 14 to 86 years. The cohort was stratified into cases or controls according to four complication groups that are to be tested: retinopathy complications (n=62), neuropathy complications (n=47), nephropathy complications (n=22), and cardiovascular complications (n=42). This classification was done according to diagnosis by the diabetes specialist after the onset of T2DM.

After assessing the anthropometric data of the study cohort (Table 1), it was seen that T2DM patients with neuropathy (p<0.001) and macrovascular (p<0.001) complications were significantly older than the control group. This indicates that T2DM-related complications are more likely to develop with age, providing us with the confidence to adjust for age during the analysis. The gender and mean BMI were not significantly different between cases and control, across all complications. The median glycated hemoglobin levels were significantly higher in the retinopathy cases (p=0.002) compared to controls. The complication groups retinopathy (p<0.001), neuropathy (p<0.001) and cardiovascular complications (p<0.001) were characterized with a longer diabetes duration as opposed to the nephropathy groups (p=0.058).

After performing QC and filtering, 39,840 SNPs were used for further testing in each category of T2DM complication. The total genotyping rate was > 0.995 across all categories. A quantile-quantile (Q-Q) plot analysis was carried out to check whether the distribution of the inflation p-values deviated from the expected distribution under the null hypothesis of no genetic association and investigate if the overall significance of the genome-wide associations is due to potential impact of population stratification. Supplementary Figure 1 presents the Q-Q plot of each respective complication, demonstrating that the genomic inflation factor was negligible in all data sets where it was 1.0 for all the categories based on the chi-squared statistics, after adjustment to age, BMI and gender. Figure 1 demonstrates the Manhattan plot of each complication, and the top 10 SNPs that contributed to the biological relevance of the respective disease is listed in Table 2.

3.1 Retinopathy complications

Gene *ACVR1C* is highly expressed in adipose tissue, and has been associated to extraocular retinoblastoma, hyperkeratosis, T2DM, obesity and anthropometric measurements, such as waist-to-hip ratio and body mass index (23–25). Interestingly, *ACVRIC* is also associated to lipid profile and glycemic markers (26–30). Similarly, gene *ZFHX4* is associated to fasting blood glucose measurement and metabolite levels (31, 32). The association with pulse pressure and blood pressure have been associated to diabetic retinopathy through arterial stiffness and vision impairment, which has been identified in multiple genes, including the *ZFHX4* gene (26, 33, 34), the *SHANK3* gene (35), and the *WNT9B* gene (34). The *SHANK3* gene, expressed in the brain, has also been associated to fibrinogen levels and platelet count, which has been reported to be risk factors in the development and progression of retinopathy (36–40).

The *ZSCAN5A* gene is expressed in the brain is associated with monocyte count, which may lead to the release of pro-inflammatory factors that interfere with endothelial cell junction integrity of the blood-retinal barrier, resulting in leucocyte infiltration in the retina (26, 37, 40, 41). The *DCP1B* gene, expressed in the brain, is associated with waist-to-hip ratio, BMI, and obesity-related traits,

TABLE 1 Demographic factors of the cohort.

	Retinopathy Complication			Neuropathy Complication			Nephropathy Complication			Cardiovascular Complication		
	Cases (n=62)	Controls (n=248)	p-value	Cases (n=47)	Controls (n=263)	p-value	Cases (n=22)	Controls (n=288)	p-value	Cases (n=42)	Controls (n=268)	p-value
Age (years; Mean ± SD)	58.00 (11.32)	56.74 (11.61)	0.445	61.70 (10.03)	56.15 (11.61)	0.002	59.18 (9.67)	56.82 (11.67)	0.358	63.11 (9.69)	56.03 (11.53)	<0.001
Gender Male Feale	34 (54.8%) 28 (45.2%)	119 (48.0%) 129 (52.0%)	0.334	22 (46.8%) 25 (53.2%)	131 (49.8%) 132 (50.2%)	0.705	12 (54.5%) 10 (45.5%)	141 (49.0%) 147 (51.0%)	0.613	22 (52.4%) 20 (47.6%)	131 (48.9%) 137 (51.1%)	0.673
BMI (kg/m^2; Mean ± SD)	31.22 (5.56)	30.92 (5.87)	0.715	31.53 (6.57)	30.87 (5.66)	0.478	30.79 (5.42)	30.99 (5.84)	0.878	29.86 (5.18)	31.15 (5.88)	0.183
HbA1c (%; Median, IQR)	7.75 (2.55)	7.01 (1.40)	0.002	7.21 (1.85)	7.10 (1.50)	0.672	7.40 (1.63)	7.10 (1.55)	0.052	7.25 (1.94)	7.10 (1.55)	0.361
Diabetes Duration, (years; Mean ± SD)*	19.95 (9.48)	13.55 (7.85)	<0.001	18.68 (10.17)	14.17 (8.10)	<0.001	18.22 (9.74)	14.61 (8.46)	0.058	19.73 (10.06)	14.08 (8.08)	<0.001

HBA1c, median glycated hemoglobin levels; IQR, Inter-quartile region; SD, standard deviation.
Pearson χ^2 was used to measure the association of categorical variables.
Independent sample t-test, presented as mean and standard deviation, or nonparametric Mann-Whitney U-test, presented as median and inter-quartile region, were used to study continuous variables.
*For each category, there were 13 individuals with missing data from each respective control group.

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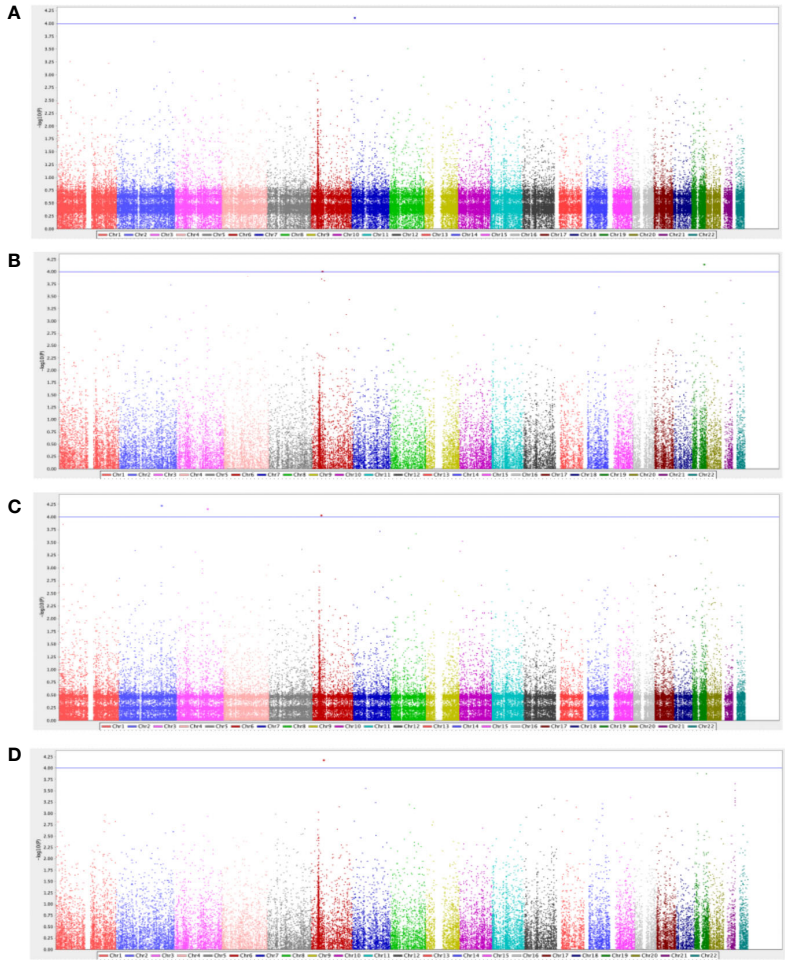

FIGURE 1
Manhattan plot for diabetes-related **(A)** retinopathy complications (n=62), **(B)** neuropathy complications (n=47), **(C)** nephropathy complications (n=22), and **(D)** cardiovascular complications (n=42). The GWAS analyses results are shown on the y-axis as -log10(p-value) and on the x-axis is the chromosomal location, adjusted for age, gender, and BMI. The blue horizontal line illustrates the suggestive genome-wide association threshold ($p<5x10^{-4}$).

all risk factors of T2DM (42–44). This *DCP1B* gene is also associated with Insulin-like growth factors (IGFs), in which transgenic mice models that elucidated that overexpression of IGF-1 in the retina resulted in variations of eye-related diseases similar to that in diabetic humans, through retinal capillaries basement membrane thickening, venule dilation, intra-retinal microvascular abnormalities, and retinal and vitreous cavity neovascularization (44, 45).

3.2 Neuropathy complications

The *GFY* gene is mainly expressed in brain tissue, and has been associated to atherosclerosis through narrowing of the peripheral arterial vasculature (46). *ADH4* gene, expressed in the liver tissue, is associated with eosinophil count, lipid measurements, Apolipoprotein A1 levels (ApoA-I), fibrinogen levels and factor VII levels (38, 39, 47–49). The association with fibrinogen is an important association, as fibrinogen participates in the coagulation process which may lead to an inflammatory process, inhibiting the growth of nerve axons and is closely related to diabetic neuropathy (50, 51). The *LRFN2* gene is expressed in the brain, and has been associated to BMI, T2DM, and obesity-related traits (26, 42, 52, 53).

Interestingly, gene *PKHD1* has been associated to intraocular pressure, brain measurement, T2DM, and metabolic markers, all risk factors associated to neuropathy (53–57). *SLC11A1* gene is expressed in the bone marrow and lymphoid tissues, and has been associated to iron metabolism (58). Using a murine model, Iron's effect on T2DM was elucidated demonstrating a positive association to motor nerve conduction velocities *via* a reduction in pro-inflammatory macrophages and an increase in anti-inflammatory macrophages in nerve sections may induce neuropathy (59). The *MATN4* encodes a protein that is involved in filamentous networks in the extracellular matrices, which is essential for axonal health and growth and may lead to nerve fiber loss (60). The *PPARA* gene has been associated to immune and inflammatory responses, as well as lipid markers, glycolytic markers, T2DM and anthropometric measurements, such as waist-to-hip ratio and body mass index, all relevant risk factors for diabetic neuropathy (40, 61–64).

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TABLE 2 Top 10 SNPs that were associated with each diabetes-related complication group in the Emirati population.

Chr	Cytoband	SNP	Gene	Risk Allele	Adjusted OR (95% CI)	Adjusted P-value
Retinopathy Complications						
2	2q21.4	rs4664229	*ACVR1C*	G	2.33 (1.48, 3.65)	2.22×10^{-4}
8	8q21.13	rs61729527	*ZFHX4*	A	4.65 (2.01, 10.69)	3.04×10^{-4}
17	17q21.32	rs4968281	*WNT9B*	A	2.18 (1.42, 3.33)	3.15×10^{-4}
22	22q13.33	rs9616915	*SHANK3*	G	0.46 (0.29, 0.71)	5.18×10^{-4}
1	1p32.3	rs61738851	*CYB5RL*	A	2.99 (1.61, 5.65)	5.46×10^{-4}
1	1q41	rs10779261	*USH2A*	G	2.09 (1.37, 3.19)	5.91×10^{-4}
19	19q13.43	rs7252603	*ZSCAN5A*	G	0.48 (0.31, 0.74)	7.55×10^{-4}
12	12p13.33	rs715146	*DCP1B*	A	3.06 (1.60, 5.86)	7.62×10^{-4}
12	12p13.33	rs1044950	*DCP1B*	A	3.06 (1.60, 5.86)	7.62×10^{-4}
12	12p13.33	rs113147414	*DCP1B*	A	3.06 (1.60, 5.86)	7.62×10^{-4}
Neuropathy Complications						
19	19q13.33	rs4802605	*GFY*	A	3.94 (2.01, 7.76)	6.99×10^{-5}
4	4q23	rs4148883	*ADH4*	A	2.52 (1.57, 4.01)	1.23×10^{-4}
6	6p21.2	rs6173100	*LRFN2*	A	5.68 (2.32, 13.8)	1.39×10^{-4}
21	21q22.2	rs11558767	*GET1*	A	3.17 (1.74, 5.77)	1.49×10^{-4}
6	6p12.2	rs2499486	*PKHD1*	G	0.38 (0.23, 0.63)	1.52×10^{-4}
2	2q35	rs17235409	*SLC11A1*	A	5.04 (2.16, 11.75)	1.85×10^{-4}
20	20q13.12	rs2072788	*MATN4*	A	2.29(1.47, 3.58)	2.68×10^{-4}
19	19q13.42	rs4644955	*TMEM86B*	A	3.35 (1.71, 6.55)	4.07×10^{-4}
22	22q13.31	rs4253772	*PPARA*	A	3.64 (1.77, 7.47)	4.34×10^{-4}
3	3Q21.2	rs78680419	*HEG1*	A	2.53 (1.50, 4.28)	4.92×10^{-4}
Nephropathy Complications						
2	2q31.2	rs72646845	*TTN*	A	38.05 (6.45, 224.4)	5.84×10^{-5}
3	3q22.1	rs61629992	*COL6A6*	A	5.26 (2.33, 11.92)	6.80×10^{-5}
6	6p21.1	rs113848006	*PI16*	G	12.91 (3.58, 46.49)	9.10×10^{-5}
1	1p36.13	rs41272737	*CROCC*	A	9.51 (2.98, 30.28)	1.37×10^{-4}
7	7q31.1	rs1799999	*PPP1R3A*	A	3.52 (1.82, 6.82)	1.91×10^{-4}
8	8q22.3	rs36027551	*DPYS*	A	17.12 (3.81, 76.95)	2.12×10^{-4}
19	19q13.41	rs143144671	*ETFB*	A	5.72 (2.24, 14.58)	2.58×10^{-4}
19	19p13.2	rs140861589	*ZNF136*	G	15.82 (3.57, 70.16)	2.80×10^{-4}
20	20p13	rs6076550	*HSPA12B*	A	14.20 (3.39, 59.52)	2.86×10^{-4}
10	10p13	rs1541010	*FRMD4A*	A	3.40 (1.75, 6.59)	2.97×10^{-4}
Cardiovascular Complications						
6	6q14.3	rs62406032	*PKHD1*	G	5.97 (2.48, 14.38)	6.52×10^{-5}
19	19p13.13	rs1078264	*MAST1*	G	2.93 (1.69, 5.08)	1.28×10^{-4}
19	19q13.33	rs480265	*GFY*	A	3.98 (1.96, 8.11)	1.31×10^{-4}
21	21q22.3	rs7279204	*PCNT*	A	3.27 (1.74, 6.12)	2.18×10^{-4}
7	7p11.2	rs146350220	*SEPT1N4*	G	11.00 (3.02, 40.06)	2.77×10^{-4}

(Continued)

TABLE 2 Continued

Chr	Cytoband	SNP	Gene	Risk Allele	Adjusted OR (95% CI)	Adjusted P-value
21	21q22.3	rs6518289	*PCNT*	A	3.18 (1.70, 5.94)	3.04×10^{-4}
15	15q25.2	rs72750868	*WDR73*	G	5.49 (2.12, 14.21)	4.47×10^{-4}
21	21q22.3	rs2839227	*PCNT*	G	2.70 (1.55, 4.71)	4.51×10^{-4}
12	12q24.31	rs28434767	*RILPL2*	A	2.51 (1.49, 4.21)	4.72×10^{-4}
21	21q22.3	rs2839223	*PCNT*	G	3.01 (1.62, 5.62)	5.22×10^{-4}

3.3 Nephropathy complications

The *TTN* gene in the skeletal muscle and has been associated to cardiac serum proteins and fractal structure of the heart, as well as T2DM and nephron-related variables, such glomerular filtration rate (65–67). While gene *PI16, DPY6, FRMD4A* and *CROCC* have not been reported to be associated with nephropathy, they have been identified in T2DM (53, 68) and obesity-related traits (69, 70). *PPP1R3A* gene is associated with T2DM and plays a crucial role in glycogen synthesis in the tubules of the kidney, leading to diabetic nephropathy.

The *ZNF136* gene is highly expressed in the kidneys, and encodes a protein that contains a Krüppel-associated box (KRAB) A-box domain, which has been associated to the development of progressive chronic kidney disease (CKD). The Glis2, a Krüppel-like zinc finger protein, mutant mice had increased cell death and basement membrane thickening in the proximal convoluted tubules, resulting in severe renal atrophy with lymphocytic inflammatory cells infiltration and renal failure (71). The *HSPA12B* gene is expressed in the kidney and urinary bladder whose pathways are related to cellular senescence and cellular response to heat stress. This gene has been associated with gamma-glutamyl transferase (GGT) levels, a marker of oxidative stress that is linked with diabetes and hypertension, both being risk factors of CKD (72, 73).

3.4 Cardiovascular complications

The *PKHD1* gene has been associated to T2DM (53), coronary artery disease (49, 74), cardiac troponin T levels (75), and obesity-related traits (44, 57, 76). While the *MAST1* gene has not been associated to cardiovascular complications, it has been reported to be linked glycated hemoglobin levels (77). Importantly, gene *GFY* has been associated to carotid plaque build, leading to cardiovascular complications (46). The *SEPT14* gene is expressed in the brain, heart, bone marrow, and lymphoid tissues, encoding a highly conserved septin family of cytoskeletal proteins that represses the accumulation of reactive oxygen species, resulting in cardiac microvascular endothelial cells apoptosis (78).

Multiple signals within the *PCNT* gene were identified. The *PCNT* gene is highly expressed in heart, and is an integral component of the microtubule-organizing proteins, which exert compressive forces on cardiomyocytes that drive the development of cardiac disorders and T2DM (53, 79). Interestingly, *PCNT* was also associated with cataract, indicating how microvascular and macrovascular complications tend to be strongly interrelated as damages of small vessels can ultimately results in heart disease manifestations in diabetes (80, 81). The *RILPL2* is highly expressed in lymphocytic cells and artery, and have been associated to obesity-related traits (43, 70), including BMI and waist-to-hip ratio, as well as peripheral arterial disease (82).

4 Discussion

For the first time, we present the top markers identified from an exome-wide association study for T2DM-related complications conducted in the Emirati population. By identifying the susceptible loci associated to high-risk patients that develop complications form T2DM, this may improve targeted therapeutic interventions and early biomarker diagnosis through a panel of genetic markers. Most of the genes identified have been reported in other GWAS studies of different ethnicities, with a biological relevance to the pathogenesis of each respective complication group. These findings provide valuable insight into the pathogenesis of T2DM driven complications and suggest novel candidate genes for future functional studies.

As per the demographic characteristics, T2DM patients with neuropathy and macrovascular complications were significantly older, with a longer diabetes duration, than the control group. The gender and mean BMI were not significantly different between cases and control, across all complications. Interestingly, the median glycated hemoglobin levels was significantly higher in the retinopathy cases (p=0.002) compared to controls, which has been reported in other studies, possibly due to the formation of thrombus, a pathophysiological basis of early diabetic retinopathy (83).

When investigating sub-phenotypes of T2DM, diabetic retinopathy has been identified to be associated with *ACVRIC* (rs4664229), *ZFHX4* (rs61729527), *WNT9B* (rs4968281), *SHANK3* (rs9616915), *ZSCAN5A* (rs7252603), and *DCP1B* (rs715146, rs1044950, rs113147414) gene. These genes have intercrossing pathways and similar genetic variants to fibrinogen levels associated to intra-vessel pressure, low platelet count, leukocyte-retinal endothelial cell adhesion, metabolite levels and glycemic markers, all important factors impacting intra-retinal microvascular abnormalities, retinal capillaries and variations of eye-related diseases (23–30, 37, 40–44). For diabetic neuropathy, gene *GFY* (rs4802605), *ADH4* (rs4148883), *LRFN2* (rs61731010),

PKHD1 (rs2499486), *SLC11A1* (rs17235409), *MATN4* (rs2072788), and *PPARA* (rs4253772) were associated or contributed to the biological relevance to the pathogenesis of the complication. Specifically, these markers have been associated to atherosclerosis, immune and inflammatory responses, AST and ApoA-I levels, iron toxicity, intraocular pressure, and compositional changes in extracellular matrices, which is essential for axonal health and growth, and may lead to nerve fiber loss in neuropathic conditions (26, 40, 42, 52–57, 61–64).

The genes that contributed to the biological relevance of diabetic nephropathy, include gene *TTN* (rs72646845), *PI16* (rs113848006), *DPY6* (rs36027551), *CROCC* (rs41272737), *PPP1R3A* (rs1799999), *ZNF136* (rs140861589), *HSPA12B* (rs6076550), and *FRMD4A* (rs1541010). The markers identified to the development of diabetic nephropathy have mainly been expressed in the kidney and urine bladder, and have been associated to nephron-related variables, such glomerular filtration rate, glycogen synthesis in the tubules of the kidney and thickening in the proximal convoluted tubules (65–67, 71–73). Cardiovascular complications in T2DM is associated to *PKHD1* (rs62406032), *MAST1* (rs1078264), *GFY* (rs480265), *SEPT14* (rs146350220), *PCNT* (rs6518289, rs2839227, rs2839223) and *RILPL2* (rs28434767). Interestingly, these markers have been associated to coronary artery disease, glycated hemoglobin levels, cardiac troponin T levels, and obesity-related traits (44, 49, 57, 74–77).

