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Insights in diabetes: Molecular mechanisms 2021

Edited by
Undurti Narasimha Das, Gundu H. R. Rao and Denis Baranenko

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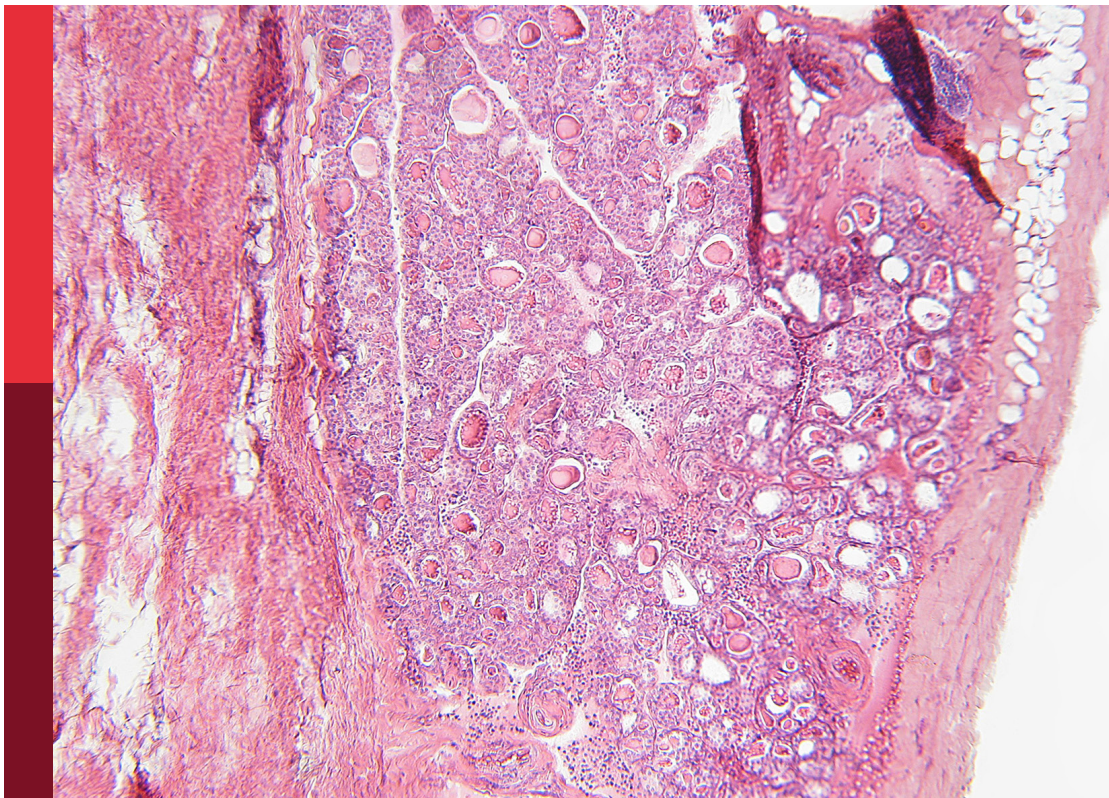

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Insights in diabetes: Molecular mechanisms 2021

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Insight Into the Metabolomic Characteristics of Post-Transplant Diabetes Mellitus by the Integrated LC-MS and GC-MS Approach- Preliminary Study

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Post-transplantation diabetes mellitus (PTDM) is a common metabolic complication after solid organ transplantation, which not only results in elevated microvascular morbidity, but also seriously impacts graft function and recipient survival. However, its underlying mechanism is not yet fully understood. In this study, an integrated liquid chromatography- mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) based-metabolomics approach was adopted to dissect the metabolic fluctuations and deduce potential mechanism associated with PTDM. 68 adult liver transplant recipients were recruited and classified as 32 PTDM and 36 non-PTDM subjects. PTDM group and non-PTDM group were well matched in gender, age, BMI, family history of diabetes, alcohol drinking history, ICU length of stay and hepatitis B infection. Peripheral blood samples from these recipients were collected and prepared for instrument analysis. Data acquired from LC-MS and GC-MS demonstrated significant metabolome alterations between PTDM and non-PTDM subjects. A total of 30 differential metabolites (15 from LC-MS, 15 from GC-MS) were screened out. PTDM patients, compared with non-PTDM subjects, were characterized with increased levels of L-leucine, L-phenylalanine, LysoPE (16:0), LysoPE (18:0), LysoPC (18:0), taurocholic acid, glycocholic acid, taurochenodeoxycholic acid, tauroursodeoxycholic acid, glycochenodeoxycholic acid, glycoursodeoxycholic acid, etc, and with decreased levels of LysoPC (16:1), LysoPC (18:2), LysoPE (22:6), LysoPC (20:4), etc. Taken collectively, this study demonstrated altered metabolites in patients with PTDM, which would provide support for enhancing mechanism exploration, prediction and treatment of PTDM.

Keywords: post-transplant diabetes mellitus, metabolomic characteristics, LC-MS, GC-MS, potential mechanism

1 INTRODUCTION

Solid organ transplantation (SOT), with more than 110,000 transplantations performed worldwide annually (1), is the treatment of choice for patients with end-stage organ failure. The short-term outcome of SOT improved remarkably due to advances in organ preservation (2), surgical techniques (3), immunosuppression regimens (4) and so on. However, metabolic complications, such as diabetes mellitus, hypertension and dyslipidemia, severely impact the long-term survival (5, 6).

Diabetes mellitus after SOT, defined as post-transplantation diabetes mellitus (PTDM), is considered to be a variant of type 2 diabetes mellitus (T2DM). PTDM is formally diagnosed at least 45 days post-transplantation and has a sudden onset within the first year post-transplantation (7). The reported prevalence of PTDM varies from 30% to 40% in liver recipients, 10% to 40% in renal recipients and 20% to 40% in other SOT recipients (8). PTDM is one of the major risk factors for diabetes-associated microvascular complications and infections, contributing to 1.63 times higher risk of graft failure and 1.87 times higher risk of mortality in SOT recipients (9).

Despite the prevalence and unfavorable outcomes associated with PTDM, the mechanism underlying PTDM is not entirely known. Over the past few decades, scientists devoted to evaluate factors affecting PTDM occurrence, such as age, gender, hepatitis infection, family history of type II diabetes mellitus, body mass index and immunosuppressive agents (10–12). Since PTDM is a serious frequent metabolic complication characterized by hepatic glucose overproduction, insulin hyposecretion and resistance, it is reasonably assumed that many metabolites and pathways are quite likely to be interrupted and play a critical role in the whole-body metabolic dysfunction. Thus, the comprehensive measurement and characterization of altered metabolites could give insights into the metabolic mechanism of PTDM.

Metabolomics is an invaluable tool for reflecting a series of biological processes underlying metabolic homeostasis and their complex association with peculiar disease, lifestyle, or genetic modifications, etc (13). Compared to targeted metabolomics focusing on well-defined metabolites, untargeted metabolomics aims at the qualitative or quantitative monitoring of all low-molecular-weight metabolites in a biological fluid and has been widely used to discover specific metabolic patterns of diseases (14). A range of analytical platforms including gas chromatography-mass spectrometry (GC-MS) (15), liquid chromatography-MS (LC-MS) (16), nuclear magnetic resonance (NMR) spectroscopy (17) and direct infusion MS (18) have been widely applied in metabolomics area. Among these, GC-MS and LC-MS are the two most powerful and commonly used analytical techniques owing to their high resolution of the chromatographic system, high sensitivity of MS detector and wide detection magnitude during the qualification and quantification of metabolites. Moreover, since no single analytical platform can cover the entire metabolome in a biological sample, the integration of GC-MS and LC-MS would serve as an appropriate strategy to capture a broader spectrum of metabolites (19, 20).

In this study, we aimed to primarily screen out the differentially expressed metabolites in PTDM and explore its potential pathophysiological mechanism by analyzing the metabolomic characteristics of PTDM recipients with the aid of the integrated liquid chromatography- mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) based-metabolomics. For the first time to our knowledge, the metabolic profiles involved in PTDM were explored, which would provide novel insights into the underlying mechanisms of PTDM from the perspective of metabolomics.

2 MATERIALS AND METHODS

2.1 Patients and Sample Collection

Adult (age ≥ 18 years) liver transplant recipients who had undergone primary liver transplantation between July 2019 and June 2020 at the Affiliated Drum Tower Hospital of Nanjing University Medical School, China were enrolled in this study. Patients were excluded if they were followed up less than one year after transplantation, underwent ABO incompatible transplantation, received a multi-organ transplantation, had diabetes mellitus prior to transplantation or developed acute rejection. The receipts received a standard triple-drug immunosuppression regimen including tacrolimus, mycophenolate mofetil and corticosteroids.

The experimental protocol was reviewed and approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (No. 2020-053-01). Signed informed consent was exempted due to the deidentified data provided to researchers and residual biosamples used.

According to the International Consensus Meeting on PTDM (7), PTDM is diagnosed at least 45 days post-transplantation using the American Diabetes Association (ADA) criteria for type 2 diabetes mellitus: with symptoms of diabetes plus random plasma glucose ≥ 200 mg/dL (11.1 mmol/L) or fasting plasma glucose ≥126 mg/dL (7.0 mmol/L) or 2-h plasma glucose after an oral glucose ≥200 mg/dL (11.1 mmol/L) during an OGTT or glycated hemoglobin (HbA1c) ≥6.5%. In this study, 32 and 36 recipients were assigned into the PTDM group and the non-PTDM group, respectively. Peripheral blood samples from these recipients were collected after overnight fasting at time of PTDM diagnosis and centrifugated at 1760 g for 10 min to prepare plasma. All the plasma samples were then divided into aliquots and stored at -80°C until analysis.

2.2 LC-MS Based-Metabolomics

2.2.1 Sample Preparation for LC-MS Based-Metabolomics

Plasma was thawed in a refrigerator at 4°C and thoroughly vortexed with seven times pure ice-cold acetonitrile for 5 min. The mixture was then centrifuged two times at 18407 g for 10 min at 4°C prior to injection into LC-MS system.

2.2.2 LC-MS Spectral Acquisition

Chromatographic separation was achieved on Shimadzu Prominence series ultra-fast liquid chromatography (UFLC)

system equipped with Phenomenex Kinetex C18 column (100×2.1 mm, 2.6μm; Phenomenex, Torrance, CA, USA) and a guard column, SecurityGuard ULTRA cartridge UHPLC C18 for 2.1 mm ID column (Phenomenex, Torrance, CA, USA). The column and autosampler were set at 40°C and 4°C, respectively. The gradient elution involved a mobile phase consisting of acetonitrile (mobile phase A) and 0.1% formic acid (mobile phase B) with a gradient program as follows: 5%-95% A, 0-20 min and 95%A, 20-23 min. The mobile phase was directly delivered into mass spectrometer at 0.4 mL/min, and the injection volume was 5 μL.

Mass spectrometry was performed on an ion trap/time-of-flight hybrid mass spectrometry with an electrospray ionization (ESI) source (IT/TOF-MS, Shimadzu, Japan). The mass spectrometer was operated simultaneously in positive and negative electrospray ionization modes by switching the interface voltage between 4.5 kV and -3.5 kV. The other parameters were set as follows: curved desorption line (CDL) temperature, 200°C; heat block temperature, 200°C; microchannel plate detector voltage, 1.65 kV; nebulizer gas (N_2), 1.5 L/min; drying gas (N_2), 10.0 L/min; collision energy, 10%, 30% and 60%. MS/MS analyses were conducted in data dependent acquisition, in which precursor ions are serially fragmented to generate their corresponding product-ion spectra. Product-ion spectra were acquired automatically in advance for a large number of ions. Furthermore, if the MS/MS information of the selected discriminating variables was missing, the product ion spectrum for these variables were acquired independently in manual mode. External calibration using the sodium trifluoroacetate was adopted to regulate the MS and MS/MS data.

2.3 GC-MS Based-Metabolomics

2.3.1 Sample Preparation for GC-MS Based-Metabolomics

The plasma was prepared with a two-step derivatization procedure, that is, alkylation and silylation, according to previous reports with a few modifications (21, 22). Briefly speaking, a 10 μL aliquot of plasma was thoroughly vortexed with ten times methanol followed by centrifuged at 18047g at 4°C for 10 min in two cycles. Then 80 μL supernatant was transferred to a brown glass vial and oximated with 25 μL methoxyamine hydrochloride (10 mg/mL in pyridine) at 4°C for 90 min. Finally, the mixture was vacuum-dried and silylated with 120 μL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) at 27°C for 120 min to separate for GC-MS analysis.

2.3.2 GC-MS Spectral Acquisition

GC-MS analysis was performed using GC/MS-QP2010 Ultra (Shimadzu Inc., Kyoto, Japan) equipped with an electron impact source operating in positive mode with the energy of 70 eV. Separation was achieved on a fused silica capillary column (Rtx-5MS; 30.0 m× 0.25 mm, 0.25 μm, Restek, USA) with a programmed temperature vaporization. The initial oven temperature was held at 70°C for 3 min, ramped to 320°C at a rate of 10°C/min, and finally held at 320°C for 2 min. The injection was performed in split mode (1: 50). Helium (>99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL/min. For mass detection, full scan with a mass range of *m/z* 45-600 was adopted, and the ion source temperature was set at 200°C.

2.4 Quality Assurance Procedure

To assure the robustness of analytical system and an acceptable level of data quality for non-targeted metabolomics, pooled QC samples, prepared by mixing equal volumes of each analyzed sample (23), were injected at the beginning of the batch to condition the analytical platform and then almost every six samples to monitor the system. The metabolic features that are detected in < 80% of QC samples (80% rule) and those with a relative standard deviation (RSD), as calculated for the QC samples, of > 30% (RSD 30% rule) were removed (20). The quality assurance procedure was performed to remove metabolic features with poor repeatability.

2.5 Statistical Analysis and Pathway Enrichment

The obtained LC-MS and GC-MS raw data files were processed using Profiling Solution version 1.1 (Shimadzu, Japan) for peak detection, matching, and alignment. After filtered by "80% rule" and "RSD 30% rule", missing values replacement and total ion intensity normalization, the resulting data was imported to SIMCA software 13.0 package (version 13.0; Umetrics, Umeå, Sweden) for multivariate statistical analysis including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Ions with variable importance in the projection (VIP) exceeding 1.0 in the OPLS-DA model and P-value adjusted by Benjamini-Hochberg method (pFDR) below 0.05 (24) were retained for further identification. Spearman correlation analysis was then applied to explore the correlations between differential metabolites and clinical indices of recipient.

The differential ions generated from LC-MS were tentatively identified based on the public online databases, such as the Human Metabolome Database (http://www.hmdb.ca) and the Metlin database (http://metlin.scripps.edu) and confidently annotated by matching retention time and mass characteristics with those of in-house standards (25); meanwhile, those from GC-MS were characterized by comparing the standard mass fragments in National Institute of Standards and Technology Research Library based on >70% similarity index (26) and confirmed with the characteristics of the authentic standards available in our lab.

To visualize and interpret the metabolic pathways related to PTDM, the differential metabolites were imported into MetaboAnalyst 5.0, which is a free web-based tool that uses the high-quality KEGG metabolic pathway database as the backend knowledge-base. Meanwhile, Cytoscape (http://www.cytoscape.org), a highly popular Java-based open source software tool, was adopted to visualize and analyze metabolite, gene and protein interaction networks. The list of differential metabolites (compound names or KEGG IDs) were first loaded in the

Metscape, a plugin for Cytoscape, to construct the compound-reaction-enzyme-gene network. Then, the network centrality parameters, such as degree, betweenness, and centroid value, were computed by CentiScaPe, another plugin for Cytoscape, to extract the core subnetwork.

3 RESULTS

3.1 Patient Characteristics

A total of 68 recipients, including 32 PTDM subjects and 36 non-PTDM subjects were recruited. The baseline demographic characteristics and clinical data of the two groups were presented in **Table 1**. PTDM group and non-PTDM group were well matched with no significant difference in gender, age, BMI, family history of diabetes, alcohol drinking history, ICU length of stay and hepatitis B infection.

3.2 Metabolomic Analysis

Typical total ion chromatograms (TICs) of PTDM and non-PTDM recipients were presented in **Supplementary Figure 1**. However, there was no visual difference in metabolic profiles between PTDM group and non-PTDM group. Therefore, PCA, an unsupervised method of multivariate analysis, was first performed to get an overview of the difference on the metabolic profiles. Outliers were checked using the Hotelling T2 range, adopting 95% and 99% confidence limits for suspect and strong outliers, respectively. Two patients from the PTDM group appeared out of Hotelling's ellipse at the 99% confidence. These two outliers shared the common feature that their liver function parameters, i.e. aspartate aminotransferase and alanine aminotransferase, were abnormal, and one of them died at the third year post-operation. Since then, the two outliers were removed, multivariate analysis was re-performed. As shown in **Supplementary Figure 2**, except two samples which lay between 95% and 99% Hotelling T2 ellipse, all of the remaining samples lay inside the 95% Hotelling T2 ellipse. Tight clustering of QC samples was observed in PCA score plots (**Figures 1A, D, G**), giving some confidence that the analytical process was running robustly providing reproducible metabolic profiles.

As depicted in PCA score plots (**Figures 1A, D, G**), there was a visual separation between PTDM and non-PTDM groups, indicating metabolic disorders in PTDM. Furthermore, supervised OPLS-DA was introduced to maximize the separation and dig out differential metabolites. All the three models produced a goodness of prediction with Q2 > 0.5 and the differences between R2Y and Q2 <0.3 (27) (**Figures 1B, E, H**). Furthermore, permutation test (200 times) and CV-ANOVA were performed to validate the generated models (28). For the permuted R2 and Q2, all the values were lower than their corresponding original ones, the intercepted value of Q2 in the vertical axis was below 0 and p-values of CV-ANOVA for all models were below 0.05 (**Figures 1C, F, I**), demonstrating high goodness of fit for the generated OPLS-DA models.

Moreover, the combination of VIP>1 and pFDR<0.05 was applied to screen out the differential metabolites between PTDM and non-PTDM. As a result, a total of 37 differential metabolites (21 from LC-MS, 16 from GC-MS) were identified. Furthermore, Spearman correlation analysis was adopted to explore the correlations between these differential metabolites and fasting plasma glucose. Based on the correlation coefficients, L-valine, LysoPE (20:4), LysoPE (18:2), LysoPC (20:2), LysoPC (18:1), LysoPC (16:0) and LysoPC (14:0) were removed because of the weak correlation (-0.3 < Spearman correlation coefficients < 0.3) (**Supplementary Figure 3**). Finally, a total of 30 differential metabolites (15 from LC-MS, 15 from GC-MS) were retained for further analysis. The detailed information including compound name, molecular formula, retention time and fold change value were shown in **Table 2** (GC-MS data) and **Table 3** (LC-MS data). These 30 differential metabolites annotated six main classes, including eight amino acids, seven glycerophospholipids, six bile acids (BAs), three carbohydrates, three long-chain fatty acids and others (**Figure 2A**). Furthermore, the contents variations of differential metabolites were depicted as a heatmap (rows correspond to metabolites, columns to samples, red and green denote increased and decreased signals in PTDM group compared with non-PTDM group) in **Figure 2B**.

3.3 Altered Pathways Related to PTDM

To explore potential metabolic pathways involved in PTDM, the differential metabolites were imported into MetaboAnalyst for functional enrichment analysis and network topology analysis. Results (**Figure 2C**) indicated that aminoacyl-tRNA biosynthesis, valine, leucine and isoleucine biosynthesis, primary bile acid biosynthesis, taurine and hypotaurine metabolism, glycine, serine and threonine metabolism, arginine biosynthesis with p-value less than 0.05 were the critical disturbed pathways involved in progression of PTDM (29, 30). To clearly elucidate the possible underlying mechanism of PTDM, a hypothetical metabolic network was reconstructed by using these differential metabolites, with the direction of the content change labeled (**Figure 3A**).

Moreover, the MetScape plugin for Cytoscape was used to construct the compound-reaction-enzyme-gene network based on the 30 differential metabolites (31). Among them, LysoPCs, and LysoPEs were regarded as category IDs. Additionally, for some metabolites, such as tauroursodeoxycholic acid (TUDCA), glycoursodeoxycholic acid (GUDCA) and glycochenodeoxycholic acid (GCDCA) were not retrieved in the MetScape plugin or KEGG database. Hence, there were 542 nodes containing 154

TABLE 1 | Clinical characteristics of the recruited PTDM and non-PTDM recipients.

Parameters	Non-PTDM	PTDM	P-value
Total N	36	32	
Sex (male/female)	28/8	22/10	0.290
Age (years)	49.92 ± 10.61	49.06 ± 9.48	0.806
BMI (kg/m^2)	22.83 ± 3.91	23.53 ± 3.31	0.323
family history of diabetes, n (%)	3	2	0.738
alcohol drinking history, n (%)	4	3	0.809
ICU length of stay (day)	3.19± 3.09	3.13 ± 1.98	0.366
hepatitis B infection, n (%)	25	27	0.826

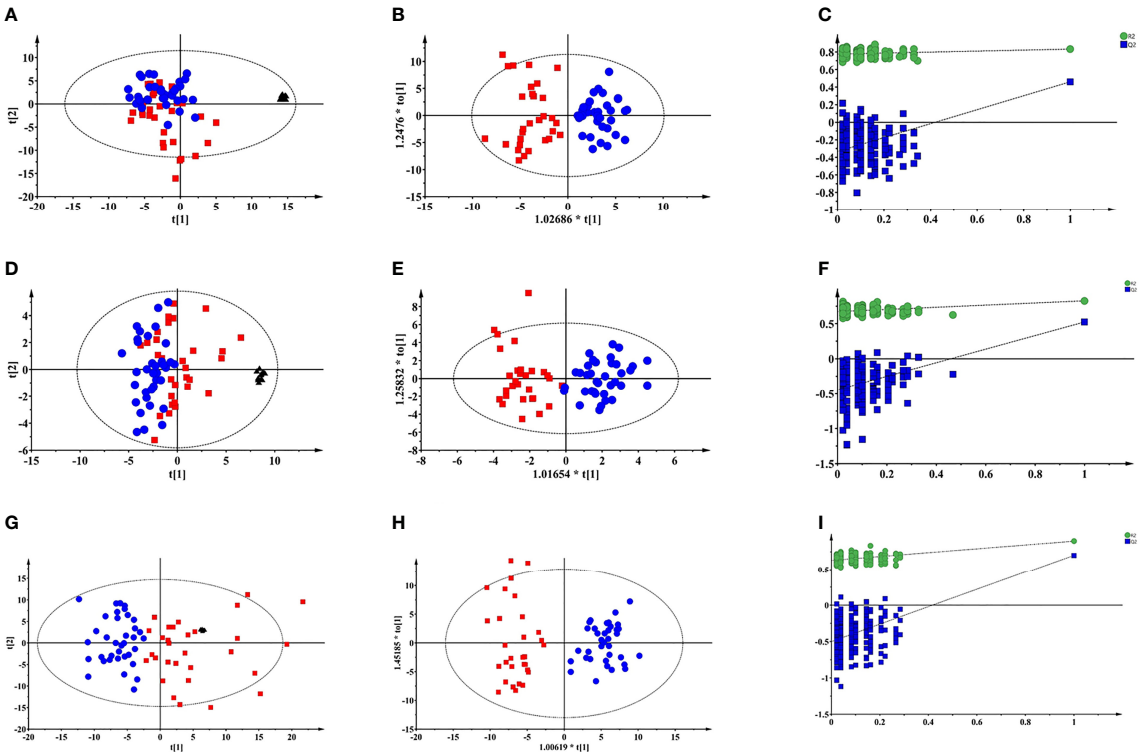

FIGURE 1 | Multivariate modelling of LC-MS and GC-MS data after log transformation and pareto scaling. **(A)** PCA score plot of LC-MS (+) data: R^2X=0.527, Q^2 = 0.322; **(B)** OPLS-DA score plot of LC-MS (+) data: R^2X=0.131, R^2Y=0.782, Q^2 = 0.502, CV-ANOVA p value = $1.0e^{-10}$; **(C)** the 200-permutation test of LC-MS (+) data; **(D)** PCA score plot of LC-MS (-) data: R^2X=0.613, Q^2 = 0.255; **(E)** OPLS-DA score plot of LC-MS (-) data: R^2X=0.165, R^2Y=0.816, Q^2 = 0.535, CV-ANOVA p value =$2.4\ e^{-9}$; **(F)** the 200-permutation test of LC-MS (-) data; **(G)** PCA score plot of GC-MS data: R^2X=0.681, Q^2 = 0.458; **(H)** OPLS-DA score plot of GC-MS data: R^2X=0.301, R^2Y=0.899, Q^2 = 0.701, CV-ANOVA p value =$8.0e^{-14}$; **(I)** the 200-permutation test of LC-MS (+) data:. Blue circles: non-PTDM; red squares: PTDM; black triangles: QC.

TABLE 2 | Differential metabolites identified by GC-MS.

Compound	Datebase ID	Formula	VIP	*p*FDR	Ion RT	Similarity	Fold change
Urea	HMDB00294	CH4N2O	1.34	<0.001	9.29	93	0.89
L-Leucine*	HMDB00294	CH4N2O	1.14	0.001	9.73	74	1.15
L-Serine*	HMDB00187	C3H7NO3	1.51	<0.001	11.03	91	1.20
L-Threonine	HMDB00167	C4H9NO3	1.44	<0.001	11.39	76	0.62
L-Proline*	HMDB00162	C5H9NO2	1.33	<0.001	13.28	93	0.84
L-Cysteine*	HMDB00574	C3H7NO2S	1.15	<0.001	13.71	74	0.69
L-Lysine	HMDB00182	C6H14N2O2	1.35	<0.001	15.59	91	0.86
L-Glutamine	HMDB00641	C5H10N2O3	1.55	<0.001	16.35	84	0.76
Deoxyribose	HMDB03224	C5H10O4	1.16	0.002	17.18	81	1.58
D-Glucose	HMDB00122	C6H12O6	1.21	0.001	17.92	90	1.16
D-Glucuronic acid	HMDB00127	C6H10O7	1.44	<0.001	18.97	83	1.33
Palmitic acid	HMDB00220	C16H32O2	1.17	0.006	19.31	92	0.87
Uric acid	HMDB00289	C5H4N4O3	1.05	0.010	19.78	87	0.88
Linoleic acid	HMDB00673	C18H32O2	1.21	0.001	22.36	77	0.86
Cholesterol	HMDB00067	C27H46O	1.35	<0.001	28.64	92	0.89

Identification was confirmed with authentic standard.

compounds, 112 reactions, 87 enzymes and 189 genes in the network (**Supplementary Figure 4**). Then, the node centrality indexes, namely degree, betweenness, and centroid value, were calculated to extract the core subnetwork displaying a critical role in the process of PTDM (32). As shown in **Figure 3B**, nine metabolites, namely linoleic acid, L-leucine, L-glutamine, L-phenylalanine, L-cysteine, cholesterol, L-serine, L-lysine, and LysoPC, were selected as hub metabolites. Meanwhile, eight

TABLE 3 | Differential metabolites identified by LC-MS.

Rt (min)	Molecular Formula	m/z	ion forms	MS/MS fragment	VIP	*p*FDR	Fold change	Identification
1.17	C9H11NO2	166.0877	$[M+H]^+$	120.0864	1.21	0.031	1.19	L-Phenylalanine
15.12	C14H28O2	227.2014	$[M-H]^-$	109.1859, 145.8610	1.45	<0.001	0.59	Myristic acid
13.40	C21H44NO7P	454.2942	$[M+H]^+$	313.2706, 436.2881	2.12	<0.001	1.44	LysoPE (16:0)
			$[M-H]^-$	196.0368, 255.2354				
14.94	C23H48NO7P	482.3258	$[M+H]^+$	341.3087, 421.2729, 464.3151	1.86	<0.001	1.26	LysoPE (18:0)
		480.3075	$[M-H]^-$	283.2683				
12.58	C24H48NO7P	494.3252	$[M+H]^+$	184.0746, 476.3165	1.94	<0.001	0.67	LysoPC (16:1)
		538.3254	$[M+HCOO]^-$	253.2237, 478.2988				
13.11	C26H50NO7P	520.3414	$[M+H]^+$	184.0755, 443.2782, 502.3330	2.23	<0.001	0.62	LysoPC (18:2)
		564.3337	$[M+HCOO]^-$	279.2348, 504.3167				
15.35	C26H5N4O7P	524.3722	$[M+H]^+$	184.0750, 311.2981, 447.2860, 506.3643	1.86	0.001	1.22	LysoPC (18:0)
		568.3622	$[M+HCOO]^-$	100.5837, 283.2678, 508.3450				
12.95	C27H44NO7P	526.2948	$[M+H]^+$	385.2803, 508.2844	1.89	<0.001	0.74	LysoPE (22:6)
		524.2744	$[M-H]^-$	196.0423, 283.2474, 327.2220				
13.13	C28H50NO7P	544.3401	$[M+H]^+$	184.0779, 485.2655, 526.3357	2.55	<0.001	0.81	LysoPC (20:4)
		588.3329	$[M+HCOO]^-$	126.9552, 303.2333, 528.3122				
10.54	C26H43NO5	448.3051	$[M+H]^+$	414.3102, 432.3129	1.89	0.001	1.54	Glycochenodeoxycholic Acid
		450.3414	$[M-H]^-$	386.3051				
9.10	C26H43NO6	464.2998	$[M-H]^-$	295.2011, 364.2687, 402.3076, 446.2918	1.56	0.001	3.67	Glycocholic Acid*
9.28	C26H45NO6S	498.2868	$[M-H]^-$	355.2611, 480.2768	1.82	0.003	5.63	Taurochenodeoxycholic Acid*
8.14	C26H45NO6S	498.2869	$[M-H]^-$	290.2154, 355.2671, 384.3029, 480.2768	2.12	<0.001	4.30	Tauroursodeoxycholic Acid
8.19	C26H45NO7S	514.2822	$[M-H]^-$	515.2866	1.12	0.028	5.14	Taurocholic Acid*
9.24	C26H43NO5	448.3051	$[M-H]^-$	386.3108, 449.3126	2.54	<0.001	2.72	Glycoursodeoxycholic Acid

Identification was confirmed with authentic standard.

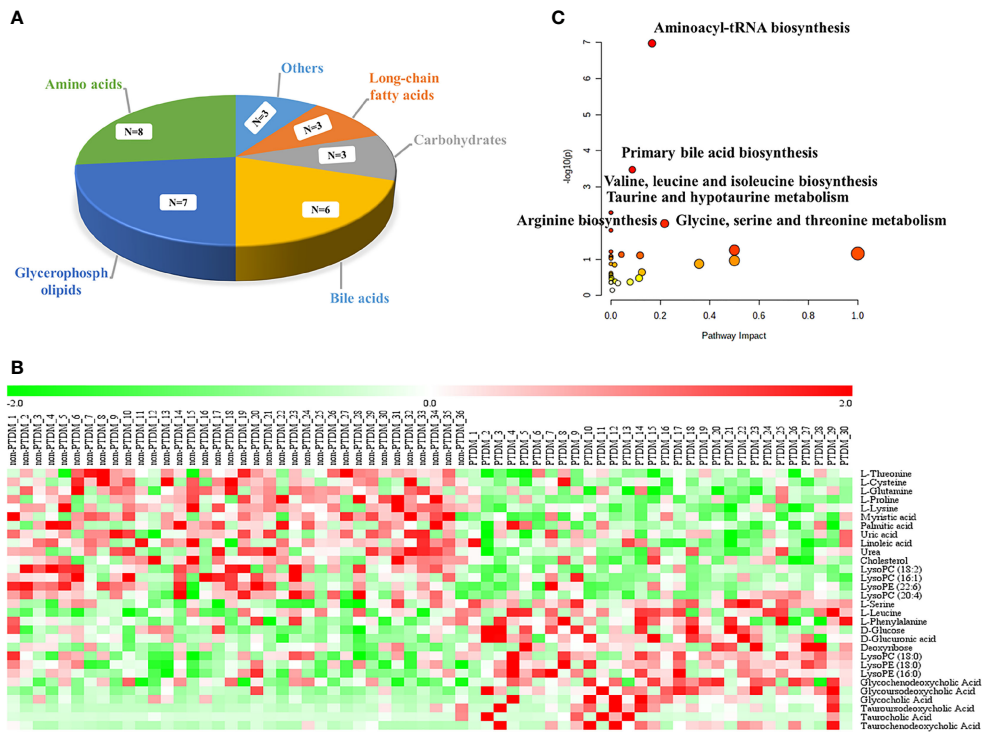

FIGURE 2 | Analysis of the differentially expressed metabolites. **(A)** classification of the 30 identified differentially expressed metabolites; **(B)** Heat map of the differentially expressed metabolites in each group. Rows, samples; columns, metabolites. The red band indicates an increased level of metabolites, while the green band indicates a decreased level of metabolites in PTDM group compared with non-PTDM group; **(C)** Summary of the altered metabolism pathways determined with MetaboAnalyst v. 5.0.

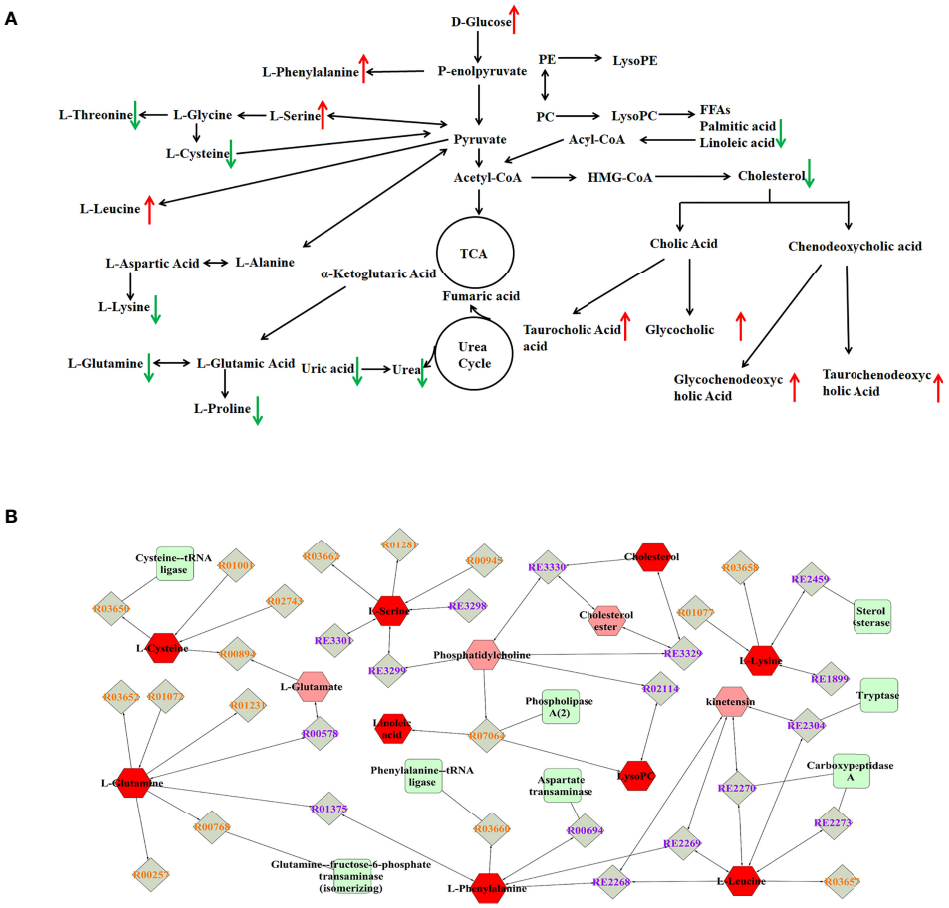

FIGURE 3 | Analysis of potential biomarkers and related pathways. **(A)** Schematic overview of metabolic pathways based on the differentially expressed metabolites. The metabolites indicated with red or green arrows represent increased or decreased levels, respectively, in PTDM group. **(B)** The compound-reaction-enzyme-gene subnetwork. Red hexagons represent the identified differentially expressed metabolites. Green squares represent enzymes which might regulate the identified metabolites. Grey diamonds represent reactions catalyzed by those enzymes.

enzymes, such as aspartate transaminase, phospholipase A2 and tryptase, were involved in this subnetwork.