The major limitation in this study is the sample size with an inadequate statistical power to be able to detect rare variants in the population pool. Moreover, the control group of the study included patients with a short duration of illness which could have contributed to a reduced power to the study. However, it is also important to note that the real period of T2DM is usually assumed to be longer than the clinically defined duration by at least several years due to a delay of diagnosis. Future studies, with a larger cohort, should adjust for duration of diabetes as it may serve as a genetic risk factor. Furthermore, the HbA_{1c} levels were recorded only at one time point, at the time of recruitment, which could have been a limiting factor. Another limiting factor is the exome microarray chip where its incompatibility with the Middle Eastern population was seen in the fact that many variants were excluded after quality control due to the identification of monomorphic markers, homozygosity due to high consanguinity, and the accumulation of deleterious recessive alleles within the gene pool of the population. In fact, approximately 82.5% did not pass the MAF cut-off, demonstrating possible missed identification of pathogenic variants. Genetic variation in population arises from new mutations occurring through generations, in which changes in MAF may occur. This is due to genetic drift or differences in fitness levels conferred by different alleles in the presence of certain environment, including population bottleneck due to high consanguinity or migration (84).

Further studies need to be conducted in a large-scale, multi-ethnic cohort to replicate the findings of this study and substantiate our current knowledge of complications associated to T2DM. Given that only 16% of the markers from the Illumina's Infinium Exome chip passed quality control assessment, this demonstrates that multiple variants were, either, monomorphic in the Arab population or were not genotyped due to the use of a Euro-centric EWAS array that limits the possibility of including targeted ethnic-specific SNPs. Our results suggest the alarming possibility that lack of representation in reference panels could inhibit discovery of functionally important loci associated to T2DM complications. Enabling global equity in the benefits of genomics will be vital for precision medicine initiatives, including risk prediction, development of therapies and implications for screening and diagnostics. Future work in diverse populations should focus on using unbiased approaches, unbiased marker discover and global genome references. This will be beneficial to better understand reproducibility and heterogeneity of effects among populations, improve the power to identify causal drivers of association signals, as well as important resources for fine-mapping of causal and rare variants.

This study has demonstrated that given that the majority of genetic studies, including the genotyping and sequencing panels, are developed based on the European ancestry, it has essentially deemed inapplicable to other ethnic groups. This foreshadows a near future where those genetic tests that are only valid for European descent be used as the blueprint for clinical applications for genetics, creating a skewed standard for ethnic minorities, such as the Middle East population. The scarcity of baseline genetic data is indicative of health inequalities that may be faced, further highlighting the urgency to ensure the inclusion of non-European descents in the genetic research movement. Hence, a microarray chip that is more inclusive to the Arab population needs to be developed and utilized to ensure that a wider spectrum of variants is included to detect rare SNPs associated within this region of the world. Further effort must be conducted to improve the representation of diverse populations in genotyping and sequencing studies to enable the unprecedented characterization of fine-scale genetic architecture and genetic susceptibilities to diseases, globally. This would allow for eventual delving into pharmacogenomics for the development of therapeutic strategies catered to the patient according to the complications experienced.

Data availability statement

The data presented in the study are deposited in the NCBI Gene expression omnibus database (accession number: GSE226084), and are available upon request from the corresponding author.

Ethics statement

An ethical request was submitted to the Dubai Health authority (DHA) whereby it was accepted under reference number DSREC-07/2020_19, and conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent before taking part in this research. All data was de-identified prior to use. The patients/participants provided their written informed consent to participate in this study.

Author contributions

HA, GT, and AH conceived the project to study diabetes-related complications in the UAE. HA, GT, and AH conceived the central research questions for the EWAS data. AM and MM initiated the first draft of the manuscript. MM conducted the analysis of the manuscripts. HA, AM, MM analyses and constructed the Figures and Tables. AM and DA were responsible for the recruitment of the patients and collecting data for the study. AM carried out the laboratory assays used in the study. HA, MM and AM provided critical review during manuscript preparation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1143067/full#supplementary-material

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Oxidative stress: The nexus of obesity and cognitive dysfunction in diabetes

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Obesity has been associated with oxidative stress. Obese patients are at increased risk for diabetic cognitive dysfunction, indicating a pathological link between obesity, oxidative stress, and diabetic cognitive dysfunction. Obesity can induce the biological process of oxidative stress by disrupting the adipose microenvironment (adipocytes, macrophages), mediating low-grade chronic inflammation, and mitochondrial dysfunction (mitochondrial division, fusion). Furthermore, oxidative stress can be implicated in insulin resistance, inflammation in neural tissues, and lipid metabolism disorders, affecting cognitive dysfunction in diabetics.

KEYWORDS

oxidative stress, obesity, cognitive dysfunction in diabetes, reactive oxygen species, insulin resistance, neuroinflammation, lipid metabolism disorders

1 Introduction

The prevalence of obesity has been on the rise globally for the last half century (1). Obesity prevalence has doubled since 1980 in more than 70 countries. Furthermore, women of all ages had a higher prevalence of obesity than men (2). Obesity causes many twenty-first-century chronic diseases worldwide and imposes enormous socioeconomic burdens (1). Numerous risk factors for chronic diseases, including cardiovascular disease (CVD) (3), type 2 diabetes (T2DM), and cognitive impairment, are influenced by obesity (4). Diabetes prevalence has been increasing, especially with T2DM, due to changes in lifestyle factors such as diet, obesity, and lack of exercise (5). The IDF Diabetes Atlas indicates that prevalence in 20–79-year-olds in 2021 was estimated to be 10.5% (536.6 million people), rising to 12.2% (783.2 million) in 2045 (6). Patients with diabetes are also at risk for complications as they age (7). Diabetes patients have been found to have an increased risk for dementia (8). In a US study, people with diabetes had an overall prevalence of dementia and cognitive impairment of 13.1% for people aged 65–74 and 24.2% for those aged 75 and over (9). Those who suffer from cognitive impairment in diabetes experience cognitive dysfunction, delayed executive, function, and impeded information processing speed, and pathology may include neuro amyloid plaques and tau protein tangles (10). There is a correlation between diabetes and cognitive impairment, which negatively affects patient quality of life (11). A cross-sectional analysis of baseline

data shows that high BMI and low mood are associated with worse cognitive function among overweight/obese elderly with metabolic syndrome (12). In older people, BMI has been associated with a higher risk of developing type 2 diabetes (13). This review aims to explore how oxidative stress processes could contribute to obesity-related cognitive dysfunction in diabetics.

Oxidative stress (OS) regulates biological components, and it has been proposed to be a mediator of the relationship between obesity and cognitive impairment in diabetes. In 1985, "oxidative stress" was introduced as a concept in redox biology and medicine (14); the concept of biological oxidative stress was defined as "an imbalance between oxidants and antioxidants in favor of oxidants, leading to a disruption of redox signaling and control and molecular damage" (15). Redox reactions contribute to regulation, where endogenous and exogenous regulatory factors, such as the number of biochemical components (oxygen, nitrogen, and sulfur), can contribute to the oxidative stress. Furthermore, these reactive species, called reactive substances, mainly reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS), stimulate the metabolic processes of cells (16). Reactive species participate in several oxidative signaling pathways, such as NF-κB, JAK-STAT, Nrf-2, and HIF-1; they are also involved in the development of several diseases, including cardiovascular diseases, cancer, and diabetes (17). The production of ROS contributes to the inflammatory response process, which leads to an increase in adipocyte size, promotes adipogenesis and lipogenesis, and adipocyte differentiation (18). Studies have demonstrated that fat accumulation is an early trigger and a fundamental cause of obesity-associated metabolic syndrome resulting in increased oxidative stress (19). Prolonged exposure of adipocytes to ROS leads to insulin-induced activation of PI3-kinase and Akt, resulting in impaired islet function and facilitated glucose transporter member 4 (GLUT4) translocation (20). Due to their sensitivity to oxidative damage, neuronal cells are especially susceptible to neurodegenerative diseases, such as diabetes-related cognitive impairment (21). Mitochondrial homeostasis plays a crucial role in maintaining neuronal and axonal energetic homeostasis. Bioenergetic deficits contribute significantly to the cognitive decline observed in aging and neurodegenerative diseases. Neurons are particularly susceptible to mitochondrial dysfunction due to their intrinsic properties (22). ROS synthesis is derived from mitochondria, and when mitochondria become dysfunctional, ROS production of ROS and oxidative stress increase, and mitochondrial maldistribution disrupts neuronal axonal energy homeostasis. Oxidative stress disrupts neurological metabolism resulting in hypoglucose metabolism in the brain (23). The brains exhibit structural changes due to an accumulation of disease-specific protein aggregates (24). These structural changes may contribute to neuronal and synaptic dysfunction, resulting in cognitive impairment (25).

2 The link between obesity and oxidative stress

Oxidative stress is produced by reactive oxygen/nitrogen species (ROS/RNS) (26). Furthermore, oxidative stress alters the balance between the production of ROS and antioxidant defenses. By-products of aerobic metabolism, ROS, can pose a health risk when exposed to stressful environments (27). ROS are primarily derived from mitochondria and electron transport chain (ETC), in which mitochondria produce adenosine triphosphate (ATP) through a series of oxidative phosphorylation processes. However, ROS also contain a variety of chemical entities, including nitric oxide, peroxynitrite, hypochlorous acid, singlet oxygen and hydroxyl radicals (28).

Several studies have shown that obesity induces the formation of oxidative stress. The high-fat diet induces oxidative stress in the white adipose tissue of rats (29). When there is a high intake of nutrients, oxidative stress increases, and inflammation is induced through signaling pathways mediated by the nuclear factor-kappa B (30). High consumption of fat-rich diets promotes mitochondrial β-oxidation of free fatty acids (FFAs), and subsequent use of cytochrome-c oxidase leading to excess electron flow increases the accumulation of ROS, ROS, and lipid peroxidation deplete vitamins and antioxidant enzymes (31). We summarise the link between obesity and oxidative stress in terms of disruption of the adipose microenvironment, chronic inflammation in obesity, and mitochondrial dysfunction (Figure 1).

2.1 Disruption of the adipose microenvironment

Obesity is an increase in lipid content in adipose tissue, manifested by an increase in the size and number of adipose cells (32). Adipose tissue can be divided into three categories: white adipose tissue (WAT), brown adipose tissue (BAT) and beige adipocytes.

White adipocytes are the primary cell type found in human adipose tissue. Energy-yielding triglycerides and cholesterol esters are stored within the sizeable intracellular lipid droplets. Leptin, adiponectin, and other adipokines are among the proteins secreted by white adipocytes.

Brown adipocytes: BAT is widely present throughout the body (33); BAT is rich in multiple lipid droplets and contains uncoupling protein 1– containing mitochondria; these adipocytes mediate thermogenic respiration (34).

Beige adipocytes: Beige adipocytes are derived from white adipocytes tissue, and browning of white adipocytes tissue can be induced by cold stimulation, exercise, and some endocrine hormones; beige adipocytes have thermogenic effects because of rich uncoupling protein 1 (35).

The different adipocytes and macrophages of the adipose tissue constitute the adipose microenvironment. Dysfunction at the WAT level may influence the development of obesity-associated metabolic complications (36). An obesity-induced immune response occurs when metabolic cells (including adipocytes) are involved (e.g., adipocytes). Overnutrition leads to adipotoxicity, which produces inflammatory factors (37). Studies have shown that macrophages infiltrate adipocytes in obese individuals and promote an inflammatory response (38). Kinase inhibitors (IKK), c-jun n-terminal kinase (JNK), and protein kinase r (PKR) can transmit nutrient signals from metabolic tissues to inflammatory cells. This

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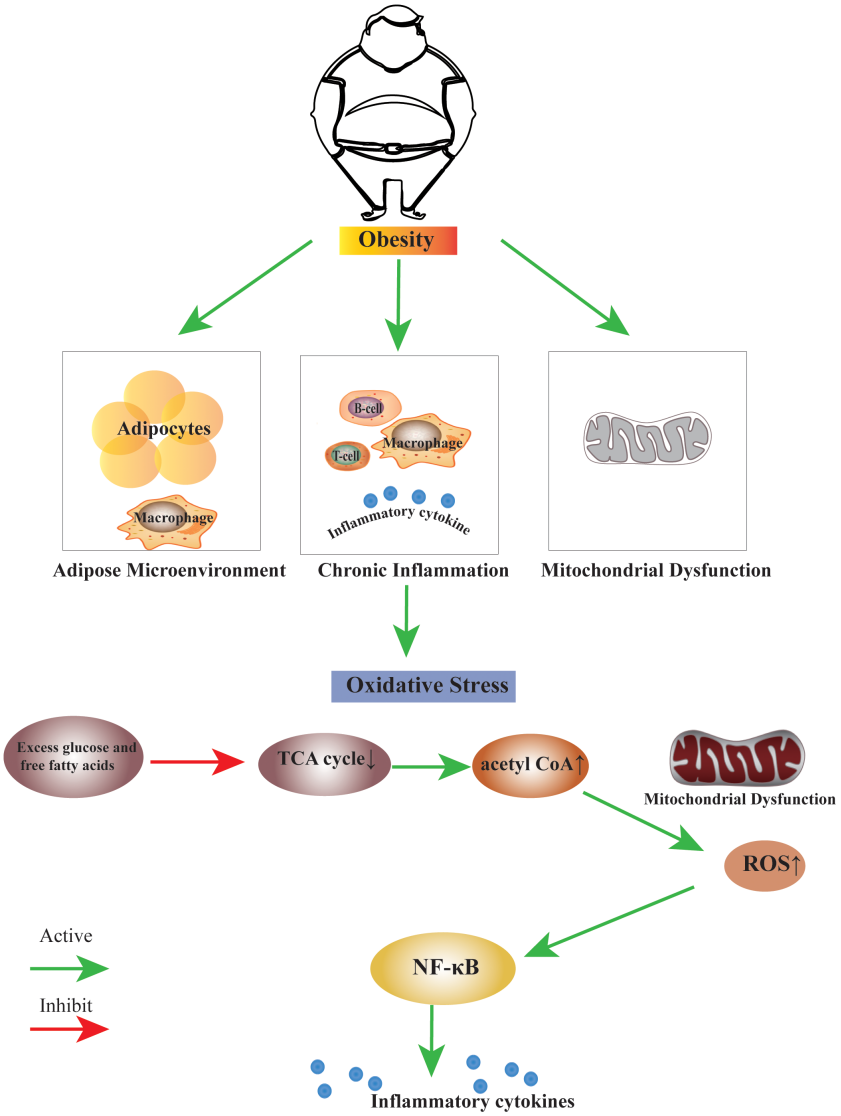

FIGURE 1
The link between obesity and oxidative stress. Obesity can induce the biological process of oxidative stress by disrupting the adipose microenvironment, mediating chronic inflammation, and mitochondrial dysfunction. The process is probably that excess glucose and free fatty acids suppress the TCA cycle, leading to an increase in the production of acetyl CoA. Excess acetyl CoA stimulates mitochondrial dysfunction, resulting in an increase of ROS within the cell, this change may activate many factors, the nuclear factor κB is the main inflammatory factor.TCA cycle, Tricarboxylic Acid cycle; acetyl CoA, Acetoacetyl coenzyme A; ROS, Reactive Oxygen Species; NF-κB, nuclear factor κB.

process is accompanied by oxidative stress, and these kinases and their downstream pro-inflammatory targeting factors can be significantly upregulated in obese subjects (39). Due to the accumulation of oxidative biomolecules in adipocytes, the homeostatic system that regulates oxidative stress and the antioxidant regulatory system are suppressed mainly in obese adipocytes. Excess ROS irreversibly damages DNA, lipids, and proteins and adversely affects cellular function (28).

2.2 Chronic inflammation in obesity

Obesity is primarily caused by an energy imbalance between excessive calories consumed and insufficient calories expended (40). Adipose tissue is regarded as an energy storage for calories and an essential endocrine organ. It produces many bioactive molecules, including chemokines and cytokines, called adipokines (or adipocytokines), when secreted by adipose tissue. They are not only regulators of systemic metabolism, but also have immunomodulatory properties (41). Adipose tissue is responsible for the production and secretion of many biologically active adipokines, including leptin, adiponectin, resistin, visfatin, and schelatin, that can lead to chronic complications (42). Obesity leads to an increase in adipocytes and enlargement of adipose tissue. The ensuing decrease in oxygen tension leads to hypoxia and massive accumulation of hypoxia-inducible factor (HIF-1) in adipocytes. Furthermore, hypoxia has been linked to adipose inflammation and macrophage infiltration (43). Studies have

shown that macrophages accumulate in adipose tissue of obese people as well as in the obese B6.V Lepob/ob mouse model, and macrophages promote the secretion and expression of adipokines, including tumor necrosis factor-alpha (TNF-α), iNOS and interleukin-6 (IL-6) (38).

Obesity is a chronic low-grade inflammatory condition, with adipose tissue infiltrated by macrophages and elevated inflammatory markers and cytokines. This low-grade chronic inflammation in adipose tissue may contribute to developing related metabolic diseases, such as insulin resistance and T2DM (44). Adipocytes produce large amounts of adipokines with inflammatory functions, such as IL-6, IL-1, and TNF-α, which induce ROS production and mediate oxidative stress (45). TNF-α is produced mainly by macrophages and is also a critical adipokine. Fat accumulation leads to adipocyte damage, leading to high production of cytokines such as TNF-α, which produces ROS in tissues and increases the rate of lipid peroxidation (46). TNF-α also activates the NF-kB signaling pathway to aggravate the inflammatory response (47). During oxidative stress, adipokines, including leptin, IL-6, and lipocalin, resist all functions (45).

Oxidative stress impairs islet beta-cell function in several ways; it significantly reduces insulin production, impairs the ability of insulinogenic vesicles to enter the plasma membrane, and reduces the response to hyperglycemia. Oxidative stress can induce islet β-cell apoptosis, and excess free radicals interfere with β-cell neogenesis (48). Oxidative stress leads to reduced GLUT4 expression and ultimately reduces insulin sensitivity by disrupting the binding of nuclear proteins to the insulin response element in the GLUT4 promoter (49). Oxidative stress was also involved in the development of diabetic encephalopathy. Oxidative stress inhibits the islet signaling system. HFD/STZ induced a significant increase in relevant oxidative stress parameters such as TBARS, NO levels, and XO activity in the brain tissue of rats compared with controls, and serum peripheral TNF-α and IL-6 inflammatory cytokine levels were significantly increased in the diabetic rats, and a similar brain AD-related miRNA expression profile was observed in the diabetic rats (50).

2.3 Mitochondrial dysfunction

Mitochondria are intracellular organelles that play an important role in the cell by metabolizing nutrients and producing adenosine triphosphate (ATP). Mitochondria regulate energy, maintenance of cellular calcium homeostasis, production and removal of reactive oxygen species, and regulation of cell death (51). Mitochondria produce energy in the form of ATP through the oxidative metabolism of nutrients, consisting of two main steps: 1) oxidation of NADH or FADH2 produced during glycolysis, TCA or β-oxidation of fatty acids, with most of the ATP being produced through the TCA cycle through the ETC; 2) oxidative phosphorylation (OXPHOS) to produce ATP. Mitochondria continuously metabolize oxygen and produce ROS during the combination of electron transport and protons in the ETC, which is the primary source of ROS (52).

Mitochondrial dysfunction can manifest itself by loss of mitochondrial membrane potential, altered ETC function, increased ROS production, and decreased oxygen consumption. There is a reduction in the efficiency of mitochondrial ATP production (53). Mitochondrial dysfunction can also occur when mitochondrial molecular dynamics is impaired. Mitochondria is a dynamic energy organelle that responds to energy demands and environmental stimuli through fusion, fission, and movement to maintain cellular homeostasis (54). Fission can also promote mitochondrial autophagy and biogenesis, two events that can occur because of mitochondrial fission (55, 56). Mitochondria generate several stress response pathways, including the mitochondrial unfolded protein response and degrading mislocalized proteins in mitochondria dysfunction (57, 58). Severely damaged mitochondria can be identified and degraded through the process of mitochondrial autophagy (59). When mitochondrial autophagy is dysregulated, ROS produced by mitochondria can activate inflammatory vesicles composed of NLRP3, the bridging protein ASC, and caspase-1, triggering inflammation. It has been reported that defects in the autophagy gene PINK1 increase NLRP3 expression and lead to brown fat dysfunction in mice (60, 61).

Studies have shown that excessive nutrient intake leads to hyperglycemia, increases ROS production, and causes mitochondrial dysfunction in adipocytes, suggesting that obesity triggers oxidative stress and mitochondrial dysfunction (57). When mitochondrial function is impaired, major adipocyte pathways are altered, resulting in decreased adipogenesis, increased lipolysis, and decreased fatty acid esterification; these alterations promote changes in insulin sensitivity (62). A study showed that high fat-induced obese (DIO) mice exhibit insulin resistance, mitochondrial dysfunction, hepatic lipid deposition, and oxidative stress (63). A study indicated that the expression of mitofusin-2 (Mfn2, a mitochondrial fusion protein) was decreased in the muscles of obese subjects or type 2 diabetics, leading to an imbalance between mitochondrial fusion and fission events and mitochondrial dysfunction, which may be involved in insulin resistance (64). Significantly elevated levels of acylcarnitine in patients with nonalcoholic fatty liver mark mitochondrial dysfunction and impaired fatty acid oxidation (65). A study indicated a decrease in mitochondrial biosynthesis in a rodent model of obesity (66). Down-regulation of mitochondrial biogenesis in obesity is associated with metabolic alterations, insulin resistance, and low-grade inflammation (67). Chronic high-fat diet feeding promotes excessive apoptosis in mouse HK-2 cells by inducing oxidative stress and mitochondrial disorders in kidney cells (68). It has been shown that a high-fat diet induces oxidative damage in the brain of obese (DIO) rats and that a high-fat diet increases lipid oxidation in the brain tissue of DIO rats as well as the level of mitochondrial ROS (69). Mitochondrial dysfunction in the brain due to obesity may lead to insulin resistance and cognitive dysfunction (70, 71). Jheng found smaller and shorter mitochondria and increased

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mitochondrial fission in the skeletal muscle of obese mice, suggesting that altered mitochondrial fission is associated with mitochondrial dysfunction in the skeletal muscle and insulin resistance (72).

A high-fat diet induces mitochondrial expansion in rodent brown fat, and excess leads to inhibition of mitochondrial fusion, resulting in fragmentation and autophagy, leading to mitochondrial dysfunction (73). The excess leads to cellular oxidative stress, which subsequently induces an inflammatory cascade response. This process is likely caused by excess glucose and free fatty acids suppressing the TCA cycle, increasing acetyl CoA. Excess acetyl CoA stimulates the mitochondria to produce excess superoxide in the electron transport chain, which increases ROS within the cell. This change may activate many factors; the nuclear factor κB is the primary inflammatory factor (74).

3 Oxidative stress and cognitive dysfunction in diabetes

Oxidative stress is an imbalance between the production of oxidants and antioxidant defenses that may result in damage to biological systems. Oxidative stress is considered one of the crucial factors in the development and progression of cognitive impairment in diabetes mellitus (75). In cognitive impairment, there are interconnections between oxidative stress, insulin resistance, neuroinflammation, and abnormal lipid metabolism (76–78) (Figure 2).

3.1 Oxidative stress and insulin resistance in diabetic cognitive dysfunction

Insulin resistance means that systemic target tissues such as fat, muscle, and liver are less sensitive to insulin and cannot properly regulate the pathological state of glucose homeostasis (79). Inflammation, dysfunction, and elevated OS levels lead to insulin signaling cascade disorder and are important triggers of insulin resistance (80–82). In pro-inflammatory conditions, activation of glial cells can lead to progressive neuronal damage (83). Additionally, insulin regulates metabolic pathways that maintain learning and memory at the brain level and glucose transport/metabolism (83). In diabetic cognitive dysfunction, insulin resistance weakens the metabolic raw material of dysfunctional neurons and affects memory function (84).Currently, the molecular mechanism of the development of insulin resistance has not been fully elucidated. Insulin receptor substrates (IRSs) work as scaffold protein driving activation of two primary insulin signaling pathways: 1) PI3K/PDK1/Akt pathway; And 2) MAPK pathway (85). The former is closely related to insulin metabolism, while the latter is mainly involved in cell growth differentiation (86).

ROS can act as both a signaling agent and a damaging agent. Low levels of endogenous reactive oxygen species play an essential role in the signaling pathway and have crucial physiological significance (87). In insulin signaling, there are redox initiation steps in which some oxidizing agents, such as hydrogen peroxide (H_2O_2), promote the phosphorylation of insulin receptors (83). In

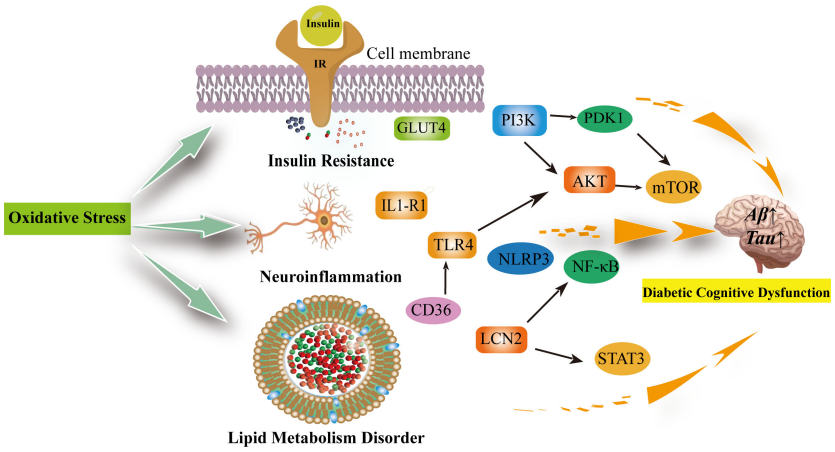

FIGURE 2
Oxidative stress can be involved in insulin resistance, neuroinflammation, lipid metabolism disorders leading to diabetic cognitive dysfunction. Oxidative stress reduces GLUT-4 expression and the translocation of GLUT-4 to the cell membrane, decreasing insulin sensitivity; In the brain, insulin activates the PI3K/PDK1/AKT and the PI3K/AKT/mTOR signaling pathway to inhibit apoptosis and promote neuronal development and survival, which are inhibited when insulin is resistant and increase the production of inflammatory factors. Neuroinflammation is associated with excessive microglia activation. activation of IL1-R1 signaling pathway, NLRP3/IL-1β signaling pathway, NK-κB signaling pathway and release of pro-inflammatory factors exacerbate neuroinflammation and neuronal damage in the brain. The inhibition of TLR4/AKT/mTOR signaling pathway inhibits cellular autophagy as well as promotes neuroinflammation and microglia apoptosis. Diabetic cognitive dysfunction is also exacerbated by the presence of impaired lipid metabolism in the brain. CD36 recognizes oxidized low-density lipoprotein receptors (TLRS) and triggers a toll-like response to stimulate sterile inflammation. Meanwhile, LCN2 is mainly produced in glial cells of the brain under oxidative stress. It promotes cellular neuroinflammation by activating the NF-kB pathway as well as the STAT3 signalling pathway to promote microglia activation. IR, Insluin resistance; GLUT4, facilitated glucose transporter member 4; PI3K, phosphatidylinositol 3' -kinase; PDK1, pyruvate dehydrogenase kinase isoform 1; AKT, Protein Kinase B; mTOR, mammalian target of rapamycin; TLR4, toll-like receptor 4; IL1-R1, interleukin 1 receptor type I; NLRP3,NLR family pyrin domain containing 3; NF-κB, Nuclear factor kappa B; LCN2, Lipocalin 2; STAT3, signal transduction and transcription 3; CD36, Platelet glycoprotein 4.

addition, it can inhibit protein tyrosine phosphatase PTP1B, which deactivates IR by dephosphorylating A-ring phosphotyrosine (88).Thus, insulin-induced H_2O_2 acts as a net positive regulator in acting insulin receptors. Furthermore, as age increases, OS levels increase and glutathione (GSH) levels decrease, which has been verified in aging models (89). In insulin resistance or T2DM mice, oxidative stress markers increased, and glutathione levels decreased in the brain(90). Previous studies have shown that brain plasticity, the ability of the brain to undergo structural and functional changes due to environmental stimulation, is carefully regulated by dietary and nutritional-dependent hormones, including insulin (91). Therefore, it can be shown that OS is closely related to the development of insulin resistance and cognitive dysfunction. Changes in insulin signals in the central nervous system can accelerate brain aging, affect brain plasticity, and promote synaptic loss and nerve degradation (92).

Oxidative stress can cause β-cell dysfunction (93). Because the antioxidant defense system of β-cell is low, OS is widely found in diabetes mellitus and plays an essential role in β-cell dysfunction (48, 93). OS can reduce the production of insulin, impair the contents of the original insulin vesicles into plasma membrane, and reduce the exocytosis of glucose into circulation (93, 94). Since apoptotic agents are highly sensitive to OS, OS can induce pancreatic cell apoptosis and lead to β-cell apoptosis (94, 95). An overload of free radicals can affect the normal metabolic pathway in β-cells, damage the K_{ATP} channel, and lead to decreased insulin secretion (93, 96). Previous studies have shown that OS activates Nf-κB, JNK/SAPK, p38 MAPK, hexosamine pathway, and toll-like receptor (TLRs), thereby impairing β-cell function (48, 93). In addition, β-cell mitochondrial dysfunction induced by oxidative stress may be an important mechanism leading to β-cell dysfunction (48, 93). β-cell dysfunction, which results from oxidative stress, can lead to insulin resistance and in turn diabetes cognitive dysfunction.

Oxidative stress can reduce insulin sensitivity in insulin-dependent cells such as adipocytes and myocytes (48). Normal GLUT-4 expression and localization are necessary to maintain these tissues' insulin sensitivity (97). Reduction of GLUT-4 expression/localization is one of the main molecular mechanisms by which oxidative stress induces insulin resistance and promotes the development of cognitive dysfunction in diabetes mellitus (48). Studies have found lower expression and localization of GLUT-4 in patients with insulin resistance and T2DM (98–100). Oxidative stress can reduce the translocation of GLUT-4 to the cell membrane (101). Long-term oxidative stress inhibits transcription factors and microscopic RNAs involved in GLUT-4 expression (101–103).

Oxidative stress can impair normal insulin signal transduction (IST) at different levels (104). Hyperglycemic-induced OS activates different stress-sensitive serine/threonine (Ser/Thr) kinases such as IKK-*β*, which phosphorylates multiple targets such as IR, IRS-1 and IRS-2, leading to adverse downstream effects, including decreased PI3K activation and insulin resistance (48, 105). Oxidative stress can damage insulin sensitivity and lead to insulin resistance and cognitive dysfunction of diabetes by downregulating the proteins involved in normal IST, such as Insulin-degrading enzyme (IDE), Biliverdin reductase-A (BVR-A), Akt, IRS, IRS-1 and GSK-3 (48, 83, 50). Therefore, IST abnormality is one of the important mechanisms of insulin resistance caused by oxidative stress.

Insulin resistance can occur in both obesity and diabetes and is manifested in peripheral and central insulin resistance (106). In the brain, insulin acts as a neuromodulator to regulate activity-dependent synaptic plasticity by activating PI3K/PDK1/Akt signaling pathways (107). Insulin can inhibit apoptosis by activating the Akt signaling pathway to promote neuronal cell survival (108). Insulin resistance is characterized by the down-regulation of insulin receptor expression and impaired IRS proteins (86). IRs are localized in both presynaptic and postsynaptic neurons (109, 110). IRs recruit and activate PI3K complexes, which subsequently activate AKT (111), AKT downstream of GLUT4 and mTOR complexes, AKT-mediated stimulation of mTOR and its downstream targets regulates protein and lipid synthesis and promotes dendritic spine formation, as well as many aspects of neuronal development, survival, autophagy, and long-term synaptic plasticity (84, 111). When oxidative stress processes activate kinases such as JNK and IKK in neurons, insulin signaling pathways become abnormal, such as the P13K/AKT pathway, thereby the downstream of the pathway is inhibited (112). Failure of insulin signaling causes tau protein hyperphosphorylation (113), in addition to increased neurotoxic Aβ deposition at specific levels of hyperinsulinemia (114), all of which can lead to decreased cognitive function in diabetes. In addition, chronic elevated blood glucose can induce inflammation and can cause insulin resistance. Pro-inflammatory mediators such as TNF-α, IL1-β and IL-6 further exacerbate the inflammatory state through feedback inhibition of insulin receptors and through feedforward mechanisms that disrupt mitochondrial function to stimulate the production of reactive oxygen species, thereby producing an inflammatory environment with reduced insulin sensitivity. This chronic inflammatory environment increases NF-κB inducible kinase (NIK), which independently impairs mitochondrial function to further promote insulin resistance (86). Pro-inflammatory cytokines secreted into the bloodstream (across the blood-brain barrier) during chronic hyperglycemia and inflammatory cytokines within the brain's innate immune system, soluble misfolded Aβ can induce inflammatory cytokines (e.g., TNF-α) through a NIK-dependent pathway that can lead to neuroinflammation and exacerbate cognitive deficits (115).

3.2 Oxidative stress and neuroinflammation in diabetic cognitive dysfunction

Neuroinflammation is the inflammatory response of factors of the central nervous system (CNS) acting on homeostasis in the body (116). This response includes distinct types of cells in the central nervous system, such as microglia and astrocytes (116, 117). Neuroinflammation aims to restore neuronal homeostasis and protect neuronal integrity (118). During the acute phase, neuroinflammation can protect neurological homeostasis, promote nerve growth, repair damaged cells, and remove protein plaques (119). During the chronic phase, neuroinflammatory-induced maladaptive results will cause neuronal damage to worsen (119). Neuroinflammation is a common pathogenic factor

in neurological disorders, including Alzheimer's disease (AD), diabetic cognitive dysfunction, and depression (120). Neuroinflammation includes various inflammatory events in the central nervous system under pathological conditions. Brain alterations in AD and diabetic cognitive dysfunction can manifest amyloid-β plaques and are associated with neuroinflammation (112). Abnormal activation of glial cells (microglia and astrocytes) can mediate neuroinflammation leading to neurodegenerative disease, and dysfunctional neurons alter the clearance of amyloid-β plaques in AD, promoting neuroinflammation and cognitive impairment (121). Obesity-induced chronic inflammation also affects the central nervous system (122), with obesity-promoting peripheral inflammation and increasing the permeability of the blood-brain barrier (BBB), and elevated levels of inflammatory mediators in diabetic patients promote neuroinflammation by triggering harmful neutrophil/microglia activation in the diabetic brain (123). Elevated levels of related proteins in inflammatory cells (e.g., lipid transport protein 2, LCN2 and tension enhancer binding protein, TonEBP) can adversely affect diabetic encephalopathy by leaking through the BBB (124).

Neuroinflammation and oxidative stress are essential in the onset and development of neurodegenerative lesions and are closely linked in their pathogenesis. ROS and RNS can further enhance intracellular signaling cascades and increase the expression of pro-inflammatory factors. On the other hand, inflammatory cells secrete active substances that produce ROS (116, 125). Therefore, neuroinflammation and oxidative stress can stimulate and interact with each other. An imbalance in redox and insufficient inflammatory response in the central nervous system causes neuroinflammation (116).

The blood-brain barrier (BBB) is a protective barrier for the CNS that prevents harmful substances from entering the brain and maintains intracerebral homeostasis by regulating the transport of essential molecules, including glucose. Pericytes and astrocytes contribute to the formation of the basal membrane of the blood-brain barrier (126, 127). Hyperglycemia increases the rate at which pericytes and astrocytes respire, producing ROS production and oxidative stress in people with diabetes (127, 128). Increased ROS further stimulates the upregulation of inflammatory cytokines and activates the NF-κB signaling pathway, leading to leakage of the blood-brain barrier (129). Neuroinflammation resulting from these injuries promotes the opening of the BBB and the influx of high blood sugar into the CNS (127). Long-term high glucose levels can cause disturbances in glucose metabolism pathways, decrease essential cofactors in redox reactions, including NADPH and NAD +, and produce advanced glycosylated end products (AGEs) (130–132). AGEs bind to AGE receptors (RAGEs) on the cell surface, producing excessive ROS (127, 133). High concentrations of ROS can initiate misfolding of proteins in neurons mitochondria, causing dysfunction of mitochondria, causing neuroinflammation, exacerbating tissue damage, and destroying neuronal regeneration (127, 134).

Mitochondria generate the energy required for almost all biological functions of the body and are an essential organelles. Neurons have high energy requirements, and neuronal mitochondria provide constant energy to neuronal cells (127, 135). Mitochondrial dysfunction causes intracellular energy, leading to inflammation and cell death (119). Previous studies have found that brain neurons are more susceptible to oxidative stress (136). Recent studies have shown that mitochondrial dysfunction plays vital role in hyperglycemic-induced neuronal damage (127, 136, 137). Oxidative stress is one of the leading causes of mitochondrial dysfunction (119). Oxidative stress can interrupt one or more mitochondrial functions, increasing membrane permeability (138). Furthermore, oxidative stress can increase neurotoxic glutamate levels, affecting mitochondrial phagocytosis (119, 139).Microglia are one of the important cells involved in neuroinflammation. They play an important role in maintaining neuronal homeostasis, neuron growth, building extra synapses, removing fragments of cells, removing protein aggregates and neuroplasticity (119, 140). Microglia can identify pathogens, protein aggregates, or fragments through pattern recognition receptors (PRRs) in pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMP), and activate phagocytosis pathogens, release cytokines, chemokines, ROS/RNS until an immune response is eliminated (141). Due to mitochondrial dysfunction, mitochondrial membrane damage releases DAMP, which initiates multiple inflammatory cascades leading to neuroinflammation. The DAMP released by mitochondria (TLR, TNF receptor, inflammasome) can be identified by PRRs of microglia, activate the TLR/NF-κ B inflammatory pathway, and promote the release of pro-inflammatory factor cytokines and chemokines (119). Inflammation caused by the DAMP released by mitochondria can lead to mitochondrial dysfunction, increase ROS, and exacerbate inflammatory circulation (119).

Neuroinflammation is closely associated with microglia hyperactivation. Studies have shown that NLRP3/IL-1β signaling may underlie the correlation between visceral obesity and cognitive impairment in humans, with high-fat diets feeding WT and NLRP3-KO mice, WT mice activating IL1R1 signaling in microglia, leading to hippocampal IL1β accumulation and neuroinflammation, and consequently cognitive impairment, while NLRP3-KO mice are protective against obesity-induced peripheral inflammation (142). Study indicated that neuroinflammation in diabetic cognitive dysfunction is associated with autophagy, continuous hyperglycemia under diabetes can trigger activation of the NF-κ B pathway and release of pro-inflammatory factors, leading to the inflammatory response, and neuronal damage (143). Pharmacological administration of mTOR inhibitors and autophagy stimulators improves inflammation *in vivo* by inhibiting NF-κ B signaling (144). In a study of Cui, they found melatonin (MLT) could improve learning and memory in diabetes-associated cognitive dysfunction mice by activating autophagy *via* the TLR4/Akt/mTOR pathway, thereby inhibiting neuroinflammation and microglial apoptosis (145). A study showed that lncRNA MEG3 overexpression significantly improved diabetic cognitive impairments by regulating the Rac1/ROS axis, and by inhibiting mitochondria-related apoptosis. In addition, MEG3 overexpression or Rac1 inhibition promoted FUNDC1 dephosphorylation and inhibited oxidative stress and neuroinflammation (146).

3.3 Oxidative stress and lipid metabolism in diabetic cognitive dysfunction

Lipids are a class of organic compounds that act as structural components of cell membranes, chemical energy sources and cell signaling molecules, involving many biological processes (147). Lipids can be divided into the following categories according to their structure: fatty acids, triglycerides, sphingolipids, phospholipids, glycolipids, sterol lipids, isopropylene enols and polyketides (148). Lipid metabolism can be defined as the synthesis, storage and breakdown of lipids (149). These processes are necessary to maintain complex homeostasis and lipid diversity and produce products involved in multiple cellular processes. The liver and adipose tissue play a key role in lipid metabolism. The liver helps digest, uptake, storage, and biosynthesis of dietary lipids, which can be exported as lipoproteins to provide energy or structural components (139). Adipose tissue can be used for long-term energy storage (150). Adipose tissue can be used for long-term energy storage (150). Insulin regulates fat storage by inhibiting or stimulating fat mobilization (151). Moreover, most body cells can synthesize cholesterol, but the amount depends on the cell's needs (152).

Lipids are abundant in the brain, accounting for about 50–60% of the dry weight, especially fatty acids, glycerophospholipids, sphingolipids, and cholesterol (153, 154). A study has shown that "adipose inclusions" or "lipid particles" can be found in AD brains (155). In neurons, oligomeric *Aβ* peptides can alter cellular cholesterol metabolism (156). Obesity and abnormal blood lipids are the main risk factors for cognitive dysfunction in diabetes mellitus (157). Physiological studies have found that cholesterol metabolism, inflammation, and innate immunity are closely related to neurodegenerative lesions (158). Previous studies have found that several risk factors for Alzheimer's disease (AD) involve genes for lipid metabolism and transport, such as *APOε4*, *CLU*, and *ABCA7* (159–161). Therefore, abnormal lipid metabolism may be important in diabetes-associated cognitive dysfunction.

Lipid types and levels in the brain are vital determinants of brain function. Studies found that human liposomes change with age and aging can cause damage to the distribution of brain lipids and cause brain dysfunction (162, 163). The increase in oxidative stress is one of the signs of aging. Redox imbalance in the body damages to the cellular mechanism (164). Increased levels of ROS and RNS in patients with DM and AD (48, 165). Significant increase in oxidized proteins and lipids in patients with the brain in AD (166, 167). Studies such as Cutler found that changes in sphingolipid and cholesterol metabolism caused by membrane-related oxidative stress can cause neurodegenerative cascades (168). Increased oxidative stress and lipid peroxidation are associated with cognitive dysfunction in diabetes mellitus. One of the most significant hypotheses for neurodegenerative lesions is the amyloid hypothesis, which holds that excessive amounts of insoluble A subtypes cause tau to be over phosphorylated, resulting in free radical generation, inflammation, and oxidative damage (169). Low-density lipid lipoprotein receptor-related protein (LRP1) is involved in the clearance of Aβ peptide. Oxidation of LRP1 will inhibit its ability to remove Aβ peptide, resulting in the accumulation of Aβ peptide in the brain (170). High concentrations of ROS can lead to increased lipid peroxidation in the brain and change membrane permeability and membrane receptor and associated enzyme activity (171). Lipid peroxidation produces active aldehydes, such as malonaldehyde (MDA) and 4-hydroxynonenaldehyde (4-HNE), which combine and modify proteins involved in metabolism, antioxidant defense systems, and axon growth. The tau protein can be modified by 4-HNE, which indirectly leads to increased entanglement of neuronal fibers (172). In addition, LRP1 is also a covalently modified protein that further leads to the production of lipid peroxidation products. These products can cause normal initiation cascade dysregulation in neurons (173).