4 DISCUSSION

As a frequent metabolic complication, PTDM seriously affects the life quality and long-term survival of recipients. However, data on the mechanism of PTDM are scarce. Beyond this, the treatment is based on expert experience rather than research-based evidence at the current stage. Metabolomics, encompassing the comprehensive and systematic profiling of multiple metabolites, is a promising approach to provide an understanding of physiological and pathological status of the living organism. Nevertheless, reports on the adoption of metabolomics to describe the metabolic profiles and pathways involved in PTDM have not been published. In this study, for the first time to our knowledge, the metabolic profiles and pathways involved in PTDM were explored by the integrated LC-MS and GC-MS based-metabolomics, which aimed to provide novel insights into the underlying pathophysiological mechanisms of PTDM. All individuals enrolled in our study were free of diabetes pre-transplantation and matched on gender, age, BMI, family history of diabetes, alcohol drinking history, ICU stay length and hepatitis to minimize confounding factors.

The integrated untargeted metabolomics revealed that 30 significantly changed metabolites, among which 15 decreased and 15 increased, possibly contributed to the development of PTDM. Based on their chemical structure, these significantly changed metabolites mainly belongs to the classes of amino acids, bile acids, glycerophospholipids and others.

Our findings highlighted several amino acids, particularly the branched-chain amino acids (BCAAs) and aromatic amino acids (AAAs), were noteworthy and might be served as biomarkers of PTDM. BCAAs (leucine, isoleucine and valine) and AAAs (tyrosine, phenylalanine and tryptophan) have been proven to be potential contributors to the development of insulin resistance and diabetes in both humans (33) and rodent models (34).

A nested case-control study in the Framingham Offspring Study comprising 2422 normoglycemic individuals followed for 12 years indicated that plasma levels of three BCAAs (isoleucine, leucine, valine) and two AAAs (tyrosine and phenylalanine) exhibited highly significant associations with the future development of T2DM (35). Furthermore, a meta-analysis focusing on dietary BCAAs intake and T2DM showed that oral BCAAs supplementation is positively associated with T2DM risk (36). The same result was found in double AAAs intake to mice (37). Our results also suggested L-phenylalanine and L-leucine increased in participants with PTDM. Since BCAAs and AAAs (expect tyrosine) are essential amino acid which must be obtained from the diet, their elevated circulating levels might be the result of excess intake and/or disruption of their catabolic process. However, epidemiological results are controversial, with some indicating that a diet high in BCAAs were positively associated with circulating levels (38), while others not (35). Since then, we inferred that the elevated circulating levels of BCAAs might arise from the hindrances to their downstream catabolism. Unlike most amino acids, whose catabolism take place in the liver, BCAAs are initially catabolized by branched-chain-amino-acid aminotransferase (BCAT) in extrahepatic tissues (such as skeletal muscle) to form branched chain α-keto acids (BCKAs) and then by branched chain α-keto acid dehydrogenase (BCKD), the rate-limiting enzyme in BCAA catabolism. Zhou M et al. (39) revealed that the enhancement of BCKD activity by administration of BT2 to BCKD deficiency obese (ob/ob) mice reduced the abundance of BCAAs and BCKAs, resulting in markedly attenuated insulin resistance. The BCAA catabolism was suggested as a potential therapeutic target for insulin resistance and T2DM. In addition, recent work revealed that elevated circulating BCAAs levels correlated with intestinal microbiota dysbiosis of the host. *Prevotella copri* and *Bacteroides vulgatus* were proven to be the main species associated with the biosynthesis of BCAAs and insulin resistance (40). Gavage with *Prevotella copri* would induce insulin resistance, aggravate glucose intolerance and augment circulating levels of BCAAs (38), while Gavage with *Bacteroides vulgatus* exerted the opposite effect (41). Similar to previous studies, the levels of L-leucine and L-phenylalanine were significantly higher in PTDM recipients than in non-PTDM ones, which might be due to BCAAs catabolism hindrance and/or host intestinal microbiota dysbiosis.

Significant variations in specific BAs species were found in our study. Simply put, the levels of taurocholic acid (TCA), TUDCA, taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), GUDCA and GCDCA were significantly higher in PTDM receipts than in non-PTDM ones. As the most frequent etiology of liver transplantation (76.5%) in our study, hepatitis B virus has been proven to alter the expression of CYP7A1, a key enzyme involved in bile acid synthesis (42). Thus, the disturbance in BA profiles has been repeated observed in hepatitis B virus-infected patients for decades (43, 44). To minimize the influence from this confounding factor, case and control subjects were well-matched in hepatitis B virus infection. What's more, in terms of aspartate aminotransferase, alanine aminotransferase, hepatic function of liver transplant recipients normally recovered within a few days, which was consistent with previous research (45). Two participants with hepatic disfunction were excluded from data analysis. Over the last few decades, BAs have attracted considerable attention in the field of diabetes, obesity, nonalcoholic fatty liver disease and so on. BAs are synthesized in hepatocytes and then undergo enterohepatic circulation with six to eight times per day in humans. Thus, BAs are detected at relatively lower levels in plasma compared with them in the liver, bile and intestine. In human, most bile acids are conjugated to glycine (G) and taurine (T) at a ratio of about 3:1. To date, it is still uncertain whether and what circulating BAs alter in patients with T2DM. For instance, a cross-sectional study including 224 T2DM patients and 102 nondiabetic individuals indicated that patients with T2DM possessed increased plasma levels of TCDCA, GCDCA, deoxycholic acid (DCA), taurodeoxycholic acid (TDCA) and glycodeoxycholic acid (GDCA), and decreased levels of CA and TCA (46). Another case-control study of age- and gender-matched T2DM versus control demonstrated elevated levels of TCA, TDCA, GDCA and DCA in T2DM subjects (47). Furthermore, a nested case-control study of 1,707 matched T2DM-control subject pairs within the China Cardiometabolic Disease and Cancer Cohort Study showed that conjugated primary BAs (GCA, TCA, GCDCA and TCDCA) and secondary BA (TUDCA) were positively related with T2DM risk, while unconjugated BAs (CA, CDCA and DCA) were inversely associated with T2DM risk (48). Accordingly, the currently human studies provided conflicting results, with some reporting certain BAs species increased in T2DM and others reporting those decreased in T2DM. Beyond this problem, considering the relatively small number of participants, the variation of BAs in PTDM recipients need to be verified in a large cohort.

What's more, we found a series of LysoPCs and LysoPEs expressed differentially in PTDM recipients. The concentration of lysoPCs in plasma, up to 100μM in healthy subjects (49), is much higher than that of lysoPEs. In plasma, LysoPCs, representing 5%-20% of total phospholipids, are mainly formed by lecithin-cholesterol acyltransferase (LCAT) in the process of transferring fatty acyl residues in sn-2 position of phosphatidylcholine to free cholesterol for the formation of cholesteryl esters, or by endothelial lipase, or by direct hepatic secretion (50). The alterations of LysoPCs species linked to T2DM have been widely studied. Significant lower levels of LysoPC (18:2), LysoPC (18:1), LysoPC (18:0), and LysoPC (17:0) were found in T2DM and impaired glucose tolerance (IGT) cohort in the population-based Cooperative Health Research in the Region of Augsburg (KORA) study. Among them, LysoPC (18:2) served as a predictor for T2DM, which was independently confirmed in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam study (51). A global lipidomics analysis of 293 Chinese individuals has also shown that LysoPC (18:0), LysoPC (18:1), and LysoPC (18:2) were all negatively correlated with HOMA-IR (52). Our finding was in agreement with above findings that LysoPC (18:2) exhibited significantly lower level in PTDM than non-PTDM recipients. Several other studies have reported inconsistent findings, i.e. diabetic men exhibited higher levels of centain

LysoPCs, including LysoPC (14:0), LysoPC (16:1), LysoPC (18:1), LysoPC (22:6), LysoPC (20:5) and LysoPC (18:3), but not including LysoPC (18:2), LysoPC (16:0) and LysoPC (18:0) (53). Our study exhibited inverse change trends of different LysoPC species with increased expression in LysoPC (18:0) and decreased expression in LysoPC (18:2), LysoPC (16:1) and LysoPC (20:4), which might be due to the opposite effects of saturated and unsaturated acyl LysoPC. Park JY et al. (54) reported that lysoPC and lysoPE species containing unsaturated fatty acids were associated with an increased risk of coronary artery disease, whereas those containing saturated fatty acids were associated with a decreased risk. Saturated LysoPCs, such as LysoPC (16:0), are a potent inflammatory mediator, while polyunsaturated acyl LysoPCs, including LysoPC (20:4) and LysoPC (22:6), can serve as an anti-inflammatory lipid mediator and inhibit the inflammation induced by saturated LysoPCs (55). In mouse models, Yea K et al. (56) have reported that the blood glucose lowering effect of LysoPCs were found to be sensitive to variations in lysoPC acyl chain length, which may elucidate the divisive findings in our study. Therefore, lysoPCs play a complex role in T2DM, especially special type of T2DM like PTDM, which needs further work to clarify.

Our study recruited liver transplant recipients to address the "real-world" problem in PTDM. The metabolomic results help to give a new sight in the mechanism of PTDM. Since the analyzed sample size was small, we speculate that PTDM might be associated with the perturbation in amino acids, bile acids and glycerophospholipids. This hypothesis provides possible research direction in the field of PTDM. In addition, a major limitation of plasma metabolomics is that all of the differential metabolites are detected in plasma, their actual origins are unclear. Further studies should investigate the highlighted pathways in relevant tissues (such as muscle and liver) and their relations to PTDM for a comprehensive understanding of its underlying mechanism.

5 CONCLUSION

In summary, the integrated LC-MS and GC-MS based-metabolomics was adopted to dig out differentially changed metabolites associated with PTDM. A total of 30 metabolites (15 from LC-MS, 15 from GC-MS) significantly altered in PTDM recipients were identified. Findings indicated that alterations in plasma metabolites, particularly amino acids, BAs and LysoPCs may contribute to the progression of PTDM. Our study offered a novel insight into the pathological mechanism of PTDM. Further studies are needed to verify these findings and to unravel the underlying mechanisms involved in PTDM.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (No. 2020-053-01). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MW, HZ, and JW designed the study protocol. MW, JX, and NY acquired the data and interpreted the results. JX and TZ wrote the original draft. MW, NY, and TZ recruited subjects. HZ and JW supervised the project and revised the original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.807318/full#supplementary-material

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N6-Methyladenosine-Related Gene Signature Associated With Monocyte Infiltration Is Clinically Significant in Gestational Diabetes Mellitus

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Objective: The objective of this study was to reveal the potential crosstalk between immune infiltration and N^6- methyladenosine (m^6A) modification in the placentas of patients with gestational diabetes mellitus (GDM), and to construct a model for the diagnosis of GDM.

Methods: We analyzed imbalanced immune infiltration and differentially expressed m^6A-related genes (DMRGs) in the placentas of patients with GDM, based on the GSE70493 dataset. An immune-related DMRG signature, with significant classifying power and diagnostic value, was identified using a least absolute shrinkage and selection operator (LASSO) regression. Based on the selected DMRGs, we developed and validated a nomogram model using GSE70493 and GSE92772 as the training and validation sets, respectively.

Results: Infiltration of monocytes was higher in GDM placentas than in control samples, while the infiltration of macrophages (M1 and M2) in GDM placentas was lower than in controls. A total of 14 DMRGs were strongly associated with monocyte infiltration, seven of which were significant in distinguishing patients with GDM from normal controls. These genes were *CD81*, *CFH*, *FABP5*, *GBP1*, *GNG11*, *IL1RL1*, and *SLAMF6*. The calibration curve, decision curve, clinical impact curve, and receiver operating characteristic curve showed that the nomogram recognized GDM with high accuracy in both the training and validation sets.

Conclusions: Our results provide clues that crosstalk between m^6A modification and immune infiltration may have implications in terms of novel biomarkers and therapeutic targets for GDM.

Keywords: gestational diabetes mellitus, N6-methyladenosine modification, immune infiltration, monocyte, nomogram

INTRODUCTION

Gestational diabetes mellitus (GDM) is a form of diabetes that is first diagnosed during pregnancy, with a worldwide prevalence of 9–21%. GDM frequently affects both short-term and long-term health in the mother and offspring, because of the diverse genetic background and epigenetic modifications that occur in response to nutritional and environmental factors (1, 2). Currently, the precise etiological mechanisms of GDM remain unclear; however, numerous studies have found that GDM is a multifactorial disease that involves genetic factors, lifestyle, and chronic inflammation. Insulin resistance (IR) and pancreatic β-cell dysfunction are regarded as essential for the pathogenesis of GDM (2, 3). Although the exact mechanisms remain to be clarified, chronic inflammation has been reported to participate in the development of IR and pancreatic β cell failure, which in turn leads to GDM (4). The placenta is a temporary organ formed during pregnancy, which serves as the only bridge connecting the mother and fetus, and has important endocrine function. Placenta-derived inflammatory cytokines, such as interleukin-1 beta (IL-1β), IL-6, IL-15, IL-10, IL-34, IL-38, and tumor necrosis factor alpha (TNF-α), can stimulate immune cells and aggravate immune and inflammatory responses, thereby exacerbating chronic inflammation and maternal IR and inducing β cell failure during pregnancy (5–11). Moreover, immune cells and inflammatory cytokines are important components of the placental microenvironment, which is essential for normal pregnancy (12, 13). Imbalanced immune infiltration in the placenta contributes to the pathogenesis and development of pregnancy-specific diseases, including GDM, and may affect GDM-related adverse pregnancy outcomes and clinical prognosis (13–15).

Chemical modifications of cellular RNAs can result in secondary structure modifications, splicing, degradation, or molecular stability, which are emerging layers of post-transcriptional gene regulation. More than 160 chemical modifications have been identified (16). N^6- methyladenosine (m^6A) RNA modification is the most prevalent type of RNA epigenetic processing (17, 18). m6A modification is mediated by its effector proteinsare in a dynamic and reversible pattern (17). m6A occurs mainly in the 3'- UTR and the vicinity of the termination codon mRNA, which is recogonized by "readers" (YTH domain family (YTHDF]1–3, and insulin-like growth factor 2 mRNA-binding proteins 1–3), catalyzed by methylases [methyltransferase-like (METTL)3/14, and Wilms' tumour 1-associated protein], and removed by demethylases [fat-mass and obesity-associated protein (FTO), and alkylation repair homolog protein 5] (19). Recent evidence indicates that perturbations of m^6A modifications dysregulate mRNA metabolism, including mRNA stability, mRNA splicing, RNA nucleation, RNA-protein teractions and mRNA translation, thereby contributing to various physiological and pathophysiological processes (20–22). Numerous m^6A modifications have been shown to regulate adipogenesis, glucose metabolism, insulin resistance, and the related chronic immune response (17, 21, 23). This suggests that m^6A modifications are implicated in the development of metabolic diseases, although the specific knowledge regarding GDM is still in its infancy.

In this study, we aimed to reveal the imbalanced immune infiltration in the placenta of patients with GDM, the differentially expressed m^6A-related genes (DMRGs) involved, as well as the crosstalk between them, and also to develop a nomogram model for the diagnosis of GDM.

MATERIALS AND METHODS

Data Collection

The human expression dataset GSE70493 was downloaded from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/). The expression data in GSE70493 contained 63 samples of the maternal placenta (GDM; n = 32 and normal glucose tolerance [NGT]; n = 31). We obtained 17,661 m^6A-related genes by crossing data from the RMBase (24) and RMvar (25) databases. The expression data in GSE92772 were obtained for validation and contained RNA profiles of maternal whole blood cells from eight GDM and eight NGT pregnant women in their second trimester. The workflow of this research is shown in **Figure 1**.

Screening of Differentially Expressed m^6A-Related Genes

Using the R software 'limma' package, we identified differentially expressed genes (DEGs) in the GSE70493 dataset, based on the criteria of $|\log2 \text{ fold change}| > 0.1$ and P value < 0.01. Heatmaps were generated using the R software 'pheatmap' package. In the case of multiple probes corresponding to the same gene, we selected the probe with the lowest P value. Genes without official symbols were removed, and all symbols were converted to symbols approved by the HUGO Gene Nomenclature Committee. We then crossed the DEGs with m^6A-related genes to obtain the DMRGs.

Functional-Enrichment Analysis

To determine the potential functions and enriched pathways of DMRGs in GDM, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were analyzed using the R software 'enrichplot' package. A P value of < 0.05 was set as the cutoff.

Evaluation of Immune Cell Infiltration in Placenta

CIBERSORTx (26) was utilized to quantify the abundance of 22 immune cell types in each sample by imputing the gene expression profiles of GSE70493. We then compared the differences in immune cell infiltration between GDM patients and healthy subjects.

Selection of Core DMRGs Correlated With Immune Infiltration

Pearson correlation coefficient (PCC) analysis was conducted to identify the DMRGs correlated with the differentially infiltrated immune cells between GDM and healthy patients. LASSO analysis, a linear regression model penalized with the L1 norm, was used to further narrow down the variables owing to its tendency to prefer solutions with fewer non-zero coefficients. A

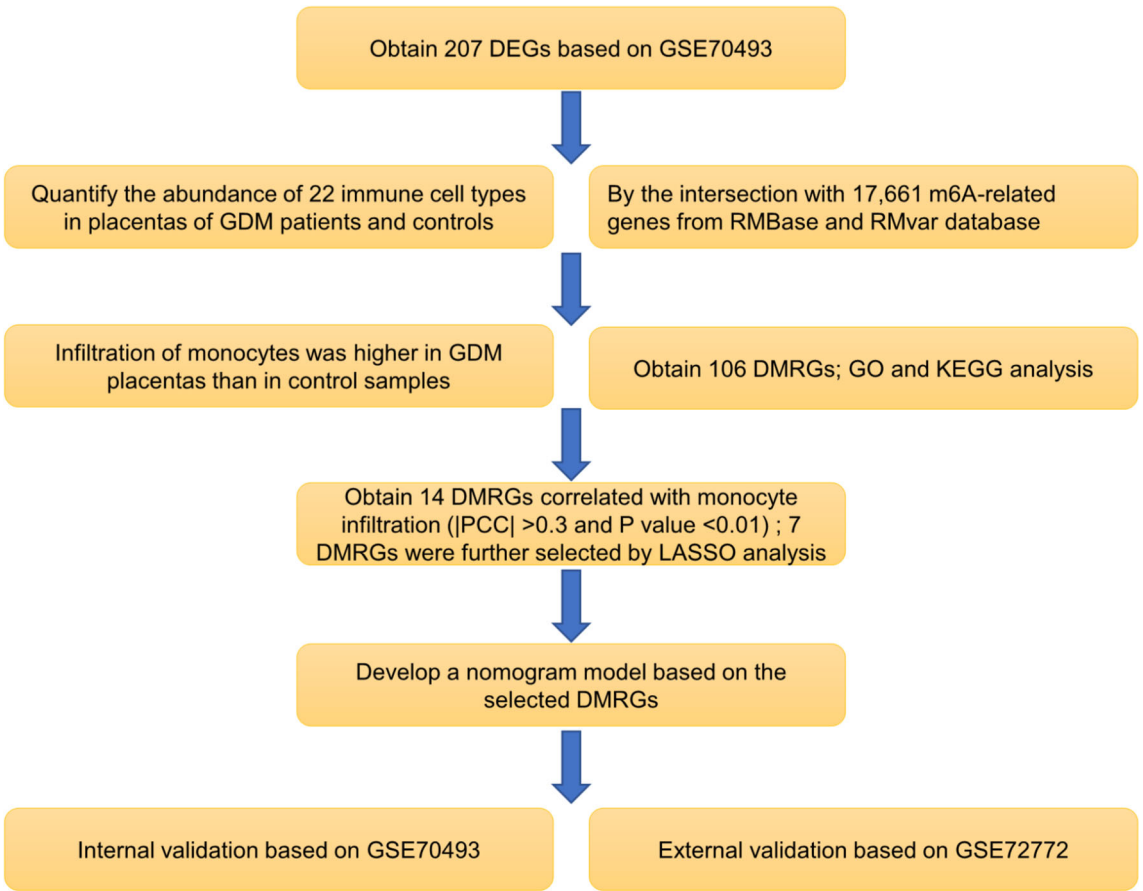


FIGURE 1 | Flowchart of the research workflow. Abbreviations are defined as follows: differentially expressed gene (DEG), gestational diabetes mellitus (GDM), differentially expressed m6A-related gene (DMRG), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Pearson correlation coefficient (PCC), least absolute shrinkage and selection operator (LASSO).

tuning parameter, lambda, was used to control the number of coefficients with a value of zero. The 10-fold K cross-validations for the centralization and normalization of selected variables to select the optimal lambda value using R software. The core DMRGs that correlated with immune infiltration were identified using LASSO analysis.

Construction and Validation of the Nomogram Model for GDM Diagnosis

A logistic regression model was considered to evaluate the performance the core m6A-related genes selected by LASSO to estimate the probability of GDM. Based on this model, we constructed a nomogram for individual predictions of GDM using R software. To validate the classification ability of the nomogram model, calibration was analyzed using a bootstrapping approach and randomly repeated 1,000 times with replacement. Decision curve analysis and clinical impact curves were used to determine clinical usefulness. The receiver operating characteristic (ROC) curve was used to evaluate the sensitivity and specificity of the nomogram. To externally validate the nomogram, we then applied the calibration, decision curve, clinical impact curve, and ROC curve analysis on GSE92772.

Statistical Analysis

Data processing and statistical analyses were performed using R software (version 4.0.3). Associations between quantitative variables were assessed using the Student's *t*-test. Spearman's rank correlation analysis was used to explore the correlations between different variables. LASSO regression, logistic regression, and nomogram development were conducted using "glmnet", "survival", and "rms" packages, respectively. *P* values < 0.05 were considered significant.

RESULTS

Landscape of DMRGs in GDM Pregnancies

Based on the criteria described above, we identified 207 DEGs in GSE70493 and obtained 106 DMRGs by crossing the DEGs with

17,661 m^{6}A-related genes. A heatmap of the DMRGs is shown in **Figure 2**. The list of all DMRGs is shown in **Table S1**. To elucidate the functions and pathways of the 106 DMRGs, we conducted enrichment analysis using R software. Based on the GO category biological process, DMRGs were mainly enriched for the terms interferon-gamma-mediated signaling pathway, antigen processing and presentation of endogenous antigen, positive regulation of leukocyte-mediated immunity, positive regulation of T cell-mediated cytotoxicity, cellular response to interferon-gamma, and cell killing (**Table S2** and **Figure 3A**). KEGG pathways were mainly enriched in viral myocarditis, type 1 diabetes mellitus (T1DM), phagosomes, cell adhesion molecules, and autoimmune thyroid disease (**Table S3** and **Figure 3B**).

Immune Cell Infiltration in the Placenta

We further explored differential immune cell infiltration in the placenta between GDM and control cases by quantifying the abundance of 22 immune cell types (**Supplementary File 1**). The results indicated that the infiltration of monocytes was higher in GDM placentas than in control samples, while the infiltration of macrophages M1 and M2 in GDM placentas were lower. No significant differences were observed among the other immune cells (**Figure 4A**). Higher propotion of M2 than M1 phenotype in GDM compared to controls was observed (**Supplementary Figure 1**).

Identification of the DMRGs Signature Related to Monocyte Infiltration

Considering the obviously high infiltration of monocytes in the GDM placentas, we calculated the PCCs of the abundance of monocytes and the expression levels of DMRGs. Fourteen DMRGs (methanethiol oxidase [*SELENBP1*], fatty acid-binding protein 5 [*FABP5*], G-protein coupled receptor 183 [*GPR183*], inhibitor of differentiation 4 [*ID4*], G-protein-coupled receptor 65 [*GPR65*], G-protein subunit γ 11 [*GNG11*], guanylate binding protein 1 [*GBP1*], complement factor H [*CFH*], tetraspanin [*CD81*], interleukin-1 receptor-like 1 [*IL1RL1*], cathepsin K [*CTSK*], sterile alpha motif domain-containing protein 9-like [*SAMD9L*], spermatogenesis associated serine-rich 2-like [*SPATS2L*], and signaling lymphocytic activation molecule family 6 [*SLAMF6*]), with a $|PCC| > 0.3$ and P value < 0.01, were selected from 106 DMRGs for further analysis (**Figure 4B**). Based on LASSO regression analysis, seven DMRGs (*CD81, CFH, FABP5, GBP1, GNG11, IL1RL1*, and *SLAMF6*) had nonzero coefficients, with a lambda coefficient of 0.1059 (**Figures 5A, B**). The count of the potential m^{6}A modification sites are shown in **Figure 5C**. The expression matrix of the seven key DMRGs, based on the 63 samples, was extracted from the dataset GSE70493. The expression levels of *CD81, CFH, GBP1, IL1RL1*, and *SLAMF6* in GDM samples were lower than those in control samples, while expression levels of *FABP5* and *GNG11* were higher in GDM placentas than those in controls (**Figures 5D, E**). As shown in **Figure 4B**, these seven DMRGs were significantly correlated with each other.

Development of the Nomogram Model

We extracted the expression matrix of the seven core DMRGs based on the training set of 63 samples extracted from dataset GSE70493. A model incorporating the DMRGs *CD81, CFH, FABP5, GBP1, GNG11, IL1RL1*, and *SLAMF6* was developed and presented as a nomogram (**Figure 6A**). The probability of GDM was accurately predicted using a calibration curve (**Figure 6B**). The decision curve (**Figure 6C**) and clinical impact curve (**Figure 6D**) revealed that our model demonstrated a positive net benefit without increasing the number of false positives. In addition, ROC curve analysis revealed that the area under the curve (AUC) was 83% (**Figure 6E**), indicating a good classification ability of the nomogram model.

Diagnostic Value of the DMRGs Signature Related to Monocyte Infiltration

Considering that screening for GDM is usually performed during 24–28 weeks of gestation, we selected GSE92772 as the validation set to evaluate the diagnostic value of the core DMRG signature, which is based on blood samples extracted during the second trimester. GSE92772 contains the expression matrix of *SLAMF6, FABP5, GBP1, GNG11, IL1RL1*, and *CD81*, without *CFH* present. In the validation set, the calibration curve (**Figure 7A**), decision curve (**Figure 7B**), and clinical impact curve (**Figure 7C**) also exhibited good performance. Moreover, the nomogram model exhibited high diagnostic value in distinguishing patients with GDM from those with NGT, with an AUC value of 85.9% (**Figure 7D**).

DISCUSSION

GDM is a common complication of pregnancy, adversely affecting both the mother and fetus (1, 2). The etiology of GDM, which involves genetic background and epigenetic modifications, remains unclear. Chronic low-grade inflammation during pregnancy can contribute to the pathogenesis of GDM by exacerbating maternal IR and inducing β cell failure (4). As an endocrine organ, the placenta derives inflammatory cytokines that stimulate immune cells and aggravates the immune/inflammatory response (12, 13). Moreover, the disturbance of immune cell infiltration in the placenta is attributed to pregnancy-specific diseases, including GDM, as well as GDM-related adverse outcomes (13–15). In this study, we found that the infiltration of monocytes was higher in GDM placentas than in control samples, while the infiltration level of macrophages (M1 and M2) in GDM placentas was lower than that in the controls. Monocyte infiltration has been shown to be crucial during inflammation. As important mediators of the innate immunity, monocytes circulate in the bloodstream and pass into tissues during the steady state and in increased quantities during inflammation (27). GDM is considered as a low-degree inflammation, and elevated levels of monocytes in the peripheral blood of patients with GDM have been previously reported (28). Based on the expression of superficial CD14 and CD16 in flow cytometry, monocytes can be divided into three subsets: classical (CD14++CD16-), intermediate (CD14+CD16++) and non-classical (CD14+CD16+) (29). Angelo et al. (28) observed increased percentage of classical monocytes, decreased frequency of intermediate monocytes in the peripheral blood of patients with

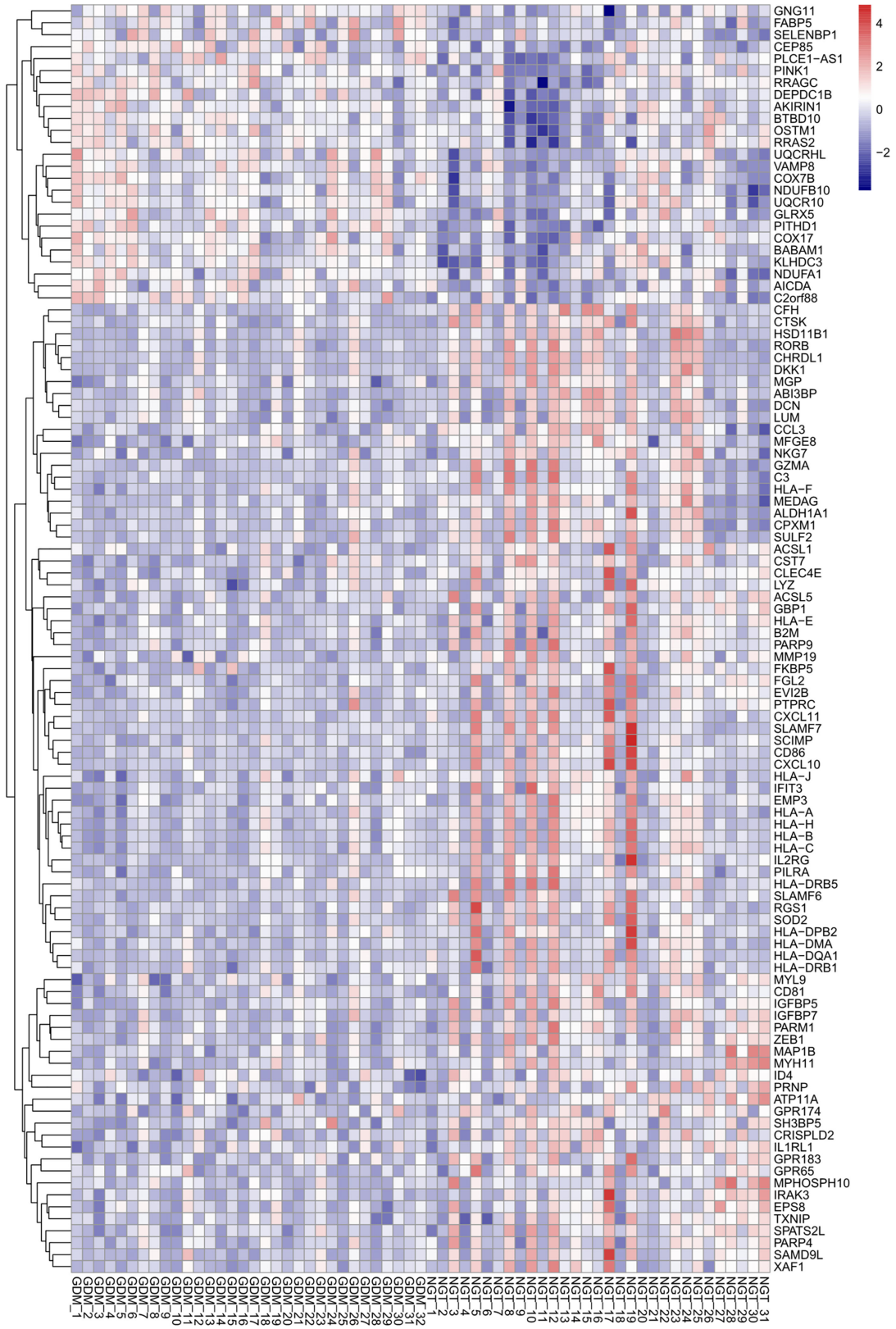
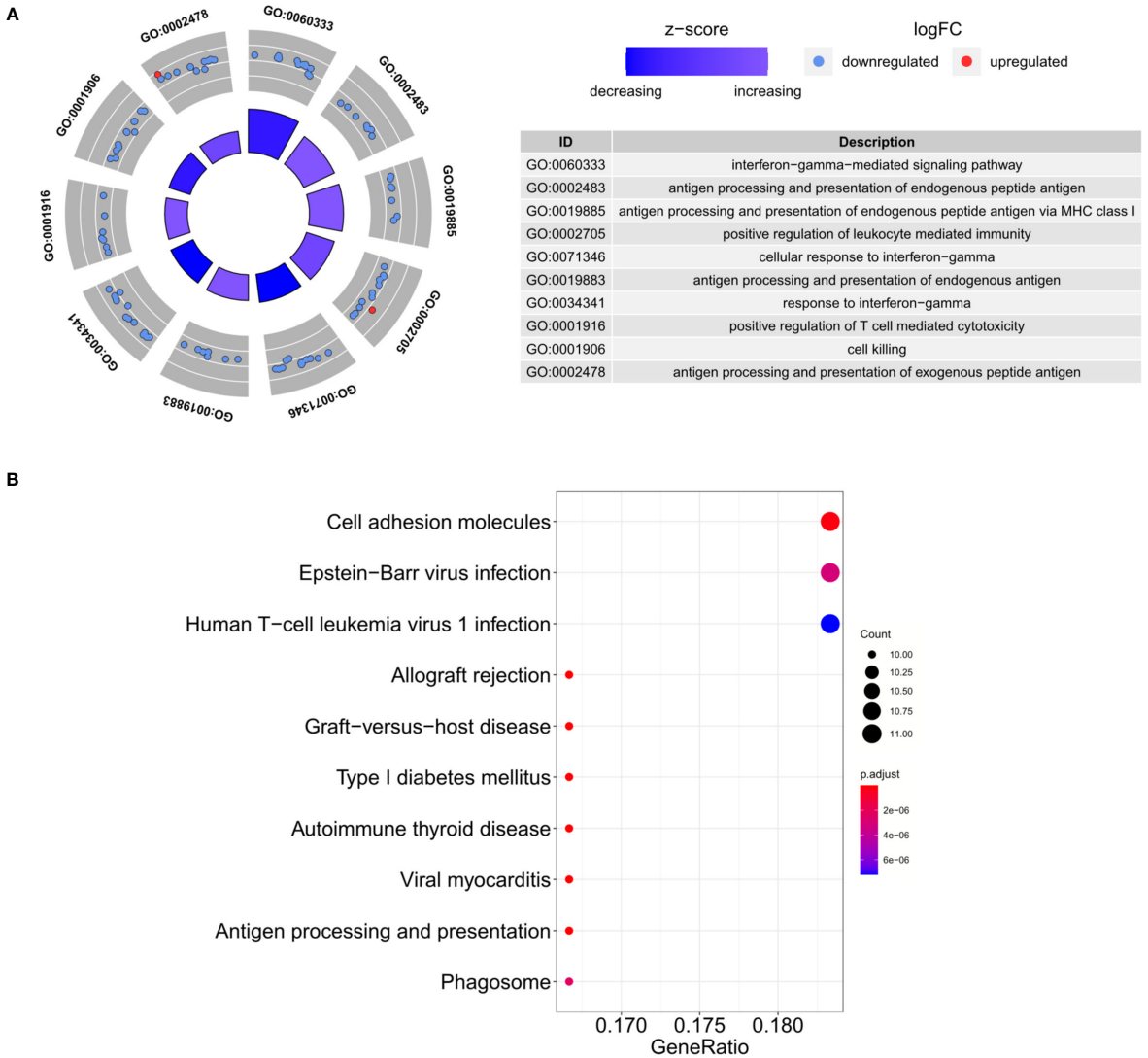


FIGURE 2 | Heatmap of differentially expressed m^{6}A-related genes. The up- and down-regulation of genes are indicated with red and blue color, respectively.



ID	Description
GO:0060333	interferon−gamma−mediated signaling pathway
GO:0002483	antigen processing and presentation of endogenous peptide antigen
GO:0019885	antigen processing and presentation of endogenous peptide antigen via MHC class I
GO:0002705	positive regulation of leukocyte mediated immunity
GO:0071346	cellular response to interferon−gamma
GO:0019883	antigen processing and presentation of endogenous antigen
GO:0034341	response to interferon−gamma
GO:0001916	positive regulation of T cell mediated cytotoxicity
GO:0001906	cell killing
GO:0002478	antigen processing and presentation of exogenous peptide antigen

FIGURE 3 | Functional enrichment analysis. **(A)** The top 10 GO biological process categories. **(B)** The top 10 KEGG pathways.

GDM compared to controls. By contraries, an increase in the intermediate subset and a decreased frequency of classical monocytes were detected in healthy pregnancy compared to non-pregnant women (30). Considering that the variation in levels of monocyte subsets may contribute to the development of inflammation in GDM, it is essential to develop new studies on this topic to validate these findings. During gestation, bone marrow-derived monocytes can migrate from the bloodstream to the uterus and differentiate into decidua-specific macrophages upon exposure to this local microenvironment (31–34). A proportion of tissue-resident macrophages is constantly replaced by blood monocytes, and the mechanisms behind these differential renewal patterns are not fully understood and may be controlled by the tissue specific microenvironment (27, 35, 36). Inflammatory stimuli often depleted macrophages and induce monocyte recruitment; these monocytes might potentially contribute to tissue-resident macrophages upon the resolution of inflammation (27). Therefore, the decrease in macrophages and increased monocytes may be due to the inflammation during GDM. Decidual macrophages are highly plastic (37). It is generally accepted that macrophages are mainly the M1 (pro-inflammatory) phenotype during the pre-implantation period, and change to M2 (anti-inflammatory) phenotype following trophoblast attachment and invasion; macrophages seem to revert to M1 phenotype at the time of delivery (37–39). Inappropriate macrophage polarization may cause adverse pregnancy outcomes (30, 37). There are controversies regarding the use of placental macrophages in describing GDM. An imbalance of M2 to M1 macrophages has been observed in the placentas of diabetic patients and rats (40), as well as in placentas of GDM patients (41). Opposing conclusions have been reported in other studies, in which macrophages maintain the M2 phenotype in GDM compared to controls (42–44). In the present study, we also

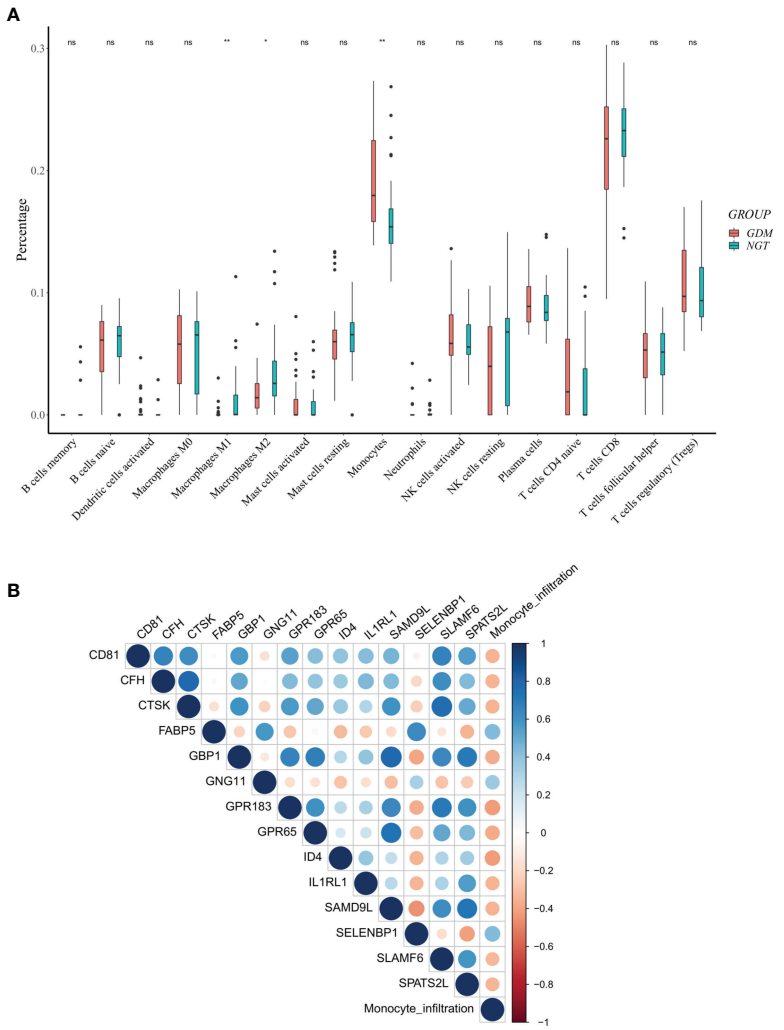


FIGURE 4 | Differentially expressed m^{6}A-related genes (DMRGs) related to monocyte infiltration in GDM Pregnancies. **(A)** Landscape of immune infiltrations in GDM Pregnancies. **(B)** Correlation among the abundance of monocytes and 14 correlated DMRGs. Asterisks denote statistical significance (ns, no significance; *p < 0.05; **p < 0.01).

observed higher propotion of M2 than M1 phenotype in GDM compared to controls (**Supplementary Figure 1**).

m^{6}A methylation plays a vital role in glucose/lipid metabolism as well as its related chronic inflammatory processes (23, 45–47). FTO moduates glucose metabolism *via* regulating forkhead box protein O1 and activating transcription factor 4 of m6A modification (46, 48). FTO also regulates adipogenesis by controlling cell cycle progression in an YTHDF2 dependent mechanism (45). METTL3 regulates lipid metabolism *via* mediating JAK1 mRNA stability an m6A-YTHDF2 dependent manner (47), and regulating NF-κB and MAPK *via* meditating m6A modification of TNF receptor associated factor 6 (49). Due to the dynamic and reversible nature, m6A methylation can be reversed by environmental stressors, including changes in nutrition. High-fat diet affecs METTL3 and FTO mRNA expression, and fasting state leads to the reduced FTO mRNA expression and increases m6A levels (50, 51). It remains unknown whether m^{6}A modifications play a role in GDM. Exploration of the crosstalk between m6A modification and GDM may provide a potential strategy for the diagnosis, prognosis and treatment. We obtained m^{6}A-related genes from the RMBase and RMvar databases and identified DMRGs based on the GSE70493 dataset. Enrichment analysis was conducted to determine the biological functions of the DMRGs. Notably, several pathways, such as type 1 diabetes mellitus and autoimmune thyroid disease, were closely correlated with the development and mal outcome of GDM. Recent studies have revealed that a small but significant population of patients with GDM develop postpartum T1DM (52, 53). Emerging evidence suggests that perturbations of the thyroid hormone signaling pathway and antibodies are associated with GDM development and adverse outcomes (54, 55). In terms of the

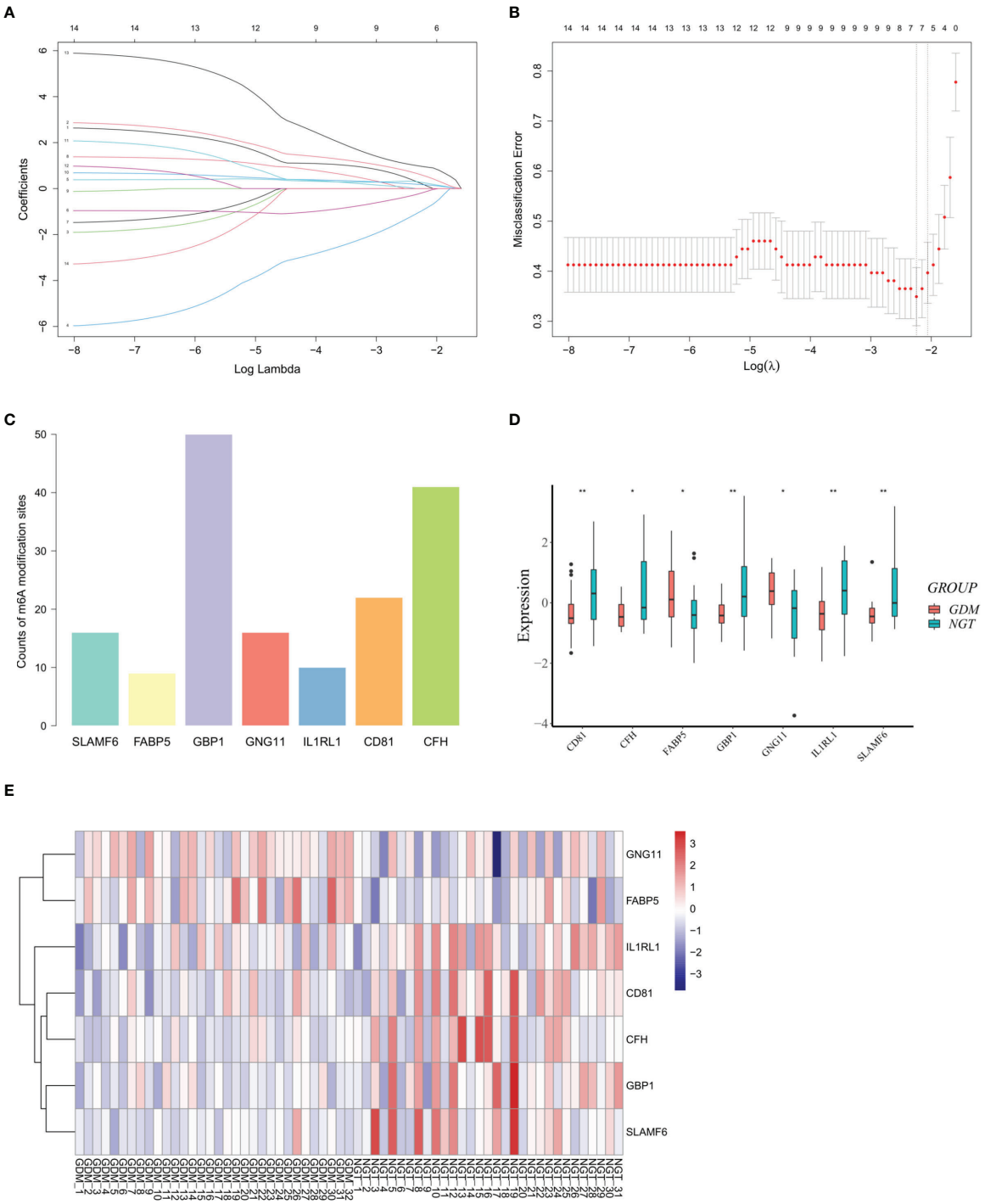


FIGURE 5 | Differentially expressed m^{6}A-related genes (DMRGs) signature selection through LASSO regression analysis. **(A)** LASSO coefficient profiles of 14 differentially expressed m^{6}A-related genes (DMRGs). The coefficient profile plot was produced against the log (lambda). **(B)** The partial likelihood deviance (binomial deviance) curve was plotted versus log (lambda) to verify the optimal lambda value. Dotted vertical lines were drawn based on the 1-SE criteria. Seven DMRGs with non-zero coefficients were selected by optimal lambda. **(C)** Counts of potential m^{6}A modification sites of the selected DMRGs. **(D)** Relative expression levels of *CD81, CFH, FABP5, GBP1, GNG11, IL1RL1*, and *SLAMF6*. **(E)** Hierarchical clustering of the expression pattern of *CD81, CFH, FABP5, GBP1, GNG11, IL1RL1*, and *SLAMF6*. Asterisks denote statistical significance (*p < 0.05; **p < 0.01).

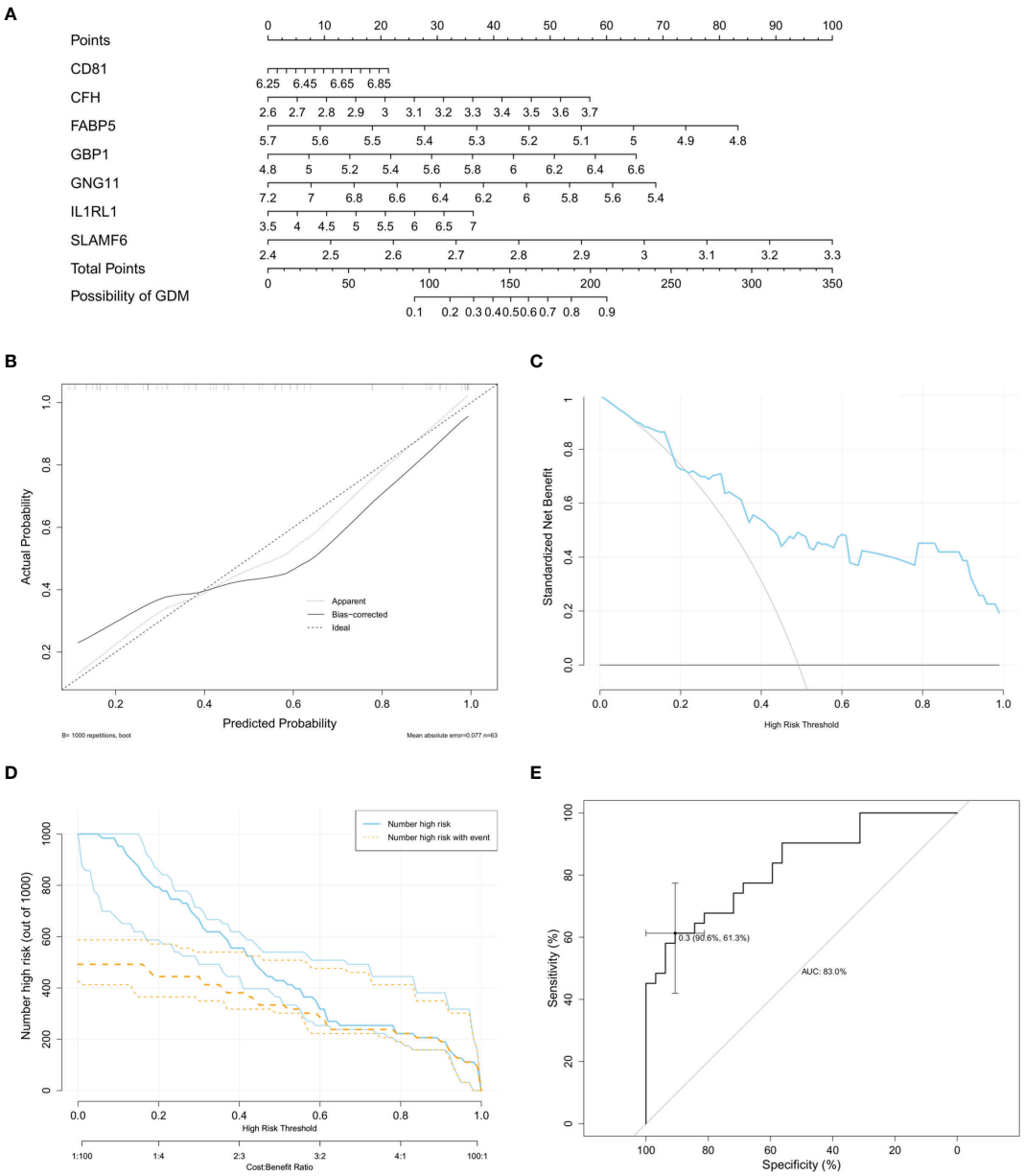


FIGURE 6 | Development and internal validation of a nomogram model for GDM based on GSE70493. **(A)** Nomogram model for patients with GDM. **(B)** Calibration curve for predicting possibility of GDM. Decision curve **(C)** and clinical impact curve **(D)** for assessing the net benefit of the nomogram. **(E)** ROC curve to assess classifying ability of the nomogram model.

GO biological process category, the DMRGs were closely related to inflammatory- and immune-related biological processes. Therefore, we suggest that, in addition to chronic inflammation, the immune response may also contribute to the pathophysiology of GDM.

As stated above, monocyte infiltration is aberrant in the placentas of patients with GDM. We obtained DMRGs related to monocyte infiltration, of which seven DMRGs (*CD81, CFH, FABP5, GBP1, GNG11, IL1RL1*, and *SLAMF6*) were selected through LASSO regression analysis to construct a nomogram. FABP5 belongs to the calycin superfamily and fatty-acid binding protein family, and serves as a gatekeeper for mitochondrial integrity to modulate regulatory T cells (Treg) and subdue immune responses (56). It has been reported that increased intra-tumoral FABP5 contributes to CD8+ T-cell infiltration and is linked to overall and recurrence-free survival, indicating that FABP5 could be an immunometabolic marker in hepatocellular carcinoma (57). Moreover, FABP5 has been observed to be enriched in classical monocytes of heart failure patients, suggesting that FABP5 contributes to monocyte

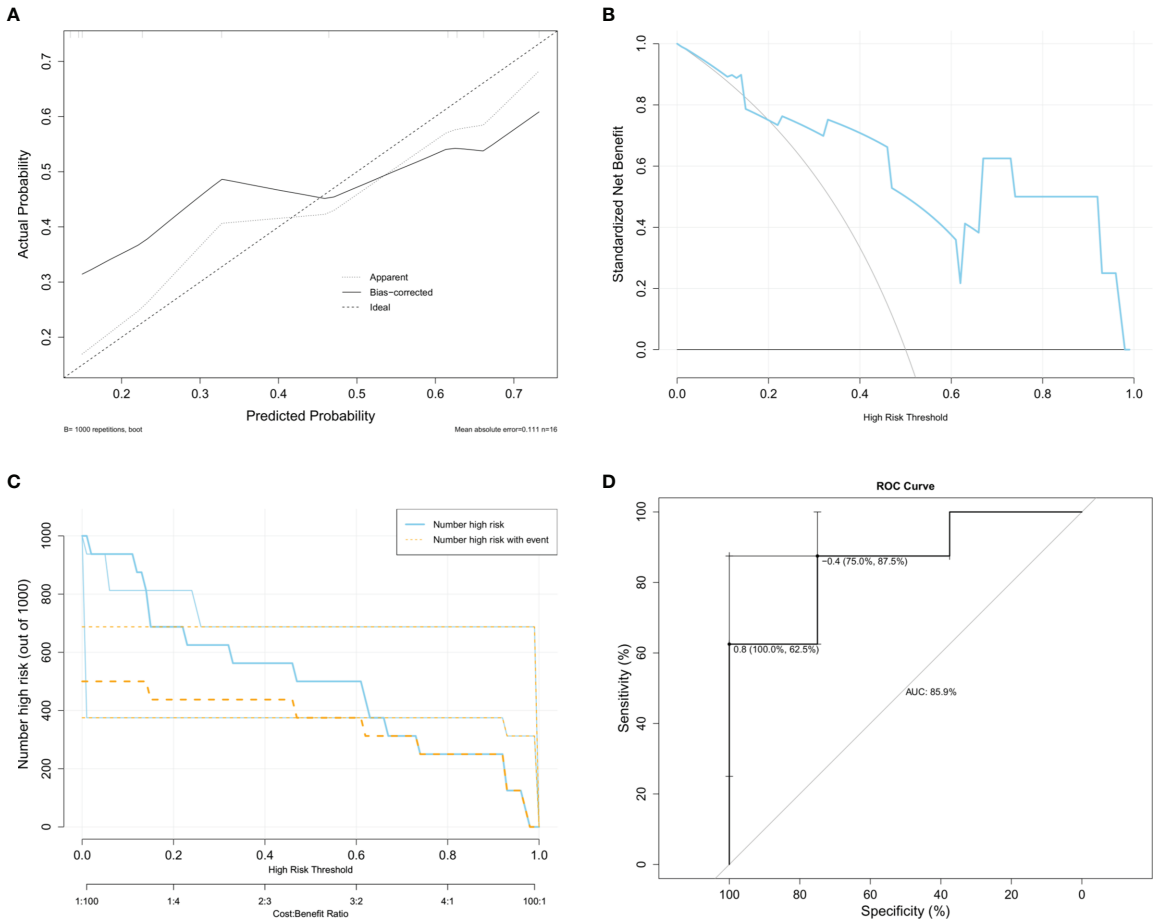


FIGURE 7 | Diagnostic value of the monocyte infiltration related DMRGs signature based on GSE92772. **(A)** Calibration curve to identify the diagnostic value for GDM. Decision curve **(B)** and clinical impact curve **(C)** for assessing the clinical usage. **(D)** ROC curve used for assessing the sensitivity and specificity of the model.

activation (58). CD81 is a tetraspanin that participates in adaptive immunity and host-virus interactions (59, 60). As an inhibitor of the alternative complement pathway, CFH protects self-surfaces from immune attacks, thereby engaging in host-virus interactions and innate immunity (61–63). GBP1 is involved in macrophage apoptosis and pyroptosis (64). Interleukin-33 (IL-33) is the only known ligand of IL1RL1, and IL1RL1/IL-33 signaling participates in various inflammatory diseases (65). SLAMF6 is expressed in a variety of immune cells and may be involved in crosstalk between different microenvironments (66–68). Finally, GNG11, a member of the guanine nucleotide-binding protein family, is involved in various transmembrane signaling systems (69, 70). The nomogram showed a robust performance in distinguishing GDM patients from normal controls in the training set (GSE70493), with an AUC of 83%. GDM diagnosis is usually confirmed by a 75 g-oral glucose tolerance test during the second trimester. GSE92772, which is based on blood samples extracted during 24–28 weeks of gestation, was selected to externally validate the diagnostic capacity of the nomogram. The nomogram model exhibited a high diagnostic value with an AUC value of 85.9%, although it lacked the expression matrix of CFH. Therefore, our findings suggest that this m^6A-related signature, correlated with monocyte infiltration, can be regarded as a novel biomarker and potential therapeutic target for GDM.

This study had a few limitations. A comprehensive analysis of the placenta and peripheral blood is warranted to verify the mRNA expression, protein expression and m^6A-modification status of CD81, CFH, FABP5, GBP1, GNG11, IL1RL1, and SLAMF6. The diagnostic ability of the nomogram model may require further validation using a larger sample size. For subsequent research, more clinical parameters regarding valuable prognosis risk characteristics should be incorporated to verify the predictive ability of the nomogram.

CONCLUSION

In this study, we analyzed the immune landscape and DMRGs in the placentas of patients with GDM. Some DMRGs were strongly associated with monocyte infiltration, which was higher in GDM placentas than in the control group. Based on seven selected

DMRGs linked to monocyte infiltration in GDM placentas, we developed and validated a highly accurate nomogram for recognizing GDM.

DATA AVAILABILITY STATEMENT

The raw data of GSE70493 and GSE92772 were obtained from public databases (https://www.ncbi.nlm.nih.gov/geo/). The processed data are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

RD: Conceptualization, Data curation, Formal analysis, Software, Writing—original draft. YW Conceptualization, Supervision, Validation, Writing—review and editing. LL: Funding acquisition, Methodology. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.853857/full#supplementary-material

Supplementary Figure 1 | Higher proportion of M2 than M1 phenotype of macrophages in GDM compared to controls.

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OPINION
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The Role of LTB4 in Obesity-Induced Insulin Resistance Development: An Overview

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INTRODUCTION

Obesity prevalence in a global context has been identified as a trigger that causes increased risk for developing chronic diseases, largely due to the presence of subclinical inflammation (1). In fact, changes in glucose and fatty-acid metabolism have been associated with diets involving excessive consumption of sucrose and saturated fat (2). This dietary pattern leads to changes in immune system activity such as an increase in macrophage infiltration and its polarization towards the M1 phenotype, which in turn deregulates the M1/M2 ratio, reducing the degree of tissue remodeling, and local homeostasis of insulin-sensitive tissues. In addition, there is a relationship between the M1/M2 imbalance and proinflammatory markers of adaptive immunity in the context of chronic diseases (3). In this regard, the role played by leukotriene B4 (LTB4), a proinflammatory lipid mediator produced from arachidonic acid (AA), has been highlighted (4). In this context, the high-fat diet (HFD) is probably a strong stimulus for LTB4 synthesis as this diet increases AA levels and consequently the production of lipid mediators in the visceral adipose tissue (5). In other words, a potent stimulus for LTB4 synthesis is possibly HFD-induced lipotoxicity. The LTB4 synthesis is enhanced in other instances than obesity since the LTB4/LTB4R1 axis is important for the immune system during an acute infection (6). In addition, LTB4 is also increased in atherosclerosis and arthritis, pathologies that are associated with chronic inflammation (7–9). Despite the increase in LTB4/LTBR1 being not only specific for obesity, it is well documented that LTB4 has a pivotal role in sustaining proinflammatory status in the context of obesity and insulin resistance (IR), due to its capacity to promote migration of the M1 macrophage when coupled to its receptor, referred to as LTB4R1 (10). Furthermore, Li and colleagues (11) have established the relationship between the LTB4-LTB4R1 system and glucose metabolism in *in vivo* and *in vitro* studies. Indeed, they have shown that LTB4 treatment impairs insulin-stimulated glucose transport by decreasing insulin-stimulated Akt phosphorylation due to IRS-1 serine phosphorylation, which in turn inhibits Glut4 translocation in L6 myocytes. In contrast, they also observed that the LTB4R1 inhibitor (CP105696) restores insulin sensitivity, as evidenced by increasing the glucose infusion rate during hyperinsulinemic-euglycemic clamp studies of C57BL rodents fed on a HFD for 14 weeks. Given the relevance obtained in proving the role played by LTB4 in the context of obesity and IR, in the current article we focus on evidence that shows LTB4 acting on inflammation developed due to HFD-induced obesity, along with the potential strategies used to mitigate the connection between LTB4-LTB4R1.

THE UNDERLYING MECHANISMS OF ACTIVATION OF THE LTB4-LTB4R1 AXIS IN INSULIN RESISTANCE

When Li and colleagues (11) achieved the inhibition of the LTB4/LTB4R1 axis, either by knock out or pharmacological inhibition of LTB4R1, and observed that LTB4 can directly promote IR, the understanding of the underlying mechanisms became intriguing. In this regard, they uncovered that the G protein-coupled receptor (Gαi) and c-Jun N-terminal kinases (JNK) activity as the mediators of LTB4/LTB4R1 deleterious effects in obesity. To elucidate the role of Gαi the authors pre-treated myocytes with the Gαi pharmacological inhibitor, pertussis toxin, that resulted in blockade of LTB4 effects to impair insulin signaling (**Figure 1**). In contrast, as LTB4-LTB4R1 can induce 307 serine phosphorylation of IRS-1 in obesity, which in turn interferes in the insulin signaling pathway, the same authors also assessed the effects of the inhibition of serine kinases IKK, ERK and JNK. While knockdown of IKK and ERK had no effects on LTB4-induced IR, the pharmacological inhibition of JNK blunted the negative effects of LTB4 in glucose transport in L6 myocytes. Taken together, the results obtained in the study by Li and colleagues indicate that LTB4 can cause IR by a mechanism involving JNK activity.

Interestingly, the relationship among LTB4, LTB4R1 and Gαi had already been addressed before by Wang and co-workers (13), who demonstrated that this axis could modulate microRNAs associated with activation of the MyD88-mediated macrophage. Indeed, macrophages harvested from the peritoneum of mice and cultured 24 h with LTB4 showed an increase in the levels of miR-155, a micro-RNA able to reduce SOCS1 expression, which in turn increases MyD88 levels allowing higher toll-like receptor (TLR) activation. They also demonstrated that LTB4-induced miR-155 expression is mediated by AP-1 since the pretreatment with AP-1 inhibitor SR11302 blunted the effects of LTB4 on miR-155 expression. In accordance, Gaudreault and coworkers (14) previously demonstrated that the LTB4-LTB4R1 axis can enhance TLR-induced AP-1 activity through TAK1 phosphorylation. Furthermore, LTB4 treatment alone is able to induce proinflammatory effects, such as the release of RANTES and IL-6 (15). Taken together, these studies highlighted that one of mechanisms by which the LTB4-LTB4R1 axis induces inflammation is through enhancing the TLR signaling pathway.

IS THERE A CORRELATION BETWEEN LTB4-LTBR1 AND POLYUNSATURATED FATTY ACIDS?

Western diets usually have a huge level of omega (ω)-6 compared to a content of ω-3; this difference achieves a ratio of 20:1 instead of 1:1 which is the recommendation for health, thus increasing risk of some diseases (16). Such imbalance might result in

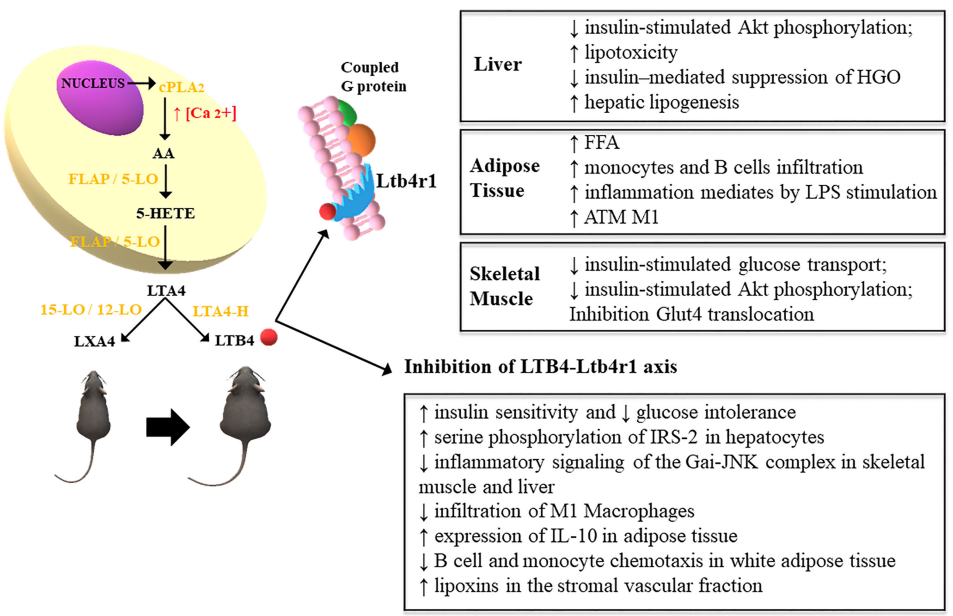

FIGURE 1 | Leukotriene synthesis occurs from the release of arachidonic acid and the action of cytosolic phospholipase A2 (cPLA2) due to the increase in intracellular calcium, which increases the expression of 5-lipoxygenase (5-LO), which translocates to the cell nucleus by interacting with 5-LO membrane anchor activating protein (FLAP). This leads to the formation of 5-hydroperoxyeicosatetraenoic acid (5-HETE) which gives rise to the formation of LTA4, converted to LTB4 by the action of the enzyme LTA4 hydrolase (LTA4-H) in obese rodents. In turn, lean animals convert LTA4 into lipoxins (LXA4) through the action of the enzyme 15/12-LO (12). The increased expression of LTB4 exerts inflammatory effects through the high-affinity interaction with its G protein-coupled receptor, Ltb4r1. This mechanism impairs the metabolism of glucose and fatty acids in the liver, TA and skeletal muscle. These effects are attenuated through the pharmacological inhibition of LTB4R1 (11).

increased inflammation, since the products derived from ω-6 such as prostaglandin E2 (PGE2) and LTB4 are strong inflammatory mediators compared to similar mediators that come from ω-3 (17). In this context, Li J. and colleagues (17) investigated the importance of ω-3 and ω-6 polyunsaturated fatty acids (PUFA) in the development of obesity and IR. For this purpose, they utilized Fat-1 transgenic mice that convert ω-6 into ω-3, i.e., these animals not only showed high levels of ω-3 but also reduction of ω-6. These alterations in the omega content increased the energy expenditure that confers to these animals a resistance to develop obesity during an HFD challenge. Interestingly, fat-1 mice exhibited increased glucose tolerance and even insulin sensitivity, i.e., these animals showed a protection to HFD-induced IR. The results also showed that compared to wild type, the transgenic mice exhibited an important reduction in LTB4 content accompanied by reduced inflammation, as evidenced by reduced levels of MCP-1 and TNF-α. Taken together, the results of their study indicate that an HFD increased ω-6 concentration and consequently LTB4 levels, which in turn induced inflammation and IR. Furthermore, PGE2, a lipid mediator derived from arachidonic acid, as well as LTB4, is increased in obesity (18). Indeed, a previous study has demonstrated that PGE2 worsens insulin resistance induced by interleukin 6 in hepatocytes (19). Despite this, we did not deeply address this point in this manuscript because it was focused on LTB4/LTB4R1. Thus, further studies are necessary to elucidate the role of each lipid mediator in the development of IR in the context of obesity.