Several studies have confirmed the presence of disorders of lipid metabolism, including SP metabolism, Trp metabolism, and GP metabolism, in both patients with cognitive impairment in diabetes or in rat models (174, 175). Lipid metabolism can regulate numerous cellular signaling pathways involved in inflammatory responses (e.g., fatty acids, diacylglycerol (DAG), sphingolipids, CD36, Lipocalin 2). Microglia lipid metabolism is specifically involved in the control of microglia activation and effector functions such as migration, phagocytosis and inflammatory signaling, and minor disturbances in microglia lipid processing are associated with altered brain function in diseases characterized by neuroinflammation (176). Furthermore, peroxides produced by lipid peroxidation interfere with the structure of the cell membrane and protein function and stimulate intracellular signaling and other pathways leading to cell death. As a type B scavenger receptor, CD36 recognizes low-density lipoprotein (LDL), oxidized phospholipids, and beta-amyloid and is also an FA transporter. By activating the innate immune response, phagocytosis, and oxidant production in microglia, cd36g orchestrates transcriptional and metabolic remodeling. By recognizing oxidized parenchymal LDL receptors (TLRs) and internalizing receptor-ligand complexes, cd36 triggers Toll-like responses to stimulate sterile inflammation, which is closely related to neuroinflammation in cognitive impairment and mild inflammation in obesity (176). Lipocalin 2 (LCN2) functions in the regulation of the immune system and inflammatory processes. LCN 2 is mainly produced in the glia of the brain under oxidative stress and can disrupt the blood-brain barrier by promoting astrocyte and brain endothelial cell damage. Studies have shown that LCN2 regulates cellular activity in the central nervous system, controlling iron accumulation, and modulating neuroinflammation by activating glial cells (177). It may regulate neuroinflammation by activating the NF-κB signaling, activating signal transduction and transcription 3 (STAT 3) pathway to activate microglia, promoting astrocyte activation, further activation of microglia, and inhibiting neuroprotective cell pathways in the brain by regulating cytokines such as IL-1β, TNF-α, and IL-6 (178). LCN 2 is associated with inflammatory responses in metabolic disorders (including obesity and insulin resistance), where the pathway involves NF-κB, C/EBP, and estrogen response elements (179, 180). Moreover, insulin induces LCN-2 expression, thought to be *via* the phosphatidylinositol 3-kinase and mitogen-activated protein

kinase signaling pathways (180). Excess ROS, RNS are highly susceptible to oxidation of lipids containing carbon-carbon double bonds, especially polyunsaturated fatty acid (PUFA), peroxides of PUFA and their reactive aldehydes, their end products-reactive aldehydes such as 4-HNE-lead to protein carbonylation, and 4-HNE and other lipophiles mediate cytokinesis through protein adduct toxicity (181). Furthermore, 4-HNE processing activates pathways including DNA damage, antioxidant, ER stress, and heat shock responses associated with neuroinflammation, insulin resistance, and other diseases (182).

4 Conclusions and perspectives

Recent epidemiological and experimental data provide evidence of a bidirectional interaction between obesity and cognitive dysfunction in diabetes. Obesity and diabetes are both risk factors for cognitive dysfunction, and cognitive dysfunction in diabetes is one of the complications of diabetes (78). Therefore, hyperglycemia in diabetic patients is a cause of cognitive dysfunction, while obesity is the primary cause. Insulin resistance, neuroinflammation, and lipid metabolism disorders are exacerbated by obesity in diabetic patients, resulting in cognitive dysfunction in diabetic patients. The physiological processes of obesity and diabetic cognitive impairment are mediated by biological processes of oxidative stress. Oxidative stress in obese tissues is mainly caused by the disruption of the adipose microenvironment, chronic low-grade inflammation, and mitochondrial dysfunction. Furthermore, oxidative stress affects insulin resistance, neuroinflammation, and lipid metabolism in diabetic brain tissue, affecting physiological and pathological processes. In addition to accelerating the destruction of neurons, aging, and tau protein deposition in the brain, diabetic brains are prone to cognitive dysfunction. The obese diabetic population should be regularly evaluated for cognitive function because obesity is a risk factor for diabetic cognitive dysfunction. Managing diabetic cognitive dysfunction disease in obese and metabolically impaired individuals requires meticulous management, including antioxidants if necessary.

Author contributions

HL and JR designed the work of review; HL, JR, and YL reviewed the literature available on this topic and wrote the paper; HL and JR contributed to the scientific writing of the manuscript; HL, JR, QW, and JW revised the manuscript. HL, JR, YL, QW, and JW contributed equally to this work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Type 2 diabetes and the risk of synovitis-tenosynovitis: a two-sample Mendelian randomization study

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Introduction: It has been shown that people with type 2 diabetes have a higher risk of synovitis and tenosynovitis, but previous studies were mainly observational, which may be biased and does not allow for a cause-and-effect relationship. Therefore, we conducted a two-sample Mendelian randomization (MR) study to investigate the causal relationship.

Method: We obtained data on "type 2 diabetes" and "synovitis, tenosynovitis" from published large-scale genome-wide association studies (GWAS). The data were obtained from the FinnGen consortium and UK Biobank, both from European population samples. We used three methods to perform a two-sample MR analysis and also performed sensitivity analysis.

Results: The results of all three MR methods we used for the analysis illustrated that T2DM increases the risk factor for the development of synovitis and tenosynovitis. Specifically, for the IVW method as the primary analysis outcome, OR = 1.0015 (95% CI, 1.0005 to 1.0026), $P = 0.0047$; for the MR Egger method as the supplementary analysis outcome, OR = 1.0032 (95% CI, 1.0007 to 1.0056), $P = 0.0161$; for the weighted median method, OR = 1.0022 (95% CI, 1.0008 to 1.0037), $p = 0.0018$. In addition, the results of our sensitivity analysis suggest the absence of heterogeneity and pleiotropy in our MR analysis.

Conclusion: In conclusion, the results of our MR analysis suggest that T2DM is an independent risk factor for increased synovitis and tenosynovitis.

KEYWORDS

diabetes mellitus, synovitis, tenosynovitis, Mendelian randomization analysis, musculoskeletal diseases, risk factors, genome-wide association study

1. Introduction

Synovitis and tenosynovitis are a group of aseptic inflammatory diseases associated with acute trauma or chronic strain. It is estimated that synovitis and tenosynovitis have a high prevalence in the population and are a common group of musculoskeletal disorders that can seriously affect personal health and work life and impose high healthcare costs on society (1–3). In 2008, the analysis of data on claims for case allowances in Brazil illustrated that the overall prevalence of synovitis and tenosynovitis was 10.9/10,000 and occurred mainly in the physically active population, being the second most common type of all musculoskeletal disorders (after back disorders) (4). If only women are considered, synovitis and tenosynovitis are the most prevalent and persistent chronic diseases. The Connecticut Department of Labor's annual report on the causes of work-related chronic diseases shows that 10% of musculoskeletal disorders are tendinopathies. For workers, the

overall prevalence of tenosynovitis was 3.1%; 5.5% in high prevalence occupations; and 2.5% in low prevalence occupations (5). Type 2 diabetes, the most common type of diabetes, accounts for 90% of all diabetes (6, 7). The global prevalence of diabetes is estimated to be 9.3% (463 million people) in 2019, rising to 10.2% (578 million people) by 2030 and 10.9% (700 million people) by 2045 (8). And 50% of these patients do not know they have diabetes. Diabetes has now been shown to be a risk factor for multiple diseases, including cardiovascular disease (9, 10), kidney disease (11), and more. Some studies have shown that people with diabetes have a higher risk of synovitis and tenosynovitis (12–14). However, these studies are mainly observational studies, which are more likely to be influenced by confounding factors. And traditional observational studies can only obtain correlational relationships, not exact causal relationships (15).

Mendelian randomization (MR) is a method that uses genetic variation as an instrumental variable (IV) for exposure to estimate the causal association between exposure and certain outcomes (15–17). MR is conceptually similar to a randomized controlled study because genetic variation is randomly assigned during gamete formation before any confounding factors interfere, and is uniformly distributed across the population (17). Furthermore, alleles are fixed across individuals and do not change with disease onset or progression. Therefore, causal inferences obtained from MR analysis are less susceptible to bias from residual confounders and reverse causality (17–21). And with the increasing abundance of genome-wide association study (GWAS) data published by large consortia, gives MR studies a sufficient sample size to analyze reliable results (22–24). Here we performed a two-sample MR study to assess the effect of T2D on the risk of synovitis and tenosynovitis.

2. Method

2.1. Study design

To obtain reliable results, mendelian randomization (MR) studies must be based on three assumptions (15–17) (Figure 1A): (1) IV is strongly correlated with exposure factors; (2) IV is not correlated with any confounding factors affecting exposure and outcome; (3) IV is not directly correlated with outcome, and his effect on outcome is only reflected through exposure (25). In this study, we performed a two-sample MR (26) analysis to explore the causal relationship between type 2 diabetes and synovitis and tenosynovitis. For the two-sample MR study, the association between variance and exposure was estimated in one dataset, and the association between variance and outcome was estimated in the second dataset. Our analysis process consisted of five main parts: (1) reading the exposure factor GWAS data; (2) selecting the appropriate instrumental variables; (3) reading the outcome GWAS data and extracting the SNPs of the aforementioned instrumental variables; (4) preprocessing the exposure factor and outcome GWAS data to make them in a uniform format; and (5) performing MR analysis and sensitivity testing. The flow chart of the whole analysis is shown in Figure 1B.

2.2. Data source

Genetic variants (SNPs) associated with type 2 diabetes were extracted from published Genome-Wide Association Study (GWAS) data published by the FinnGen Consortium, using the "Type 2 diabetes" phenotype in this study. The GWAS included 215,654 Finnish subjects, including 32,469 cases and 183,185 controls. The pooled data for tenosynovitis and synovitis were obtained from the GWAS phenotyped "M65 Synovitis and tenosynovitis" published by the UK biobank, which was derived from a European sample of 361,194 subjects, including 2,812 cases and 358,382 controls. Our MR study was conducted using publicly available studies or shared datasets and therefore did not require additional ethical statements or consent.

2.3. Selection of IV

For the first assumption, "IV is highly associated with exposure", we selected SNPs from the European GWAS under the genome-wide significance threshold ($p < 5 \times 10^{-8}$) associated with exposure interest as potential SNPs. we then used the clump function ($r^2 = 0.001$, kb = 10,000) to remove selected single nucleotide linkage disequilibrium (LD) between selected single nucleotide polymorphisms (SNPs). These SNPs were excluded from the subsequent analysis. We used the F-statistic to assess weak instrumental variable effects (27). When the F-statistic is <10, we consider the genetic variation used to be weak IV, which may have some bias on the results. Then for the second assumption, "IV is not associated with confounding factors". We further examined whether these SNPs were associated with potential risk factors such as BMI, smoking, and hyperlipidemia by using a comprehensive web-based genotype-phenotype association database "PhenoScanner" (http://www.phenoscanner.medschl.cam.ac.uk). For SNPs associated with confounding factors, we manually performed culling. At the genome-wide significance level ($p < 5 \times 10^{-8}$), we removed SNPs associated with these potential confounders. For the third assumption, "IV is not associated with outcome," we needed to manually remove SNPs associated with outcome ($p < 5 \times 10^{-8}$). After extracting the remaining SNPs from the outcome data, we performed harmonization to ensure that the effects of IVs on exposure and outcome corresponded to the same effect alleles while excluding SNPs with palindromic sequences that could not determine the orientation and incompatible SNPs. The last remaining SNPs were used as IVs for the next MR analysis.

2.4. MR analysis

To avoid the effect of potential pleiotropy, we used three different MR methods (inverse variance Weighted (28) (IVW), MR-Egger regression (29) and weighted median (30)) to assess the causal effect between T2DM and synovitis and tenosynovitis. The results of the IVW method were used as the main results. In the hypothesis of IVW, we considered that all SNPs were not polyvalent (all were valid IVs). In addition, considering

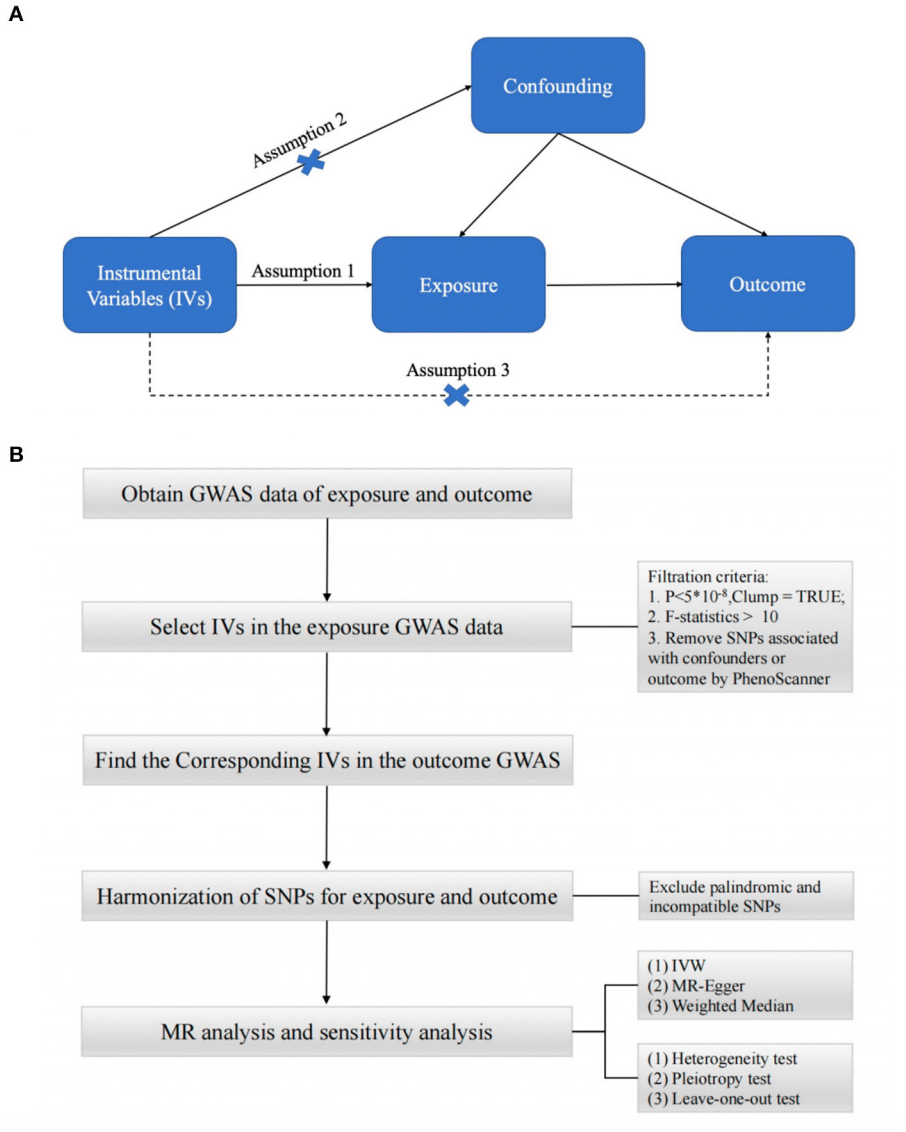

FIGURE 1
(A) Three assumptions of Mendelian randomization. **(B)** Flow chart of Mendelian randomization.

that the results of GWAS were done after standardization for multiple phenotypes, we considered a positive relationship between outcome and exposure. Briefly, the IVW method assumes that all IVs are valid IVs, the weighted median method allows 50% of the IVs to violate the IVs assumption, and MR-Egger allows all IVs to violate the IVs assumption. Furthermore, in MR-Egger's hypothesis framework, we consider the existence of the intercept and use it to assess pleiotropy. If this intercept term is 0, the results of the MR-Egger regression model are very close to IVW; however, if the intercept term is very far from 0, it indicates that these IVs may have horizontal pleiotropy. MR-Egger and Weighted median were used as complements to IVW estimation. These methods, although less efficient (wider CI), can provide reliable estimates under a wider range of conditions.

2.5. Sensitivity analysis

To demonstrate the reliability of our results, we performed a sensitivity analysis to detect potential horizontal pleiotropy and heterogeneity in our analysis. Cochran's Q test was used to detect potential heterogeneity. Cochran's Q statistic assessed the heterogeneity between genetic variants and considered heterogeneity when $p < 0.05$. And we plotted funnel plots based on the results. Subsequently, MR-Egger intercept tests were performed to provide estimates of horizontal pleiotropy ($p < 0.05$ was considered as the presence of an intercept and horizontal pleiotropy). MR-PRESSO analysis was performed to further analyze the pleiotropy and to look for sources of pleiotropy (31). A leave-one-out analysis was also performed to assess whether causality was depending on or biased toward any single SNP.

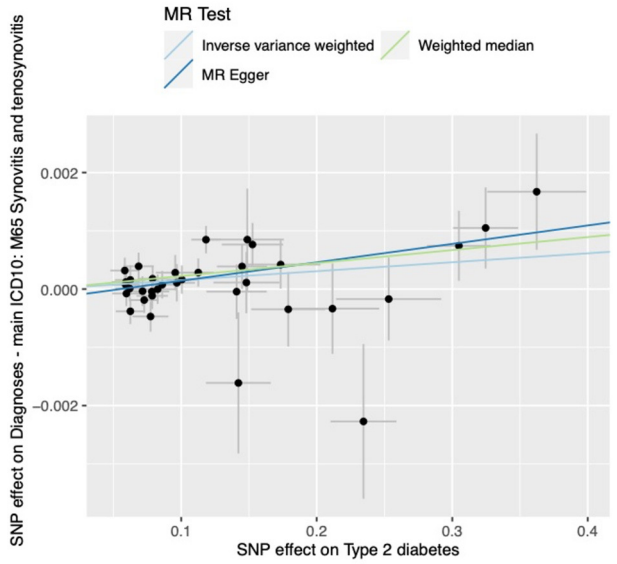

FIGURE 2
Scatterplot of MR analysis.

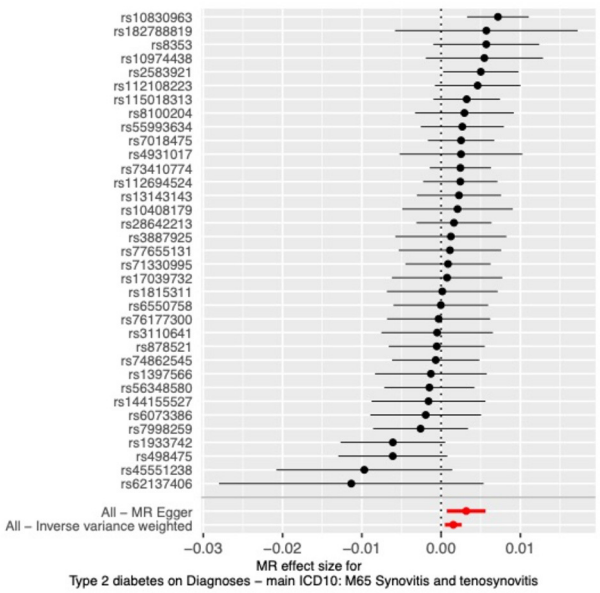

FIGURE 3
Forest plot of MR analysis.

All statistical analyses were performed using the "TwoSampleMR" package (https://github.com/MRCIEU/TwoSampleMR) of R software (version 4.1.3).

3. Results

3.1. Instrumental variables

Through the above process of screening, we finally selected 35 SNPs as IVs for the final analysis. All IVs were performed with an F-statistic > 10, indicating a low probability of weak IV bias. The details information on all the IVs is displayed in Appendix 1.

3.2. MR analysis

The results of all three MR methods we used for the analysis illustrated that T2DM increases the risk factor for the development of synovitis and tenosynovitis. Specifically, for the IVW method as the primary analysis outcome, OR = 1.0015 (95% CI, 1.0005 to 1.0026), $P = 0.0047$; for the MR Egger method as the supplementary analysis outcome, OR = 1.0032 (95% CI, 1.0007 to 1.0056), $P = 0.0161$; for the weighted median method, OR = 1.0022 (95% CI, 1.0008 to 1.0037), $p = 0.0018$. In addition, based on the results of the MR analysis, we plotted scatter plots (Figure 2) and forest plots (Figure 3).

3.3. Sensitivity analysis

To further verify the reliability of the results, we performed a sensitivity analysis to examine the heterogeneity and pleiotropy of MR. The results of Cochran's Q test showed no heterogeneity in IVs ($p > 0.05$), and the funnel plots we plotted are shown

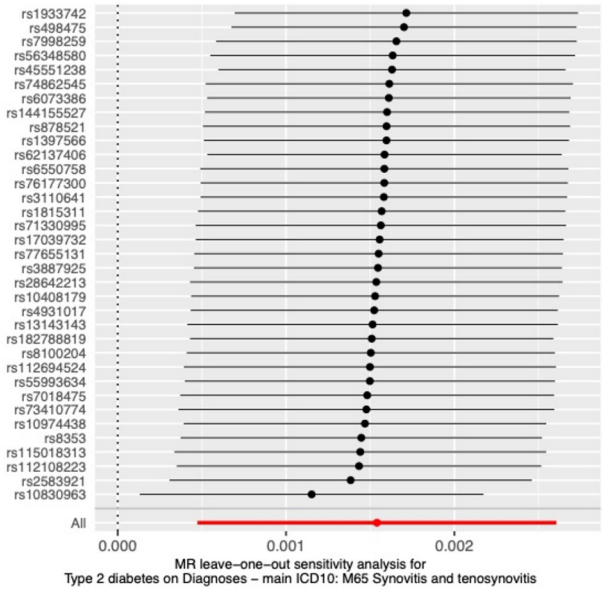

FIGURE 4
Sensitivity analysis by the leave-one-out method.

in Appendix 2. No significant pleiotropy or SNPs with outliers ($P > 0.05$) were found in the MR-PRESSO analysis. The results of the MR-Egger intercept test also showed no pleiotropy in our analysis ($p = 0.16$). The results of the leave-one-out test showed that causality did not rely on or bias any single SNP (Figure 4).