Increased levels of free fatty acids are certainly one of the mechanisms involved in the proinflammatory state that link obesity to insulin resistance (20). Indeed, palmitate, a saturated fatty acid, can induce proinflammatory M1 macrophage polarization, which in turn promotes insulin resistance (21). Thus, palmitate has been extensively used to induce inflammation and insulin resistance (22, 23). In contrast, oleate, an unsaturated fatty acid, can improve insulin sensitivity (24, 25). In this context, a study performed by Pardo and colleagues (26) observed that preincubation with culture medium of RAW 264.7 macrophages treated with palmitate was able to decrease insulin-induced IR and Akt phosphorylation in hepatocytes, while such effect was not observed if macrophages were loaded with oleate. They also showed that preincubation with palmitate increased proinflammatory cytokines in macrophages, which in turn induced ER stress in hepatocytes, while these effects were not seen with oleate preincubation. In addition, the culture medium of macrophages pretreated with oleate exhibited lower levels of LTB4 compared to the culture medium incubated with palmitate. In contrast, when LTB4 was added to oleate in preincubation of macrophages, the protection of insulin signaling was missing in hepatocytes. Thus, this study has confirmed by *in vitro* studies ω-3 is able to attenuate LTB4 (26). In accordance, a recent study *in vivo* showed that HFD enriched with ω-3 (eicosapentaenoic acid) downregulated LTB4 levels and inflammation in visceral adipose tissue of mice (27). In summary, these data point out those strategies that decrease ω-6/ω-3 ratio are promising to lower LTB4 levels, thus preventing inflammation and IR in obesity.

LTB4/LTB4R1 AXIS PROMOTES PROINFLAMMATORY PHENOTYPE OF B CELLS WHICH IN TURN ORCHESTRATE OVERALL INFLAMMATION IN ADIPOSE TISSUE IN OBESITY

Despite macrophages being the most abundant immune cells infiltrated into tissues, other immune cells such as leukocytes, lymphocytes, neutrophils, and eosinophils, are also present and may participate in the inflammatory process associated with obesity and IR. In this context, Nishimura and coworkers (28) demonstrated that CD8+ effector T cells are increased, while CD4+ helper and regulatory T cells decrease in adipose tissue in obesity. Furthermore, the activation of CD8+ T cells allows the recruitment of macrophages and their polarization toward M1, which evidences a role of adaptive immunity in the development of IR associated with obesity. In addition, another cell of the adaptive immune system that plays a role in obesity-induced IR is the B lymphocyte, since it is in adipose tissue in obesity and its genetic depletion reduces HFD-induced IR (29, 30).

However, the underling mechanisms related to B cell recruitment to adipose tissue were not uncovered until an article published in 2018 (3). In their elegant study, Ying W and colleagues by using knock out mice models and immune cell transplants, showed that the LTB4/LTB4R1 axis drives the attraction of B2 cells to adipose tissue during HFD feeding and directly stimulates a proinflammatory phenotype in these cells, promoting IR. They also demonstrated that B2 cells orchestrate IR by inducing Th1 response in lymphocytes and macrophage polarization towards M1. Despite this, the authors also concluded that macrophages can also induce IR by other mechanisms than this connected to B2 cells, since macrophage depletion achieved by using clodronate resulted in more pronounced effects in insulin sensitivity. Furthermore, their study also evidenced that the pivotal source of LTB4 are adipocytes because B cells still accumulate in the adipose tissue after depletion of macrophages and T cells. However, as adipose tissue also presents eosinophiles, neutrophiles and other immune cells (3, 31), we cannot rule out their participation, besides adipocytes, in LTB4 synthesis during obesity development. Also, as TNF-α, IL-6, and IL-1β increase in obesity by several mechanisms such as TLR4 activation, ER stress, among others (32–34), it is reasonable to assume that the increased levels of these cytokines can also enhance LTB4 synthesis and action. We also hypothesized that LTB4 and proinflammatory cytokines may work in a positive feedback loop. However, this point deserves its own review article. Taken together, these results unveiled that different immune cells orchestrate adipose tissue inflammation during obesity development, and shed light on LTB4R1 as a potential therapeutic target to improve IR.

CONCLUSIONS

In conclusion, the studies of the Li and colleagues group discussed in this manuscript highlighted that several types of immune cells coordinate adipose tissue inflammation during obesity development and that LTB4/LTB4R1 has an important role in the inflammation-induced IR by a mechanism that involves JNK activation. Thus, further studies should investigate potential strategies to blunt LTB4-LTB4R1 (3, 11) (**Figure 1**). We also addressed articles regarding the relationship between PUFAs and LTB4 (17, 26). These data allow a conclusion that a high ω-6/ω-3 ratio increases LTB4 levels, i.e., collaborates with development of inflammation and IR in obesity (16), thus strategies that lower ω-6/ω-3 ratio are promising to reduce LTB4 levels and therefore deserve further investigation.

AUTHOR CONTRIBUTIONS

IC and AO contributed to discussion, and wrote, reviewed, edited manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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A Hypothesis From Metabolomics Analysis of Diabetic Retinopathy: Arginine-Creatine Metabolic Pathway May Be a New Treatment Strategy for Diabetic Retinopathy

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Diabetic retinopathy is one of the serious complications of diabetes, which the leading causes of blindness worldwide, and its irreversibility renders the existing treatment methods unsatisfactory. Early detection and timely intervention can effectively reduce the damage caused by diabetic retinopathy. Metabolomics is a branch of systems biology and a powerful tool for studying pathophysiological processes, which can help identify the characteristic metabolic changes marking the progression of diabetic retinopathy, discover potential biomarkers to inform clinical diagnosis and treatment. This review provides an update on the known metabolomics biomarkers of diabetic retinopathy. Through comprehensive analysis of biomarkers, we found that the arginine biosynthesis is closely related to diabetic retinopathy. Meanwhile, creatine, a metabolite with arginine as a precursor, has attracted our attention due to its important correlation with diabetic retinopathy. We discuss the possibility of the arginine-creatine metabolic pathway as a therapeutic strategy for diabetic retinopathy.

Keywords: diabetic retinopathy, metabolomics, biomarker, creatine, arginine, mechanism

Abbreviation: DR, Diabetic retinopathy; T2DM, type 2 diabetes mellitus; PKC, protein kinase C; Cr, creatine; PCr, phosphocreatine; CK, creatine kinase; NMR, nuclear magnetic resonance; MS, mass spectrometry; LC, liquid chromatography; GC, gas chromatography; HILIC-MS, hydrophilic interaction chromatography-mass spectrometry; FIA–MS, Flow-injection analysis-mass spectrometry; CE, capillary electrophoresis; HPLC, high-performance liquid chromatography; UPLC, ultra-high performance liquid chromatography; CE, Capillary electrophoresis; PDR, proliferative diabetic retinopathy; OIR, oxygen induced ischemic retinopathy; GC-MS, gas chromatography mass spectrometry; UPLC-MS, ultra-performance liquid chromatography-mass spectrometry; TCA, tricarboxylic acid cycle; LC-MS, liquid chromatography-mass spectrometry; HILIC-MS Hydrophilic interaction chromatography-mass spectrometry; UPLC-Q-Axis Orbiter-MS, ultra-performance liquid chromatography-quadrupole-Exactive Orbitrap-mass spectrometry; IDO, indolamine-2,3-dioxygenase; UACR, albumin/creatinine; KEGG, Kyoto Encyclopedia of Genes and Genomes; NOS, nitric oxide synthase; NO, nitric oxide; EDHF, endothelium-derived hyperpolarizing factor; GAA, guanidinoacetate; Hcy, homocysteine; ECs, endothelial cells; EC, endothelial cell; LPC, Lysophosphatidylcholine; ICAM-1, increased intercellular cell adhesion molecule-1.

INTRODUCTION

Diabetic retinopathy (DR) has been recognized as the main cause of blindness worldwide, with about one-third of all diabetes patients developing diabetic retinopathy (1). The retina is metabolically active and transmits electrochemical signals from photoreceptors to the brain *via* neurons, supported by glial cells and vascular tissue (2). The entire process relies on highly complex coordination between the various cell types, and the blood-vision barrier plays a key role (3, 4). The accumulation of glycation end products, oxidative stress, polyol pathway and protein kinase C (PKC) activation are the main pathogenesis of DR. This changes the normal interaction between cells and causes serious blood vessel abnormalities leading to damaging of the blood-retinal barrier and neuronal function (5–9). Diabetic retinopathy is difficult to cure, diagnosis and drug intervention in the early stages of diabetic retinopathy can effectively prevent or slow down the progression of disease. Therefore, identification of biomarkers associated with disease progression can be very helpful.

Metabolomics is the analysis of a large number of endogenous small molecules. It provides the overall metabolic profile of a biological sample as opposed to genomics and proteomics, which provide the profiles for DNA/RNA and proteins alone, respectively (10–12). The methods of analysis used in metabolomics are mostly classified into two categories: targeted metabolomics and non-targeted metabolomics (13–15). In contrast to targeted metabolomics, which focuses only on changes in specific metabolites, non-targeted metabolomics is designed to capture much more metabolite information to compare these high-throughput data under normal vs. disease states (15–17). Non-targeted metabolomics approaches can thus discover potential biomarkers of diseases and provide an effective basis for diagnosing and treating them (18–20).

Arginine, a semi-essential amino acid, involved in many biological processes such as creatine biosynthesis and the urea cycle, is one of the strongest insulin secretagogues, which induce insulin release from pancreatic β cells (21). Additionally, arginine is a substrate for nitric oxide synthase (NOS) and can produce NO, which exerts a significant influence on the health of the vascular endothelial cells as well as the kidneys (22, 23). Creatine (Cr) can be either be synthesized endogenously within the body or extrinsically derived from foods like meat, fish, etc. (24). Cr, phosphocreatine(PCr), and creatine kinase (CK) isoenzymes are responsible for maintaining the ATP pool (25). Therefore, creatine is one of the leading sports supplements (26). As research continues, Creatine has been found to have multiple physiological effects, including anti-inflammatory (27–29), antioxidant (30–33), neuroprotective (34), reduce homocysteine (Hcy) (35–37), and anti-diabetic (34).

This review aims to summarize the progress of metabolomics studies in diabetic retinopathy and to explore common research platforms for metabolomics. We also summarize the current knowledge of known metabolomics biomarkers of diabetic retinopathy based on literature and analyze the metabolic pathways involving those biomarkers. In addition, we discuss the creatine-arginine metabolic network as a potential area for finding new treatment strategies.

METABOLOMICS ANALYSIS PLATFORM

Metabolomics analysis platform can be divided into two main types, nuclear magnetic resonance (NMR) spectroscopy (38) and mass spectrometry (MS) (39, 40). Using different instruments and platforms, typically 50 to as many as 5000 different metabolites can be identified at any given time. No technique so far has been successful in identifying all metabolites in a single run or analysis, and most metabolomics studies use only one platform or multiple tandems. Due to the complementarity between NMR (41) and MS (42), researchers often use combinations of NMR and MS as well as employ the current method to enhance research quality and expand the metabolome coverage (43–46).

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy can measure the behavior of an atom's nucleus when subjected to a magnetic field (47, 48). Currently, instruments that use 500 and 600 MHz frequencies are the most widely used instruments to detect these signals and are the optimal choice for their sensitivity and manufacturing cost. It is worth noting that the resolution of these signals increases when the magnetic field strength is higher (49).

NMR spectroscopy applies to both liquid/gas phase samples as well as tissue samples (50, 51). It carries several advantages, for example, it requires less sample preparation and the detection process is non-destructive to the sample, so it can be reused for other studies. Moreover, NMR has high reproducibility and good quantitative performance, allowing the measurement of the number of protons under a given condition which allows for direct comparison with spectral data (52). However, the primary disadvantage of NMR is its lower sensitivity compared with MS. NMR can identify nearly 50 metabolites in serum/plasma samples and approximately 200 in urine (53).

Mass Spectrometry (MS)

Mass spectrometry is an analytical method that measures the ion-to-mass ratio based on the ionization of components in the samples by an ion source, and is widely used in the detection of metabolites (54–56). The sample can be directly analyzed by mass spectrometry, or in tandem with other separation methods to obtain mass spectra, such as liquid chromatography (LC) (57–59), gas chromatography (GC) (60, 61), hydrophilic interaction chromatography-mass spectrometry (HILIC-MS) (62), Flow-injection analysis-mass spectrometry(FIA–MS) (63), or capillary electrophoresis (CE) (64, 65). It should be noted that no single method can separate all metabolites simultaneously, as some metabolites are difficult to ionize, and in some cases, mass number limitations prevent mass spectrometry techniques from measuring all metabolites (66). LC has been most widely used because of its better separation. Especially, high-performance

liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UPLC) have become increasingly popular (67–69). GC also offers high separation, but it is unable to measure metabolites with poor thermal stability (70). Capillary electrophoresis (CE) has a long history of use. Its application is mainly limited by its poor sensitivity, which has been greatly improved by the introduction of the CE-ESI interface (71, 72).

Compared to NMR, MS has a much higher sensitivity and is therefore able to measure a wider range of metabolites (40, 43, 73, 74). In particular, UPLC offers excellent chromatographic separation, high speed, and high sensitivity, allowing the detection of thousands of metabolites within a short time (75–78). HPLC tandem MS plays a huge contribution in research that requires high throughput, such as natural drug development and disease biomarker identification (79–83).

BIOMARKERS FOR DIABETIC RETINOPATHY

Vitreous Humor Biomarkers

Tomita et al. (84) analyzed the metabolites of vitreous humor in 43 proliferative diabetic retinopathy (PDR) patients, and 21 controls using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) with significant differences in creatine. The authors found that patients with PDR had lower levels of creatine and higher levels of glycine in the vitreous humor than controls. They also verified in an oxygen induced ischemic retinopathy (OIR) model that reduced creatine levels correlate with retinal vascular proliferation and demonstrated that oral creatine caused a significant reduction in retinal vascular proliferation (p=0.0024), opening the possibility for a new therapeutic strategy for diabetic retinopathy. Wang et al. (85) identified potential DR biomarkers in vitreous humor using gas chromatography mass spectrometry (GC-MS). Vitreous humor samples were gathered from 28 type-2 diabetes patients with PDR as well as 22 non-diabetic patients with macular fissure. They found 15 potential biomarkers in the vitreous humor, namely pyruvate, ornithine, uric acid, pyroglutamic acid, creatinine, L-leucine, L-alanine, L-threonine, L lysine, L-valine, L-phenylalanine, L-isoleucine, L-glutamine, inositol, and hydroxylamine. These are mainly involved in various metabolic pathways such as gluconeogenesis, ascorbate-aldose metabolism, valine-leucine-isoleucine biosynthesis, and arginine-proline metabolism.

A non-targeted metabolomics study on vitreous humor from patients with DR showed changes in glucose metabolism as well as activation of the pentose phosphate pathway. Glass fluid samples from PDR patients (n=9) and normal subjects were kept as controls (n=8) and were analyzed by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). A variety of metabolites were found to be potential biomarkers, including xanthine, pyruvate, proline, and guanine (86). Paris et al. (62) used liquid chromatography-mass spectrometry (LC-MS) and hydrophilic interaction liquid chromatography (HILIC)-mass spectrometry to analyze the vitreous humor of PDR patients (n=9), non-diabetes control patients (n=11), and OIR mouse model. They found significant changes in the levels of octanoyl carnitine, propionyl carnitine, hexanoyl carnitine, acetylcarnitine, palmitoylcarnitine, elaidic/vaccenylcarnitine, allantoin, glutamate, lysine, and arginine. Barba et al. (87) analyzed the vitreous humor of a total of 22 patients suffering from PDR and 22 non-diabetic patients and found that the content of lactate and glucose among the PDR patients was higher than that in non-diabetic patients, while that of galactitol and ascorbic acid was lower when compared with that in non-diabetic patients. The reduced galactitol level was attributed to activation of the polyol pathway.

Plasma Biomarkers

Plasma metabolomics of 124 DR patients and 32 controls were explored using GC–MS, and UPLC–MS. They identified glutamine and glutamic acid as new biomarkers for the prediction of DR (88). A plasma metabolomics analysis based on GC–MS demonstrated that 2,4-dihydroxybutyric acid (DHBA), 3,4-DHBA, ribonic acid, and ribitol are risk markers for DR progression as these metabolites are associated ($P < 0.042$) with DR (89). Another plasma metabolomics study using GC-MS identified 11 potential biomarkers of diabetic retinopathy, namely 1,5-gluconolactone, 1,5-anhydroglucitol, gluconic acid, lactose/cellobiose, maltose/trehalose, 2-deoxyribonic acid, 3,4-dihydroxybutyric acid, erythritol, mannose, ribose, and urea. The samples for this study were acquired from 40 patients undergoing non-proliferative diabetic retinopathy (NPDR) and 40 patients suffering from T2DM without retinopathy. Metabolic pathway analysis indicated a remarkable enrichment of the pentose phosphate pathway, which could explain the NADPH production against oxidative stress (49). Sumarriva et al. performed plasma metabolomics research showed that compared to diabetes controls, the metabolism of multiple amino acids, such as leukotrienes, niacin, pyrimidine, and purine, changed in DR patients. Arginine, citrulline, glutamic γ-semialdehyde, and de-hydroxy carnitine were critical members in the above pathways differences (90). Li et al. (91) employed GC-MS in the study of plasma metabolomics in 25 patients with PDR, 39 patients with NPDR, and 24 patients with NDR, and found 10 metabolites with significant differences: β-hydroxybutyrate, methylmalonic acid, citric acid, pyruvate, glucose, stearic acid trans-oleic acid, L-aspartate, linoleic acid, and arachidonic acid.

Serum Biomarkers

Xuan et al. (92) studied 43 patients with diabetic retinopathy and 44 normally controlled serum lipomics using UPLC-MS. Significant differences were found in the following 14 lipid metabolites: Lysophosphatidylcholine(LPC)(14:0) LPC (14:0), LPC (16:0) LPC (14:0), LPC (16:0), LPC (16:1), LPC (18:0), LPC (18:1), LPC (18:2), LPC (18:3), LPC (18:4), LPC (20:0), LPC (20:3), LPC (20:4), LPC (20:5), LPC (22:3), and LPC (22:6). These provide a basis for the discovery of lipid biomarkers in diabetic retinopathy. Xuan et al. (93) in their study used multi-platform techniques to analyze serum samples from 111 diabetic patients

without retinopathy (NDR=111) and 350 diabetic patients with retinopathy (n=350). The DR-induced metabolic changes were usually linked to glycolytic metabolism, tricarboxylic acid cycle (TCA) metabolism, urea cycle metabolism, polyol metabolism, amino acid metabolism, and lipid metabolism. Following a systematic screening using univariate analysis, 2-piperidone and 12-HETE were recognized as potential biomarkers for DR. 12-HETE, an eicosanoid-like acid, is the leading product of human 12-lipoxygenase (LOX), inducing endoplasmic reticulum stress in human retinal endothelial cells. Studies show that 12-LOX is involved in retinal microvascular disorders of DR (94–96). A study based on widely targeted metabolomics evaluated serum metabolites from 69 type 2 diabetes mellitus (T2DM) patients with DR and 69 T2DM patients without DR. The biomarkers of diabetic retinopathy identified using a UPLC-MS system were linoleic acid, nicotinuric acid, ornithine, and phenylacetylglutamine. In particular, this research developed a new multidimensional network of biomarker systems and the area under the curve (95% CI) of this system is an exploration of the biomarker determination method (97).

Zhu et al. (98) studied the serum metabolomics of 21 PDR patients and 21 diabetic patients without retinopathy (NDR) patients. A total of 63 significant changes in metabolites were found using LC-MS. Fumaric acid, uridine, acetic acid, and cytidine (area under curve 0.96, 0.95, 1.0, and 0.95, respectively) are considered potential biomarkers of PDR. A serum metabolomics study of 24 patients with PDR, 22 patients with NPDR, and 35 healthy human control groups demonstrated that compared with the control group, indolamine-2,3-dioxygenase (IDO) expression was enhanced among patients with NPDR, while the levels of kynurenine, kynurenic acid, and 3-hydroxy kynurenine were higher in PDR patients. The authors speculated that diabetic retinopathy might be related to IDO and tryptophan metabolites (99). Serum samples from patients with NPDR (n=123), PDR (n=51), and NDR (n=143) were profiled by targeted mass-spectrometry-based metabolomics. After multivariate analyses, 16 metabolites were found to show profound changes, including tetradecenoylcarnitine (C14:1), hexadecanoylcarnitine (C16), lysine, methionine, tryptophan, tyrosine, total dimethyarginine, phosphatidylcholine diacyl C32:2, phosphatidylcholine diacyl C34:2, phosphatidylcholine diacyl C36:2, phosphatidylcholine diacyl C38:6, phosphatidylcholine diacyl C40:6, phosphatidylcholine acyl-alkyl C36:5, phosphatidylcholine acyl-alkyl C42:3, hydroxysphingomyeline C22:1 and sphingomyeline C24:0 (63).

Aqueous Humor Biomarkers

Wang et al. (85) analyzed and identified potential DR biomarkers in aqueous humor of 23 patients suffering from PDR and 25 patients with non-diabetic cataracts. Eight metabolites, namely D-glyceric acid, isocitric acid, threonine, d-glucose, inositol, L-lactic acid, citrulline, and fructose 6-phosphate, were found to be significantly different in the aqueous humor by comparative analysis.

A metabolomics study based on NMR was carried out on the aqueous humor samples from diabetic patients with cataracts (n=13), DR patients with cataracts (n=14), and elderly cataracts (n=7). Metabolites such as lactate, succinate, 2-hydroxybutyrate, aspartamide, dimethylamine, histidine, threonine, and glutamine showed significant changes. Pathway analysis showed that DR might be related to alanine, aspartic acid, and glutamate metabolic pathways (100). The information of DR biomarker was listed in **Table 1**.

KEGG ENRICHMENT ANALYSIS

We enriched the above potential biomarkers according to the types of biological fluids, intending to comprehend the relationship between biomarkers and diseases. Enrichment analysis by metaPA and Kyoto Encyclopedia of Genes and Genomes (KEGG) showed that metabolic pathways enriched in the different biological fluids are unique (**Figure 1**). It is worth mentioning that arginine-related metabolism was both enriched in vitreous humor, plasma, serum, and aqueous humor. This suggests that arginine has a critical effect on diabetic retinopathy.

DISCUSSION

Biomarkers can provide early warning signs in patients with serious diseases. Therefore, they help in the early diagnosis of the disease so that effective treatment can be made available to the patient at the earliest. In this review, we have summarized the known potential biomarkers for DR, in a variety of biological samples, including vitreous humor, plasma, serum, and aqueous humor, from research done in recent years. Through enrichment analysis, we found that arginine-related metabolic pathways were abnormal in a variety of biological fluids.

Arginine Biosynthesis-Related Metabolites Are Significantly Elevated in DR Patients

The urea cycle is a part of the arginine biosynthesis pathway, and the arginase enzyme can cleave arginine to generate urea and ornithine. Ornithine can be converted into citrulline, and then citrulline is produced through a series of reactions to arginine (101). The metabolites of the urea cycle seem to have some association with DR. The metabolites of the urea cycle seem to have some association with DR.The levels of ornithine (85, 97, 102), arginine (62, 90, 102), citrulline (85, 90, 102), proline (86), and argininosuccinate (102) were significantly elevated in DR patients (**Figure 2**) (73). The above content expands our understanding of the pathogenesis of DR. The changes in the metabolites of the urea cycle, especially arginine, are significantly associated with DR.

Arginine is involved in many biological processes and is also the substrate of nitric oxide synthase (NOS) and arginase, producing nitric oxide (NO) and urea, respectively (103). NO is a vasodilator that exerts a significant influence on vascular endothelial health, while arginine induces the release of insulin in pancreatic β cells (**Figure 3**) (104). In addition, animal experiments using DR mouse models and bovine retinal endothelial cells cultivated by high glucose revealed the role of arginine metabolism as a mediator for DR (105, 106).

TABLE 1 | The information of diabetic retinopathy biomarker.

Study	Samples	Platform	Number (cases/model and controls)	Potential biomarkers	Pathways
Tomita et al. (84)	Vitreous humour	UPLC-MS	43 PDR and 21 non-diabetic epiretinal membrane	Creatine, succinate, glycine, lactate, pyruvate, proline, allantoin, urate, citrulline, ornithine, dimethylglycine, N-acetylserine, α-ketoglutarate	Glycine, serine, arginine and proline amino acid metabolism
Wang et al. (85)	Vitreous humour	GC-TOF-MS	28 PDR and 22 non-diabetic patients with macular fissure	Pyruvate, ornithine, uric acid, pyroglutamic acid, creatinine, L-leucine, L-alanine, L-threonine, L lysine, L-valine, L-phenylalanine, L-isoleucine, L-glutamine, inositol, and hydroxylamine	Gluconeogenesis, ascorbate-aldose metabolism, valine-leucine-isoleucine biosynthesis, and arginine-proline metabolism
Haines et al. (86)	Vitreous humour	UPLC-MS	9 PDR and 8 non-diabetic patients	Xanthine, pyruvate, proline, and guanine	Unclear
Paris et al. (62)	Vitreous humour	LC-MS and HILIC-MS	9 PDR and 11 non-diabetic patients	Octanoylcarnitine, propionylcarnitine, hexanoylcarnitine, acetylcarnitine, palmitoylcarnitine, elaidic/vaccenylcarnitine, allantoin, glutamate, lysine, and arginine	Unclear
Barba et al. (87)	Vitreous humour	NMR	22 PDR and 22 non-diabetic patients	Lactic acid, glucose, galactitol, and ascorbic acid	Unclear
Rhee et al. (88)	Plasma	GC–TOF–MS and UPLC–Q–TOF–MS	124 DR and 32 NDR	Glutamine and glutamic acid	Unclear
Curovic et al. (89)	Plasma	GC-MS	141 DR and 504 NDR	2,4-dihydroxybutyric acid (DHBA), 3,4-DHBA, ribonic acid, and ribitol	Unclear
Chen et al. (49)	Plasma	GC-MS	44 NPDR and 40 NDR	1,5-Anhydroglucitol, 1,5-gluconolactone, 2-deoxyribonic acid, 3,4-dihydroxybutyric acid, erythritol, gluconic acid, lactose/cellobiose, maltose/trehalose, mannose, ribose, and urea	Pentose phosphate pathway
Sumarriva et al. (90)	Plasma	LC-MS	83 DR and 90 NDR	Arginine, citrulline, glutamic γ-semialdehyde, and dehydroxycarnitine	The metabolism of multiple amino acids, leukotrienes, niacin, pyrimidine, and purine
Li et al. (91)	Plasma	GC-MS	25 PDR, 39 NPDR, and 24 NDR	Pyruvate, L-aspartate, β-hydroxybutyrate, methylmalonic acid, citric acid, glucose, stearic acid trans-oleic acid, linoleic acid, and arachidonic acid	Unclear
Xuan et al. (92)	Serum	UPLC - MS	44 PDR and 43 non-diabetic patients	LPC (14:0), LP (16:0), LPC (14:0), LPC (16:0), LPC (16:1), LPC (18:0), LPC (18:1), LPC (18:2), LPC (18:3), LPC (18:4), LPC (20:0), LPC (20:3), LPC (20:4), LPC (20:5), LPC (22:3), and LPC (22:6)	Unclear
Xuan et al. (93)	Serum	GC-MS, LC-MS	350 DR and 111 NDR	2-Piperidone and 12-HETE	Unclear
Zuo et al. (97)	Serum	UPLC-MS	69 DR and 69 NDR	Linoleic acid, nicotinuric acid, ornithine, and phenylacetylglutamine	Unclear
Zhu et al. (98)	Serum	LC-MS	44 NPDR and 40 NDR	Fumaric acid, uridine, acetic acid, and cytidine	Alanine, aspartate and glutamate metabolism, caffeine metabolism, beta-alanine metabolism, purine metabolism, cysteine and methionine metabolism, sulfur metabolism, sphingosine metabolism, and arginine and proline metabolism
Munipally et al. (99)	Serum	HPLC	24 PDR, 22 NPDR, and 35 healthy human control group	kynurenine, kynurenic acid, and 3-hydroxy kynurenine	Tryptophan metabolites
Yun et al. (63)	Serum	LC-MS and FIA-MS	123 NPDR, 51 PDR, and 143 NDR	Tetradecenoylcarnitine, hexadecanoylcarnitine, lysine, methionine, tryptophan, tyrosine, total Dimethyarginine, phosphatidylcholine diacyl C32:2, phosphatidylcholine diacyl C34:2, phosphatidylcholine diacyl C36:2, phosphatidylcholine diacyl C38:6, phosphatidylcholine diacyl C40:6, phosphatidylcholine acyl-alkyl C36:5, phosphatidylcholine acyl-alkyl C42:3, hydroxysphingomyeline C22:1, and phingomyeline C24:0	Unclear

(Continued)

TABLE 1 | Continued

Study	Samples	Platform	Number (cases/model and controls)	Potential biomarkers	Pathways
Wang et al. (85)	Aqueous humor	GC-TOF-MS	23 PDR and 25 NDR	D-glyceric acid, isocitric acid, threonine, d-glucose, inositol, L-lactic acid, citrulline, and fructose 6-phosphate	Unclear
Jin et al. (100)	Aqueous humor	NMR	13 diabetic patients with cataract, 14 DR with cataract, and 7 elderly cataract	Lactate, succinate, 2-hydroxybutyrate, aspartamide, dimethylamine, histidine, threonine, and glutamine	Alanine, aspartic acid and glutamate metabolic pathways

LC-MS, liquid chromatography-mass spectrometry; HPLC, ultra-performance liquid chromatography; UPLC-MS, ultra-performance liquid chromatography-mass spectrometry; UPLC–Q–TOF–MS, ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry; GC-MS, gas chromatography mass spectrometry; GC-TOF-MS, gas chromatography quadrupole time-of-fight mass spectrometry; HILIC-MS hydrophilic interaction chromatography-mass spectrometry; NMR, nuclear magnetic resonance; FIA–MS, flow-injection analysis-mass spectrometry; UPLC-Q-Axis Orbiter-MS, ultra-performance liquid chromatography-quadrupole-Exactive Orbitrap-mass spectrometry; DR, diabetic retinopathy; NDR, diabetic patients without retinopathy; PDR, proliferative diabetic retinopathy; NPDR, non-proliferative diabetic retinopathy; LPC, Lysophosphatidylcholine.

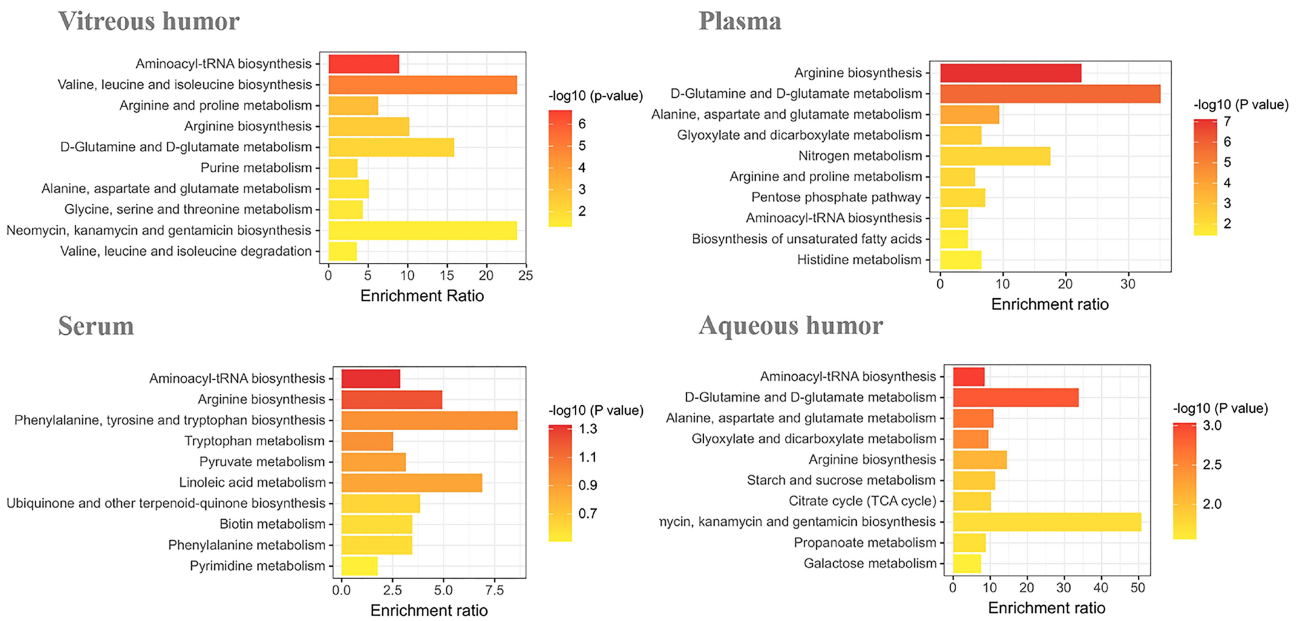

FIGURE 1 | Enrichment analysis of DR potential biomarkers in vitreous humor, plasma, serum, and aqueous humor.

Arginine-Creatine Metabolic Pathway May Be a New Therapeutic Strategy for DR

Meanwhile, another biomarker that caught our attention, creatine, a product of arginine metabolism. Unlike the elevated levels of arginine, creatine levels were significantly lower in patients with DR (84, 85). Thereby, we put forward a hypothesis that the reduced conversion of arginine to creatine leads to metabolic changes in DR patients with increased arginine levels and decreased creatine levels. Callback of this metabolic change, may be a new treatment strategy for DR. There is no strong evidence for this hypothesis, but there is substantial research supporting the positive effects of creatine supplementation on DR.

Creatine can be either be synthesized endogenously within the body or extrinsically derived from foods like meat, fish, etc. (24). There are two steps in creatine biosynthesis. The first step is to catalyze arginine and glycine with L-arginine glycine amidinotransferase (AGAT; EC 2.1.4.1) to produce ornithine and guanidinoacetate (GAA). This step mainly occurs in the kidney and is mostly distributed in the mitochondrial intermembrane space (107). The second step is the methylation of GAA in the amidino group for producing Cr through the action of S-adenosyl-l-methionine: N-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2) (108), the liver is possible to be the principal organ contributing this reaction (109, 110). Approximately two-thirds of Cr is phosphorylated to form PCr, a key agents of cellular energy regeneration (111, 112). Cr, PCr,

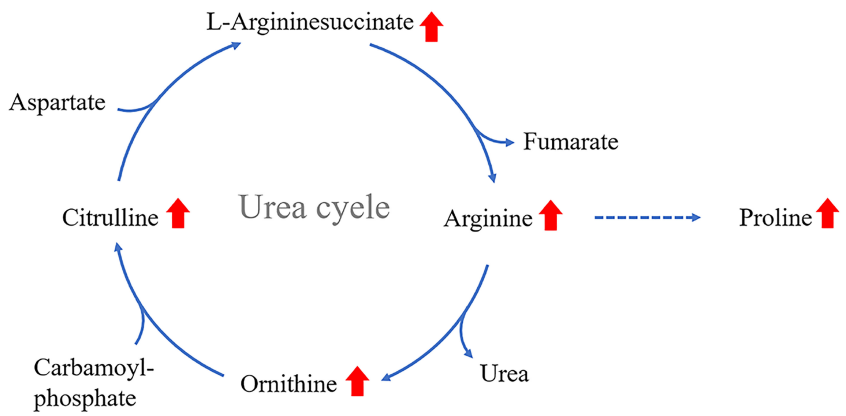

FIGURE 2 | Increased levels of proline, ornithine and arginine in the vitreous humor of PDR patients; arginine levels are elevated in the serum of severe DR patients; citrulline levels are elevated in the aqueous humor of DR patients.

and creatine kinase (CK) isoenzymes are responsible for maintaining the ATP pool (25). This is critical for some organs with high energy demands, like retina, skeletal or cardiac muscle, retina, spermatozoa, and brain (113).

AGAT is the rate-limiting enzyme in creatine biosynthesis, simultaneous reduction in mRNA content, enzyme levels, and AGAT enzyme activity when endogenous sources or dietary Cr supplementation (114). This feedback inhibition of AGAT by Cr is most pronounced in the kidney and pancreas, which are the major tissues for GAA production (115). Research shows that ingestion of creatine supplements reduces the rate of creatine biosynthesis (116).GAA, catalyzed by GAMT to generate creatine, is an important intermediate in creatine biosynthesis. Deficiency of GAMT will cause GAA accumulation and lead to axonal hypersprouting and apoptosis (117). There are no reports of abnormal GAA levels in DR patients.

Studies have shown that creatine supplementation can help improve hyperglycemia (34) and improve glycemic control in

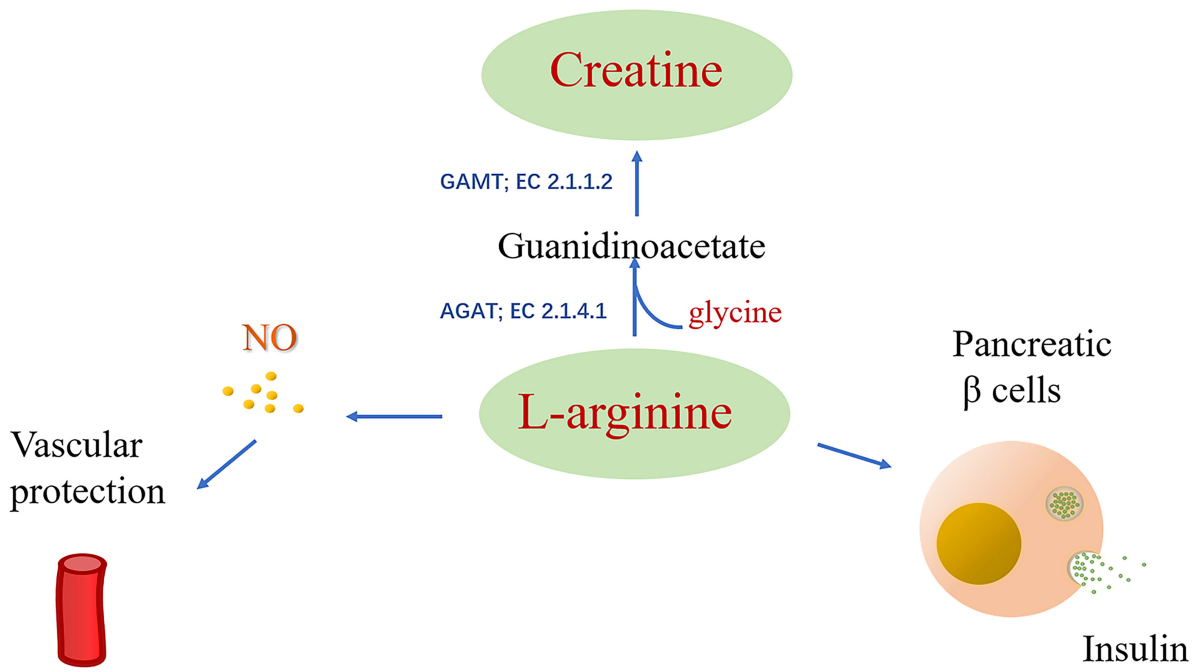

FIGURE 3 | Arginine is catalyzed by the substrate of nitric oxide synthase (NOS) to produce NO, and arginine can induce the release of insulin from pancreatic β cells.

patients with type 2 diabetes (118). In mice, lower creatine levels could be ascribed to the vascular proliferation of the retina under the OIR model (p=0.027) with the use of retinal metabolomics. Moreover, it was seen that this vascular proliferation could be reversed after the administration of oral creatine *via* anti-VEGF (84). Tomita et al. found that a decrease in creatine was accompanied by an increase in glycine levels in OIR mice, this results consistent with the vitreous humor of PDR patients (84). Glycine is involved in the biosynthesis of creatine, the amidine group of arginine is transferred to glycine to generate ornithine and GAA, and then GAA is catalyzed by GAMT to generate creatine. Increased glycine appears to be protective for DR, and glycine has proven anti-glycation and anti-diabetic properties (119, 120). Moreover, glycine significantly upregulated the mRNA expression of PEDF (an angiogenesis inhibitor) (121). However, in the study by Tomita et al., arginine was not significantly different in the vitreous humor of PDR patients and the retina of OIR mice. In previous studies, arginine was reported to be significantly elevated in plasma and vitreous humour (62, 90, 102).

Mitochondria are the primary site of production ATP and the main source of cellular energy. The number of mitochondria in a cell depends on its energy demand (122). Mitochondrial dysfunction due to overproduced of ROS in hyperglycemic states (122, 123), and make a major impact on tissues with high energy demands, such as the retina (111). Study shows persistent hyperglycemia leads to reduced mitochondrial respiration (124), Cr-Pcr system is essential for energy-demanding tissues and cells due to the maintenance of adequate ATP pools (111).

Another study showed that creatine enhanced the functional capillary density in skin and recruitment in post-occlusive reactive hyperemia (35, 125). The author speculates that creatine may help increase the bioavailability of epoxyeicosatrienoic acid (EET), thereby improving endothelium-derived hyperpolarizing factor (EDHF) stimulation and microvascular dilation (125). Apart from this, the potential therapeutic effect of creatine on the nervous system also deserves attention. It has been reported that creatine protects against neurotoxicity and oxidative stress (30, 31). Oxidative stress is one of the biggest risk factors for diabetic retinopathy. An animal experiment demonstrated that creatine has a significant antioxidant effect and indicated that creatine supplementation may become a treatment strategy for neurodegenerative diseases caused by oxidative stress (34, 126). Besides, creatine administration significantly attenuated abnormal glucose tolerance, and is considered to delayed the onset of diabetes

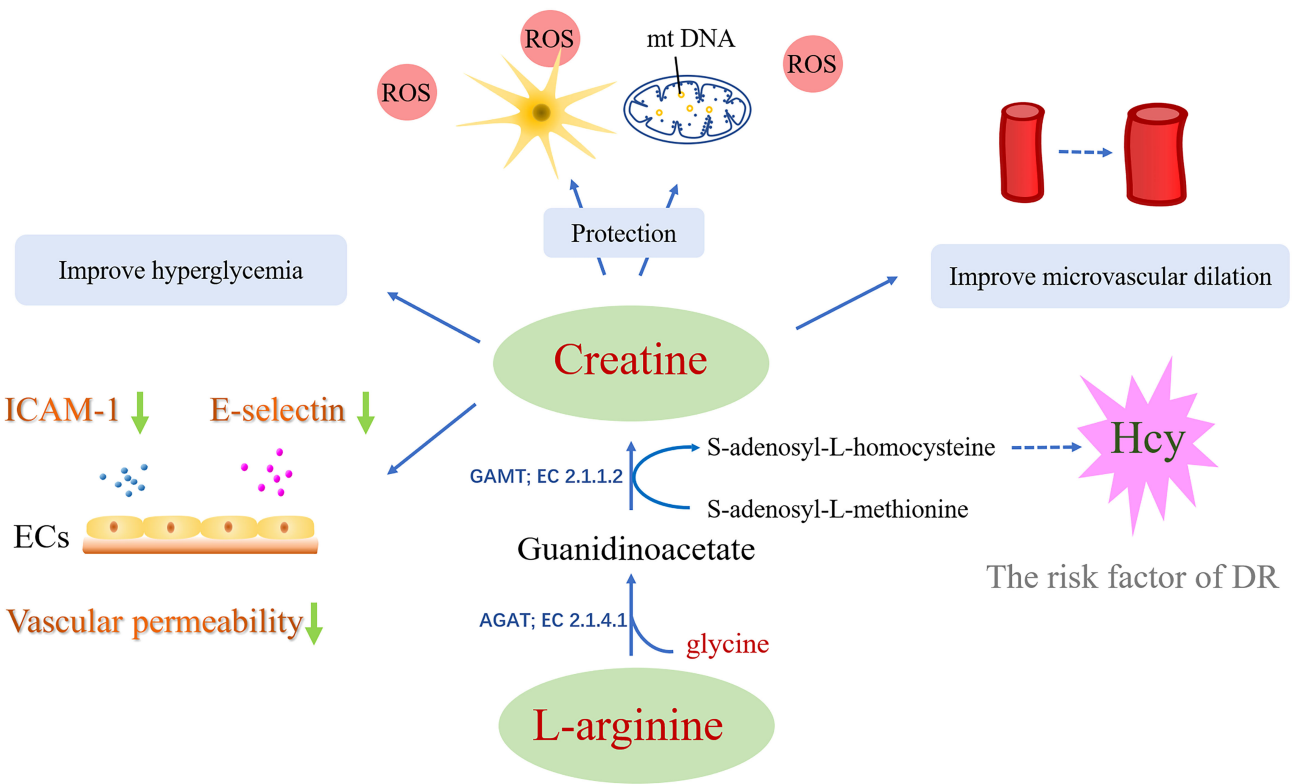

FIGURE 4 | The approach of creatine in treating diabetic retinopathy: i) Creatine has the potential to act as an anti-inflammatory aid and provide vascular protection. ii) Creatine has a significant antioxidant effect and protects mtDNA and nerve cells from cytotoxicity induced by oxidative stress. iii) Creatine may help increase the bioavailability of epoxyeicosatrienoic acid, thereby improving microvascular dilation. iv) Creatine may reduce the formation of Hcy. v) Creatine supplementation can help improve hyperglycemia.

(34). Studies have shown that creatine exhibits resistance to oxidation, which is effective in protecting mtDNAs from oxidative stress-elicited cytotoxicity (127, 128). Suggestively, creatine could provide a way for the effective management of diseases involving oxidative stress (126–128).

Synthesis of creatine yields homocysteine as a byproduct, which is an amino acid that contains sulfhydryl groups. S-adenosylmethionine (SAM) is demethylated to generate creatine as well as S-adenosyl homocysteine (SAH). SAH hydrolase (SAHH) enzyme then hydrolyzes SAH to Hcy. A correlation has been reported between the increase in Hcy expression and an aggravated risk for diverse DR, including blood retinal barrier dysfunction, inflammation, and mitochondria dysfunction (129–131). Replenishment of creatine has been demonstrated to save the SAM input (132–134) given about 40–70% expenditure of entire methyl groups by the creatine synthesis (134), which can diminish the Hcy formation (133) and may help reduce the possibility of developing DR.

In addition, creatine can reduce acute inflammation induced by carrageenan, whose action is identical to that of butazepine, a non-steroidal anti-inflammatory drug (27). Research done by Nomura et al. on pulmonary endothelial cells (ECs) revealed that after the administration of 0.5 mM creatine, the endothelial cell (EC) expressions of E-selectin and Intercellular Adhesion Molecule-1 were suppressed. Moreover, the serotonin-and H2O2-elicited permeability of endothelium was also prominently reduced upon creatine (5 mM) replenishment. These observations suggested that the administration of creatine makes the membranes more stable, and the ECs less leaky (28). Associations between DR and increased intercellular cell adhesion molecule-1 (ICAM-1), E-selectin expressions, and enhanced permeability "leakiness" of the endothelium have been reported several times (135). It shows that creatine has the potential to act as a protector of the vascular system and as an inflammation inhibitor (**Figure 4**).

CONCLUSION

In recent years, researchers have identified many potential DR biomarkers, which are not yet used for clinical diagnosis. Further research is required to clarify their molecular mechanisms in DR. In this review, we have discussed the known biomarkers of diabetic retinopathy, which can help in predicting and preventing DR in the future. Furthermore, we suggest that the arginine-creatine metabolic pathway may be a new strategy for the treatment of diabetic retinopathy.

AUTHOR CONTRIBUTIONS

YS, LK, A-HZ, YH, HS, and G-LY analyzed the data. YS wrote the paper. A-HZ and X-JW revised the paper. All the authors read and approved the final manuscript.

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Understanding the Mechanism of Dysglycemia in a Fanconi-Bickel Syndrome Patient

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Fanconi–Bickel Syndrome (FBS) is a rare disorder of carbohydrate metabolism that is characterized mainly by the accumulation of glycogen in the liver and kidney. It is inherited as an autosomal recessive disorder caused by mutations in the *SLC2A2* gene, which encodes for GLUT2. Patients with FBS have dysglycemia but the molecular mechanisms of dysglycemia are still not clearly understood. Therefore, we aimed to understand the underlying molecular mechanisms of dysglycemia in a patient with FBS. Genomic DNA was isolated from a peripheral blood sample and analyzed by whole genome and Sanger sequencing. CRISPR-Cas9 was used to introduce a mutation that mimics the patient's mutation in a human kidney cell line expressing GLUT2 (HEK293T). Mutant cells were used for molecular analysis to investigate the effects of the mutation on the expression and function of GLUT2, as well as the expression of other genes implicated in dysglycemia. The patient was found to have a homozygous nonsense mutation (c.901C>T, R301X) in the *SLC2A2* gene. CRISPR-Cas9 successfully mimicked the patient's mutation in HEK293T cells. The mutant cells showed overexpression of a dysfunctional GLUT2 protein, resulting in reduced glucose release activity and enhanced intracellular glucose accumulation. In addition, other glucose transporters (SGLT1 and SGLT2 in the kidney) were found to be induced in the mutant cells. These findings suggest the last loops (loops 9-12) of GLUT2 are essential for glucose transport activity and indicate that GLUT2 dysfunction is associated with dysglycemia in FBS.

Keywords: Fanconi-Bickel syndrome (FBS), dysglycemia, glucose transporter 2 (GLUT2), clustered regularly interspaced short palindromic repeats (CRISPR)- Cas9, sodium-glucose transport protein 2 (SGLT2)

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INTRODUCTION

The classical phenotype of Fanconi-Bickel Syndrome (FBS) was initially described by 1 (1). GLUT2 mutations were first described in three FBS patients, including the original patient in 1997 (2). More than 100 FBS cases with different *SLC2A2* mutations; nonsense, missense, Fs/InDel, intronic, and compound heterozygous variants have been reported so far (3–8). *SLC2A2* gene consists of 11 exons

and 10 introns and encodes for the GLUT2 transmembrane protein (524 amino acids) (SLC2A2-201 ENST00000314251.8) (9). GLUT2 is a low affinity facilitated glucose transporter and expressed in tissues that have a role in glucose homeostasis. GLUT2 in human and rat liver is considered the primary transporter for glucose uptake and storage as glycogen during the feeding state, and to release glucose either by glycogenolysis or gluconeogenesis during the fasting state (10, 11). Glycogen storage, post-prandial hyperglycemia and fasting hypoglycemia in FBS patients can be explained due to a disturbance in glucose transport and metabolism in the liver. Moreover, GLUT2 in the kidney releases filtered glucose into the blood circulation. Previous studies suggested that GLUT2 dysfunction in the kidney is associated with glycogen storage and glycosuria, which are the main symptoms found in FBS patients (12, 13). Furthermore, GLUT2 is the major glucose transporter in the rat beta cells and is suggested to play a role in glucose uptake and insulin secretion. However, GLUT2 is expressed at low levels in human beta cells, and its role is not well studied and is still controversial (3, 14, 15). FBS patients develop dysglycemia (glucose intolerance, post-prandial hyperglycemia, fasting hypoglycemia, transient neonatal diabetes, frank diabetes mellitus, and gestational diabetes) with different severity regardless of the type of mutation. The molecular mechanisms of dysglycemia in FBS are not well understood (4). In this study, we aimed to mimic a patient's GLUT2 mutation in human embryonic kidney cells (HEK293T) to investigates the role of GLUT2 in dysglycemia associated with FBS.

MATERIALS AND METHODS

Patient Recruitment and Genetic Analysis

This study was approved by the Institutional Review Board for the Protection of Human Subjects, Sidra Medicine, Qatar and written informed consent was obtained for the study. Clinical information was collected and genomic DNA of the patient and parents was isolated from peripheral blood samples using QIAamp DNA Blood Maxi Kit (Qiagen). Whole-genome sequencing (WGS 30x) using the Illumina HiSeq platform was performed. Sanger sequencing was used to confirm the mutation in the patient and both parents using primers (**Table S1**). Snapgene software was used for Sanger sequencing analysis.

CRISPR-Cas9

Clustered Regularly Interspaced Short Pallindromic Repeats (CRISPR)-Cas9 system was used for GLUT2 gene modification in human embryonic kidney cells (HEK293T). Different guide RNAs (gRNAs) close to the patient mutation identified by the PAM sequence (NGG) were designed (**Table S1**). The gRNA construct containing the 20 nucleotides target CRISPR sequence (crRNA) and the tracer sequence (tracrRNA) was generated. The genome-editing protocol described by Lee et al. (16) was used with the few changes to introduce GLUT2 edits in HEK293T cells. To form the gRNA, sense and antisense oligonucleotides with BbsI overhangs (**Table S1**) were phosphorylated with T4 polynucleotide kinase. The Cas9 plasmid (pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230; http://n2t.net/addgene:42230; RRID : Addgene_42230)) was digested with BbsI and purified from a 1% agarose gel using a gel extraction kit (QIAEX II). Subsequently, ligation of the digested pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid with the annealed gRNAs were performed at 16°C overnight, using T4 DNA ligase (Invitrogen). The gRNA-Cas9 plasmid was transformed into chemically competent TOP10 bacteria (ThermoFisher), and screened on LB agar plates supplemented with 100 μg/mL ampicillin. Positive Cas9-gRNA plasmids were validated by Sanger sequencing using CRISPR_Seq primers (**Table S1**) following plasmids extraction using QIAprep Spin Miniprep Kit (QIAGEN). Further amplification of the positive plasmids was performed using Endofree Plasmid Maxi Kit (QIAGEN). To detect the most efficient gRNA, HEK293T cells were used. In short, HEK293T cells were transfected with the different gRNA-Cas9 plasmids using FuGENE® HD Transfection Reagent (Promega). Eighty thousand cells were seeded in a 24-well plate, and 1ug of gRNA-Cas9 plasmid was transfected with 3.4 μl of FuGENE HD transfection reagent. Genomic DNA was extracted from the transfected HEK293T cells after 2-3 days using Genomic DNA QuickExtract (EpiCentre, Madison, WI, USA) and amplified by Amplitaq reaction at 55°C using genomic GLUT2 forward and reverse primers (**Table S1**), and purified following the manufacturer's protocol (QIAquick PCR Purification Kit (50), QIAGEN)). T7 endonuclease assays were performed to detect heteroduplex DNA resulting from gene editing (one wild-type and one mutant DNA strand). 200 ng of purified DNA was denatured and annealed in a thermomixer (10 minutes at 95°C, followed by a gradual decrease in temperature to 25°C). Reannealed DNA was then mixed with an enzyme master mix containing 0.5 μL of T7 Endonuclease I (New England Biolabs), 0.2 μL of 10× NEBuffer #2, and 1.3 μL of sterile distilled water and incubated at 37°C for 60 minutes. The reaction was stopped immediately after incubation by the addition of 6 μL of an EDTA-containing stop solution. The entire reaction was loaded on a 2% agarose gel and stained with SyberSafe. The mutations introduced by the individual gRNAs were further analyzed by Sanger sequencing of bacterial colonies following TA cloning (TOPO® TA Cloning® Kit, Invitrogen) using M13 primers (**Table S1**). gRNA with the highest editing activity was used to transfect HEK293T cells for single cell originated clone isolation and expansion. Lastly, Sanger sequencing of several HEK293T cell colonies was performed to identify the specific gene edits generated. The mutation in the selected clone was confirmed in the first three passages to exclude any contamination from wild-type cells.

Growth Assay

To visually monitor cell growth, 300,000 wild-type (WT)/mutant HEK293Tcells were seeded in 60 mm culture dishes, and images were taken by a 10X microscope on days 1 and 4. Edu Cell Proliferation Assay (EdU-647, Merck Millipore) was performed on day 2 using 60,000 WT/mutant cells in 4-well glass chambers slides. Edu (50 μM) was added to the test chambers and

incubated for 3 hours. The cells were fixed with 4% PFA for 15 minutes and permeabilized with 0.5% Triton X-100 in PBS for 20 minutes. Then, 100 µL of reaction cocktail was added for 30 minutes and kept in the dark, followed by washing with 3% BSA in PBS and stained with DAPI. Fluorescence images of stained and fixed cells were acquired using a 60x oil objective in an Eclipse Ti inverted microscope (Nikon, Tokyo, Japan) fitted with a CSU-X1A confocal spinning disk unit (Yokogawa, Tokyo, Japan), a Visitron Systems (VS) – Laser Merge System Laser Combiner including VS-ViRTEx experiment control unit, and a pco.edge 4.2 scientific CMOS camera (PCO AG, Kelheim, Germany). Images were collected in the VisiView (Visitron Systems GmbH, Puchheim, Germany) and analyzed with Fiji/ImageJ (NIH, Bethesda, MD) (17, 18). Briefly, 16-bit confocal fluorescence images were converted into 8-bit images, and individual cell nuclei were manually selected using the freehand selection tool. Selected regions of interest (ROI) were added to the ROI manager, and background-subtracted mean intensities were calculated and plotted for each cell type.

Flow Cytometry

We used 50,000 WT/mutant HEK293T cells to measure the expression of GLUT2. Cells were fixed with 4% PFA for 15 minutes and then permeabilized for 20 minutes. A small portion of each cell type was kept unstained, while the remaining cells were incubated with 10µl of anti-hGLUT2 PE-conjugated mouse IgG2a antibody (R&D SYSTEMS, FAB14148) or 5µl of PE mouse IgG1 control antibody (400112, BioLegend) and remaining volume of 100µl of Brilliant staining buffer (BD Biosciences) for 15 minutes in the dark. The stained cells were washed once with staining buffer (BioLegend), and the signal was measured on a BD Symphony A5 instrument.

qRTPCR

The expression of other glucose transporters (SGLT1, SGLT2, and GLUT1) in WT and mutant HEK293T cells was examined quantitatively. RNA from WT/mutant HEK293Tcells was extracted following the manufacturer's protocol (RNeasy Mini Kit, Qiagen) and normalized to 2 µg for cDNA synthesis. Three µL cDNA products were added to Fast 96-Well reaction plates (0.1 mL) (Applied Biosystems) and amplified using SYBR^{R} Green PCR master mix (Applied Biosystem) at primer-specific Tm (**Table S1**). The mRNA levels were quantified using a QuantStudio^{TM} 12K Flex SystemBlock 96-well instrument.

Glucose Uptake Assay

To test the glucose uptake activity, 500,000 WT/mutant cells were incubated in low glucose medium for 3 hours, followed by incubation with 1 mM 2-DG for 1 hour. Cells were lysed with extraction buffer at 85°C for 40 minutes. Next, the reaction mixtures were neutralized with 10 µL neutralizing buffer. Intracellular glucose levels were analyzed as recommended by the manufacturer (Glucose Uptake Assay Kit (Colorimetric, Abcam). The absorbance was measured at 412 nm using a microplate reader (Flaoster, Omega).

Statistical Values

All results were assessed three times, and the average of three values is given as the Standard deviation. P-value was calculated using two-tailed t-test. P-value <0.05 is considered as a significant difference.

RESULTS

Clinical Information and Genetic Testing Results

We report a 2-year-old Palestinian boy born to consanguineous parents with FBS (**Figure 1A**). He was born full term by normal vaginal delivery with a birth weight of 2.8 Kg (3rd centile) and length 49 cm (15th centile). Maternal history was significant for gestational diabetes mellitus. His newborn screening showed high galactose levels with normal GALT activity. He was followed up by a metabolic team and started on a special formula feed since birth. On day 18 after birth, the patient presented with poor feeding, vomiting, and polyuria. His biochemical tests showed severe metabolic acidosis with electrolyte imbalance, glycosuria, proteinuria, and phosphaturia (**Table 1**). In addition, the patient displayed dysglycemia (fasting hypoglycemia and post-prandial hyperglycemia, with low levels of C-peptide and insulin). HbA1c levels were high, and diabetes

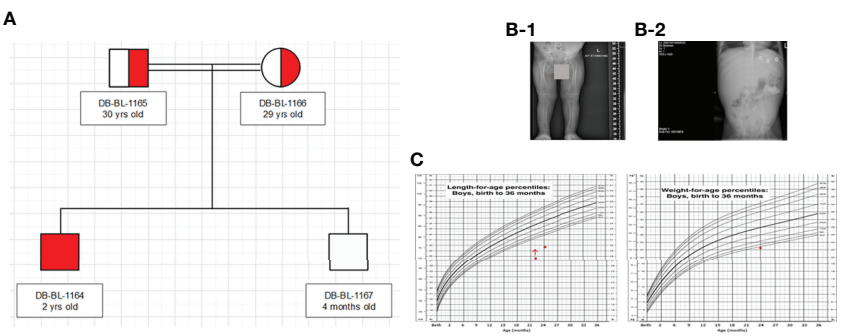

FIGURE 1 | Clinical characteristics of patient: **(A)** Family Pedigree **(B)** Radiological findings (x-ray showed rickets (1), and hepatomegaly (2). **(C)** Growth charts (According to CDC length chart, patient has short stature (dot) responding to growth hormone (red arrow), and underweight (dot).

mellitus Type 1 evaluation was negative for all autoantibodies (**Table 1**). At the age of 5 months, the patient was found to have hepatomegaly (**Figure 1B-2**) and impaired liver function tests (**Table 1**) with liver biopsy showing stage 1 fibrosis. The lipid profile was normal except for elevated triglyceride levels (**Table 1**). In addition, the patient had failure to thrive and also developed rickets (**Figure 1B-1**). An MRI of the brain showed features suggestive of anterior pituitary (adenohypophysis) hypoplasia. As for the full pituitary hormonal tests, they were normal except for low IGF-1 levels (**Table 1**) and the patient was diagnosed with growth hormone (GH) deficiency following a GH provocation test (data not shown). Recombinant GH therapy was started for the patient and monitored over six months, where he showed an increase in height to 16cm/year compared to 3 cm/year before therapy (**Figure 1C**). In addition, the patient received multiple medications for electrolyte imbalance (sodium bicarbonate, potassium, phosphorous, and vitamin D). The patient's whole genome sequencing revealed a homozygous mutation (c.901C>T, R301X) (NM_000340) in the *SLC2A2* gene, with both parents being carriers of the same mutation. The mutation was confirmed by Sanger sequencing of patients DNA (**Figure 2**). **Figure S1** illustrates the expected truncated GLUT2 topology for the patient.

CRISPR-Cas9 Technology to Mimic GLUT2 Patient's Mutation in HEK293T Cells

We cloned four different gRNAs into a Cas9-expressing plasmid to edit GLUT2 in HEK293T cells (**Figure S2**). gRNA3 was found to be the most efficient to edit GLUT2 (**Figure S3**) with different insertion and deletion mutations in GLUT2 (**Figure S4**). The selected HEK293T colony displayed a deletion mutation of 11 nucleotides, located two nucleotides after the patient mutation site (c. 901 C>T, R301X) (**Figure 3**). This resulted in a frameshift in GLUT2 and introduced a stop codon at c. 1164. Both the mutant and the WT cell lines were monitored by Sanger sequencing for few passages, all of which revealed a clean and identical sequences (**Figure 3**). Mutant cell line showed only the recurrent deletion trace; no WT nor other traces was detected, and therefore the cell line was characterized with a homozygous mutation in GLUT2 close to the mutation site identified in the patient. These cells were subsequently used to evaluate the impact of GLUT2 mutation on glucose transport activity.

Proliferation Rate of WT and Mutant Cells

We cultured the same number of WT and mutant HEK293T cells in complete DMEM medium supplemented with 10% fetal bovine serum and 1% P/S to assess the difference in growth. Both cell lines displayed similar morphology; however, the GLUT2 mutant cells grew slower than the WT cells at day 1 and 4 (**Figure S5**), suggesting that the mutation of GLUT2 affected cell growth. To further explore this possibility, we performed Edu incorporation assays to investigate the difference in proliferation rate between WT and mutant cells. The Edu signal (red fluorescence) was strong in wild-type cells when compared to mutant cells (**Figure 4**, top panel). Quantification of the intensity of the Edu signal suggested that wild-type cells proliferated at a

TABLE 1 | Summary of biochemical tests for the patient.

Investigation	Test value	Normal range
Electrolyte levels and Urine analysis		
Serum phosphorus (mmol/L)	0.80	0.93-1.64
Serum calcium (mmol/L)	1.7	2.2 -2.7
Serum Sodium (mmol/L)	132	134-146
Serum Potassium (mmol/L)	3.1	3.5-5.0
BUN (mmol/L)	2.6	1.2-6.3
Creatinine (µmol/l)	25	35-58
Urinalysis	Proteinuria (+2) glycosuria (+3) Phosphaturia	
Liver function tests		
Alanine amino transferase (ALT) (IU/L)	82	8-22
Aspartate transaminase (AST) (IU/L)	110	0-30
Alkaline phosphatase (IU/L)	410	48-95
Blood glucose tests		
Fasting glucose (mmol/l)	2.1	3.5-5.5
2 hours post OGTT (mmol/l)	20	7.8-11.1
C-Peptide (ng/ml) [At Diagnosis]	0.33	0.78-5.19
Insulin (pmol/l) [At Diagnosis]	6	111-1153
HbA1c% [At Diagnosis]	8.1	4.8-6.0
Diabetes mellitus Type 1 evaluation (GAD65, Insulin, IA-2, ZnT8 Abs test)	Negative	–
Miscellaneous Hormone Profile		
TSH (mIU/L)	3.34	0.4-4.0
PTH intact (pmol/l)	1.8	2.0- 6.8
IGF-1 (mcg/dl)	<3.0	27.4-113.5
Lipid profile		
Cholesterol (mmol/l)	3.5	<5.18
Triglyceride (mmol/l)	3.2	<1.7
High Density Lipoprotein (HDL-C) (mmol/l)	0.7	>1.17
Low Density Lipoprotein LDL (mmol/l)	2	<2.6

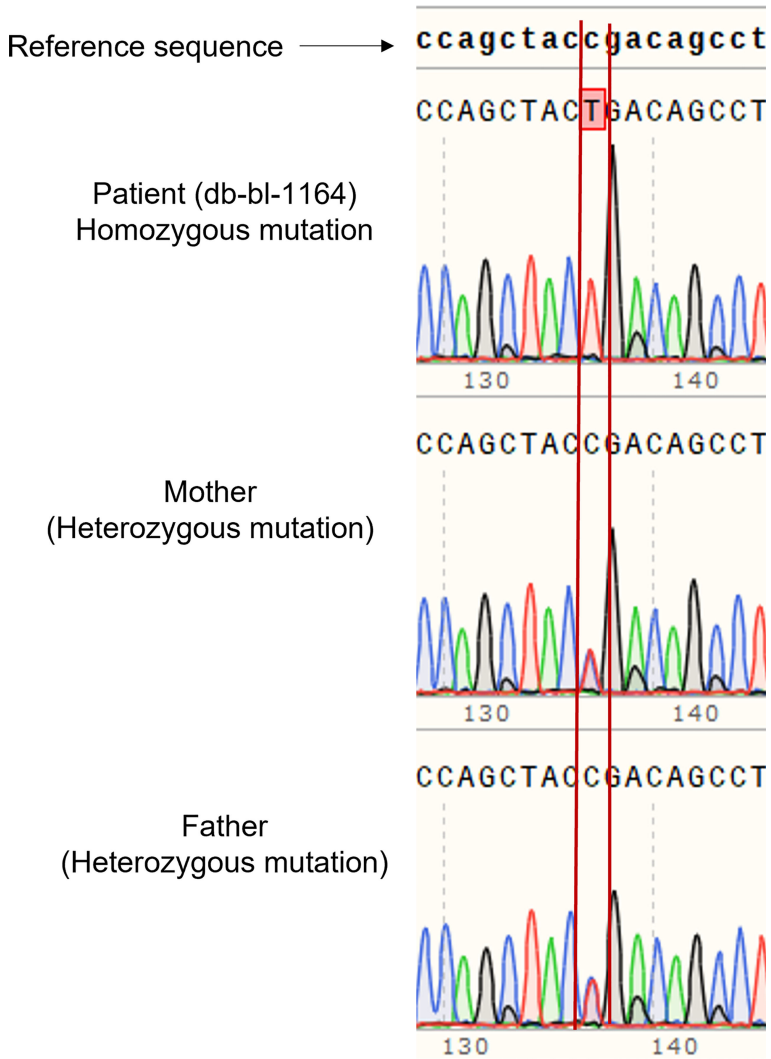

FIGURE 2 | Genetic analysis of patient and parents. Sanger sequencing of DNA of the patient showed homozygous mutation of *SLC2A2* (c.901C>T, R301X), and the parents are carriers.