4. Discussion

In contrast to previous observational studies, the MR analysis we performed aimed to investigate the causal relationship between

T2DM and the risk of synovitis and tenosynovitis. To our knowledge, this is the first two-sample MR study to examine the causal relationship between T2DM and the risk of synovitis and tenosynovitis using large GWAS data. By MR analysis, we found that T2DM increases the risk of synovitis and tenosynovitis in the population. Moreover, there was no heterogeneity or pleiotropy in our study, and the results of the sensitivity analysis suggest that our results are reliable.

Previous studies are still controversial in stating whether T2DM increases the risk of synovitis and tenosynovitis in the population. Results from an analysis of the Taiwan Health Insurance Claims Database illustrated that diabetes mellitus was significantly associated with the occurrence of stenosing flexor tenosynovitis (SFT) (RR, 1.74; 95% CI 1.54-1.97) (12). Cross-sectional studies from Arabia (32) and Amman, Jordan (33), also indicate a greater probability of tenosynovitis than the general population. These studies show that T2DM increases the risk of synovitis and tenosynovitis in people of other races. Although most studies support that T2DM increases the risk of developing tenosynovitis and synovitis, there are also studies that illustrate that the incidence of tenosynovitis does not differ significantly among patients with T2DM (34). In addition, all of these studies were low on the evidence-based medical evidence scale, with the potential for various serious risks of bias. However, our MR analysis largely avoided confounding factors and had an effect similar to that of a randomized controlled trial. Moreover, the sample size included in the analysis was large, giving sufficient evidence to resolve the controversy. In addition, all data included in our study were derived from the European population, avoiding the bias of population heterogeneity.

There are fewer studies on the effect of diabetes on the risk associated with musculoskeletal disorders. A previous MR demonstrated T2DM as an independent risk factor for carpal tunnel syndrome (35). The results of all three MR methods analyzed in our study indicate that T2DM increases the risk of synovitis and tenosynovitis, and there was no significant heterogeneity or pleiotropy in the results of the analysis. We can use the results of the MR analysis to screen for people at risk in advance. That is, people with diabetes are more likely to develop synovitis and tenosynovitis, and for patients with diabetes we may be able to avoid the development or further progression of synovitis and tenosynovitis through early prevention and screening. In addition, synovitis and tenosynovitis of the hand have previously been suggested in studies as clinical and diagnostic tools for diabetic patients (32). Our findings provide some degree of justification for realizing this possibility.

However, we have some limitations in this study. First, both GWAS datasets we included in our study were derived from European populations, which to some extent limits the generalization of the results to other populations (e.g., Asians and Africans). Therefore, our findings should be used with caution when preparing for application to other populations. Second, our exposure data are for the "M65 Synovitis and tenosynovitis" phenotype, including systemic synovitis and tenosynovitis, without stratification by specific disease type and severity, patient gender, or age. Third, we excluded only SNPs associated with known confounders, such as BMI, blood lipids, and BMI-related characteristics (arm fat mass, arm fat removal, and waist circumference), and other unknown confounders need to be further investigated. Finally, it should be noted that SNPs refer to the biological function of an individual and cannot fully replace the T2DM phenotype. Whereas T2DM is genetically as well as environmentally, lifestyle, and epigenetic modifications, our results can only partially explain the causal effect of T2DM on synovitis and tenosynovitis.

5. Conclusion

In conclusion, we performed MR analysis using data from a large sample of GWAS analyses, and the results of our analysis showed that T2DM is an independent risk factor for increased synovitis and tenosynovitis. And the results of our sensitivity analysis proved that the results of our MR analysis are stable and reliable.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: UK-Biobank: https://www.nealelab.is/uk-biobank; the FinnGen: https://www.finngen.fi/en.

Author contributions

Conceptualization: YL. Methodology: JG and CP. Writing: JG and QH. Review and editing: CP and QH. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1142416/full#supplementary-material

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Nonalcoholic fatty liver disease and type 2 diabetes: an observational and Mendelian randomization study

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Introduction: Nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes mellitus (T2DM) are both chronic multisystem diseases that cause tremendous health burdens worldwide. Previous epidemiological studies have found a bidirectional relationship between these two diseases; however, their causality remains largely unknown. We aim to examine the causal relationship between NAFLD and T2DM.

Methods: The observational analysis included 2,099 participants from the SPECT-China study and 502,414 participants from the UK Biobank. Logistic regression and Cox regression models were used to examine the bidirectional association between NAFLD and T2DM. Two-sample Mendelian randomization (MR) analyses were conducted to investigate the causal effects of the two diseases using summary statistics of genome-wide association studies from the UK Biobank for T2DM and the FinnGen study for NAFLD.

Results: During the follow-up, 129 T2DM cases and 263 NAFLD cases were observed in the SPECT-China study, and 30,274 T2DM cases and 4,896 NAFLD cases occurred in the UK Biobank cohort. Baseline NAFLD was associated with an increased risk of incident T2DM in both studies (SPECT-China: OR: 1.74 (95% confidence interval (CI): 1.12–2.70); UK Biobank: HR: 2.16 (95% CI: 1.82–2.56)), while baseline T2DM was associated with incident NAFLD in the UK Biobank study only (HR: 1.58). Bidirectional MR analysis showed that genetically determined NAFLD was significantly associated with an increased risk of T2DM (OR: 1.003 (95% CI: 1.002–1.004, $p< 0.001$)); however, there was no evidence of an association between genetically determined T2DM and NAFLD (OR: 28.1 (95% CI: 0.7–1,143.0)).

Conclusions: Our study suggested the causal effect of NAFLD on T2DM development. The lack of a causal association between T2DM and NAFLD warrants further verification.

KEYWORDS

nonalcoholic fatty liver disease, type 2 diabetes, Mendelian randomization, China, UK Biobank

Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as an abnormal accumulation of fat in the liver without significant alcohol intake (1). With a global estimated prevalence of 25%, it is the most prominent cause of liver disease worldwide (2). Due to its high potential in developing liver fibrosis, liver cirrhosis, and hepatocellular carcinoma, NAFLD has already caused considerable clinical and health burdens worldwide (3, 4).

NAFLD is a multisystem disease that can affect extrahepatic organs and regulatory pathways, causing other chronic diseases and related complications (5). Over the past decade, compelling observational studies have demonstrated that NAFLD and type 2 diabetes mellitus (T2DM) are two pathologic conditions that frequently coexist, and there seems to be a bidirectional relationship between them (6). The presence of NAFLD substantially increases the risk of incident T2DM, and such risk parallels the severity of NAFLD (7–9). However, the studies targeting baseline NAFLD and incident T2DM have been mostly conducted in the Asian population, and relevant evidence in the Western population is lacking (10). On the other hand, the prevalence of NAFLD is more than twofold higher in patients with T2DM than in the general population. This association remains similar among different races, but the strength of the association seems to be stronger in white Europeans than in Asians (11).

Although the bidirectional relationship between NAFLD and T2DM has been widely reported, the causality remains largely uncertain due to potential confounding factors or the reverse causation bias within observational studies (12). As a result, it is crucial to dissect the causal relationship between NAFLD and T2DM to better understand the disease etiology and inform effective diagnostic, therapeutic, and preventive strategies.

In recent years, Mendelian randomization (MR) analysis, a form of instrumental variable (IV) analysis, has profoundly equipped researchers with tools for estimating causal inference between exposures and outcomes (13). This approach carries two merits of minimizing confounding and diminishing reverse causality because genetic variants are randomly allocated at conception (thus unrelated to self-adopted and environmental factors) and cannot be modified by the development and progression of the disease. Bidirectional MR is an extension of traditional MR in which the exposure–outcome causal relationship was explored from both sides, providing an efficient way to ascertain the direction of a causal relationship (14).

In this study, we first examined the observational association between T2DM and NAFLD in both the Chinese and White populations. We then investigated the direction of the causal relationship using the bidirectional MR method based on online genome-wide association study (GWAS) data.

Methods

Study population

SPECT-China (registration number ChiCTR-ECS-14005052; www.chictr.org.cn) is a population-based study investigating the prevalence of metabolic diseases and risk factors in East China. A stratified cluster sampling method was used to select a sample in the general population at 23 sites across Shanghai, Zhejiang, Jiangsu, Anhui, and Jiangxi Province. The sampling process was stratified according to rural/urban areas and economic development. Chinese citizens aged 18 years old and above who have lived in their current residence for 6 months or longer were selected for our study. After excluding those with acute illness, severe communication problems, or unwilling to participate, a total of 6,899 subjects were included in the SPECT-China study from February to June 2014 (15).

Between January and June 2019, the participants were invited to attend a first-round follow-up visit. A total of 2,171 participants attended the follow-up survey. We then excluded 72 participants with missing liver ultrasound results at baseline or in the follow-up, leaving 2,099 eligible participants for further analysis (Supplementary Figure S1).

The study protocol was approved by the Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (approval number 2013 (86)). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration. Informed consent was obtained from all participants in the study.

The UK Biobank (UKB) is a population-based prospective cohort study, including more than 500,000 community-dwelling adults aged 37–73 years across the UK between 2006 and 2010 (https://www.ukbiobank.ac.uk/). We declare that all data are publicly available in the UKB repository (16). The UKB received ethical approval from the UK National Health Service, the National Research Ethics Service North West, the National Information Governance Board for Health and Social Care in England and Wales, and the Community Health Index Advisory Group in Scotland. All participants provided written, informed consent. This study was approved by the UK Biobank (application number 77740).

Data collection and measurements

Sociodemographic characteristics, medical history, family history, and lifestyle factors were obtained through our questionnaire. Regional economic status was assessed by the gross domestic product per capita at each site and categorized into high and low economic status according to the national level in 2013 ($6,807 per capita from the World Bank) (17). Well-trained and experienced staff conducted anthropometric measurements according to a standard protocol at each study site, providing us with weight, height, waist circumference, and blood pressure. Venous blood samples were drawn after overnight fasting for at least 8 h. These samples were immediately stored at −20°C and sent to the central laboratory by air on dry ice within 4 h.

Instrumental variables

First, we extracted independent SNPs for diabetes from the summary statistics of GWAS, which were publicly downloadable

from the IEU OPEN GWAS PROJECT (https://gwas.mrcieu.ac.uk/), with the same Batch ID of "ukb-b." We then explored the FinnGen dataset, which had no overlapped participants with the dataset mentioned before, for GWAS summary statistics of NAFLD. The FinnGen Biobank GWAS was performed by the FinnGen team (https://r4.finngen.fi/) and is available on the IEU OPEN GWAS PROJECT. Finally, we pruned the genetic variants within a 250-kb window to include independent SNPs (r2< 0.1). All the SNPs used in the study are shown in Supplementary Table S1.

Definition of variables

In the SPECT-China dataset, current smoking was defined as having smoked at least 100 cigarettes in one's lifetime and currently smoking cigarettes. T2DM was determined by fasting plasma glucose at ≥7.0 mmol/l and/or HbA1c ≥48 mmol/mol (6.5%) and/or a self-reported previous diagnosis by healthcare professionals according to the 2010 ADA criteria (18). Liver fat accumulation (steatosis) was detected by ultrasound; the presentation of steatosis included increased liver echogenicity, stronger echoes in the hepatic parenchyma as compared with the renal parenchyma, vessel blurring, and narrowing of the lumen of the hepatic veins according to the criteria of Saadeh et al. (19). NAFLD was defined as ultrasound evidence of fatty liver and the exclusion of secondary causes (having a history of excessive consumption (30 g/day in men and 20 g/day in women) of pure alcohol, self-reported viral hepatitis, and using medications associated with secondary NAFLD (corticosteroids, amiodarone) (20).

In the UK Biobank dataset, T2DM and NAFLD were ascertained using linkage with hospital inpatient records. The date and cause of hospital admissions were obtained from record linkage to Health Episode Statistics (England and Wales) and the Scottish Morbidity Records (Scotland). We defined outcomes according to the International Classification of Diseases, edition 10 (ICD-10): E11 for T2DM and K76.0 for NAFLD after the exclusion of viral hepatitis (B15–B19).

We alternatively used the fatty liver index (FLI), a noninvasive algorithm for identifying liver steatosis at baseline. The formula of FLI was as follows:

$$FLI = \frac{(e^{0.953*loge(triglycerides)+0.139*BMI+0.718*loge(ggt)+0.053*waistcircumference-15.745})}{(1+e^{0.9533*loge(triglycerides)+0.139*BMI+0.718*loge(ggt)+0.053*waistcircumference-15.745})}*100$$

I was expressed as a value ranging from 0 to 100, and previous studies have validated that it matched the observed percentages of patients with hepatic steatosis accurately (21). NAFLD was based on an FLI ≥ 60 and the exclusion of viral hepatitis, excessive alcohol consumption (alcohol consumption: ≥ 30 g/day for male participants and 20 g/day for female participants), or aspartate transaminase or alanine aminotransferase > 500 U/L (11).

Statistical analysis

Continuous variables were expressed as mean (standard deviation), and categorical variables were described as percentages (%). Characteristics of the study sample were compared by the *t*-test for continuous variables and Pearson's Chi-square test for categorical variables. The multivariate Cox regression model was used in the UK Biobank dataset, and the logistic regression model was used in the SPECT-China dataset to evaluate the association between NAFLD and diabetes. The follow-up time was calculated from the baseline date to the diagnosis of outcome, death, or the censoring date (30 May 2022), whichever came first. The model was adjusted for age, gender, education level, living area, smoking status, drinking status, economic status, and BMI. Family history of diabetes was additionally adjusted when assessing the association between baseline NAFLD and incident T2DM.

For the two-sample MR method, we mainly performed an inverse-variance–weighted (IVW) MR analysis to verify the causal association between NAFLD and diabetes. Moreover, we applied MR-Egger regression and the weighted median approach. If there was no evidence of directional pleiotropy (*p*-value for MR-Egger intercept > 0.05), the estimate from the IVW method was considered the most reliable indicator.

A two-tailed $p< 0.05$ was considered statistically significant. All analyses were performed using R version 4.2.0 and SPSS software version 26.0.

Results

Baseline characteristics

Table 1 shows the baseline characteristics of the participants. In the SPECT-China dataset, a total of 2,099 participants (mean age ± SD: 53.54 ± 11.35 years) were included in the final analyses. Participants who developed diabetes were relatively older, richer, had a lower education level, and tend to have a habit of drinking. They also had a higher level of BMI, were more likely to have a family history of diabetes, and had a higher prevalence of NAFLD. Meanwhile, compared with those who did not develop NAFLD during follow-up, participants with incident NAFLD were more likely to be women, have a habit of drinking and smoking, have a higher economic status, live in urban areas, and have a higher level of BMI. Further analysis of the UK Biobank dataset showed similar results (Table 1; Supplementary Table S2).

Association between diabetes and NAFLD

Figure 1 demonstrates the association between baseline NAFLD and incident T2DM. After adjusting for age, gender, education level, living area, smoking status, drinking status, economic status, and BMI, we found that baseline NAFLD was associated with a significantly higher risk of incident T2DM both in the SPECT-China dataset (OR: 1.74 (95% confidence interval (CI):1.12–2.70); $p = 0.013$) and the UK Biobank dataset (HR: 2.16 (95% CI: 1.82–2.56); $p< 0.001$). This result remained unchanged when we additionally used FLI to define NAFLD (HR: 1.76 (95% CI: 1.71–1.81); $p< 0.001$).

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TABLE 1 Baseline characteristics of the study population.

	Participants without baseline T2DM		Participants without baseline NAFLD	
	Incident T2DM	No incident T2DM	Incident NAFLD	No incident NAFLD
SPECT-China				
Number of participants	129	1,707	263	882
Age at recruitment (years)	57.67 ± 8.60*	52.38 ± 11.52	52.90 ± 10.92	52.03 ± 10.92
Gender (%)				
Women	58.1%	58.3%	42.6%*	35.8%
Men	41.9%	41.7%	57.4%*	64.2%
Current smoking (%)	29.5%*	19.3%	24.3%*	17.6%
Current drinking (%)	31%	32%	38.1%*	29.3%
Economic status (%)				
High	89.9%*	80%	90.9%*	77.7%
Low	10.1%*	20%	9.1%*	22.3%
Living area (%)				
Rural	65.9%	61.2%	74.5%*	64.7%
Urban	34.1%	38.8%	25.5%*	35.3%
College education or above (%)	10.1%*	22.1%	16.7%	19.2%
BMI (kg/m^2)	26.43 ± 3.45*	23.81 ± 3.23	24.27 ± 2.96*	22.30 ± 2.66
Family history of diabetes (%)	15.5%*	9.8%		
Baseline NAFLD (%)	43.4%*	19.2%		
Baseline T2DM (%)			8.4%	6.5%
UK Biobank				
Number of participants	30,274	458,610	4,896	496,867
Age at recruitment (year)	59.2 ± 7.3*	56.2 ± 8.1	56.8 ± 7.9*	56.5 ± 8.1
Gender (%)				
Women	41.8*	55.7	53.1*	54.4
Men	58.2*	44.3	46.9*	45.6
Smoking status (%)				
Never	45.3*	55.7	45.9*	54.9
Previous	40.8*	33.9	39.4*	34.6
Current	13.9*	10.4	14.7*	10.5
Drinking status (%)				
Never	8.1*	4.1	6.7*	4.4
Previous	6.1*	3.3	7.0*	3.6
Current	85.8*	92.6	86.3*	92.0
Townsend deprivation index	−0.4 ± 3.4*	−1.4 ± 3.0	−0.4 ± 3.4*	−1.3 ± 3.1
Living area (%)				
Rural	11.2*	15.0	12.1*	14.7
Urban	88.8*	85.0	87.9*	85.3

(Continued)

TABLE 1 Continued

	Participants without baseline T2DM		Participants without baseline NAFLD	
	Incident T2DM	No incident T2DM	Incident NAFLD	No incident NAFLD
College Education or above (%)	21.0*	33.1	21.4*	32.2
BMI (kg/m^2)	31.4 ± 5.6*	27.0 ± 4.5	31.3 ± 5.7*	27.4 ± 4.8
Family history of diabetes (%)	24.6*	13.5		
Baseline NAFLD (%)	0.4*	0.1		
Baseline T2DM			8.1*	2.6

Continuous variables were expressed as mean ± SD, and categorical variables were described as a percentage (%). Characteristics of the study sample were compared by the t-test for continuous variables and Pearson's Chi-square test for categorical variables.
*p< 0.05, significantly different from that in the nonprogressor group.
NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus.

Figure 2 shows the association between baseline T2DM and incident NAFLD. After multivariable adjustment, we observed that baseline T2DM is significantly associated with incident NAFLD (HR: 1.58 (95% CI: 1.42–1.77); p< 0.001) in the UK Biobank dataset. However, this association was not significant in the SPECT-China dataset (OR: 1.13 (95% CI: 0.64–1.99); p = 0.669). Further adjustment for total cholesterol, triglycerides, and systolic blood pressure did not attenuate these results (Supplementary Table S3).

Bidirectional MR analysis

When analyzing online GWAS datasets using the IVW method, we found that genetically instrumented NAFLD was consistently associated with a higher risk of T2DM (OR: 1.003 (95% CI: 1.002–1.004); p< 0.001), but there was no association between genetically instrumented T2DM and NAFLD (ORs ranged from 3.13 to 345.8, all p > 0.05) (Table 2). Pleiotropy bias was not detected in these analyses (both p > 0.05).

Discussion

In the observational analysis, we found a significant association between baseline NAFLD and an increased risk of incident T2DM in the SPECT-China and the UK Biobank datasets. The association between baseline T2DM and increased risk of incident NAFLD was observed in the UK Biobank dataset but not in the SPECT-China dataset. Further bidirectional two-sample MR analysis showed consistent evidence that genetically instrumented NAFLD increased T2DM risk while the T2DM→NAFLD relationship was unlikely to be causal.

Over the past decade, several population-based studies have focused on the relationship between NAFLD and T2DM. Almost all of the studies that have used noninvasive imaging techniques (predominantly ultrasonography) to diagnose NAFLD have shown that NAFLD increases the risk of incident T2DM (7–9), echoing our results. However, all of these studies are conducted in the Asian population, mainly South Koreans, and evidence derived from other populations is lacking. Therefore, we conducted the

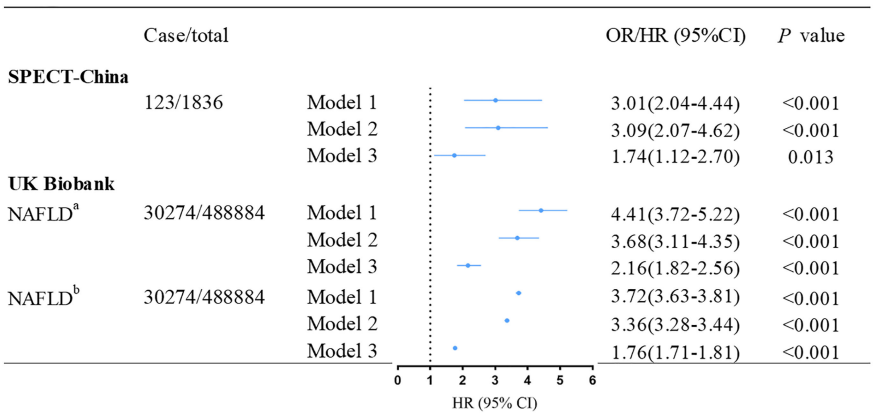

FIGURE 1
Association between baseline NAFLD and incident T2DM in SPECT-China and UK Biobank. [a]NAFLD was ascertained according to hospital inpatient records. [b]NAFLD was defined by fatty liver index. Model 1 was adjusted for age, and gender. Model 2 was further adjusted for education level, living area, smoking status, drinking status and economic status. Model 3 was additionally adjusted for BMI and family history of diabetes based on Model 2. OR, odd ratio; HR, hazard ratio; CI, confidence interval; NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus.