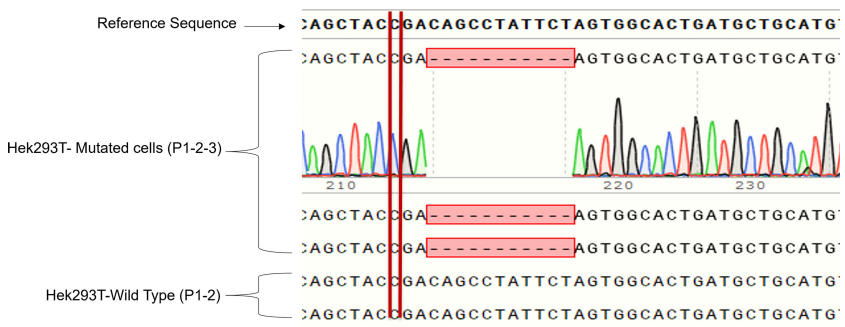

FIGURE 3 | Sanger sequencing of the first three passages of sorted HEK293T cells with GLUT2 mutation. The deletion mutation was confirmed in the first three passages of the sorted cell colony to validate cell genotype.

significantly faster rate than the mutant cells (**Figure 4**, lower panel).

GLUT2 Expression in Wild-Type and Mutant HEK293T

The expression of GLUT2 protein in wild-type and mutant cells was monitored using flow cytometry. Surprisingly, the expression of GLUT2 was significantly increased in the mutant cells in comparison to wild-type cells (**Figure 5**).

The Expression of Other Glucose Transporters in HEK293T Cells

We were interested in investigating the expression of other glucose transporters in the GLUT2 mutant cells. Therefore, the gene expression of SGLT1, GLUT1, and SGLT2 was assessed using qRT-PCR. We amplified an equal amount of cDNA from normalized high-quality RNA (2µg) extracted from WT and mutant HEK293T cells. The mutant cells displayed a slight increase in the expression of SGLT1 (**Figure 6**). We were unable to detect any expression of SGLT2 in WT cells, while the same transporter was expressed at relatively higher levels in the mutant cells (**Figure 6**). The expression of GLUT1 was slightly reduced in the mutant cells compared to WT cells (**Figure 6**). Thus, our results suggest that mutation of the *SLC2A2* gene (GLUT2) in HEK293T cells results in the overexpression of a dysfunctional GLUT2 protein and enhanced expression of SGLT2, which could result in increased accumulation of intracellular glucose.

Glucose Uptake in WT and Mutant HEK293T Cells

To study the effect of the GLUT2 mutation on glucose uptake, cells were incubated with 1 mM 2-DG. Interestingly, the intracellular levels of 2-DG were significantly higher in the mutant cells compared to WT cells (**Figure 7**). To determine whether the increased accumulation of glucose in the mutant cells was due to an increase in SGLT2 expression (**Figure 6**) or due to the inhibition of GLUT2-mediated glucose export, the glucose uptake assay was

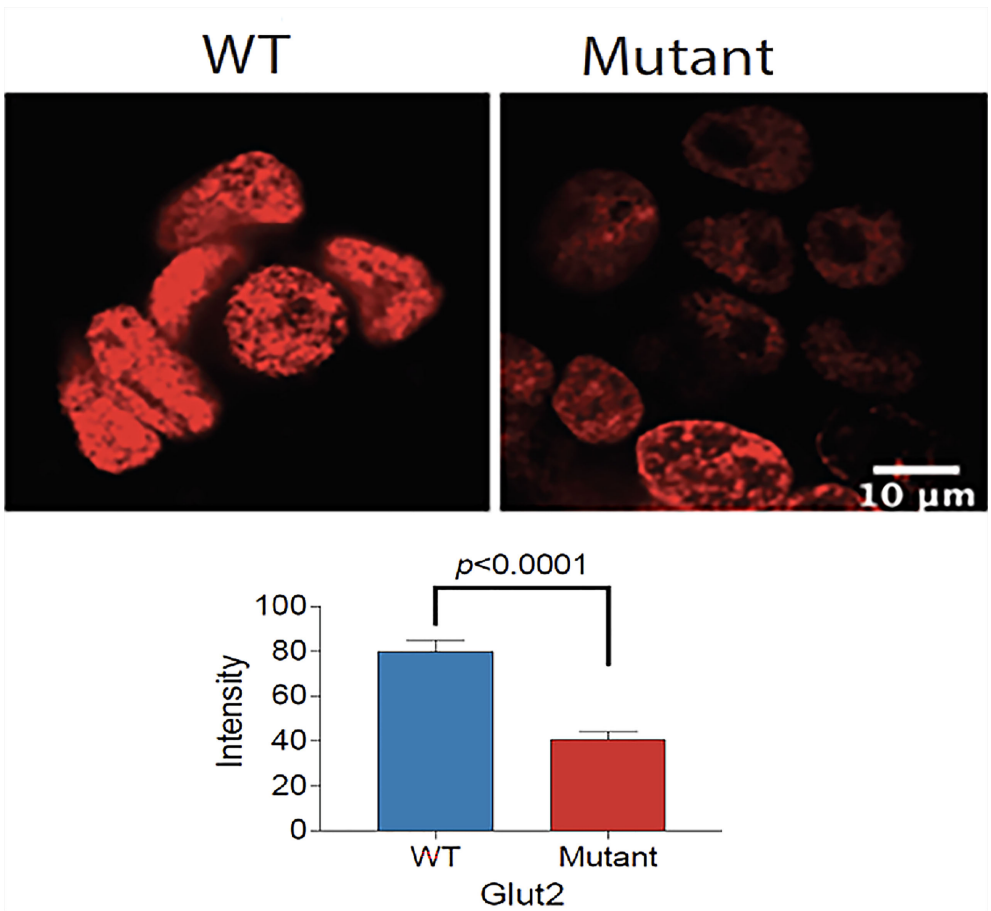

FIGURE 4 | Reduced proliferation of GLUT2 mutant cells. Top panel: Confocal fluorescence images of WT and mutant GLUT2 expressing HEK293T cell nuclei showing reduced Edu incorporation. Lower panel: Graph showing Edu intensity in the nuclei of WT and mutant GLUT2 expressing HEK293T showing significantly lower Edu intensities ($p<0.0001$). Analysis based on single cell nuclei measurement.

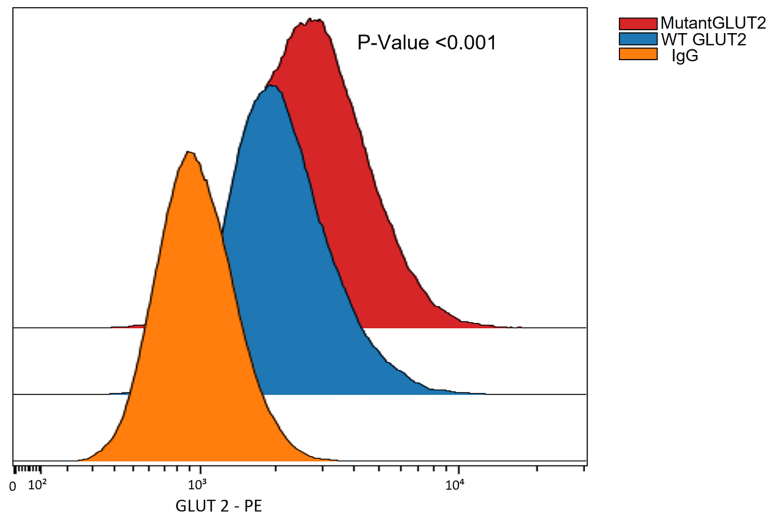

FIGURE 5 | Flow cytometry to assess the expression of GLUT2 protein in WT and mutant HEK293T cells. The expression of GLUT2 was significantly increased in the mutant cells. P-Value <0.001 using nonparametric Mann-Whitney test.

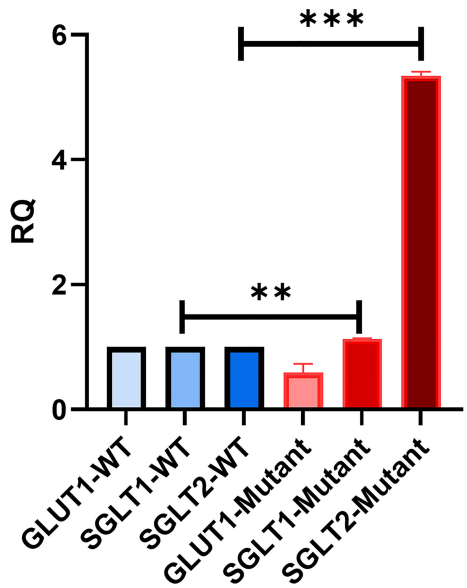

FIGURE 6 | qRT-PCR to assess the expression of other glucose transporters in HEK293T. GLUT2 mutation stimulates the expression of SGLT2. Significant induction of SGLT1 and SGLT2 in mutant cells in comparison to WT cells. Down-regulation of GLUT1 expression in mutant cells in comparison to WT cells. P-value was calculated using two-tailed t-test and presented with a "*" in the graph. **P values less than 0.01; ***P values less than 0.001.

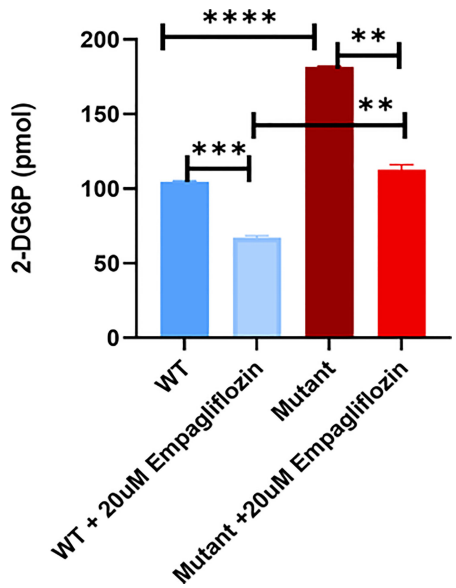

FIGURE 7 | Glucose uptake test in WT and mutant HEK293T cells using 2-DG. Mutant cells have significantly increased glucose accumulation in comparison to WT. Treatment of the cells with the SGLT2 inhibitor (Empagliflozin) confirmed the GLUT2 release activity in the mutant cells is disturbed. P-value was calculated using two-tailed t-test and presented with a "*" in the graph. **P values less than 0.01; ***P values less than 0.001; ****P values less than 0.0001.

repeated in the presence of the SGLT2 inhibitor empagliflozin. As illustrated in **Figure 7**, the SGLT2 inhibitor reduced glucose accumulation in both cell lines. Importantly, the accumulation of intracellular glucose remained higher in the mutant cells even in the presence of the inhibitor, suggesting that the mutant GLUT2 protein is less efficient in glucose export. Thus, we suggest that the last four transmembrane domains (domains 9-12) of GLUT2 are essential in glucose export activity in kidney cells, and treatment with SGLT2 inhibitors could attenuate the dysglycemia observed in FBS patients.

DISCUSSION

Dysglycemia is observed in virtually all patients with FBS at some stage of their lives. The pattern of dysglycemia in FBS patients can range from fasting hypoglycemia, post-prandial hyperglycemia, and diabetes mellitus. However, the underlying biochemical and molecular mechanisms of dysglycemia are not well understood. This study aimed to understand how disturbances in GLUT2 function is associated with dysglycemia in an FBS patient. For the first time, we use CRISPR-Cas9 genome editing to mimic the mutation identified in a FBS patient in HEK293T cells, a human embryonic kidney cell line. In the kidney, the main role of GLUT2 is to transport glucose back to the circulation, thereby preventing glucose loss. Using this novel FBS model, we demonstrate that the gene edited cells accumulate more glucose than the wild-type cells, likely as a result of reduced glucose export. Our results could therefore explain, at least in part, the accumulation of glycogen in the kidneys in some FBS patients.

A recent study aimed at understanding the molecular mechanisms of dysglycemia in FBS used HEK293 cells transfected with constructs expressing either wild-type or FBS-associated mutant GLUT2. All mutants displayed the same fructose uptake activity as the WT protein, except for p.Thr198Lys, which displayed a small decrease in uptake activity (19). Although informative, this work was based on the overexpression of mutant proteins on top of wild-type endogenous GLUT2 expressed in HEK293. In contrast, the current study aimed to understand the molecular mechanisms of dysglycemia in FBS patients by mimicking the GLUT2 mutation we recently identified in a patient. To achieve this aim, we used CRISPR-Cas9 gene editing to introduce a mutation in *SLC2A2* in HEK293T cells. One of our gRNAs introduced a deletion mutation close to the mutation found in our patient, thereby causing a frameshift and a stop codon at nucleotide 1164 (**Figure 3**). The mutant cells displayed slower proliferation than the wild-type cells, which suggest that GLUT2 could affect cell growth (**Figure 4**). This is an interesting observation as most patients with FBS have short stature and failure to thrive and thus needs further investigations.

Proximal tubular cells augment glucotoxicity during hyperglycemia, either by increased glucose reabsorption or intracellular glucose accumulation (20). Song et al. suggested that the inhibition of SGLT1 results in mild glycosuria that is enhanced in response to SGLT2 inhibition (21). Chhabra et al. reported glycosuria in mice with hypothalamic melanocortin deficiency due to decreased GLUT2 expression (22). In addition, Hinden et al. showed that the inhibition of the cannabinoid-1 receptor (CB1R) leads to down-regulation of GLUT2. Hence, the translocation of GLUT2 to the apical membrane of renal proximal tubular cells (RPTCs) was affected, causing a decrease in glucose reabsorption and glycosuria in diabetic mice (23). Moreover, de Souza Cordeiro et al. specifically knocked out GLUT2 in mice and reported that GLUT2 dysfunction was associated with glycosuria and improved glucose tolerance (24). Another study showed an increase in glucose uptake by the apical movement of GLUT2 in rats treated with streptozotocin to induce diabetes; this effect disappeared in response to overnight fasting (25). In addition, a separate study demonstrated that the expression and activity of SGLT2 and GLUT2 were enhanced in human exfoliated proximal tubular epithelial cells (HEPTECs) isolated from patients with type 2 diabetes mellitus in comparison to healthy controls (26). Recently, Jiang et al. explained the potential role of renal SGLT2 and GLUT2 in the pathology of gestational diabetes mellitus (GDM) in a mouse model exposed to a high-fat diet (27).

During euglycemia, glucose reabsorption in the kidneys occurs primarily *via* SGLT2 and secondarily through SGLT1 (28). GLUT2 and GLUT1 release glucose across the basolateral membrane into the bloodstream. Glycosuria occurs once the glucose levels in the blood exceed the maximum re-absorptive capability of the kidneys. During hyperglycemia, SGLT2 and SGLT1 activities are enhanced due to increased glucose glomerular filtration. Moreover, protein kinase C stimulates the translocation of GLUT2 to the brush border membrane, which may increase glucose reabsorption (28). Wu et al. concluded that the last four transmembrane domains (domains 9 to 12) play a major role in glucose transport activity using Xenopus oocytes and mammalian cells (29). The latter conclusion is supported by the data reported in the current study. The GLUT2 mutant generated in our study contains a premature stop codon upstream of transmembrane domains 9-12 and the mutant protein displays reduced glucose transport activity (**Figure 7**).

In our study, mutation of GLUT2 in HEK293T cells resulted in the upregulation of GLUT2 protein (**Figure 5**). This was a rather surprising observation, since the gene editing introduced a premature stop codon in GLUT2. Thus, the exact nature of the GLUT2 protein in the edited cells will require further studies.

We found that the mRNA levels of SGLT2 were very low in wild-type HEK293T cells (**Figure 6**), which is consistent with a previous finding showing that the mRNA and protein levels of SGLT2 are minimal in the proximal tubular cell line (HK-2) in comparison to human kidney cells (30). Interestingly, we found that the mRNA levels of SGLT2 were increased in cells expressing mutant GLUT2 (**Figure 6**). Moreover, the accumulation of intracellular glucose was increased in the mutant cells (**Figure 7**). One possibility for the increased accumulation glucose in the mutant cells could be the enhanced expression of SGLT2 in these cells. To test this hypothesis, the glucose uptake assay was repeated in the presence of a clinically used SGLT2 inhibitor. As expected, the inhibitor attenuated glucose accumulation in both wild-type and mutant cells. However, the accumulation of glucose in the mutant cells remained higher than the wild-type cells even in the presence of the SGLT2 inhibitor, supporting our hypothesis that the GLUT2 mutant cells have a defect in glucose export.

In conclusion, our results support the notion that the last four transmembrane domains of GLUT2 (domains 9-12) are vital for glucose transport activity and suggest that disturbances in GLUT2 expression and/or function could contribute to the dysglycemia observed in FBS. It will be very important to explore if the intracellular accumulation of glucose that we observe in our gene edited HEK293T cells also results in the accumulation of glycogen, similar to the accumulation of glycogen in the kidneys observed in some FBS patients. In

addition, future work will focus on using CRISPR-Cas gene editing to introduce FBS-associated GLUT2 mutations in other metabolically active human cells, including pancreatic beta and liver cell lines. Such studies will allow us to better understand the role of GLUT2 in FBS, and hopefully help in the development of better treatment options for FBS patients.

The limitation of our study is that we have only studied a single patient with a GLUT2 mutation. Similar studies should be undertaken in more patients with other GLUT2 mutations. Another limitation of our study is that we were unable to determine if the accumulation of glucose in our GLUT2 mutant HEK293T cells resulted in increased accumulation of glycogen in these cells. The most accurate method to monitor glycogen accumulation in non-liver cells is metabolic labeling using radioactive glucose. Unfortunately, the strict regulations controlling the use of radioactive materials in Qatar prevented us from performing these studies. Such studies should however be explored with collaborators outside Qatar in the future.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are not publicly available to protect patient confidentiality but are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was approved by the Institutional Review Board for the Protection of Human Subjects, Sidra Medicine, Qatar and written informed consent was obtained for the study. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Writing the Manuscript: SS, MS, JE, and KH. CRISPR: SS, MS, IM, IH, MA, and KH. Patient recruitment and clinical data collection: SS, BH, and KH. Flow cytometry: SS, IP, SM, J-CG, and KH. Edu assay: SS, AB, SN, KB, and KH. WGS and Sanger sequencing: SS, IM, NS, and KH. Rest experimental work: SS, AA, JE, and KH. All authors read, edited, and approved the final manuscript for submission.

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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.2022.841788/full#supplementary-material

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ORIGINAL RESEARCH
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Plasma miR-193b-3p Is Elevated in Type 2 Diabetes and Could Impair Glucose Metabolism

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Objective: To explore differentially expressed miRNAs in type 2 diabetes and their potential cellular functions.

Methods: We screened plasma miRNAs by miRNA array analysis and validated them by TaqMan real-time PCR in 113 newly diagnosed, untreated type 2 diabetes cases and 113 healthy controls. Low-abundance plasma proteins encoded by miR-193b-3p target genes were explored in this study population. We further investigated the potential cellular functions of the differentially expressed miRNAs in HepG2 cells.

Results: miR-193b-3p was differentially expressed in type 2 diabetes cases compared to healthy controls (fold change = 2.01, P = 0.006). Plasma levels of triosephosphate isomerase (TPI1, a protein involved in the glycolytic pathway) decreased in type 2 diabetes cases (fold change = 1.37, P = 0.002). The effect of miR-193b-3p on TPI1 was verified by transfection of miR-193b-3p into HepG2 cells. miR-193b-3p inhibited the expression of YWHAZ/14-3-3ζ in the PI3K-AKT pathway, subsequently altering the expression of FOXO1 and PCK1. After transfection, cells were incubated in glucose-free medium for another 4 h. Glucose levels in medium from cells with elevated miR-193b-3p levels were significantly higher than those in medium from negative control cells (P = 0.016). In addition, elevated miR-193b-3p reduced glucose uptake by inhibiting insulin receptor (IR) and GLUT2 expression.

Conclusion: Plasma miR-193b-3p levels increased in type 2 diabetes cases, and TPI1 levels decreased in both plasma and HepG2 cells with increased miR-193b-3p levels, while extracellular lactate levels did not significantly changed. Moreover, miR-193b-3p may affect glucose metabolism by directly targeting YWHAZ/14-3-3ζ and upregulating the transcription factor FOXO1 downstream of the PI3K-AKT pathway.

Keywords: type 2 diabetes, microRNA, proteomics, glucose metabolism, case-control study

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INTRODUCTION

Type 2 diabetes has been a growing global problem in last decades. Its main pathological mechanisms include insulin resistance in muscle, adipose, and liver tissues combined with dysfunction and subsequent failure of insulin-producing pancreatic beta cells (1–3). The global diabetes prevalence in 2019 is estimated to be 9.3% (463 million people), rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045. China has 116.4 million people with diabetes, causing a heavy medical and economic burden (4)..

MicroRNA (miRNA) molecules are short non-coding RNAs that mediate RNA silencing and post-transcriptional regulation of gene expression, negatively regulate the abundance of specific proteins, and then control numerous cellular and biological processes including metabolism (5). Accumulating evidence also suggests that miRNAs play an important role in cellular metabolic regulation (e.g., let-7 family), adipocyte differentiation (e.g., miR-133a), pancreatic development (e.g., miR-375, miR-26a-5p), and insulin biosynthesis, secretion, and signaling (e.g., miR-375, miR-7) (6, 7). For the cellular glucose metabolism, miRNAs play a pivotal role by targeting the key rate-limiting enzymes of relevant pathways to fine-tune control of metabolic homeostasis. Aberrant expression of these miRNAs can result in an over or under expression of key enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6PC), contributing to the etiology of diabetes (8). Dysfunction in multiple tissues that control glucose homeostasis and insulin sensitivity often occurs years before diagnosis (9, 10). Diabetes is generally diagnosed based on elevated plasma glucose level which does not distinguish stage progression of diabetes (8). Recent studies have shown that miRNAs can be detected in circulating blood and can be future biomarkers for diagnosis of diabetes states (11). Plasma miRNAs, such as miR-122, have been shown to be differentially expressed at the progressive glycemic impairment stage (12), and miR-144-3p was found elevated in newly diagnosed diabetes (13). Previous studies explored the cellular functions of miRNAs or circulating miRNAs separately, and few of them have delved into the potential function of circulating miRNAs (14–18).

Proteins are important molecules in cellular functions. With the development of mass spectrometry proteomic approaches, studies have shown that low-abundance plasma proteins, such as adiponectin and resistin (19–21), are associated with diabetes risk (22, 23). Subsequent studies have also shown that these low-abundance proteins are involved in the development and progression of diabetes and are direct effector molecules in tissue or cellular dysfunction (24–26). miR-375 has been found to play an important role in the development and progression of diabetes by targeting messenger RNA (mRNA) transcripts and regulating protein expression (6, 7, 27, 28). However, most previous studies have explored the differences in plasma miRNA levels and plasma protein levels separately, and few have examined the differences in plasma miRNA and protein levels in the same study population or investigated their potential associations and underlying biological mechanisms in the development of type 2 diabetes.

Therefore, in the present study, we first explored the plasma miRNA profiles in 113 pairs of age- and sex-matched newly diagnosed, untreated diabetes cases and controls. Second, we examined the plasma proteomic profiles in the same study population and performed further bioinformatic analysis of miRNA target genes and signaling pathways enriched with the target genes. Finally, we verified the associations between miRNA and protein levels through *in vitro* experiments. We further explored the potential mechanism of the differential expression of plasma miRNAs in the development of type 2 diabetes through miRNA transfection and molecular biology experiments. The detailed workflow of this study is shown in **Supplementary Figure 1**.

MATERIALS AND METHODS

Study Design and Population

The Dongfeng-Tongji (DFTJ) cohort was established in 2008 and enrolled 27,009 retired employees of Dongfeng Motor Corporation (DMC) who resided in Shiyan city, Hubei, China. Participants completed epidemiological questionnaires, provided blood samples, and participated in physical examinations at baseline enrollment in 2008. The participants were invited to participate in a follow-up examination in 2013, and the follow-up rate was 96.2% (n=25,978). Detailed information on the DFTJ cohort is described elsewhere (29). Participants were defined as having type 2 diabetes if they had a fasting plasma glucose level of ≥ 7.0mmol/L and/or a hemoglobin A1c (HbA1c) level of ≥ 6.5% (30). Individuals with type 2 diabetes who did not have cardiovascular disease or cancer and did not take any antidiabetic medication were selected as cases. Accordingly, a 1:1 age- and sex-matched population of individuals without diabetes, cancer, cardiovascular diseases, and medication use was selected as the control population. Finally, a total of 113 case-control pairs were enrolled in the present study. The detailed characteristics of the cases and controls are presented in **Table 1**. The 113 pairs of case-control samples were randomly divided into a screening group (n = 15 pairs) and a validation group (n = 98 pairs) to explore differentially expressed miRNAs. Considering the amount of plasma used for protein screening, twenty-five pairs of samples were randomly selected for protein screening, and an equal number of validation group samples were used for miRNA screening. The characteristics of the participants in the validation group are shown in **Supplementary Table 1**.

All participants gave written informed consent. The study protocol was approved by the Ethics and Human Subject Committee of the School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, and Dongfeng General Hospital, DMC.

Plasma Sampling and RNA Isolation

Approximately 5 ml of venous blood was collected from each participant, placed in an EDTA-anticoagulant tube and centrifuged at 1000 × g for 10 min. Plasma was carefully transferred to an RNase-free tube and stored at −80°C until use. Prior to isolating miRNAs from plasma, we transferred the

TABLE 1 | Characteristics of the participants.

Variables	DM cases (n=113)	Controls (n=113)	*P*
Male, n (%)	53 (46.9)	53 (46.9)	1.0
Age, years	61.1 (7.1)	61.1 (7.1)	0.948
Smoking, n (%)			
Never	79 (70.5)	77 (68.1)	0.814
Current	21 (18.8)	25 (22.1)	
Ever	12 (10.7)	11 (9.7)	
Drinking, n (%)			
Never	80 (71.4)	73 (65.2)	0.525
Current	27 (24.1)	31 (27.7)	
Ever	5 (4.5)	8 (7.1)	
Physical activity, n (%)	102 (91.1)	100 (88.5)	1.0
BMI, kg/m^2	25.3 (3.4)	22.9 (3.0)	<0.001
WHR	0.89 (0.06)	0.86 (0.06)	0.001
FPG, mmol/L	8.9 (2.4)	5 (0.4)	<0.001
HbA1c, %	6.5 (1.5)	5.3 (0.3)	<0.001
TG, mmol/L	2 (3.2)	1.3 (0.8)	0.022
LDLC, mmol/L	2.5 (0.9)	2.8 (0.8)	0.005
HDLC, mmol/L	1.5 (0.5)	1.6 (0.4)	0.477
TC, mmol/L	4.8 (1.5)	4.8 (1.1)	0.964
SBP, mmHg	137.7 (22.6)	135.8 (22.4)	0.522
DBP, mmHg	80.0 (13.1)	80.0 (13.0)	0.364
Neutrophil, 10^9/L	3.7 (1.4)	3.3 (1.2)	0.058
Lymphocyte, 10^9/L	2 (0.8)	1.7 (0.5)	0.003
Monocyte, 10^9/L	0.4 (0.2)	0.3 (0.2)	0.046
Eosnophils, 10^9/L	0.2 (0.2)	0.1 (0.1)	0.014
Basophil, 10^9/L	0.2 (0.2)	0.1 (0.2)	0.297
WBC, 10^9/L	6 (1.8)	5.4 (1.4)	0.008
RBC, 10^{12}/L	4.5 (0.4)	4.5 (0.5)	0.205
PLT, 10^9/L	183.3 (54.8)	196.9 (51.4)	0.056
Family history of diabetes, n (%)	9 (8.2)	8 (7.1)	0.685

WHR, waist-to-hip ratio.

supernatant to a new tube after a brief centrifugation. Total RNA was isolated from plasma using a miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacturer's instructions. In total, 200 µl of plasma was used for the entire miRNA extraction. Approximately 1.6 × 108 copies/µl of synthetic Caenorhabditis elegans cel-miR-39 (Qiagen) was added to the denatured plasma samples as an internal control for the validation study. RNA sample concentrations were quantified using a NanoDrop ND-1000 (Nanodrop, USA).

MiRNA Microarray Analysis

A MiRCURY LNA™ MicroRNA Array (Exiqon: 7th generation) was used for initial microarray screening. RNA samples were labeled and hybridized according to Exiqon's manual. Scanned images were imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicate miRNAs were averaged and miRNAs with intensities of ≥ 30 in all samples were selected to calculate the normalization factor. Expression data were processed using median normalization. After normalization, miRNAs that were significantly differentially expressed between the two groups were identified based on the fold change and P values. Volcanic Plots were used to visually indicate miRNAs with significant differences. Clustering was performed to show distinguishable miRNA (Fold Change >= 1.5, *P*-value <= 0.05) expression profiling among samples. After applying the Benjamini-Hochberg false discovery rate (FDR) correction for multiple comparisons, a P value of < 0.05 was considered a statistically significant difference. The R Statistical Software (http://www.r-project.org/), the ggplot2 Package (31), and pheatmap Package (32) were also used for the analysis of Volcanic Plots and Clustering, respectively. The microarray data have been submitted to the Gene Expression Omnibus (GEO) database (GSE134998; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134998).

Quantification of Plasma miRNA

miRNA validation was carried out using TaqMan real-time quantitative PCR (qPCR). Reverse transcription (RT) was performed using miRNA-specific stem-loop RT primers and a MicroRNA RT Kit (Life Technologies) following the manufacturer's instructions. The resulting cDNA was diluted and used immediately for qPCR or stored at −20°C until use. miRNA expression levels were measured by real-time qPCR in a ViiA ^{7} Real-Time instrument (Life Technologies) using TaqMan® Universal Master Mix (Life Technologies). The miRNA expression levels were normalized to those of cel-miR-39 and determined by the equation $2^{-\Delta Ct}$, where ΔCt=cycle threshold (Ct) (miRNA) – Ct (cel-miR-39).

Bioinformatic Analysis

The miRNA target sites in mRNA were predicted with miRDB (http://mirdb.org/), miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) and TargetScan (http://www.targetscan.org/). Pathway enrichment analysis of the target genes was

performed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database resource (http://www.genome.jp/kegg/). Bioinformatic analysis of the pathway enrichment results was performed with Database for Annotation, Visualization and Integrated Discovery (DAVID) tools (http://david.abcc.ncifcrf.gov/).

Plasma Proteomics

The 113 case-control pairs were randomly divided into a preliminary screening dataset and a validation dataset. In the preliminary screening dataset, twenty-five plasma samples from each group were pooled into five samples, and isobaric tagging for relative and absolute quantification (iTRAQ)-based protein expression profiling was performed to identify proteins. The significantly and differentially expressed proteins were validated in the validation dataset by multiple reaction monitoring mass spectrometry (MRM-MS) with liquid chromatography-mass spectrometry (LC-MS), and synthetic peptides of beta-galactosidase (Sangon Biotech) were added to the denatured plasma samples as internal standard peptides.

Cell Culture and miRNA Transfection

All *in vitro* experiments were performed in human hepatoma HepG2 cells procured from the National Infrastructure of Cell Line Resource, Beijing, China. The identity of all cell lines was confirmed by short tandem repeat profiling at the time of procurement in July 2018. Cells were maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100μg/ml streptomycin under conditions of 5% CO2/95% air at 37°C. HepG2 cells were transfected with either the mimic negative control (mimic_NC, 50nM) or the miRNA mimic (50nM) (RiboBio) with Lipofectamine 3000 and Plus Reagent (Invitrogen) according to the manufacturer's instructions. When used, the miRNA inhibitor and inhibitor negative control were transfected at a dose of 100nM.

RNA Isolation, qRT-PCR, and Western Blotting

After transfection for 48 h, cells were lysed in radio immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors. Total RNA was isolated from both cells and media using Invitrogen TRIzol (Life Technologies). Then, RNA (2μg) was reverse transcribed using random hexamers, and the expression levels of genes were measured with specific primers (**Supplementary Table 2**) and Applied Bio systems SYBR Green Master Mix (Life Technologies). Proteins were isolated from both cells and media for *in vitro* experiments by cold acetone sedimentation. Protein samples (30μg) were analyzed by Western blotting (primary antibodies are listed in **Supplementary Table 3**).

Glucose Production Assay and Lactate Measurements

HepG2 cells were transfected with the miRNA mimic or inhibitor for 48 h. Prior to termination of culture, cells were incubated in 12h serum starvation conditions in DMEM without glucose plus L-glutamine (Cat No: 11966025, Gibco) and then were incubated 4h in the same medium supplemented with 10% FBS. Extracellular lactate levels were estimated by a lactate assay kit (Sigma-Aldrich), and glucose levels were estimated by the glucose oxidase method.

Glucose Consumption and Intracellular Glycogen Content Measurements

After HepG2 cells were transfected with the miRNA mimic or inhibitor and cultured in high-glucose DMEM for 48 h, the median glucose levels were measured by the glucose oxidase method. Cell viability was assessed with a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies). Glucose consumption was normalized by the CCK-8 assay. The anthrone-sulfuric acid colorimetric method was used to measure the intracellular glycogen content, which was normalized to the protein content (Pierce™ Rapid Gold BCA Protein Assay Kit, Life Technologies). To estimate the effect of insulin, after 24 h of transfection, cells were serum starved for 12 h and then incubated for 2 h with insulin (100nM).

Statistical Analysis

Differences in clinical characteristics, plasma miRNA levels, and plasma protein levels between type 2 diabetes cases and controls were evaluated by chi-square test for categorical variables, by Student's t test for normally distributed data or by Mann–Whitney U test for skewed data. Correlations between plasma miRNA profiles or protein expression levels (log transformed and normally distributed) and clinical measurements were evaluated by Pearson correlation analysis. Multivariate logistic regression models were used to calculate the odds ratios (ORs) and 95% confidence intervals (CIs). All bars in figures indicate the mean ± standard deviation (SD) values, and data were analyzed using ANOVA with a *post hoc* test.

RESULTS

Characteristics of the Study Population

As shown in **Table 1**, BMI, waist-hip ratio (WHR), and levels of fasting plasma glucose (FPG), HbA1c, and triglyceride (TG) were significantly higher but the levels of low-density lipoprotein cholesterol (LDLC) were lower in type 2 diabetes cases than in controls. Compared with controls, type 2 diabetes cases had elevated white blood cell counts. Similarly, neutrophil count, lymphocyte count, and eosinophil count were significantly higher in type 2 diabetes cases than in controls.