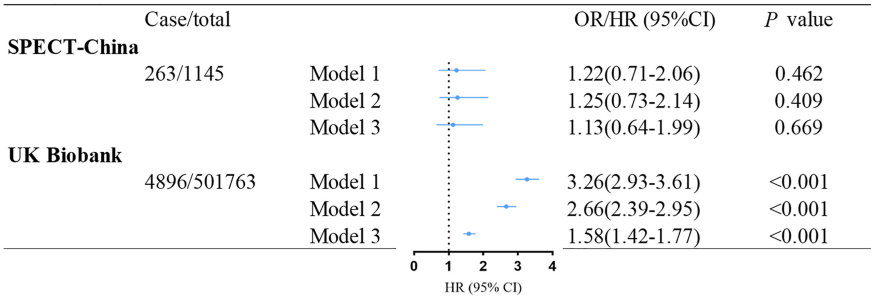

FIGURE 2
Association between baseline T2DM and incident NAFLD in SPECT-China and the UK Biobank. Model 1 was adjusted for age and gender. Model 2 was further adjusted for education level, living area, smoking status, drinking status, and economic status. Model 3 was additionally adjusted for BMI based on model 2. OR, odd ratio; HR, hazard ratio; CI, confidence interval; NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus.

analysis in the UK Biobank cohort of European descent, and similar results were found. Since the diagnosis of NAFLD in the UK Biobank depended on hospital admission records, the prevalence of NAFLD was only 0.98%, which was significantly lower than the average prevalence worldwide. To reduce this selection bias, we further used the FLI to assess the association between NAFLD and incident diabetes, and the results remained unchanged.

On the other hand, several studies point to T2D as a risk factor for NAFLD as well as the progression toward NASH, fibrosis, and HCC (22–24). In our study, the relationship between T2DM and NAFLD was found in the UK Biobank dataset but not in the SPECT-China dataset. This result requires verification in larger cohorts as it may be specific to the Chinese population or just a chance finding due to the small sample size.

However, an observational study is not capable of investigating the causal effect between these two diseases and has many limitations. First of all, the golden standard for diagnosing NAFLD is liver biopsy, which is not feasible in epidemiological studies. Using liver ultrasound and FLI instead may cause diagnostic bias. Moreover, though we carefully adjusted for various confounders, bias from residual and unmeasured confounding may still exist. Therefore, we further conducted bidirectional MR analysis to minimize the effects of confounding factors and elucidate the causal effect between these two diseases, where we found a NAFLD→T2DM relationship. A previous study revealed that genetically predicted higher circulating ALT and AST were related to an increased risk of T2DM (25). Another study found a weak association between genetically instrumented hepatic

TABLE 2 MR estimates of the causal association between T2DM and NAFLD.

	Effect size (95% CI)	*p*-value
T2DM → NAFLD		
IVW (fixed effects)	28.1 (0.7–1,143.0)	0.08
IVW (multiplicative random effects)	28.1 (0.3–2,386.2)	0.14
MR-Egger	345.8 (0.01–24,295,450.8)	0.31
Weighted median	3.13 (0.01–886.1)	0.69
Test for heterogeneity: p = 0.015 (IVW) and p = 0.013 (MR-Egger)		
Test for horizontal pleiotropy: MR-Egger intercept = −0.01 (−0.05 to 0.03), p = 0.63		
NAFLD→T2DM		
IVW (fixed effects)	1.003 (1.002–1.004)	<0.001
IVW (multiplicative random effects)	1.003 (1.001–1.005)	0.002
MR-Egger	1.01 (1.02–1.00)	0.18
Weighted median	1.003 (1.002–1.005)	<0.001
Test for heterogeneity: p = 0.016 (IVW) and p = 0.366 (MR-Egger)		
Test for horizontal pleiotropy: MR-Egger intercept = 0.994 (0.990 to 0.998), p = 0.224		

The effect size was presented as the odds ratio and its 95% confidence interval.
CI, confidence interval; T2DM, type 2 diabetes mellitus; NAFLD, nonalcoholic fatty liver disease; IVW, the inverse-variance–weighted (IVW) method; MR, Mendelian randomization.

steatosis and two glycemic traits: fasting glucose and fasting insulin. This study also demonstrated that a one-standard deviation (SD) increase in CT-measured hepatic steatosis led to a 30% increased risk of T2D (26). Since circulating ALT and AST are markers for NAFLD and the glycemic trait is a marker for T2DM, our results are in accordance with their results and provided a more direct supplement.

The NAFLD→T2DM causality is biologically plausible. The key features of NAFLD are hepatic lipid accumulation and hepatic inflammation. In the early period of NAFLD, an elevated hepatic lipid availability combined with the inadequate adaptation of mitochondrial function owing to lipid oxidation could induce the hepatic production of DAG and ceramides, affecting hepatic insulin resistance (27). Moreover, patients with NAFLD have moderate increases in total bile acids (28). Primary bile acids are produced in the liver from cholesterol and then secreted into the intestine as glycine and taurine conjugates. The intestinal microbiota then converts primary bile acids into secondary bile acids, which interact with various nuclear receptors in the intestine such as farnesoid X receptor (FXR) and Takeda G protein-coupled membrane receptor 5 (TGR5) (29, 30). These interactions play an important role in insulin clearance and the regulation of hepatic lipid and glucose metabolism. Insulin clearance is decreased in patients with NAFLD, causing less sensitivity to insulin. By contrast, in the later stages of liver disease, the activation of Toll-like receptor 4 (TLR4) by lipopolysaccharide induces inflammation, ceramide biosynthesis, and insulin resistance (31). With *de novo* ceramide synthesis, ceramides derived from palmitic acid are the most potent at decreasing insulin action and causing insulin resistance (32, 33). These mechanisms worked together in triggering insulin resistance, which is the key pathology of T2DM.

The major strengths of this study include the investigation of the bidirectional association between T2DM and NAFLD among the same Chinese population and validation from a large European cohort. More importantly, Mendelian randomization analysis was further performed to determine the causal effects. There are also several limitations to our study. First, liver biopsy, the current gold standard for diagnosing hepatic steatosis was not feasible in a large epidemiological study. Using a blood marker equation to define liver steatosis may not be accurate enough, and ultrasound has limited sensitivity in detecting minor amounts of fatty infiltration. Second, in the SPECT-China study, OGTT 2-h postprandial glucose was not available to be included in the definition of T2DM. Consequently, some potential patients with T2DM might be misclassified. Third, though we carefully adjusted for various confounders, bias from residual and unmeasured confounding may still exist. Forth, the Mendelian randomization analysis was restricted to volunteers of European ancestry, mostly White British, and the number of SNPs for NAFLD is small. Therefore, whether these findings could generalize to other populations need further research. Moreover, although pleiotropy tests were conducted in our study, there may still be potential SNPs associated with unknown phenotypes.

Conclusions

In summary, results from both observational analysis and bidirectional MR analysis suggest a potentially causal NAFLD→T2DM relationship. Our findings raise public awareness of the intrinsic link between NAFLD and T2DM and emphasize the intervention strategies that target the prevention of T2DM by active management and treatment of NAFLD.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Shanghai Ninth People's Hospital, Shanghai JiaoTong University School of Medicine (approval number 2013(86)) UK National Health Service, National Research Ethics Service North West, National Information Governance Board for Health and Social Care in England and Wales, and Community Health Index Advisory Group in Scotland. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YL, BW and NW conceptualized this paper. YTY and YFY analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1156381/full#supplementary-material

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Epigenetic modification in diabetic kidney disease

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Diabetic kidney disease (DKD) is a common microangiopathy in diabetic patients and the main cause of death in diabetic patients. The main manifestations of DKD are proteinuria and decreased renal filtration capacity. The glomerular filtration rate and urinary albumin level are two of the most important hallmarks of the progression of DKD. The classical treatment of DKD is controlling blood glucose and blood pressure. However, the commonly used clinical therapeutic strategies and the existing biomarkers only partially slow the progression of DKD and roughly predict disease progression. Therefore, novel therapeutic methods, targets and biomarkers are urgently needed to meet clinical requirements. In recent years, increasing attention has been given to the role of epigenetic modification in the pathogenesis of DKD. Epigenetic variation mainly includes DNA methylation, histone modification and changes in the noncoding RNA expression profile, which are deeply involved in DKD-related inflammation, oxidative stress, hemodynamics, and the activation of abnormal signaling pathways. Since DKD is reversible at certain disease stages, it is valuable to identify abnormal epigenetic modifications as early diagnosis and treatment targets to prevent the progression of end-stage renal disease (ESRD). Because the current understanding of the epigenetic mechanism of DKD is not comprehensive, the purpose of this review is to summarize the role of epigenetic modification in the occurrence and development of DKD and evaluate the value of epigenetic therapies in DKD.

KEYWORDS

epigenetic modification, diabetic kidney disease, metabolic disorder, biomarker, noncoding RNA

Introduction

Diabetic kidney disease (DKD) is a common complication of diabetes and a major cause of end-stage renal disease (ESRD), which seriously affects the quality of life of patients (1–4). The main pathological features of DKD are glomerular sclerosis, podocyte detachment, epithelial-mesenchymal transition (EMT)/endothelial-to-mesenchymal transition (EndMT)/macrophage-myofibroblast transition (MMT), excessive extracellular matrix (ECM) and renal tubular fibrosis. These pathological changes affect glomerular and tubular function, leading to the progression of

proteinuria and decreased glomerular filtration capacity. A long-term hyperglycemic environment in diabetics induces metabolic disorders, oxidative stress and hemodynamic changes. Although these symptoms occur with genetic mutations, they are, to a greater extent, related to epigenetic variations (5). For instance, studies have found that even after a long period of strict glycemic control, patients with diabetes may still develop complications due to early high glucose (HG) exposure (4, 6, 7). This metabolic memory phenomenon has been shown to be related to DNA methylation and histone acetylation at the promoter, which suggests that epigenetic modifications are subsumed in the pathological process of diabetes and affect patients' conditions over a long period of time (8, 9). Therefore, a deeper understanding of the epigenetic modifications in DKD can help to better understand the pathogenesis of the disease and provide potential predictive and therapeutic targets for DKD treatment. In the current study, we conducted a comprehensive analysis and introduction of DKD-related epigenetic mechanisms and epigenetic therapies based on searching the published literature from PubMed (https://pubmed.ncbi.nlm.nih.gov) and Web of Science (http://www.webofknowledge.com/databases). Our aim is to encourage more clinicians and researchers to pay attention to the function of epigenetic modifications in the occurrence and development of DKD and conduct laboratory, preclinical and clinical studies on the development of epigenetic drugs and therapeutic strategies for DKD.

The pathogenesis of DKD

Diabetic patients often have high blood pressure, high blood lipids, high uric acid and obesity, all of which may lead to kidney damage (10, 11). The pathogenesis of DKD is complex. The main pathological characteristics of DKD are glomerulosclerosis and renal fibrosis (12, 13). An impaired glomerular filtration barrier is the primary cause of albuminuria. Renal fibrosis and albuminuria are important causes of renal function loss, which is the consequence of multiple factors and mechanisms. DKD-associated renal fibrosis is defined by the excessive deposition of ECM caused by various adverse stimuli (14–17). Understanding the pathogenesis of DKD may help to prevent, slow down, or even reverse DKD. Figure 1 briefly summarizes the pathogenesis of DKD.

Metabolism disorders

Glucose, lipid and hormone metabolism disorders caused by HG exposure may lead to the accumulation of advanced glycation end products (AGEs) and the activation of protein kinase C (PKC) (18–22). AGEs can activate related signaling pathways, such as the nuclear factor kappa-B (NF-κB) and transforming growth factor β (TGF-β) pathways, promote EMT/EndMT, and result in glomerular podocyte loss and progressive glomerulosclerosis (23–27). Activated PKC may decrease endothelial nitric oxide synthase (eNOS) production, which not only activates NF-κB-mediated inflammatory pathways but also stimulates the production of vascular endothelial growth factor (VEGF), inducing endothelial dysfunction and further (26, 28–30).

Oxidative stress

The HG environment activates polyols, PKC, hexosamine and other pathways, leading to an increase in the oxidative stress

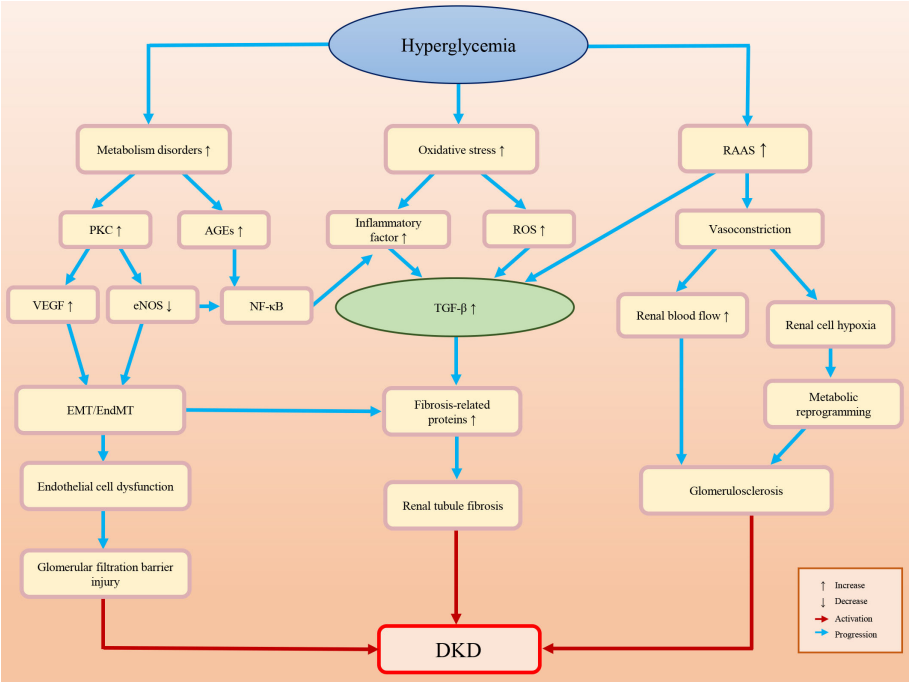

FIGURE 1
Briefly summarizes and illustration of the pathogenesis of DKD.

response and reactive oxygen species (ROS) (31–33). ROS mediate various signaling pathways, such as TGF-β, adenosine 5′ monophosphate-activated protein kinase (AMPK) and nuclear factor-erythroid 2-related factor 2 (Nrf2), which pertain to the cell cycle, cell proliferation, autophagy, inflammation and oxidative stress (33–35). In DKD, the activation of ROS can promote podocyte apoptosis and inflammatory factor release, and activate the renal fibrosis signaling pathways, which results in renal fibrosis and the decline of glomerular filtration function (23, 25, 27).

Inflammation

Diabetes is often accompanied by chronic inflammation. The expression levels of inflammatory factors (e.g. tumor necrosis factor-α (TNF-α), interleukin-6, interferon γ (IFN-γ) and interleukin-17) are elevated in DKD patients (14–16, 36–41). Abnormal expression of these cytokines may activate renal fibrosis-related signaling pathways, induce EndMT/EMT/MMT, and promote the accumulation of ECM, which ultimately stimulates the expression of fibrosis-related proteins (e.g. α-smooth muscle actin (α-SMA) and connective tissue growth factor (CTGF)) and glomerulosclerosis (42–47).

Hemodynamic change

Diabetic patients' kidneys are always exposed to an HG environment for a long time. The long-term high level filtration load may induce glomerular feedback dysfunction and aggravate glomerular sclerosis (48). The renin-angiotensin-aldosterone system (RAAS) can also be activated by HG exposure (e.g., the products of HG-induced metabolic disorders and oxidative stress-induced ROS) (49). The RAAS not only induces the constriction of blood vessels in the kidney, but also upregulates TGF-β fibrosis-associated pathways and inflammation (49–53). The decline in blood flow and oxygen delivery at the glomerular filtration barrier after renal vasoconstriction may promote glycolysis and metabolic reprogramming and produce metabolites (e.g., lactate and L-serine) (54–58). These metabolites are associated with multiple cellular behavior variations, such as mitochondrial damage, histone modification, and the activation of the renal cell fibrosis-related signaling pathway, which may affect cell senescence and survival, increase inflammation reflection, induce podocyte damage, endothelial cell dysfunction, and renal tubular cell fibrosis, and further aggravate kidney damage (59–64).

The epigenetic modification of DKD

DNA methylation in DKD

DNA methylation is a significant epigenetic regulatory mechanism. DNA methylation is catalyzed by a family of DNA methyltransferases that transfer a methyl group from S-adenyl methionine to the carbon of a cytosine residue (65). DNA methylation can change chromosome structure, conformation, stability and the interaction mode between DNA and protein, thereby participating in a variety of regulatory mechanisms (e.g., gene transcription and imprinting, cell differentiation and fibrosis) (66–70).

DNA methylation is associated with DKD. VanderJagt et al. found that many methylation modifications occur from prediabetes to diabetes. Among these methylation modifications, six genes are associated with DKD, which may induce inflammation and immunity and break urate homeostasis (71). By comparing the DNA methylation of kidney proximal tubule cells in 10-week-old db/db mice with that in normal mice, Marumo et al. considered that at the early stage of DKD, several potentially functional genes were significantly methylated, e.g., angiotensinogen (*Agt*) and claudin 18 (*Cldn18*), which may alter the progression of DKD (72). Park et al. indicated that there are extensive methylation differences in DKD kidneys, among which the change in TNF-α methylation has a close connection with kidney function decline (73). In addition, the application of reversed-phase high performance liquid chromatography (RP-HPLC) to determine DNA methylation levels in peripheral blood mononuclear cells also revealed differences in genomic methylation levels between patients with renal dysfunction and patients with simple diabetes (74, 75). These abnormal changes may be a response to a chronically hyperglycemic environment. Furthermore, the degree of methylation in DKD varies from stage to stage. Lecamwasam et al. collected blood samples from diabetic patients with chronic kidney disease (CKD) and indicated that differential methylation patterns of 5'-C-phosphate-G-3' (CpG) sites are associated with different stages of CKD. Of note, relative to the early CKD group, the cysteine-rich secretory protein 2 (*CRISP2*) gene promoter carried 12 hypermethylated CpG sites in the late CKD group, which may lead to oxidative stress in inflammatory pathways (76).

Histone modification in DKD

Histones are an important component of nucleosomes and a general term for alkaline proteins that bind to DNA (77–79). The free N-terminus at the end of histones can undergo various modifications, including acetylation, methylation, phosphorylation, and ubiquitination (80). Once histones are modified, the function of chromatin will be changed: first, the charge of amino acids will be changed, and the affinity between histones and DNA will be decreased; second, binding to specific surfaces and regulating transcriptional activity will also be changed (79). Figure 2 briefly summarizes the histone epigenetic modifications and their regulatory roles in DKD.

Histone methylation

Histone methylation is a process in which methyl groups are transferred to lysine and arginine residues in the histone tail by histone methyltransferase (81). Histone methylation is a dynamic and reversible process because the methylation of histones can be erased by histone demethylases (82). Histone methylation plays a

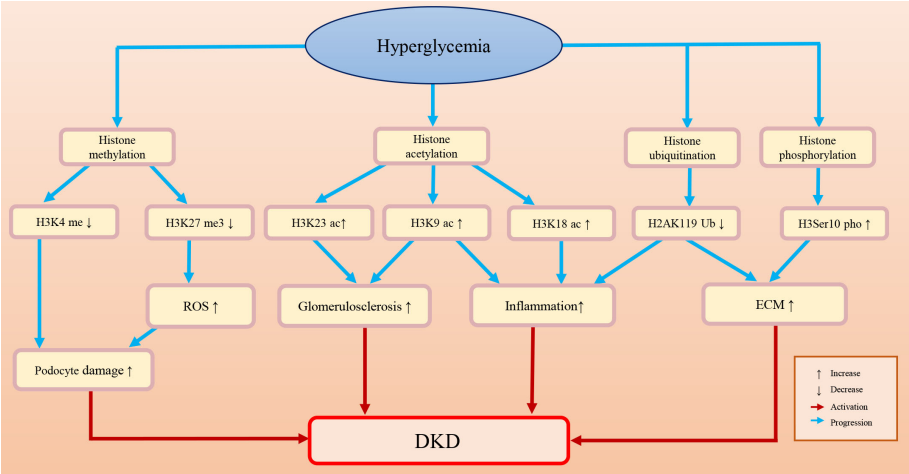

FIGURE 2
The histone epigenetic modifications and their roles in DKD.

regulatory role similar to that of DNA methylation. Whether it functions in transcriptional repression or activation depends on the methylation degree and the modification site.