Plasma miRNA Profiles in Type 2 Diabetes Cases and Controls

Plasma miRNA profiles in 15 case-control pairs were assessed by miRNA array screening (**Figure 1**). Among the 1934 detected miRNAs, 167 were differentially expressed between the two groups. The results of cluster analysis of differentially expressed miRNAs are shown in **Figure 2A**. We selected top

12 miRNAs (fold change and *P* values are listed in **Table 2**) for validation in a larger population (98 type 2 diabetes cases and 98 matched controls) using TaqMan real-time PCR, and the expression levels of 4 miRNAs were successfully measured (To improve the repeatability, we excluded 8 miRNAs for further analysis. Because the plasma levels of these miRNAs were below the instrument detection limit when we validated the 12 miRNAs by PCR.). The detailed expression profiles of miR-193b-3p, miR-26b-3p, miR-197-5p, and miR-4739 are shown in **Figure 2 (E. b)**. As the results indicate, the plasma levels of miR-193b-3p were significantly elevated in type 2 diabetes cases compared with that in controls (fold change =2.01, $P = 0.006$).

Multivariate logistic analysis suggested that plasma miR-193b-3p levels were significantly associated with elevated diabetes risk (OR: 2.11, 95% CI: 1.02-4.37) after adjustment for age, sex, BMI, smoking status, drinking status, and family history of diabetes. Further adjustment for total cholesterol (TC) levels and systolic blood pressure (SBP) slightly enhanced the association (OR: 2.28, 95% CI: 1.05-4.91). Similarly, after further adjustment for white blood cell count, red blood cell count, and platelet (PLT) count, the miR-193b-3p level was consistently associated with increased type 2 diabetes risk (OR: 2.25, 95% CI: 1.05-4.84) (**Table 3**). In addition, plasma miR-193b-3p levels were significantly related to TC, TG, FPG, HbA1c, and lymphocyte count ($P < 0.01$) (**Table 4**).

Target Genes of miR-193b-3p

Based on three commonly used target algorithm tools (TargetScan, miRDB, and miRTarBase), we performed pathway enrichment analysis on genes predicted by at least two of the tools. The PI3K-AKT signaling pathway was predicted with an FDR of < 0.05 (**Supplementary Figure 2**). Three genes including SOS Ras/Rho guanine nucleotide exchange factor 2 (SOS2), the GTPase KRas (KRAS) (33), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) (34) were potential direct targets of miR-193b-3p in the PI3K-AKT signaling pathway and have been reported to be involved in cellular glucose metabolism. Therefore, we speculated that miR-193b-3p might regulate cellular glucose metabolism by directly targeting SOS2, YWHAZ, and KRAS.

Plasma Protein Profiles in Type 2 Diabetes

In total, 815 proteins were detected in pooled plasma by iTRAQ. The differentially expressed proteins are presented in **Figure 2B**. The mRNAs encoding 35 of these proteins were predicted to be miR-193b-3p targets by three commonly used target algorithm tools (TargetScan, miRDB, and miRTarBase) (**Supplementary Figure 3**). Identified proteins encoded by target genes are listed in **Supplementary Table 4**. Differentially expressed proteins are shown in **Table 5**. Triosephosphate isomerase (TPI1, P60174), profilin-1 (PFN1, P07737), talin-1 (TLN1, Q9Y490), and coactosin-like protein (COTL1, Q14019) were selected for further validation in a larger population (fold change > 1.2, $P < 0.05$, and coverage of identified peptides > 50%). Proteins with low coverage of identified peptides, including O15511, P21333, and B3GN61 were not selected for validation (**Table 6**). The fold change values and *P* values of the other 28 proteins are shown in

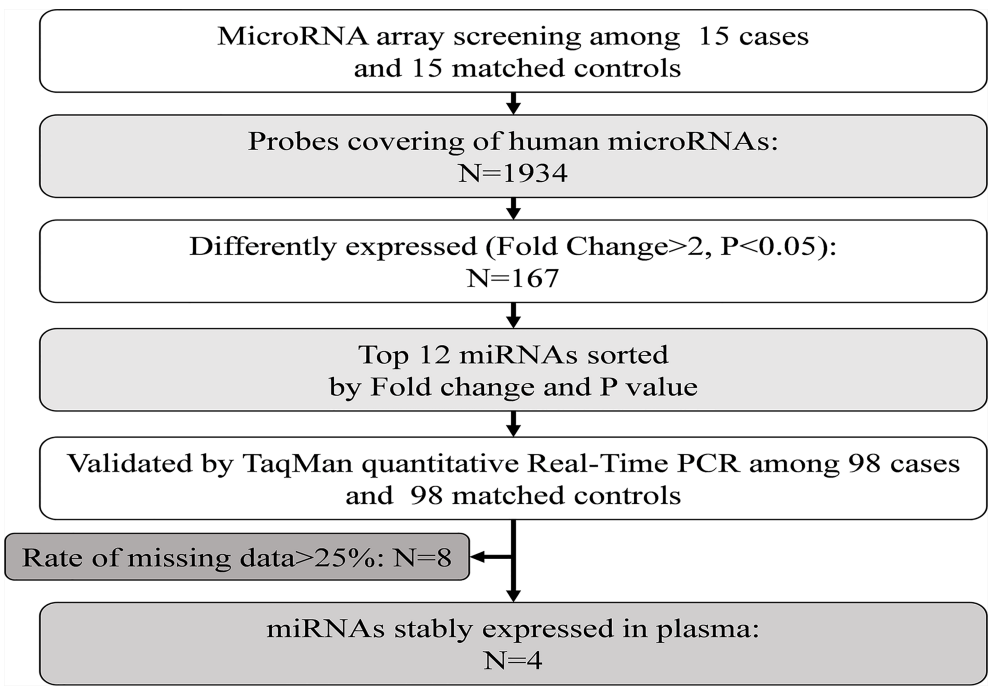

FIGURE 1 | Study design for the plasma miRNA profile in type 2 diabetes cases and controls. PCR, polymerase chain reaction.

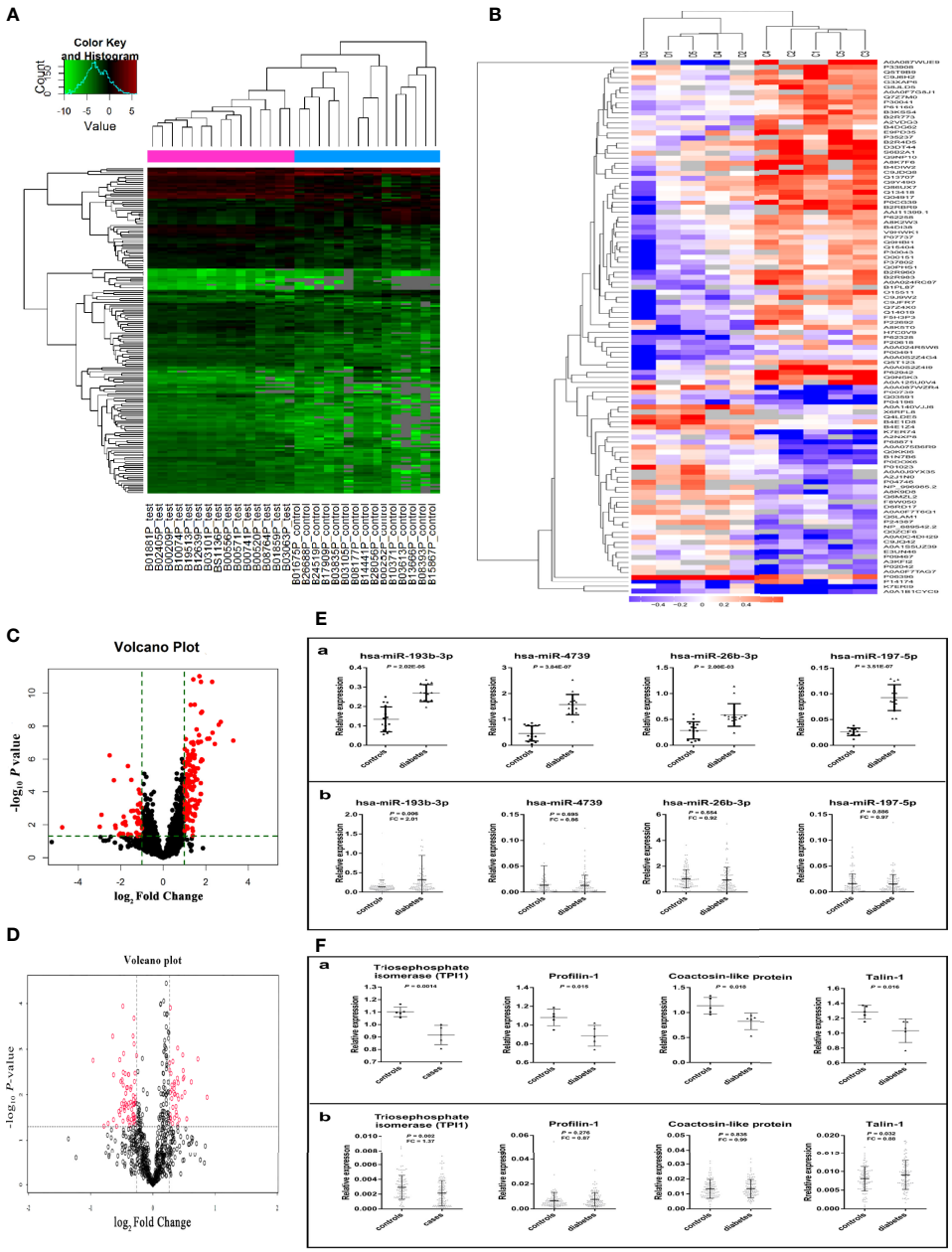

FIGURE 2 | Results of miRNAs and protein screening and validation. **(A)** shows the results of cluster analysis of differently expressed miRNAs. Sample numbers with the test suffix are type 2 diabetes cases (like B01881P_test). Sample numbers with the control suffix are healthy controls (like B01675P_control). **(B)** shows the results of cluster analysis of differently expressed proteins. C1-C5: type 2 diabetes cases, D1-D5: healthy controls. **(C)** The volcano plot of miRNAs in array screening. **(D)** The volcano plot of proteins. (**E**. a) Differential expression of miRNA in plasma screened by miRNA microarray chip. (**E**. b) Differential expression of miRNA in plasma validated by Taqman PCR. (**F**. a) The different expression of 4 plasma proteins in preliminary screening group. (**F**. b) The different expression of 4 plasma proteins in validation group. (The figures were created by graphPad and R Statistical Software).

Supplementary Table 5. KIT and YWHAZ were predicted by three databases, but YWHAZ was not differentially expressed between the two groups, and the coverage of identified peptides for KIT was less than 50%.

Two peptides per protein were selected for relative quantification of protein levels in plasma from 98 controls and 98 type 2 diabetes cases. The Q1/Q3 transitions of target proteins and the internal standard protein (beta-galactosidase) are shown in **Table 7**. As shown in **Figure 2 (F. b)**, plasma TPI1 levels significantly decreased (fold change = 1.37; $P = 0.002$) but talin-1 levels marginally but significantly increased (fold change = 1.13; $P = 0.032$) in type 2 diabetes cases. However, no significant difference was observed for plasma levels of profilin-1 and coactosin-like protein. A non-significant negative correlation

TABLE 2 | Differently expressed miRNAs selected to validation.

miRNAs	Fold change	P value	FDR	CV	
				Controls	Diabetes
miR-3591-5p	0.17	5.99E-07	4.22E-05	0.5	0.28
miR-122-3p	0.32	2.66E-06	1.00E-04	0.43	0.38
miR-193b-3p	2.02	2.45E-07	2.02E-05	0.48	0.16
miR-26b-3p	2.11	1.00E-04	2.00E-03	0.58	0.37
miR-300	2.44	5.06E-10	1.56E-07	0.52	0.12
miR-217	2.52	7.96E-07	5.30E-05	0.43	0.3
miR-3926	2.66	1.46E-11	1.38E-08	0.43	0.15
miR-641	2.68	1.31E-07	1.13E-05	0.51	0.28
miR-593-5p	3.13	1.04E-07	1.03E-05	0.61	0.27
miR-105-5p	3.22	3.52E-08	5.40E-06	0.65	0.28
miR-4739	3.45	1.59E-09	3.84E-07	0.65	0.25
miR-197-5p	3.53	1.35E-09	3.51E-07	0.28	0.27

CV, coefficient of variance, FDR, False Discovery Rate, FDR is calculated from Benjamini Hochberg FDR.

between plasma miR-193b-3p and TPI1 was observed (**Supplementary Figure 4**).

We further explored the difference in miR-193b-3p and protein levels between type 2 diabetes cases and controls in males and females (**Figure 3**). No significant differences in plasma miR-193b-3p levels were observed between males and females, while TPI1 levels significantly reduced in females but not in males.

Effects of miR-193b-3p on TPI1 Expression and Glycolysis in HepG2 Cells

Since TPI1 mRNA was predicted to be the direct target of miR-193b-3p, a miR-193b-3p mimic was transfected into HepG2 cells to evaluate the effects of miR-193b-3p on TPI1 gene expression. MiR-193b-3p was overexpressed in the mimic group compared with the mimic_NC group, inhibitor group, and inhibitor negative control group (**Figure 4A**). However, cell viability did not differ between the four groups (**Supplementary Figure 5A**). We further examined the mRNA and protein levels of TPI1 in transfected cells. The TPI1 protein level (**Figure 4B**) significantly decreased in mimic group compared with other groups ($P < 0.01$), while the TPI1 mRNA levels did not significantly differ between the mimic group and the negative control group (**Supplementary Figure 5B**). Since TPI1 is an important isomerase in the cellular glycolytic process (35), we further measured extracellular lactate levels. As shown in **Supplementary Figure 5C**, lactate levels did not significantly differ between the mimic group and other treatment groups.

MiR-193b-3p Reduced YWHAZ and SOS2 Expression Levels, Subsequently Altering PCK1 Expression and Gluconeogenesis in HepG2 Cells

Bioinformatic analysis indicated that miR-193b-3p might directly target SOS2, YWHAZ, and KRAS and thus regulate cellular glucose metabolism. *In vitro*, mRNA levels of YWHAZ significantly decreased in the miR-193b-3p mimic group compared to miRNA mimic_NC (P=0.049) and miRNA inhibitor group (P=0.007) 48 h after miR-193b-3p mimic transfection (**Figure 4C**), while SOS2 and KRAS levels did not significantly change (**Supplementary Figures 5D, E**). Moreover, the protein levels of YWHAZ and SOS2 were significantly decreased in the miR-193b-3p mimic group (**Figure 4E**) compared to other groups ($P < 0.01$). YWHAZ has been shown to regulate the expression of FOXO1 (36, 37). In addition, as a transcription factor in the PI3K/AKT signaling pathway, FOXO1 has been shown to increase the expression of genes encoding proteins involved in gluconeogenesis, including phosphoenolpyruvate carboxykinase (PCK1) and glucose 6-phosphatase (G6PC) (38, 39). Therefore, we further measured the expression levels of FOXO1, PCK1, and G6PC. Although mRNA levels of FOXO1, PCK1, and G6PC did not significantly change (**Supplementary Figures 5F–H**), the protein levels of FOXO1 and PCK1 were significantly increased in the mimic group compared with other groups (**Figure 4E**, $P < 0.01$). As

TABLE 3 | Association of 4 miRNAs with T2D risk in validation population.

	Model 1		Model 2		Model 3	
	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
miR-193b-3p	2.11 (1.02,4.37)	0.046	2.28 (1.05,4.91)	0.036	2.25 (1.05,4.84)	0.038
miR-4739	1.00 (0.70,1.44)	0.991	1.01 (0.70,1.44)	0.975	1.05 (0.73,1.52)	0.793
miR-26b-3p	0.80 (0.58,1.12)	0.194	0.81 (0.57,1.15)	0.237	0.87 (0.59,1.28)	0.471
miR-197-5p	0.92 (0.66,1.27)	0.602	0.94 (0.67,1.30)	0.689	0.96 (0.68,1.35)	0.824

Model 1: adjusted for sex, age, BMI, smoking status, drinking status, physical activities and family history of diabetes.
Model 2: adjusted for variables in model 1 and TC (total cholesterol) and SBP (systolic pressure).
Model 3: adjusted for variables in model 2 and WBC (white blood cell count), RBC (red blood cell count) and PLT count.

TABLE 4 | Pearson correlation coefficients of plasma miRNAs correlation to clinical biochemical measurements and blood cell counts was analyzed in healthy participants (n=49).

	miR-193b-3p	miR-4739	miR-26b-3p	miR-197-5p
Clinical biochemical measurements				
SBP	0.101	0.065	0.250**	0.207**
DBP	0.083	-0.034	0.141	0.049
TG	0.283**	-0.021	0.126	0.12
LDLC	0.008	-0.005	0.228**	-0.035
HDLC	-0.118	-0.003	-0.101	-0.004
CHOL	0.185*	-0.033	0.177*	-0.03
FPG	0.274**	0.009	-0.133	-0.098
HbA1c	0.237**	-0.033	0.218*	-0.032
Blood cell counts				
Neutrophil	0.023	-0.088	0.254**	0.019
Lymphocyte	0.277**	0.067	0.11	0.045
Monocyte	0.105	0.004	0.031	0.114
Eosnophils	-0.054	0.021	0.047	0.075
Basophil	-0.045	-0.042	-0.036	-0.029
RBC	0.012	-0.048	0.161*	0.057
PLT	-0.011	0.011	0.297**	0.036

** indicates correlations statistically significant: *P < 0.05, **P < 0.01.*

shown in **Figure 4D**, when cells were incubated in glucose-free medium for another 4 h after transfection, the glucose level in the medium of the mimic group was significantly increased compared to those in the medium of the other groups (mimic vs. mimic_NC: P =0.002; mimic vs. inhibitor: P = 0.026).

MiR-193b-3p Altered Glucose Uptake in HepG2 Cells

Since SOS2 is an important upstream factor in the PI3K/AKT signaling pathway (40, 41), we further estimated the glucose consumption and intracellular glycogen content of the transfected cells after incubation in high-glucose DMEM for another 48 h. Both glucose consumption (P = 0.032) and the intracellular glycogen content (P = 0.009) were significantly decreased (**Supplementary Figures 5I, J**) in the miRNA overexpression group compared to miRNA knockout group (mimic vs. inhibitor), whereas the expression levels of glycogen synthase kinase (GSK3A) and glycogen synthase (GYS2) did not significantly change (**Supplementary Figures 5L, K**). Furthermore, we investigated factors affecting glucose uptake and found that the protein levels of insulin receptor (IR) and glucose transporter 2 (GLUT2) were significantly decreased in the miR-193b-3p mimic group compared to other groups (**Figure 4F**). To further explore the effects of miR-193b-3p on insulin-mediated glucose uptake, we estimated glucose consumption and the intracellular glycogen content 2 h after insulin stimulation. Both were significantly decreased in the mimic group compared to negative control group (P = 0.015 and 0.049, respectively) (**Supplementary Figures 5O, P**).

DISCUSSION

It has demonstrated that miRNAs play an important role in the glucose metabolism (8). MiRNAs in circulating blood could be potential biomarkers of diabetes (11). Based on a case-control study we found that plasma miR-193b-3p levels increased in newly diagnosed, untreated diabetes cases. The *in vitro* experiments indicated that elevated levels of miR-193b-3p in cells may impair glucose metabolism by inhibiting the expression of SOS2 and YWHAZ/14-3-3ζ in the PI3K-AKT pathway. These findings provided new evidence to the important role of miRNAs in the diabetes development.

Recent studies focused on delineating circulating miRNA profiles to find new disease biomarkers (9, 15). Previous studies showed that plasma levels of miR-122 (42), miR-126

TABLE 5 | Differentially Expressed Proteins Identified by iTRAQ Analysis.

Accession	Description	Peptides Coverage	Case vs. Control	
			Fold change	*P* value
Q4LDE5	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1(SVEP1)	0.31	1.41	0.004
P60174	Triosephosphate isomerase 1 (TPI1)	61.85	0.83	0.001
P07737	Profilin-1 (PFN1)	73.57	0.82	0.015
Q9Y490	Talin-1 (TLN1)	53.56	0.81	0.016
Q7Z7M0	Multiple epidermal growth factor-like domains protein 8 (MEGF8)	6.12	0.8	0.001
Q14019	Coactosin-like protein (COTL1)	51.41	0.73	0.018
O15511	Actin-related protein 2/3 complex subunit 5 (ARPC5)	38.41	0.71	0.016

Accession was the number of proteins in Uniprot database.Peptides coverage: The number of amino acids in the peptide detected by mass spectrometry accounted for a proportion of the total number of amino acids in the protein.

TABLE 6 | Differentially Expressed Proteins Identified by iTRAQ Analysis.

Accession	Description	Peptides Coverage	Case vs. Control	
			Fold change	*P* value
Q4LDE5	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1(SVEP1)	0.31	1.41	0.004
P60174	Triosephosphate isomerase 1 (TPI1)	61.85	0.83	0.001
P07737	Profilin-1 (PFN1)	73.57	0.82	0.015
Q9Y490	Talin-1 (TLN1)	53.56	0.81	0.016
Q7Z7M0	Multiple epidermal growth factor-like domains protein 8 (MEGF8)	6.12	0.8	0.001
Q14019	Coactosin-like protein (COTL1)	51.41	0.73	0.018
O15511	Actin-related protein 2/3 complex subunit 5 (ARPC5)	38.41	0.71	0.016

Accession was the number of proteins in Uniprot database.Peptides coverage: The number of amino acids in the peptide detected by mass spectrometry accounted for a proportion of the total number of amino acids in the protein.

(43, 44), and circulating exosomal miR-20b-5p (9) elevated in type 2 diabetes cases and may affect insulin function. Furthermore, a previous study showed that plasma miR-193b-3p levels significantly increased in a prediabetic population and glucose-intolerant mice (16). Moreover, circulating miR-193b-3p levels returned to baseline levels in glucose-intolerant mice receiving chronic exercise therapy intervention. In addition, polycystic ovary syndrome (PCOS) patients with impaired glucose metabolism had increased serum miR-193b-3p levels compared with PCOS patients with normal glucose tolerance (45). However, the potential mechanism of miR-193b-3p in cellular glucose metabolism has not been extensively explored. Limited evidence suggested that miR-193b-3p controls adiponectin production in human white adipose tissue (46), which is strongly and inversely associated with diabetes risk (25).

In the present study, plasma miR-193b-3p levels were related to TG, HbA1c, and FPG levels in healthy controls. In addition, SOS2, KRAS, and YWHAZ/14-3-3ζ in the PI3K-AKT signaling pathway were associated with glucose metabolism, as shown by miR-193b-3p target gene enrichment analysis (23, 34, 40). Currently, *in vitro* studies on glucose metabolism mainly focused on hepatogenic cells, most commonly human hepatocellular carcinoma HepG2 cells (47); therefore, we further explored the effect of miR-193b-3p on glucose metabolism in HepG2 cells.

YWHAZ/14-3-3ζ, a member of the 14-3-3 protein family, is a direct target of miR-193b-3p in MCF-7 cells (48) and can directly downregulate the expression of FOXO1, an important transcription factor downstream of the PI3K-AKT pathway (37, 49). In the present study, we validated the association of miR-193b-3p with YWHAZ at both mRNA and protein levels. Moreover, we found that as miR-193b-3p level increased, the protein levels of FOXO1 and PCK1 also elevated. Similarly, glucose output from the cells increased, consistent with previous findings indicating that FOXO1 increased the expression of genes encoding proteins involved in gluconeogenesis, including PCK1 and G6PC (38, 39). In summary, as shown in **Supplementary Figure 6**, miR-193b-3p can target YWHAZ/14-3-3ζ and subsequently upregulate transcription factor FOXO1 downstream of the PI3K-AKT pathway, which increases PCK1 expression, having a potential effect on enhancing gluconeogenesis. Further experiments are required to prove this hypothesis.

The present study also showed that the protein level of SOS2 decreased as the level of miR-193b-3p increased. SOS2 has been reported to be involved in positive regulation of Ras proteins (50)

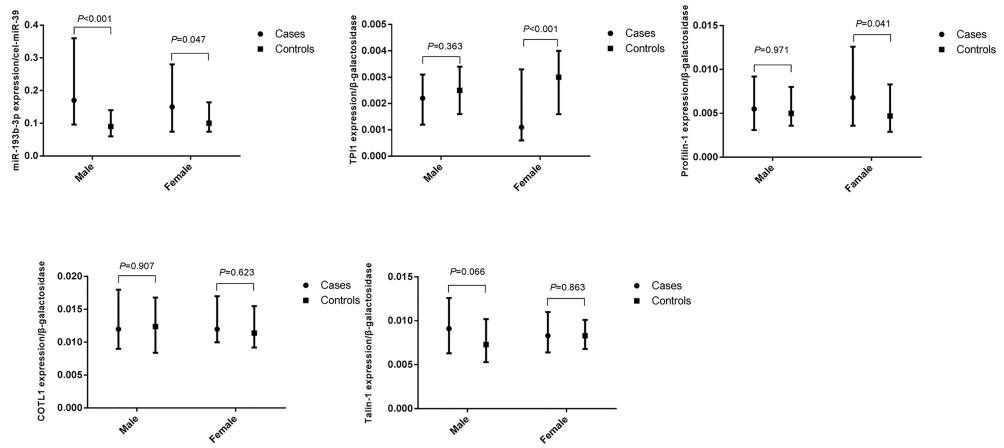

FIGURE 3 | Differences of plasma miR-193b-3p and proteins between healthy controls and type 2 diabetes cases in groups according to gender. (The figures were created by graphPad).

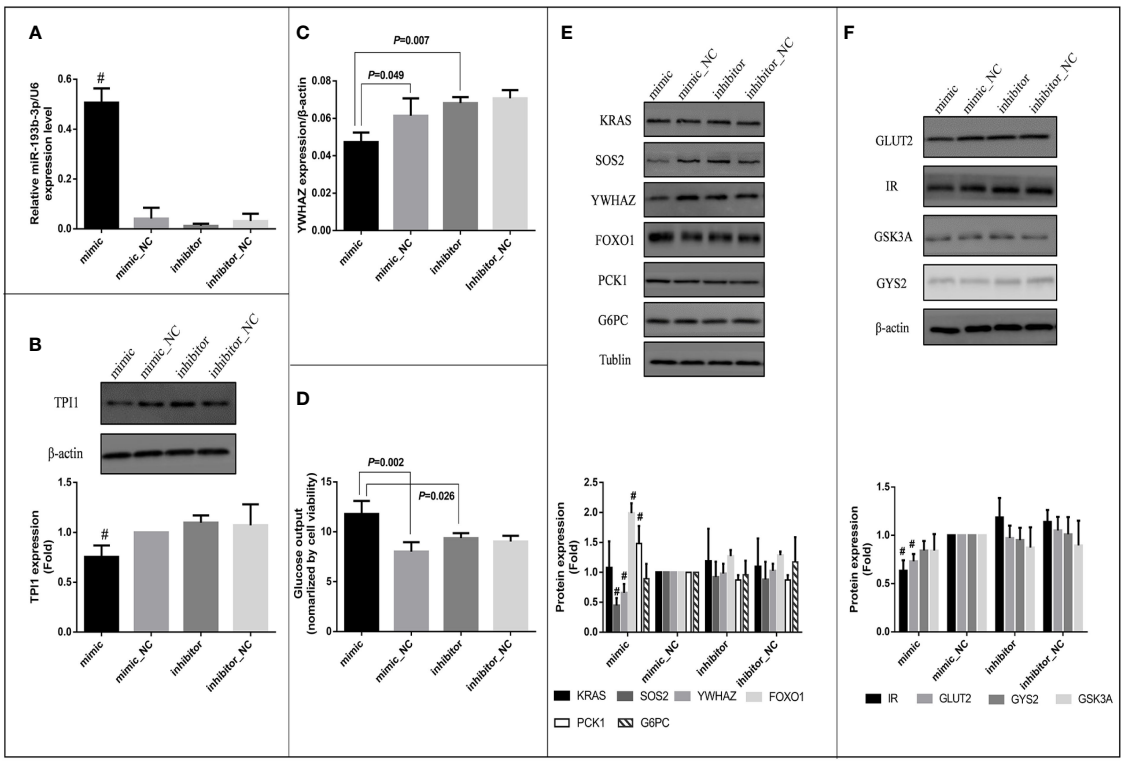

FIGURE 4 | Effects of miR-193b-3p on glucose metabolism in HepG2 cells. Mimic: HepG2 cells transfected with miR-193b-3p mimic; mimic_NC: cells transfected with negative control of mimic; inhibitor: cells transfected with miR-193b-3p inhibitor; inhibitor_NC: cells transfected with negative control of inhibitor. **(A)** The expression level of miR-193b-3p after transfection; **(B)** Representative Western blot of TPI1 and β-actin after transfection. **(C)** The mRNA expression level of YWHAZ. **(D)** The level of extracellular glucose after transfection. **(E)** Representative Western blot of KRAS, SOS2, YWHAZ, FOXO1, PCK1, G6PC, and Tublin after transfection. **(F)** Representative Western blot of GLUT2, IR, GSK3A, GYS2, and β-actin after transfection. The bar graphs of figure **(B)**, **(E)** and **(F)** showed the fold change of protein levels (cells transfected with mimic negative control as reference group) quantified by Image Pro Plus, # indicates P <0.01 for protein level in mimic group compared with all the other groups. (The figures were created by graphPad).

as an important upstream factor in the PI3K-AKT pathway and can cooperate with other factors to activate the PI3K-AKT pathway in (51). Therefore, SOS2 may not significantly affect the expression of AKT (a serine/threonine kinase), which can be activated by phosphatidylinositol 3-kinase (PI3K) and plays an important role in processes of glucose metabolism, including glycogen synthesis (49). Accordingly, the expression of GSK3A and GYS2 did not significantly change with elevated miR-193b-3p levels, which were directly affected by AKT (52).

In addition, decreased expression levels of IR and GLUT2 proteins were observed, but the mRNAs encoding these proteins were not predicted to be direct targets of miR-193b-3p in the miRNA-target databases. These reductions may be due to enhanced gluconeogenesis. Increased intracellular gluconeogenesis reduces the concentration gradient of glucose across the membrane, thereby decreasing GLUT2 expression and glucose uptake (53). In addition, the 14-3-3 protein has been reported to inhibit the expression of insulin receptor substrate 1 (IRS1), leading to insulin resistance (54), although the exact mechanism needs to be further explored.

We observed that as the miR-193b-3p levels increased, the TPI1 levels decreased in plasma and cells. Given that TPI1 is an enzyme involved in glycolysis without apparent tissue specificity (reference from Expression Atlas: https://www.ebi.ac.uk/gxa/home), we cannot attribute its plasma down regulation to a specific cell type yet. Based on the results of gene enrichment analysis and liver being one of the main organs involved in the regulation of glucose metabolism, we chose the hepatocytes to explore the potential function. For the potential mechanism of decreased level of TPI1 in HepG2, On the one hand, miR-193b-3p may directly target TPI1 mRNA through noncanonical binding mode, as previously mentioned (55). On the other hand, the central glycolytic enzyme TPI1 has been reported to play a key role in linking energy with redox metabolism during the stress response and in cancer, and the pyruvate kinase (PK) substrate phosphoenolpyruvate (PEP) can inhibit TPI1 activity in the feedback regulation system of glycolysis (35). In the present study, the protein levels of PCK1 increased in cells as miR-193b-3p levels elevated. Together with GTP, PCK1 catalyzes the formation of PEP from oxaloacetate and releases carbon dioxide and GDP (56). Thus, miR-193b-3p might indirectly affect cellular function of TPI1.

In vitro cell experiments, the expression of miR-193b-3p in the miRNA mimic group increased more than 5~10-fold

TABLE 7 | Q1/Q3 transitions of 4 target proteins selected for the MRM experiments.

Accession	Protein Name	Peptide Sequence	Q1/Q3 (m/z)	DP	CE
P00722	β-galactosidase	GDFQFNISR	542.3/489.3	70.6	28.4
			542.3/636.3	70.6	28.4
		VDEDQPFPAVPK	671.3/587.2	80.1	33
			671.3/755.4	80.1	33
P07737	Profilin-1	DSPSVWAAVPGK	607.3/913.4	75.4	30.7
			607.3/301.2	75.4	30.7
		TFVNITPAEVGVLVGK	822.5/968.6	91.1	38.5
			822.5/1069.6	91.1	38.5
P60174	Triosephosphate isomerase	SNVSDAVAQSTR	617.8/562.3	76.2	31.1
			617.8/934.5	76.2	31.1
		VVLAYEPVWAIGTGK	801.9/1057.6	89.6	37.7
			801.9/928.5	89.6	37.7
Q14019	Coactosin-like protein	EVVQNFAK	312.2/130	53.9	14.6
			312.2/147.1	53.9	14.6
		FALITWIGENVSGLQR	902.5/473.3	96.9	41.3
			902.5/959.5	96.9	41.3
Q9Y490	Talin-1	AVASAAAALVLK	542.8/914.6	70.7	28.4
			542.8/685.5	70.7	28.4
			362.2/147.1	57.5	17.3
			362.2/260.2	57.5	17.3
		GLAGAVSELLR	543.3/617.4	70.7	28.4
			543.3/716.4	70.7	28.4

Accession was the number of proteins in Uniprot database, Q1: parent ion, Q2: transition; DP=Declustering Pressure; CE=Collision Pressure.

compared to the mimic_NC group, while the expression of SOS2 and KRAS on RNA level did not significantly change. However, significant differences in protein expression levels and glucose output were observed. Previous studies indicated that miRNAs can negatively regulate gene expression by targeting specific mRNA transcripts and inducing their degradation or translational repression (39). According to the results of this study, and based on the transcripts analyzed, we suggest that miR-193b-3p inhibit the translation rather than inducing the degradation of target mRNAs encoding proteins including IR and GLUT2 that mediating glucose uptake. Our results illustrated the effects of miR-193b-3p overexpression on cellular function, however, the detailed effects of miR-193b-3p on protein and the effects of cytokines on downstream proteins and the potential mechanism needed to be further explored. Regarding the effect of miR-193b-3p on glucose metabolism, we only observed changes in glucose output and consumption. More studies are needed to explore this issue.

A strength of this study is that we selected newly diagnosed type 2 diabetes who did not receive any pharmacological treatment. Thus, we were able to exclude the potential effects of pharmacological treatment on miRNA profiles and, to some extent, reduce the potential confounding factors. In addition, we used a multidimensional "omics" approach in this population-based study to identify differentially expressed proteins associated with miR-193b-3p and to gain clues for further functional studies. Finally, we provided insight into the potential mechanism of miR-193b-3p in the development of diabetes.