Histone methylation is an important epigenetic modification in DKD. In diabetic mice, upregulation of TGF-β may promote the recruitment of the histone H3 lysine 4 (H3K4) methylation methyltransferase SET7/9 and upregulate the expression levels of H3K4me and p21. This may lead to glomerular cell injury, severe glomerular sclerosis, albuminuria, and a decreased glomerular filtration rate (83). Histone methylation also affects podocyte survival and function. The foot processes of podocytes are attached to the basement membrane of the glomeruli. Foot process effacement and simplification can lead to proteinuria, which is a sign of podocyte injury (84, 85). Adjacent podocytes connect through the slit diaphragm and form an important barrier for glomerular filtration proteins (86). Therefore, the structure and arrangement of podocytes are very important to kidney function (85). PAX transcription activation domain interacting protein (PTIP) is a part of the H3K4 methyltransferase complex (87). Lefevre et al. found that the H3K4 trimethylation (H3K4me3) level declined in PTIP knockout mouse podocytes, which may affect the transcription of the neurotrophic tyrosine kinase receptor type 3 (*Ntrk3*) gene, resulting in podocyte development disorder and abnormal podocyte arrangement and eventually leading to tubulointerstitial fibrosis and glomerulosclerosis (88, 89). Furthermore, PTIP can interact with dachshund homolog 1 (DACH1) and be recruited by DACH1 to its promoter-binding sites. In podocytes, DACH1-PTIP recruitment can repress transcription, limit promoter H3K4me3, and affect the transcription of downstream genes (89–92). Cao et al. found that DACH1 played a safeguard role in podocytes. DACH1 expression is dramatically decreased in DKD patients, which may result in proteinuria. In DACH1 knockdown podocytes combined with hyperglycemia, DACH1-PTIP promoter binding was reduced, transcriptional repression was lost, and the H3K4me3 expression level was increased (88).

Decreased expression of H3K27me3 in DKD may aggravate podocyte injury and fibrosis. Enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), a methyltransferase, can regulate podocyte oxidative stress and renal injury in diabetes (93, 94). DKD patients often have metabolic disorders and high levels of AGEs. Liebisch et al. found that in podocytes of diabetic mice, high levels of AGEs can downregulate EZH2 expression levels, decrease H3K27me3 levels, and induce podocyte injury (95). Siddiqi et al. found that in diabetic rats, depletion of EZH2 may decrease H3K27me3 levels and increase glomerular thioredoxin interacting protein (TxnIP) expression levels, which may promote ROS accumulation, increase matrix production, and lead to podocyte injury and proteinuria (96). Similarly, Ye et al. studied the safeguard role of H3K27me3 and EZH2 in a rat DKD model and indicated that in rat renal mesangial cells, TGF-β downregulated the expression of EZH2, decreased the enrichment of the epigenetic repressive mark H3K27me3 at the fibrotic gene promoter (e.g., Serpin family E member 1 (*Serpine1*) and C-C motif chemokine ligand 2 (*Ccl2*), and increased fibrosis protein expression and renal fibrosis (97). Ubiquitously transcribed tetratricopeptide repeat on chromosome X (UTX) is a demethylase that removes dimethyl and trimethyl groups from H3K27 (98). UTX expression is increased in the renal tubules of diabetic mice and DKD patients (99). Increased UTX may promote the transcription of inflammatory factor genes and DNA damage. However, administration of the H3K27 demethylase inhibitor GSK-J4 alleviated inflammatory damage to renal tubules in diabetic mice (99).

Glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) play pivotal roles in DKD-associated fibrosis and inflammation. GR and MR are expressed in a variety of renal cells (e.g., podocytes, endothelial cells and fibroblasts). The absence of GR may induce renal fibrosis and albuminuria (100, 101). Overactivation of MR may lead to endothelial dysfunction, renal fibrosis, and renal water and salt metabolism dysfunction (102, 103). Water and salt metabolism disorder is a common metabolic

abnormality in DKD patients (104). Disturbance of renal water and salt metabolism may lead to sodium retention, blood pressure elevation, glomerular sclerosis, and tubulointerstitial fibrosis (102). The expression levels of GR and MR are closely related to epigenetic modification. Disruptor of telomeric silencing-1 (Dot1) is a histone lysine methyltransferase whose function and activity are regulated by GR (104). When MR is deficient, GR can modify Dot1 methyltransferase activity through the serum/glucocorticoid-regulated kinase 1 (Sgk-1) and aldosterone (a corticosteroid)-dependent signaling pathways, thereby relaxing chromatin in relevant locations and promoting transcription to compensatively increase epithelial sodium channel expression (104–107). In this way, kidney salt retention can be regulated and the filtration function of the kidney can be ensured.

Histone acetylation

Histone acetylation usually occurs on lysine residues. Lysine is positively charged, and DNA is negatively charged. Under normal conditions, histone proteins and DNA are tightly bound by interaction. When histone acetylation occurs, acetyl-coenzyme A is transferred to the lysine side chain, which disrupts the interaction between histones and DNA and leads to nucleosome structure relaxation and a subsequent increase in accessibility to transcription factors (108, 109).

Histone acetylation plays an important role in the onset of DKD. Chen et al. found increased acetylation of H3K9 and H3K18 in the renal cortex of diabetic mice, which is related to inflammatory responses and glomerulosclerosis (110). Sufyan et al. found that the increased acetylation of H3K9 and H3K23 is associated with albuminuria and glomerulosclerosis in a mouse model (83). Lizotte et al. found that H3K9/14 acetylation was associated with insulin resistance, podocyte apoptosis and kidney injury (111).

Histone deacetylases (HDACs) are epigenetic regulatory factors that can reverse the histone acetylation process. HDACs can be divided into four groups according to their homology: class I includes HDAC1/2/3/8; class II includes HDAC4/5/6/7/9/10; class III includes sirtuin (SIRT)1-7; and class IV includes HDAC11 (112). Wang et al. found that the expression of HDAC2/4/5 was increased in streptozotocin (STZ)-induced diabetic rats and db/db mice, and the increased expression of HDAC4 exacerbated inflammation and led to podocyte injury (113).

HDAC3, as a profibrotic factor, plays a pivotal role during the genesis of DKD (114). The expression level of HDAC3 is upregulated in renal tubular epithelial cells of DKD mice (115). Klotho protein protects the kidney by regulating the expression of fibrinogen and prevents renal fibrosis by inhibiting profibrotic signaling pathways (e.g., TGF-β/small mothers against decapentaplegic (Smad) and wingless/integrated (Wnt)/β-catenin (115, 116). HDAC3 may modulate the expression of Klotho. Chen et al. found that HDAC3 promotes renal fibrosis by inhibiting the transcription of the antifibrotic protein Klotho (115). HDAC3 also regulates macrophage function, promotes macrophage M2 polarization activation and leads to MMT, which is a marker of renal fibrosis (117, 118). HDAC3 inhibitors can reverse M2 polarization and the phagocytic activity of macrophages and alleviate renal fibrosis (115, 118).

SIRT3 plays a protective role in DKD-related kidney injury. In DKD patients, HG can downregulate SIRT3, which inhibits the activity of antioxidant enzymes, aggravates oxidative stress, induces mitochondrial dysfunction and leads to the accumulation of metabolic substances such as ROS (60, 61). These variations cause a series of changes in kidney cells, including metabolic reprogramming and immunoreaction fibrosis, and eventually induce kidney damage (61, 119–121). Protein Kinase B (AKT) is involved in apoptosis and proliferation by regulating the phosphorylation of forkhead box O (FoxO) (122). High levels of SIRT3 may inhibit the activity of the Akt/FoxO signaling pathway and reduce oxidative stress and renal tubular epithelial cell apoptosis (123). SIRT3 also plays a role in maintaining endothelial cell homeostasis (124). Srivastava et al. reported that SIRT3 is involved in the regulation of EndMT, and SIRT3 deficiency in mouse endothelial cells may induce/aggravate renal fibrosis. However, renal fibrosis can be relieved by the overexpression of SIRT3 (124).

Histone ubiquitination

Ubiquitin is a protein with a highly conserved sequence (125). Histone ubiquitination often occurs at specific lysine residues in the C-terminal tails of histone H2A and histone H2B (126). Three enzymes are involved in the process of histone ubiquitination. First, the ubiquitin molecule is activated by E1 (ubiquitin-activating enzyme) in an ATP-dependent manner; then, the activated ubiquitin moiety forms a complex with E2 (ubiquitin-conjugating enzyme) with the assistance of E1, and the complex is transferred to the target protein with the assistance of specific E3 (ubiquitin ligase) (127). The process of ubiquitination is dynamic and reversible, and deubiquitination enzymes can reverse this process (128). The ubiquitin proteasome system is involved in the degradation of many types of proteins, which is associated with the regulation of a series of cell behaviors and the occurrence of diseases (129, 130).

In diabetic patients, ubiquitin A-52 residue ribosomal protein fusion product 1 gene (UbA52), which is associated with renal tubular injury, and the UbA52 expression level can be upregulated in response to increasing concentrations of glucose (131, 132). Abnormal ubiquitination modifications have also been observed in DKD models. Increased H2A ubiquitination and decreased H2B ubiquitination levels have been observed in HG-treated mesangial cells. In addition, these histone ubiquitination changes may enhance the activation of TGF-β and influence the pathogenesis of DKD (126, 133). Histone ubiquitination can regulate the expression of downstream proteins by changing their occupancy in the promoter region and thus promote renal fibrosis. For example, decreased occupancy of H2AK119 monoubiquitination (H2AK119Ub) at the *TGF-β* and monocyte chemoattractant protein-1 (*MCP-1*) promoters may upregulate TGF-β pathway-related factors in diabetic kidneys, activate fibrosis-related signals, and accelerate renal fibrosis (134). Intriguingly, histone methylation has been shown to be cross-regulated by histone ubiquitination. Goru et al. found that in diabetic kidneys, decreased occupancies of H2AK119Ub may increase occupancies of histone H3K36 dimethylation (H3K36me2) marks on the promoter of *SET7/9*

and upregulate the protein SET7/9 expression. Of note, the increased expression level of SET7/9 can increase the promoter occupancies of H3K4me2 on the promoter of collagen type I alpha 1 (*COL1A1*), which may lead to ECM deposition in the kidney and renal fibrosis (135).

Currently, ubiquitin proteasome system-related proteasome inhibitors have been approved for cancer therapy with good efficacy. However, studies on histone ubiquitination modification in DKD are few, and related drug development remains in the experimental stage. Aspirin and Carbobenzoxyl-L-leucyl-L-leucyl-L-leucine (MG132) are potential proteasome inhibitors. Aspirin can prevent and alleviate renal fibrosis in diabetic animals by increasing histone H2AK119Ub and reducing SET7 deposition-induced ECM (136). MG132 alleviates oxidative stress-induced damage to the kidney by inhibiting diabetes-increased proteasome activity and upregulating Nrf2 (137). Although these drugs are in the preclinical stage, proteasome inhibitors have shown promising therapeutic potential in DKD treatment.

Histone phosphorylation

Histone phosphorylation is a central step in chromosome coagulation, transcriptional regulation, and DNA damage repair during cell division (78, 138, 139). In DKD mice and patients, the increase in histone H3 serine 10 (H3Ser10) phosphorylation may upregulate vascular cell adhesion molecule 1 (VCAM-1), promote glomerular endothelial activation, and activate DKD fibrosis and inflammation progression (26, 140). Histone phosphorylation is associated with albuminuria. Sayyed et al. found that glomerulosclerosis and albuminuria were associated with increased H3Ser10 phosphorylation, and the process of histone phosphorylation could be reversed. Ccl2 blockade can prevent the progression of DKD by blocking H3Ser10 phosphorylation (83). Moreover, Tikoo et al. found that resveratrol (a kind of polyphenol) can prevent kidney disease progression by reducing H3 dephosphorylation in diabetic rats (141, 142).

NcRNA changes in DKD

Long noncoding RNA (LncRNA) and DKD

LncRNAs are a class of RNA molecules whose transcript length exceeds 200 nt (143). Instead of encoding proteins, lncRNAs regulate cell behaviors by influencing gene transcription and protein translation (144). LncRNAs are associated with the occurrence and development of a variety of diseases. In DKD, lncRNAs are involved in renal fibrosis, inflammation, podocyte injury, albuminuria and other pathological processes in direct or indirect ways (145).

LncRNAs are crucial during the genesis of DKD. LncRNAs can affect protein expression by targeting microRNAs (miRNAs) and related signaling pathways. miR-96-5P regulates the expression of fibronectin, which is involved in renal fibrosis. It has been observed that the expression level of miR-96-5P is downregulated in HG-treated renal tubular epithelial cells and DKD mouse models (146). LncRNA GAS5 can bind to miR-96-5p and inhibit its expression, thereby promoting renal fibrosis (146). HG may stimulate the expression of lncRNA NR_038323. In STZ-induced DKD mice, lncRNA NR_038323 may interact with miR-324-3p, which upregulates the expression of dual-specificity protein phosphatase-1 (DUSP1), downregulates the expression of collagen I, collagen IV and fibronectin, and significantly improves renal fibrosis and glomerular hypertrophy (147). In the early stage of DKD, the expression level of lncRNA CYP4B1-PS1-001 is significantly downregulated. However, the enforced expression of lncRNA CYP4B1-PS1-001 can inhibit the proliferation and fibrosis of murine mesangial cells by interacting with nucleolin (148, 149). LncRNA SOX2OT can exert renal protective effects by inhibiting renal fibrosis (150, 151). In DKD mice, overexpression of lncRNA SOX2OT may alleviate hyperglycemia, decrease the expression of fibronectin, suppress collagen-related interstitial fibrosis, enhance the autophagy of mesentery cells, and significantly inhibit the proliferation and fibrosis of mesentery cells (150).

LncRNAs are also associated with proteinuria. The expression of lncENST00000436340 is increased in DKD patients. It has been demonstrated that lncENST00000436340 may promote the degradation of polypyrimidine tract binding protein 1 (PTBP1) by enhancing its binding to mRNA, which regulates cytoskeletal rearrangement, and leads to podocyte injury and urine protein (152). LncRNA DLX6-AS1 is highly expressed in DKD patients and podocytes cultured in HG. cAMP-response element binding protein (CREB) can target DLX6-AS1, and overexpression of CREB may increase the level of DLX6-AS1. High levels of DLX6-AS1 may disrupt the podocyte structure, increase kidney inflammation, and induce albuminuria (153).

MiRNA and DKD

miRNAs are a class of small and highly conserved noncoding RNAs that regulate protein expression at the posttranscriptional level by interacting directly with the 3'UTR of target genes (154). miRNAs also participate in the pathogenesis of DKD. It has been demonstrated that the expression of miRNA-5b-181p is decreased in a DKD mouse model, and supplementation with miRNA-5b-181p-mimic may reduce albuminuria and alleviate abnormal mesentery expansion (155). Since miRNA can be stably present in urine in the form of exosomes, it can be used as a biomarker to predict the progression of DKD. It has been reported that the expression of miR-342b, miR-30 and miR-2a is significantly increased in the urinary exosomes of DKD patients (156).

miR-33 and miR-21 play significant roles in renal fibrosis. miR-33 can promote fibrosis by activating the TGF-β/Smad inflammatory pathway (157, 158). In a folate-treated mouse model, miR-33 deletion enhanced fatty acid oxidation, reduced lipid accumulation, and protected mouse kidneys from fibrosis (159). miR-21 expression is upregulated in DKD patients. It has been demonstrated that miR-21 in the exosomes of renal tubular cells can target the phosphatase and tensin homolog (PTEN)/AKT pathway and promote renal fibrosis (160). TGF-β/Smad3 mediates the upregulation of miR-21 in renal tubular epithelial cells, which in turn positively regulates the expression of ECM and α-SMA in TECs and fibrotic kidneys (161). The TGF-β/Smad3 pathway also

induces the expression of renal tubule collagen I, promotes ECM accumulation and accelerates renal fibrosis by promoting the expression of miR-192 (162, 163).

Fibroblast growth factor receptor 1 (FGFR1) plays a key role in the anti-EndMT process and in reducing kidney fibrosis (164, 165). Koya et al. performed a series of studies on DKD-related EndMT and found that there was EndMT-related crosstalk between miR-29, miR-let-7 family members and FGFR1 (166–168). Overactivation of the TGF-β/Smad signaling pathway may decrease the expression of miR-29, which promotes the transcription of the inflammatory factor IFN-γ and inhibits FGFR1, leading to a downregulation of FGFR1-dependent miR-let-7 (166, 169, 170). The decreased expression of miR-let-7a enhances glycolysis, increases lactic acid and ROS accumulation, turns on metabolic reprogramming and leads to EndMT (54, 55, 167). Furthermore, N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) can maintain endothelial homeostasis and protect the kidney from fibrosis by activating FGFR1 and miR-let-7 (167, 171–173).

Circular RNA (CircRNA) and DKD

CircRNAs are a class of single-stranded closed-loop RNAs that mainly exist in the cytoplasm or exosomes. Functionally, circRNAs can interact with proteins and other RNAs by acting as microRNA sponges and regulate transcription in either a competitive or noncompetitive fashion; in some cases, circRNAs can also be translated into polypeptides and perform regulatory functions (174–176).

CircRNA profiles vary with different physiological states, so they can be used as biomarkers and therapeutic targets of diseases. The most common function of circRNAs in DKD is serving as molecular sponges through targeting miRNA and functional proteins, such as SIRT6, SRY-Box Transcription Factor 6 (SOX6), TGF-β1 and NF-κB. CircRNAs are widely involved in DKD-related oxidative stress, inflammation, ECM accumulation and renal fibrosis (177). Qin et al. found that the HG environment can increase the expression levels of circ_0123996 and SOX6 and decrease the expression of miR-203a-3p in mesenchymal cells. Silencing circ_0123996 can suppress cell proliferation and alleviate inflammation and fibrosis (178). Ge et al. found that after exposing mesangial cells to a similar HG environment as in DKD patients, the expression of circ_0000064 was increased (179). Knockdown of circ_0000064 may inhibit the expression of fibrosis-related proteins, such as type I collagen, type IV collagen, and fibronectin (25, 179). Table 1 summarizes the DKD-related circRNAs. Studies of the function of circRNAs in DKD remain at the animal and cell experimental stages, and to date, no circRNA drug has been approved for the clinical treatment of DKD.

DKD therapy

Current therapies in DKD

Currently, the main therapeutic strategies for DKD are to alleviate or avoid proteinuria by controlling blood glucose and blood pressure and enhancing renal filtration capacity. Since the

TABLE 1 DKD related circRNA.

Circ RNA	Experimental object	Change	Pathway	Effect	Reference
Circ_0000064	Renal tubular epithelial cells	↑	miR-2-532p↓ ROCK3↑	Oxidative stress↑ Apoptosis↑ Fibrosis↑	(180)
	Human renal mesangial cells	↑	miR-424-5p↓ WNT2B↑	Proliferation↑ Inflammation↑ ECM accumulation↑	(181)
	Mouse mesangial cells	↑	miR-30c-5p↓ Lmp7↑	Oxidative stress↑ Inflammation↑ ECM accumulation↑	(182)
Circ_EIF4G2	NRK-52E cells	↑	miR-218↓ SERBP1↑	Fibrosis↑	(183)
Circ_15698	Mouse mesangial cells	↑	miR-185↓ TGF-β↑	ECM accumulation↑	(177)
Circ_AKT3	Mouse mesangial cells	↓	miR-296-3p↑ E-cadherin↓	Apoptosis↑ ECM accumulation↑	(184)
CircRNA_0000491	Mouse mesangial cells	↑	miR-101b↓ TGFβRI↑	ECM accumulation↑	(185)
	Mouse mesangial cells	↑	miR-455-3p↓ HMBG1↑	Apoptosis↑ Inflammation↑ Oxidative stress↑ Fibrosis↑	(186)

(Continued)

TABLE 1 Continued

Circ RNA	Experimental object	Change	Pathway	Effect	Reference
Circ_0037128	Human mesangial cells	↑	miR-17-3p↓ AKT3↑	Proliferation↑ Fibrosis↑	(187)
	Podocytes	↑	miR-31-5p↓ KLF9↑	Podocytes injury↑	(188)
Circ_0080425	Mouse mesangial cells	↑	miR-24-3p↓ FGF11↑	Proliferation↑ Fibrosis↑	(189)
	Human umbilical vein endothelial cells	↑	miR-140-3p↓ FN1↑	Cell dysfunction↑	(190)
CircRNA_010383	Mouse glomerular mesangial cells Mouse tubular epithelial cells	↓	miR-135a↑ TRPC135↓	ECM accumulation↑	(191)
CircRNA_0000309	Podocytes	↓	miR-188-3p↓ GPX4↑	Proliferation↑ Fibrosis↑ Podocytes apoptosis↑	(192)
Circ_HIPK3	Rat mesangial cells	↑	miR-185↓ TGF-β↑ Cyclin D1↑	Proliferation↑	(193)
Circ_0114428	Glomerular mesangial cells	↑	miR-185-5↓ Smad3↑	Proliferation↑ Fibrosis↑ EMT↑	(194)
Circ_ACTR2	Human renal mesangial cells	↑	miR-205-5p↓ HMGA2↑	Proliferation↑ Inflammation↑ ECM accumulation↑ Oxidative stress↑	(195)
Circ_AOK1	Human glomerular epithelial cells	↑	miR-520h↓ Smad3↑	Proliferation↑ Fibrosis↑ EMT↑	(196)
Circ_0123996	Mouse mesangial cells	↑	miR-149-5p↓ Bach1↑	Proliferation↑ Fibrosis↑	(197)
	Human mesangial cells	↑	miR-203a-3p↓ SOX6↑	Proliferation↑ Inflammation↑ Fibrosis↑	(178)
Circ_0068087	Renal tubular epithelial cells	↑	miR-106a-5p↓ ROCK2↑	Apoptosis↑ Inflammation↑ Oxidative stress↑ Fibrosis↑	(198)
Circ_0125310	Mesangial cells	↑	miR-422a↓ IGF1R↑ P38↑	Proliferation↑ Fibrosis↑	(199)
Circ_WBSCR17	Renal tubular epithelial cells	↑	miR-185-5p↓ SOX6↑	Apoptosis↑ Inflammation↑ Fibrosis↑	(200)
Circ_000166	Renal tubular epithelial cells	↑	miR-296↓ SGLT2↑	Fibrosis↑	(201)
Circ_0037128	Renal tubular epithelial cells	↑	miR-497-5p↓ NFAT5↑	Inflammation↑ Oxidative stress↑ Fibrosis↑	(202)
Circ_0003928	Renal tubular epithelial cells	↑	miR-506-3p↓ HDAC4↑	Oxidative stress↑ Apoptosis↑	(203)
Circ_SMAD4	Mouse glomerular mesangial cells	↓	miR-377-3p↑ BMP7↓	Inflammation↑ ECM accumulation↑ Apoptosis↑	(204)

(Continued)

TABLE 1 Continued

Circ RNA	Experimental object	Change	Pathway	Effect	Reference
Circ_0123996	Mesangial cells	↑	miR-203a-3p↓ SOX6↑	Proliferation↑ Inflammation↑ Fibrosis↑	(205)
Circ_0008529	Renal tubular epithelial cells	↑	miR-485-5p↓ WNT2B↑	Apoptosis↑ Inflammation↑	(206)
Circ_0000285	Podocytes	↑	miR-654-3p↓ MAPK6↑	Podocytes injury↑	(207)
Circ_LRP6	Mouse glomerular mesangial cells	↑	miR-205↓ HMGB1↑ TLR4↑ NF-κB↑	Proliferation↑ Oxidative stress↑ Inflammation↑ ECM accumulation↑	(208)
Circ_NUP98	Human glomerular mesangial cells	↑	miR-151-3p↓ HMGA2↑	Oxidative stress↑ Inflammation↑ Fibrosis↑	(209)
Circ_HIPK3	Renal tubular epithelial cells	↓	miR-326↑ miR-487a-3p↑ SIRT1↓	Proliferation↓ Apoptosis↑	(210)
Circ_0060077	Renal tubular epithelial cells	↑	miR-145-5p↓ VASN↑	Apoptosis↑ Oxidative stress↑ Inflammation↑ Fibrosis↑	(211)
Circ_TLK1	Human mesangial cells	↑	miR-126-5p↓ miR-204-5p↓ AKT↑ NF-κB↑	Inflammation↑ Oxidative stress↑ ECM accumulation↑	(212)
Circ_FBXW12	Human mesangial cells	↑	miR-31-5p↓ LIN28B↑	Inflammation↑ Oxidative stress↑ ECM accumulation↑	(213)
Circ_0003928	Renal tubular epithelial cells	↑	miR-151-3p↓ Anxa2↑	Apoptosis↑ Inflammation↑	(214)
Circ_0000181	C57BL/6 mice	↑	miR-667-5p↓ NLRC4↑	Inflammation↑	(215)
Circ_LARP4	Mouse mesangial cells	↓	miR-424↑ Bax↓	Apoptosis↑ Fibrosis↑	(216)
Circ_0054633	Human umbilical vein endothelial cells	↑	miR-218↓ ROBO1↑ HO-1↑	Vascular endothelial cell dysfunction↓	(217)
Circ_ITCH	Rat mesangial cells	↓	miR-33a-5p↑ SIRT6↓	Inflammation↑ Fibrosis↑	(218)

↑, upregulation; ↓, downregulation; ROCK, Rho kinase; WNT2B, Wnt family member 2B; Lmp7, large multifunctional protease 7; SERBP1, SERPINE1 mirna binding protein 1; TGFβRI, TGFβ-receptor type I; HMGB1, high mobility group box 1; KLF9, kruppel-like factor 9; FGF11, fibroblast growth factor 11; FN1, fibronectin 1; TRPC135, transient receptor potential cation channel 135; GPX4, glutathione peroxidase 4; HMGA2, high-mobility group AT-hook 2; Bach1, BTB and CNC homology 1; Sox6, SRY-Box Transcription Factor 6; IGF1R, type 1 insulin-like growth factor receptor; SGLT2, sodium-glucose cotransporter 2; NFAT5, nuclear factor of activated T cells 5; HDAC, histone deacetylase; BMP7, bone morphogenetic protein 7; MAPK6, mitogen-activated protein kinase 6; TLR4, toll-like receptor 4; SIRT, sirtuin; VASN, vasorin; LIN28B, Lin-28 homolog B; Anxa2, annexin A2; NLRC4, NOD-like receptor family CARD domain-containing protein 4; ROBO1, roundabout 1; Bax, Bcl-2 associated X protein; HO-1, heme oxygenase-1.

direct cause of DKD in diabetic patients is high blood glucose, lowering blood glucose is the priority for controlling the progression of DKD. Some hypoglycemic drugs also have therapeutic effects on renal disorders. For example, SGLT2 inhibitors (e.g., empagliflozin) not only reduce the tubule reabsorption of glucose but also improve the kidney filtration capacity and delay the progression of kidney disease by reducing glomerular pressure and albuminuria (219). Overactivation of the RAAS may trigger glomerular hypertension, which in turn promotes the constriction of bulbar arterioles, damages endothelial cells, and leads to albuminuria. Therefore, the use of antihypertensive drugs can significantly prevent renal dysfunction while maintaining normal blood pressure (220). RAAS inhibitors are widely used drugs for the treatment of DKD and have been proven to be effective in all stages of DKD (220–222). Table 2 summarizes the main regular drugs for DKD treatment.

Potential epigenetic therapies in DKD

Presently, studies of epigenetic drugs for DKD mostly remain at the animal experimental stage, and histone acetylation inhibitors are a research hotspot. We summarized the potential epigenetic therapies for DKD in Table 3. HDACIs have been widely studied in tumors and approved for the treatment of cutaneous T-cell lymphoma and multiple myeloma. HDACIs also have a protective effect against diabetic kidney damage. For example, HDAC2 expression is increased in diabetic rats, and administration of trichostatin A (TSA) may decrease ECM-related protein and mRNA expression and prevent (262). TSA also inhibits the activity of the class II type of HDAC, which plays a similar role in blocking EMT. Xu et al. found that the expression of HDAC5 was increased in the renal tubules of diabetic mice. After TSA administration, the expression of HDAC5 was decreased and the accumulation of ECM was alleviated (264). Valproate (VPA), sodium butyrate (NaB), and vorinostat are all HDACIs that inhibit class I and II HDACs (265). VPA is a branched short-chain fatty acid that can alleviate the damage to renal tubules in STZ-induced diabetic rats, reduce autophagy and stress, reduce proteinuria, and prevent kidney fibrosis (258, 259, 266). NaB is another branched short-chain fatty acid that can reduce inflammation and oxidative damage and relieve albuminuria in diabetic rats (260, 267). Vorinostat can relieve oxidative stress in STZ-induced diabetic rats, and decrease renal tubular cell proliferation and glomerular matrix production (261, 268).

TABLE 2 Regular therapies of DKD.

Drug	Drug class	Research category	DKD related outcome	Reference
Dulaglutide Liraglutide	GLP-1 agonist	Approved medication	Urinary albumin/creatinine ratio↓ Albuminuria↓	(223–225)
Dapagliflozin Canagliflozin Empagliflozin	SGLT2 inhibitor	Approved medication	Blood pressure↓ Weight↓ Glomerular pressure↓ GFR↑ Albuminuria↓	(226–229)
Sitagliptin Linagliptin	DPP-4 inhibitor	Approved medication	Blood glucose↓ Oxidative stress↓ Inflammation↓ Glomerular injury↓ Albuminuria↓	(172, 230–234)
Captopril Losartan Telmisartan Irbesartan	ACEI and ARB	Approved medication	Blood pressure↓ GFR↑ Albuminuria↓	(220–222, 235)
Finerenone	Mineralocorticoid (Aldosterone) receptor Antagonists	Approved medication	Renal fibrosis↓ Inflammation↓	(236–239)
Spironolactone	Aldosterone receptor antagonists	Approved medication	Blood pressure↓ Inflammation↓ Albuminuria↓	(240, 241)
Sevelamer	AGEs antagonist (phosphate binders)	Approved medication	Inflammation↓	(242)
Pirfenidone	TGF-β inhibitor	Approved medication	Fibrosis↓	(243, 244)
Ruboxistaurin	PKC inhibitor	Clinical trial	Fibrosis↓ Albuminuria↓	(245, 246)
Atrasentan	ETR antagonist	Clinical trial	Fibrosis↓ Albuminuria↓ Blood pressure↓	(247–250)
AcSDKP	Endogenous peptide	Animal experiment	Fibrosis↓	(168, 172, 234, 251, 252)
Fasudil	ROCK inhibitor	Animal experiment	Inflammation↓ Fibrosis↓ Glomerulosclerosis↓	(253–256)
FPS-ZM1	RAGE inhibition	Animal experiment	Glomerular nephrin↑ Inflammation↓ Fibrosis↓ Podocyte injury ↓	(257)

↑, upregulation; ↓, downregulation; GLP-1, glucagon-like peptide-1; SGLT2, sodium-glucose cotransporter 2; DPP-4, dipeptidyl peptidase 4; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; AGEs, advanced glycation end products; TGF-β, transforming growth factor β; PKC, protein kinase C; ETR, endothelin receptor; GFR, glomerular filtration rate; FPS-ZM1, 4-chloro-N-cyclohexyl-N-(phenylmethyl)-benzamide; RAGE, receptor for advanced glycation end products.

TABLE 3 Epigenetic therapies of DKD.

Type	Drug	Applications	DKD related research status	Treatment outcomes in DKD	Reference
HDACI	VPA	Approved for use in epilepsy	Animal experiment	Apoptosis↓ Fibrosis↓ Kidney injury↓	(258, 259)
HDACI	NaB	In a clinical trial to treat schizophrenia	Animal experiment	Fibrosis↓ Apoptosis↓ Inflammation↓ DNA damage↓ Albuminuria↓	(260, 261)
HDACI	TSA	Pre-clinical	Animal experiment	Fibrosis↓ Albuminuria↓	(262)
HDACI	Vorinostat	Approved for use in cutaneous T cell lymphoma	Animal experiment	Oxidative stress↓ ECM↓ Albuminuria↓	(261)
HDAC	SIRT3	Pre-clinical	Animal experiment	Oxidative stress↓ Kidney injury↓	(123)
H3K27 demethylase inhibitors	GSK-J4	Pre-clinical	Animal experiment	Inflammation↓ Fibrosis↓ Glomerulosclerosis↓ Albuminuria↓	(263)

↑, upregulation; ↓, downregulation. HDACI, histone deacetylase inhibitor; VPA, valproate; TSA, trichostatin A; SIRT, sirtuin.

Although HDACIs have great potential in the treatment of DKD, their drawbacks, such as adverse effects and poor tolerance, should not be ignored (265, 269, 270). For life-threatening diseases such as cancer, side effects such as nausea, vomiting, and liver toxicity are acceptable. However, whether the application of HDACIs is a good choice for chronic diseases such as DKD should be discussed with great deliberation. In addition, the specificity of HDACIs is poor. Because class I, II and IV HDACs are all dependent on zinc for enzymatic reactions, and most HDACIs target the zinc domain, HDACIs have broad spectrum effects (commonly called pan-HDACIs) (270, 271).

Conclusion and perspectives

Epigenetic modifications are common in diseases and some epigenetic variations are highly specific in a certain disease or a certain stage of disease, which provides us with potential therapeutic targets in clinical treatments (272, 273). Presently, many studies have confirmed the role of epigenetics in DKD. In this review, we concluded the evidence for epigenetic modifications associated with DKD by summarizing the relevant literature, and we found that epigenetic modifications are involved in the inhibition/activation of a variety of pathogenic signaling pathways. Epigenetic variations affect multiple renal cell functions, such as the activity of GR and glucose metabolism (274, 275). In particular, epigenetic variation-induced EndMT/EMT processes are pivotal in the genesis of DKD, which are the core events in kidney fibrosis (Figure 3). Epigenetic modifications are a consequence of exposure to HG and contribute to the progression of DKD. Since DKD is the result of multiple factors and their complex interactions, different epigenetic modifications may contribute to the same outcome through different signaling pathways and mechanisms. However, most of the existing epigenetic studies have focused on the effect of a single variation on the changes in the signaling pathway to promote or mitigate the occurrence of DKD processes. Therefore, drugs or biomarkers designed for a single target are probably not accurate, and the joint use of multiple epigenetic drugs targeting different epigenetic variations should be considered in future DKD treatment. In addition, most of these studies were conducted in diabetic animals or cell models under HG conditions, but we believe that the human body environment is more complex and that more influential factors and mechanisms should be involved in DKD than animals and cells. Therefore, additional solid experimental and clinical trial data from clinical specimens and patients are eagerly anticipated.

In recent years, epigenetic detection technology has developed rapidly. With the wide application of high-throughput sequencing technology in the clinic, the detection of epigenetic changes (mainly DNA methylation and noncoding RNA profiles) in kidney tissues or the peripheral blood of DKD patients has become easier, faster and cheaper to implement (276, 277). These sequencing results are of great value for the

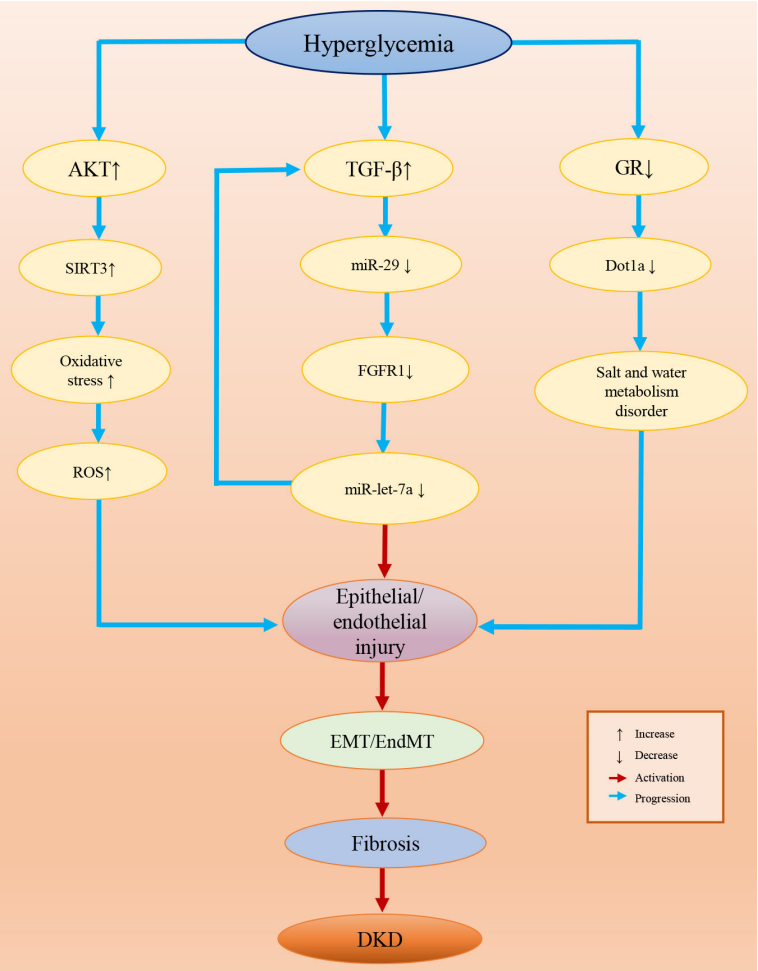

FIGURE 3
Epigenetic variation-induced EndMT/EMT processes in DKD.

precision diagnosis and drug development of DKD. Moreover, the CRISPR–Cas9 system is being tried as a novel tool for editing a specific epigenetic variation, which is a potential approach for the prevention and treatment of DKD (276, 278, 279).

Author contributions

WS and DY conceived the manuscript. ZL and DY drafted the manuscript. ZL drew the figures. ZL and WW proofread the manuscript and made revisions. LL and XA collected the references. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Glossary

Abbreviation	Full name
DKD	diabetic kidney disease
ESRD	end-stage renal disease
EMT	epithelial-mesenchymal transition
EndMT	endothelial-to-mesenchymal transition
MMT	macrophage-myofibroblast transition
HG	high glucose
ECM	extracellular matrix
AGEs	advanced glycation end products
PKC	protein kinase C
NF-κB	nuclear factor kappa-B
TGF-β	transforming growth factor β
eNOS	endothelial nitric oxide synthase
VEGF	vascular endothelial growth factor
ROS	reactive oxygen species
AMPK	adenosine 5′ monophosphate-activated protein kinase
Nrf2	nuclear factor-erythroid 2-related factor 2
TNF-α	tumor necrosis factor-α
IFN-γ	interferon γ
α-SMA	α-smooth muscle actin
CTGF	connective tissue growth factor
RAAS	renin-angiotensin-aldosterone system
Agt	angiotensinogen
Cldn18	claudin 18
RP-HPLC	reversed-phase high performance liquid chromatography
CKD	chronic kidney disease
CpG	5'-C-phosphate-G-3'
CRISP2	cysteine-rich secretory protein 2
PTIP	PAX transcription activation domain interacting protein
DACH1	dachshund homolog 1
Ntrk3	neurotrophic tyrosine kinase receptor type 3
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
TxnIP	thioredoxin interacting protein
Serpine1	serpin family E member 1
Ccl2	C-C motif chemokine ligand 2
UTX	ubiquitously transcribed tetratricopeptide repeat on chromosome x
GR	glucocorticoid receptor
MR	mineralocorticoid receptor

(Continued)

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Abbreviation	Full name
Dot1	Disruptor of telomeric silencing-1
Sgk-1	serum/glucocorticoid-regulated kinase 1
H3K	histone H3 lysine
me3	trimethylation
me2	dimethylation
SIRT	sirtuin
HDAC	histone deacetylase
STZ	streptozotocin
Smad	small mothers against decapentaplegic
Wnt	wingless/Integrated
AKT	protein Kinase B
FoxO	forkhead box O
UbA52	ubiquitin A-52 residue ribosomal protein fusion product 1 gene
Ub	monoubiquitination
MCP-1	monocyte chemoattractant protein
COL1A1	collagen type I alpha 1
MG132	carbobenzoxyl-L-leucyl-L-leucyl-L-leucine
VCAM-1	vascular cell adhesion molecule 1
Ser	serine
DUSP1	dual-specificity protein phosphatase-1
PTBP1	polypyrimidine tract binding protein 1
CREB	cAMP-response element binding protein
PTEN	phosphatase and tensin homolog
FGFR1	fibroblast growth factor receptor 1
AcSDKP	N-acetyl-seryl-aspartyl-lysyl-proline
SOX6	SRY-Box Transcription Factor 6
SGLT2	sodium-glucose cotransporter 2
TSA	trichostatin A
VPA	valproate
NaB	sodium butyrate
ROCK	Rho kinase
WNT2B	Wnt family member 2B
Lmp7	large multifunctional protease 7
SERBP1	SERPINE1 mirna binding protein 1
TGFβRI	TGFβ-receptor type I
KLF9	kruppel-like factor 9
FGF11	fibroblast growth factor 11
FN1	fibronectin 1

(Continued)

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Abbreviation	Full name
TRPC135	transient receptor potential cation channel 135
GPX4	glutathione peroxidase 4
HMGA2	high-mobility group AT-hook 2
Bach1	BTB and CNC homology 1
IGF1R	type 1 insulin-like growth factor receptor
NFAT5	nuclear factor of activated T cells 5
BMP7	bone morphogenetic protein 7
MAPK6	mitogen-activated protein kinase 6
HMGB1	high mobility group box 1
TLR4	toll-like receptor 4
VASN	vasorin
LIN28B	Lin-28 homolog B
Anxa2	annexin A2
NLRC4	NOD-like receptor family CARD domain-containing protein 4
ROBO1	roundabout 1
HO-1	heme oxygenase–1
Bax	Bcl-2 associated X protein
GLP-1	glucagon-like peptide-1
DPP-4	dipeptidyl peptidase 4
ACEI	angiotensin-converting enzyme inhibitor
ARB	angiotensin receptor blocker
ETR	endothelin receptor
GFR	glomerular filtration rate
FPS-ZM1	4-chloro-N-cyclohexyl-N-(phenylmethyl)-benzamide
RAGE	receptor for advanced glycation end products
lncRNA	long noncoding RNA
miRNA	microRNA
circRNA	circular RNA

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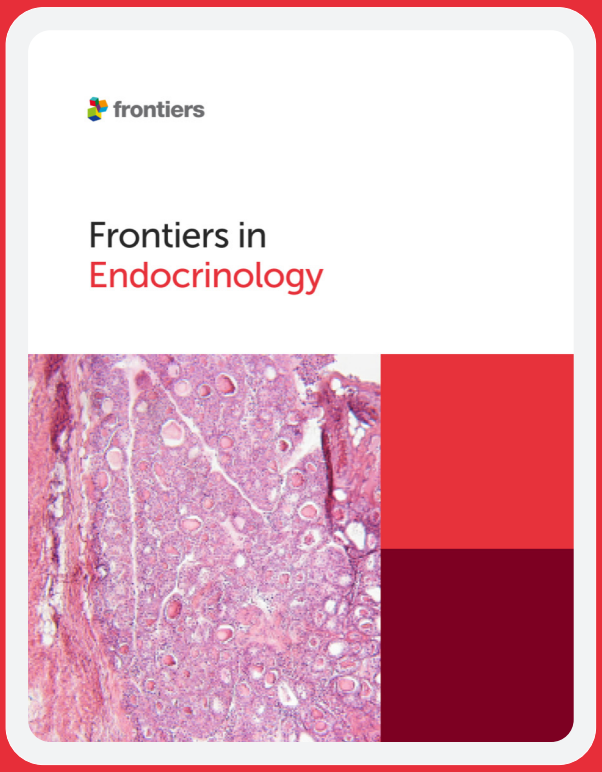

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