There were several limitations should be considered. Firstly, miRNAs in plasma or serum are packaged in extracellular vesicles or bound to various proteins, including lipoproteins and argonaute proteins (57), and the concentration of most miRNAs in plasma are relatively low. In the present study, we screened 12 miRNAs with obvious differential expression for verification, finally four miRNAs were successfully measured and one miRNA with differential expression was validated. This might be partly due to the limited sample size in screen and validation. Secondly, we were unable to determine the source and target tissues or cells of these miRNAs. This limitation of the present study requires further research to distinguish the forms of miRNA present in plasma. Thirdly, the DFTJ cohort study was conducted in a population of middle-aged and older individuals; thus, whether these findings are applicable to other populations remains to be determined. Fourthly, in the present large study population it is difficult to perform oral glucose tolerance tests; therefore, diagnosis of diabetes based on the criteria of FPG ≥ 7.0mmol/L and/or HbA1c ≥ 6.5% may lead to misclassification. However, the positive association of miR-193b-3p with diabetes risk might not be attenuated but instead enhanced. Fifthly, "Fifthly, before testing the glucose synthesized by gluconeogenic pathway, cells were incubated in serum- and glucose-free medium for 12 h and were then incubated an additional 4 h without glucose, so that glycogen stores can be completely consumed. However, in our experimental design, we erroneously added 10% serum in the last 4 h of incubation, so we cannot atribute glucose appereance in the medium completely to gluconeogenesis, while it might be better to further study the source of increased glucose by vivo experiments or using stable isotopes. Finally, in the present study it is difficult to obtain liver tissue from diabetic patients for *in vitro* functional experiments, therefore, we investigated the effects of miR-193b-3p on glucose metabolism in HepG2 cells. However it has indicated that the HepG2 cells have relatively low similarity with human tissue (58), therefore, the functional effects of miRNA in other cell lines need to be further verified.

In conclusion, miR-193b-3p was differentially expressed in plasma of type 2 diabetes cases. With miR-193b-3p levels

increased, TPI1 levels decreased both in plasma and in HepG2 cells. In addition, miR-193b-3p may affect glucose metabolism by directly targeting YWHAZ/14-3-3ζ and upregulating the FOXO1 transcription factor downstream of the PI3K-AKT pathway. Based on the results of observed in HepG2, the effects of miR-193b-3p on glucose metabolism in other tissues, such as skeletal muscle and adipose tissue, might be similar. However, it remians to be eluciated in further studies. Additionally, miR-193b-3p was verified as a plasma biomarker of diabetes, the expression of miR-193b-3p in extracellular vesicle in plasma needed test to explore the origin and destination of miR-193b-3p.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics and Human Subject Committee of the School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, and Dongfeng General Hospital, DMC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HH and MH conceived and designed the study. HH performed experiments, MZ, ZL, HN, JH and ZC participated in experiments. HH did the statistical analysis and drafted the manuscript. JY, HG, XZ, HY, TW, MH checked the data extraction. All authors critically revised the manuscript for important intellectual content. MH obtained fundings and supervised the study. MH has full access to all of the data 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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.814347/full#supplementary-material

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Conflict of Interest: Author HY is employed by Dongfeng Central Hospital, Dongfeng Motor Corporation.

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Role of Glucagon and Its Receptor in the Pathogenesis of Diabetes

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Various theories for the hormonal basis of diabetes have been proposed and debated over the past few decades. Insulin insufficiency was previously regarded as the only hormone deficiency directly leading to metabolic disorders associated with diabetes. Although glucagon and its receptor are ignored in this framework, an increasing number of studies have shown that they play essential roles in the development and progression of diabetes. However, the molecular mechanisms underlying the effects of glucagon are still not clear. In this review, recent research on the mechanisms by which glucagon and its receptor contribute to the pathogenesis of diabetes as well as correlations between *GCGR* mutation rates in populations and the occurrence of diabetes are summarized. Furthermore, we summarize how recent research clearly establishes glucagon as a potential therapeutic target for diabetes.

Keywords: glucagon, diabetes, pathogenesis, glucagonocentric hypothesis, glucagon receptor, glucagon-like peptide 1

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1 INTRODUCTION

Diabetes is a metabolic disorder characterized by hyperglycemia resulting from an absolute deficiency of insulin secretion (type 1 diabetes, T1D), or a combination of insulin resistance and an inadequate compensatory insulin secretion (type 2 diabetes, T2D) (1). However, each type of diabetes in animals and humans is accompanied by hyperglucagonemia (2–4), so glucagon excess is more critical to the development of diabetes than insulin deficiency (4, 5). Increasing evidence indicates that blocking glucagon and glucagon receptor (GCGR) can relieve hyperglycemia in animals and humans, clearly establishing the important roles of glucagon and GCGR in the pathogenesis of diabetes (6, 7).

Glucagon is a linear peptide containing 29 amino acids. It is secreted by islet α cells and mainly targets the liver cells (8). GCGR is a G-protein-coupled receptor (GPCR) mainly detected in islet β cells and liver cells (9). After glucagon specifically binds to GCGR, it promotes liver glycogen breakdown and increases blood glucose levels to stimulate insulin release (10, 11). Glucagon-like peptide 1 (GLP-1), mainly expressed in intestinal L cells, activates glucagon-like peptide-1 receptor (GLP-1R) to adjust metabolism (12, 13). Glucagon and GLP-1 are derived from the same biosynthetic precursor proglucagon and are involved in the regulation of lipid and cholic acid metabolism, thereby playing pivotal roles in glucose metabolism and the pathogenesis of diabetes (7, 12, 13).

In this review, we explore the controversial relationships between glucagon and metabolic disorders associated with diabetes based on recent research with an emphasis on recent evidence supporting the important role of glucagon. We also elucidate the correlation between *GCGR*

mutations in populations and the occurrence of diabetes. Furthermore, we summarize drug strategies to provide a new basis for the treatment of diabetes.

2 CONTROVERSY REGARDING THE ROLE OF GLUCAGON IN METABOLIC DISORDERS ASSOCIATED WITH DIABETES

2.1 Insulinocentric Theory

The debate over the relative roles of hormones in the regulation of diabetes-related metabolic disorders has spanned decades. In 1921, the discovery of insulin was regarded as one of the greatest breakthroughs in the history of medicine. This led to the establishment of the insulinocentric view, which proposes that all diabetes-related metabolic disorders are directly caused by a lack of insulin secretion (14). Glucagon was not yet characterized and accordingly was not associated with these metabolic disorders. The insulinocentric theory was accepted for over half a century until Unger et al. proposed the bihormone theory at a conference in 1975 (15, 16).

2.2 Bihormonal Regulation

According to the theory of bihormonal regulation, diabetes results from the abnormal secretion of both insulin and glucagon (15, 16). Some metabolic disorders associated with diabetes are directly caused by insulin deficiency, such as elevated lipolysis, increased proteolysis, and decreased glucose utilization. Others, such as decreased glycogen synthesis, increased ketogenesis, elevated hepatic glycogenolysis, and gluconeogenesis, are direct effects of excess glucagon (15–18) (**Figure 1**). Glucagon has glucogenic, ketogenic, and gluconeogenic functions and mediates severe endogenous hyperglycemia and hyperketonemia under a state of insulin deficiency; thus, it is a direct cause of the substantial increases in the levels of glucose and ketone in severe presentations of diabetes (19). In patients with diabetes with relatively steady levels of insulin, a rise in glucagon causes hyperglycemia and glycosuria (17). Glucagon suppression may be an effective adjunct to routine antihyperglycemic therapy in patients with diabetes (20–22).

2.3 Glucagonocentric Hypothesis

Glucagonocentric hypothesis was proposed by Unger et al. based on the following evidence: (a) hyperglucagonemia is present in all forms of diabetes; (b) marked hyperglucagonemia is caused by perfusing anti-insulin serum to the normal pancreas; (c) during a total insulin deficiency, all metabolic manifestations of diabetes can be suppressed by glucagon suppressors, like somatostatin, and in global *Gcgr* knockout (*Gcgr*$^{-/-}$) mice, demonstrating that β cell destruction does not cause diabetes (4). Thus, compared with insulin deficiency, glucagon excess plays a more essential role in the development of diabetes.

Gcgr $^{-/-}$ mice were designed to further understand the role of GCGR in the development of diabetes; these mice do not respond to glucagon at any concentration, and their fasting blood glucose levels are lower than those of wild-type mice. These knockout mice exhibit enhanced glucose tolerance and elevated insulin sensitivity during insulin tolerance testing (23). When β cells of *Gcgr* $^{-/-}$ mice were destroyed by streptozotocin (STZ) and insulin secretion was inhibited, animals did not develop hyperglycemia, suggesting that *Gcgr* $^{-/-}$ mice do not develop T1D, even under a state of insulin deficiency (24). After the transient repair of defective *Gcgr* with an adenovirus vector, the blood glucose levels of the mice increased after β cell destruction (25). When *Gcgr* was inactivated again, blood glucose levels returned to normal, suggesting that in the absence of glucagon, insulin deficiency does not result in abnormal blood glucose levels, and that the

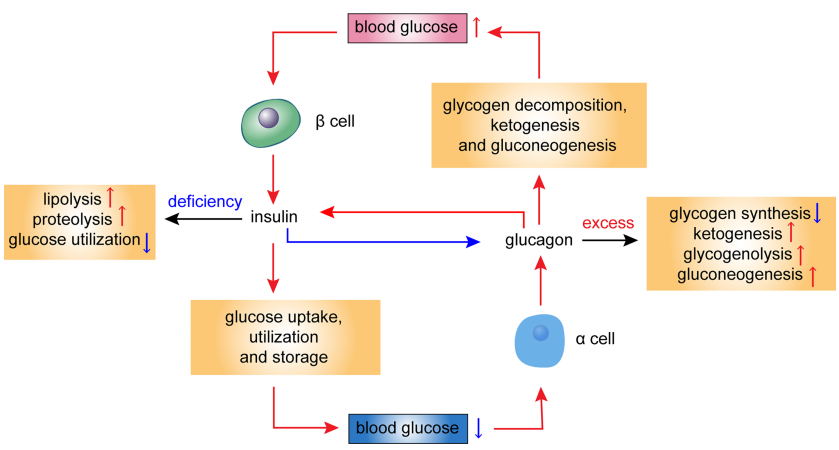

FIGURE 1 | Hormonal regulation of glucose homeostasis in the islet cells. This diagram illustrates the metabolic effects of glucagon and insulin. Blood glucose levels influence secretion of insulin and glucagon. Insulin deficiency leads to elevated lipolysis, increased proteolysis, and decreased glucose utilization, while excess glucagon leads to decreased glycogen synthesis, increased ketogenesis, elevated glycogenolysis, and gluconeogenesis. Red arrows refer to a stimulatory effect, while blue arrows refer to an inhibitory effect.

abnormal blood glucose concentration caused by insulin deficiency can be restored by eliminating the effect of glucagon (25). Hence, blocking *Gcgr* can restore hyperglycemia in rodent models with insufficient insulin secretion; however, this effect requires a certain number of β cells (26). Active GLP-1 was identified in pancreatic perfusate from *Gcgr* $^{-/-}$ but not wild-type mice (27), and FGF21 acts additively with GLP-1 to prevent insulinopenic diabetes in mice lacking glucagon action (28), which further reduces the risk of *Gcgr* $^{-/-}$ mice developing diabetes. On the contrary, *Gcgr* knockout implies that glucagon cannot function normally, which can cause a series of metabolic problems, such as hyperglucagonemia and compensatory hyperplasia of α cells (23, 29, 30). Therefore, the above phenomena should be monitored in the development of GCGR antagonists. The therapeutic potential of GCGR is not fully recognized and should be a basis of further studies; however, the established animal models provide an effective means for the development of strategies to reduce the incidence of diabetes.

3 MECHANISM BY WHICH GLUCAGON AFFECTS INSULIN SECRETION

In healthy people, high blood glucose stimulates β-cell insulin secretion, and glucagon secretion is suppressed; low blood glucose inhibits β-cell insulin secretion, and glucagon secretion is stimulated (**Figure 1**). Nevertheless, hyperglucagonemia was present in patients with diabetes, including T1D (31) and T2D (32). No significant difference of plasma glucagon level was found between T1D and T2D (31, 32). Absolute deficiency or relative deficiency of insulin secretion weakened the inhibition of insulin on glucagon (4).

Glucagon's role in intra-islet paracrine regulation is essential. Svendsen et al. (27) used isolated perfused pancreas from wild-type, *Glp-1r* knockout, diphtheria toxin-induced proglucagon knockdown, β cell-specific *Gcgr* knockout, and *Gcgr*$^{-/-}$ mice to examine glucagon-induced insulin secretion. They found that paracrine glucagon actions are required for maintenance of normal insulin secretion, and intra-islet glucagon signaling involves the activation of both GCGR and GLP-1R. Loss of either GCGR or GLP-1R does not change insulin responses, whereas combined blockage of both receptors significantly reduces insulin secretion (27). Additionally, *Gcgr* $^{-/-}$mice show normal blood glucose levels and increased glucagon levels in glucose-stimulated insulin secretion (GSIS) tests after treatment with 10 mM (33) or 12 mM (27) glucose. This is similar to levels observed in control mice, suggesting that the insulin-promoting effect of glucagon is achieved mainly *via* GLP-1R. However, as the cognate downstream receptor of glucagon, the physiological significance of β-cell GCGR remains subtle. Zhang et al. (34) states that glucagon potentiates insulin secretion *via* β-cell GCGR at physiological but not high concentrations of glucose, and β-cell GCGR activation promotes GSIS more than GLP-1R in high fat diet. These findings indicate that GCGR contributes to glucose homeostasis maintenance during nutrient overload. These studies emphasized the indispensable roles of GCGR on β cells in mediating both the glucose balance and catabolic state and implied that GCGR is closely related to the pathogenesis of diabetes. Accordingly, studies of the mechanisms by which GCGR regulates insulin secretion are of great significance.

In pancreatic β cells, GLUT2, a glucose transporter protein, is required for GSIS (35). Glucose binding to GLUT2 is a key pathway leading to increased ATP levels, deionization, increased intracellular calcium concentration, and enhanced insulin exocytosis. GLUT1 expression decreased in *Gcgr*$^{-/-}$ mice but increased in wild-type mice after glucose stimulation (36). As a paracrine hormone, glucagon binds to GCGR with high affinity, while also exerting a "spillover" effect by binding to GLP-1R with low affinity (37). After glucagon binds to GCGR and GLP-1R on β cells, the activated receptors engage the G protein Gαs, which stimulate the generation of cyclic adenosine monophosphate (cAMP) (34, 38–40). The response of glucagon to glucose mainly depends on cAMP signaling in islet β cells and the increased cAMP level promotes insulin release (39, 41) (**Figure 2**).

4 ASSOCIATION OF *GCGR* MUTATIONS WITH DIABETES IN VARIOUS POPULATIONS

T2D, also called non-insulin dependent diabetes mellitus, is a common disorder with complex traits. Multiple genomic scans have identified different loci associated with T2D, including a locus on chromosome 17q24-25 (42, 43) and *GCGR* on chromosome 17q25, which might be explained by linkage identified in the same region (44). GCGR mediates glucose homeostasis by binding to glucagon and may contribute to the pathogenesis of T2D and the development of β-cell dysfunction, resulting in a deficient insulin response in some patients with T2D. Further studies are needed to determine the effect of hepatic glucagon resistance on metabolic disorders and its association with the occurrence of diabetes. Chronic hyperglycemia increases the protein expression of GCGR in the liver and decreases downstream glucagon signaling, leading to liver glucagon resistance (45, 46). *GCGR* mutations may be related to hyperglucagonemia *via* the impairment of endogenous glucagon autofeedback, to high hepatic glucose output in T2D *via* elevated glycogenolysis and/or gluconeogenesis, and to abnormal insulin secretion *via* the glucagon resistance of β cells in T2D.

GCGR is regarded as a candidate gene for the pathogenesis of T2D and *GCGR* mutations with similar frequencies have been found associated with T2D (47). Polymorphisms in the *GCGR* gene are associated with T2D in Caucasians (48). The Gly40Ser variant of GCGR (c.118G >A) causes a change from glycine (at the 40th amino acid residue) to serine. In French and Sardinian familial T2D groups, 5% and 8% of randomly selected patients with diabetes, respectively, showed Gly40Ser mutations. These percentages are substantially higher than the frequencies of any other candidate gene mutations reported previously (47). Gough et al. examined patients from three geographically distinct regions in the United Kingdom and the Gly40Ser mutation was present in

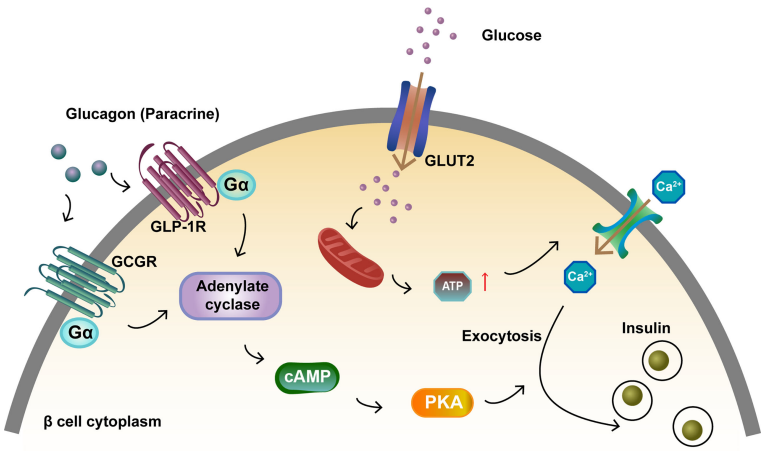

FIGURE 2 | Activation of GCGR and GLP-1R to promote insulin secretion in islet β cells. Glucagon binds to GCGR and GLP-1R on β cells and the activated receptors engage the G protein Gαs. This results in adenylate cyclase activation and cAMP formation. Glucose binds to GLUT2, which increases ATP levels and intracellular calcium concentration, and enhances insulin exocytosis. The increase in intracellular cAMP levels activates PKA, which also promotes insulin exocytosis.

15/691 patients with T2D and 1/425 geographically matched controls (48), suggesting that individuals with the Gly40Ser mutation may be predisposed to T2D. GCGR mutation frequencies have been examined in other populations and regions. However, the Gly40Ser mutation was not detected in studies involving subjects of Japanese (49–52), Finnish (53), Dutch (54), Utahans (55), German (54, 56), Russian (57), Indian Tamil (58), Han Chinese (59), Taiwanese (60), Brazilian (61), and Italian (44) descents. Another study (62) conducted in different areas of Sardinia did not find low insulin secretion in the population carrying this mutation in contrast to the earlier 1995 study (47). It showed that the Gly40Ser variation was not related to T2D in the Sardinian population and that its frequency varied among regions in Sardinia. Although no such association was found in Brazil, reduced insulin secretion was observed in Gly40Ser carriers (61). Based on a genetic analysis of 64 children with diabetes, the Gly40Ser mutation may be associated with T2D susceptibility in China (63). It reduces the binding of GCGR and glucagon and insulin secretion; this observation led Hansen to hypothesize that the Gly40Ser mutation in GCGR can lead to the abnormal functioning of islet β cells and may predispose carriers to diabetes, possibly by impairing glucagon-mediated signaling and decreasing the sensitivity of the target tissues to glucagon (64).

In addition to the relationship between the Gly40Ser mutation and T2D, an elevated frequency of *GCGR* mutations has been found in probands from multiple (affected sibling pair) families with T1D, also known as insulin-dependent diabetes; however, the lack of preferential transmission from heterozygous parents to affected siblings with T1D suggests population stratification (48). Overall, this Gly40Ser mutation may promote islet β-cell dysfunction, resulting in deficient insulin responses in patients with diabetes.

Together, these findings suggest that the contribution of *GCGR* to diabetes may vary and mutations in this gene play only a small role in determining the susceptibility of an individual to diabetes and the observed genetic heterogeneity of diabetes. Given the heterogeneity of the disease, the importance of *GCGR* for diabetes susceptibility may vary among ethnicities owing to the differences in genetic and environmental factors. *GCGR* is a polymorphic gene. The absence of a *GCGR* polymorphism (Gly40Ser) at one site does not rule out mutations associated with susceptibility to diabetes in other regions. For example, in addition to Gly40Ser, homozygous missense mutations (P86S) have been found in *GCGR*; these mutations contribute to the formation of an ineffective GCGR, resulting in hyperglycemia and extreme α-cell proliferation (65). Recent studies have reported 250 missense variants in human *GCGR* (66, 67). *GCGR* shows lower allelic diversity and fewer missense variants and variants with trait associations than the other class B1 GPCRs. These observations support the crucial role of the glucagon system in metabolism and indicate that the predominant signaling pathway mediating the physiological effects of GCGR is the one mediated by Gαs. These findings provide a clear link between molecular mechanisms and clinical phenotypes. The metabolic phenotypes related to several missense variants of *GCGR* have been investigated in case studies and in studies of genetically engineered animals, including V368M and V369M (68, 69). Further research is needed to explore the relationship between *GCGR* variants and diabetes.

5 GLUCAGON-RELATED THERAPIES FOR DIABETES

Several emerging glucagon-based therapies are under pre-clinical and clinical development.

5.1 GCGR Antagonism

GCGR antagonism has been proposed as a pharmacological approach to treat T1D or T2D, including the use of small molecule antagonists, monoclonal antibodies (mAb) against

GCGR, and antisense oligonucleotides that reduce expression of the receptor (70–73). Relevant clinical trials have shown that they can reduce blood glucose levels through inhibition of glucagon action (74–76); however, several adverse effects, such as increased LDL-cholesterol (LDL-c), ALT level, and bodyweight, have been observed (74, 77).

5.1.1 GCGR Antagonists

Several GCGR antagonists have been developed to improve glucose tolerance, insulin secretion, and glucose control in animals (78, 79), and have shown remarkable efficacy in patients with T2D, such as MK-0893, MK-3577, LY2409021 and LGD-6972 (76, 80–82). They upregulate circulating GLP-1 level by promoting intestinal L-cell proliferation and GLP-1 production in T2D (82). MK-0893 and MK-3577, which were advanced to phase II clinical trials, led to robust glucose lowering in patients with T2D; however, their adverse effects, such as increased LDL-c and ALT level, have hindered their clinical development (83–86). LY2409021 significantly reduced blood glucose and HbA1c levels with a lower risk of hypoglycemia (80, 81), but it increased liver fat (87). LGD-6972 is an allosteric GCGR antagonist, structurally different from other small molecule GCGR antagonists. It was well tolerated at all tested doses and did not cause hypoglycemia (88, 89), but additional details on biochemical differentiation are lacking and this compound does not appear to be under active clinical development (71).

5.1.2 GCGR mAbs

With the cessation of clinical trials of GCGR antagonists and better understanding of the protein structure of GCGR, antibodies against GCGR have been developed. GCGR mAbs have good specificity, strong targeting, and are relatively easy to source. They can not only return blood glucose and HbA1c to normal levels when administered to mice with T1D not treated with insulin (73), as well as patients with T1D (90), but also show a strong hypoglycemic effect in mice and monkeys with T2D (91, 92). They can even induce β cell regeneration by the transdifferentiation of a portion of pancreatic α cells or δ cells into β cells (93). REMD 477 is a fully competitive mAb against GCGR. A single dose of REMD-477 significantly reduces insulin requirement in patients with T1D, which improves glycemic control in patients without serious adverse reactions (90). Another GCGR mAb, REGN1193, has good safety and tolerability, but transient elevation of transaminases was also observed (94). Overall, GCGR mAbs are promising for improving glycemic control and have great research promise.

5.1.3 GCGR Antisense Oligonucleotides (GR-ASO)

GR-ASO inhibits the effect of glucagon mainly by decreasing the expression of GCGR mRNA (95). The intraperitoneal administration of GR-ASO to db/db mice and Zucker diabetic fatty (ZDF) rats decreases (nearly normalizes) non-fasting blood glucose levels (95). GR-ASO improves β-cell function (i.e., improves the insulin response to intraperitoneal glucose stimulation) and substantially improves glucose tolerance in normal and ZDF rats. However, *Gcgr*$^{-/-}$ mice and other animals treated with GR-ASO show extensive islet α-cell proliferation and significantly elevated circulating proglucagon-related peptide levels (96). Recently, ISIS-GCGRRx (76), IONIS-GCGRRxN (97), and ISIS 325568 (98) have been shown to attenuate glucagon-stimulated hepatic glucose production and glucose fluctuations. They support the treatment of GR-ASO in patients with T2D.

5.2 GLP-1R Agonists

The most well-characterized biological function of GLP-1 is to potentiate glucose-dependent insulin secretion, which makes the GLP-1R an attractive target in the treatment of T2D (99). Thus, GLP-1R agonists are clinically used as anti-diabetic drugs (100). Glucagon not only acts to antagonize insulin in the fasting state but also functions in the fed state and promotes insulin secretion to maintain normal blood glucose levels (34). The insulin-promoting properties of glucagon are mediated by GCGR and GLP-1R in β cells (27, 33, 101); however, GLP-1R is the main receptor to exert an insulin-stimulating effect (101). It is reasonable to assume that even with *GCGR* mutations in β cells, glucagon binding to GLP-1R exerts an insulin-promoting effect that can reduce blood glucose concentrations in patients with diabetes. Although GLP-1R agonists have been used for the treatment of diabetes, their efficacy is limited by target receptor desensitization and downregulation *via* the recruitment of β-arrestins (102, 103). GLP-1R agonists with decreased β-arrestin-2 recruitment have shown promising effects in recent preclinical and clinical studies (104). Understanding the mechanisms of action may resolve these issues with the application of GLP-1R agonists.

5.3 GCGR and GLP-1R Co-Agonists

Owing to the traditional view that the main effect of glucagon is to increase blood glucose levels, the idea of increasing glucagon concentration as a means of reducing glucose levels initially met resistance. Nevertheless, the action of glucagon on GCGR and GLP-1R (regulators of insulin secretion and energy metabolism) has a significant effect on systemic glucose homeostasis (105). On the one hand, GCGR and GLP-1R co-agonists can activate GLP-1R to promote insulin secretion and then reduce blood glucose. On the other hand, they can activate GCGR, promote lipid metabolism and reduce body weight (106–108). Since human islets have more mixed α-β cell interfaces, the ratio of GCGR to GLP-1R may be particularly vital to human islet function (8, 109). SAR425899 is a novel polypeptide with a co-excitatory effect on GCGR and GLP-1R, which can reduce blood glucose and HbA1c levels and reduce body weight in patients with T2D; however, it has an adverse effect on the gastrointestinal tract (110). It also improves postprandial blood glucose control by significantly enhancing β cell function and slowing glucose

absorption rate (111). These findings highlight the possible clinical relevance of dual agonist peptides that simultaneously stimulate the synthesis of GCGR and GLP-1R and may drive the development of novel antidiabetic drugs.

6 CONCLUSIONS

In this review, we provide a clear overview of various theories of hormonal regulation of diabetes, with a focus on the essential roles of glucagon and its specific receptor in the pathogenesis of diabetes. Although GCGR and glucagon play important roles in diabetes, the mechanisms and role of mutations still needs to be explored. We summarized the pleiotropic effects of glucagon, future research prospects, and the development of novel therapeutic strategies. This area of research remains challenging but exciting. Further research on islet α cells, glucagon, and GCGR signaling pathways is expected to provide a basis for developing new strategies for diabetes prevention.

AUTHOR CONTRIBUTIONS

YJ wrote the manuscript. GS designed and critically reviewed the manuscript. SS critically revised the manuscript. YL and LF supervised the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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The m^{6}A Methyltransferase METTL3 Ameliorates Methylglyoxal-Induced Impairment of Insulin Secretion in Pancreatic β Cells by Regulating MafA Expression

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Methylglyoxal, a major precursor of advanced glycation end products, is elevated in the plasma of patients with type 2 diabetes mellitus. Islet β-cell function was recently shown to be regulated by N^6-methyladenosine (m^{6}A), an RNA modification consisting of methylation at the N6 position of adenosine. However, the role of m^{6}A methylation modification in methylglyoxal-induced impairment of insulin secretion in pancreatic β cells has not been clarified. In this study, we showed that treatment of two β-cell lines, NIT-1 and β-TC-6, with methylglyoxal reduced m^{6}A RNA content and methyltransferase-like 3 (METTL3) expression levels. We also showed that silencing of METTL3 inhibited glucose-stimulated insulin secretion (GSIS) from NIT-1 cells, whereas upregulation of METTL3 significantly reversed the methylglyoxal-induced decrease in GSIS. The methylglyoxal-induced decreases in m^{6}A RNA levels and METTL3 expression were not altered by knockdown of the receptor for the advanced glycation end product but were further decreased by silencing of glyoxalase 1. Mechanistic investigations revealed that silencing of METTL3 reduced m^{6}A levels, mRNA stability, and the mRNA and protein expression levels of musculoaponeurotic fibrosarcoma oncogene family A (MafA). Overexpression of MafA greatly improved the decrease in GSIS induced by METTL3 silencing; silencing of MafA blocked the reversal of the MG-induced decrease in GSIS caused by METTL3 overexpression. The current study demonstrated that METTL3 ameliorates MG-induced impairment of insulin secretion in pancreatic β cells by regulating MafA.

Keywords: METTL3, methylglyoxal, N^6-methyladenosine, pancreatic β cells, insulin secretion

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a public health problem of considerable magnitude that is characterized by hyperglycemia, insulin resistance, and gradual exhaustion of insulin secretion from pancreatic β cells (1). Methylglyoxal (MG), a highly reactive dicarbonyl product of glucose metabolism, is believed to be the most important precursor of advanced glycation end products (AGEs). We (2) and others (3, 4) have demonstrated that plasma MG levels are significantly higher in T2DM patients than in control individuals. MG may be involved in the development of DM and diabetic complications by acting as either a precursor of AGEs or a direct toxin (5–7). MG reduced islet β-cell insulin secretion both *in vivo* and *in vitro* (8–10); however, the molecular mechanism by which MG treatment results in decreased insulin secretion has not been elucidated.

N^6-Methyladenosine (m^6A), the most frequent mRNA modification in eukaryotes, has garnered wide interest in recent years because of its roles in regulating mRNA splicing, output, translation, and stability (11, 12). m^6A levels are mainly regulated by methyltransferases such as methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14), as well as demethylases, fat mass, obesity-associated protein (FTO), and α-ketoglutarate-dependent dioxygenase homolog 5 (ALKBH5) (13).

The m^6A modification is essential for the physiological function of pancreatic β cells (14). Levels of m^6A RNA in the pancreatic islets and plasma of patients with T2DM were markedly lower than those in control subjects (14–18). These changes in m^6A levels were attributed to decreases in METTL3 and METTL14 expression (14, 18–20) and an increase in FTO expression (16). Musculoaponeurotic fibrosarcoma oncogene family A (MafA), a key regulator of insulin gene transcription, is markedly decreased in the β cells of patients with T2DM (21). Wang et al. showed that METTL3 specifically targets MafA and regulates its protein expression (18). However, to the best of our knowledge, it remains unclear whether the m^6A modification is involved in MG-induced dysfunction of β-cell insulin secretion. Therefore, the present study was designed to explore the connection between MG and m^6A levels and to clarify the mechanisms underlying the role of the m^6A RNA modification in MG-induced β-cell dysfunction.

MATERIALS AND METHODS

Cell Culture

The mouse insulinoma β-cell lines NIT-1 and β-TC-6 were purchased from Procell Life Science and Technology Co. (Wuhan, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, California, USA) containing 10% fetal bovine serum (Gibco, California, USA). NIT-1 and β-TC-6 cells were treated with 1 mM of MG (Sigma, Missouri, USA) for the duration indicated in each experiment. This concentration of MG was selected based on previous studies (9, 10), which showed that 1 mM of MG sufficiently decreased islet β-cell function *in vitro*.

Cell Transfection

Small interfering RNAs (siRNAs) targeting METTL3 (si-METTL3), the receptor for advanced glycation end products (RAGE, si-RAGE), glyoxalase 1 (Glo-1, si-Glo-1), and MafA (si-MafA), as well as a negative control siRNA (si-NC), were synthesized by Riobio Technology Co. (Guangzhou, China). The siRNA sequences were as follows:

siRNA METTL3-1: 5′-GGACTCGACTACAGTAGCT-3′;
siRNA METTL3-2: 5′-CAAGTATGTTCACTATGAA-3′;
siRNA METTL3-3: 5′-GACTGCTCTTTCCTTAATA-3′;
siRNA RAGE-1: 5′-GCATTCAGCTGTTGGTTGA-3′;
siRNA RAGE-2: 5′-CCACTGGAATTGTCGATGA-3′;
siRNA RAGE-3: 5′-CCAGCAGCTAGAATGGAAA-3′;
siRNA Glo-1: 5′-CTATGAAGTTCTCGCTCTA-3′;
siRNA Glo-2: 5′-GCAAACGATGCTAAGAATT-3′;
siRNA Glo-3: 5′-AGAAGACAGCATGGACGTT-3′;
siRNA MafA-1: 5′-TCAACGACTTCGACCTGAT-3′;
siRNA MafA-2: 5′-TGATGAAGTTCGAGGTGAA-3′;
siRNA MafA-3: 5′-GATGAAGTTCGAGGTGAAG-3′.

Lipofectamine 3000 reagent (Invitrogen, California, USA), Opti-MEM medium (Gibco, California, USA), and siRNAs were mixed and incubated at room temperature for 15 min and then added to cells and incubated for 36 h. Three siRNA sequences were synthesized for each target gene, and the siRNA targeting METTL3-2, RAGE-1, Glo-1-2, and MafA-3 with the highest inhibition efficiencies were selected for subsequent experiments (**Supplementary Figure S1**).

Recombinant adenovirus constructs with either METTL3 (Ad-METTL3) or an empty vector (Ad-NC) and pCDNA3.1 plasmids carrying either MafA (pCDNA-MafA) or the empty vector (pCDNA) were synthesized by HanBio Technology Co. (Shanghai, China). Cells were infected with Ad-NC or Ad-METTL3 for 48 h. Cells were transfected with pCDNA or pCDNA-MafA using Lipofectamine 3000 reagent (Invitrogen, California, USA) for 48 h.

m^6A RNA Methylation Quantification

m^6A RNA methylation was quantified using the m^6A RNA Methylation Quantification Kit (Abcam, Cambridge, UK). Total RNA was extracted from NIT-1 and β-TC-6 cells using TRIzol reagent (Tiangen, Beijing, China). Briefly, the negative control, positive control, and 200 ng of sample RNA were added to the designated wells. Diluted capture antibody, detection antibody, and diluted enhancer solution were then added to each well. The m^6A content was quantified colorimetrically; the absorbance at 450 nm was measured using a microplate reader; and the m^6A content was calculated based on a standard curve. The percentage of total RNA containing m^6A was calculated using the formula provided by the manufacturer.

Quantitative Real-Time PCR

Total RNA was extracted and reverse transcribed using a reverse transcription kit (Tiangen, Beijing, China) according to the

manufacturer's instructions. Gene expression was analyzed by qPCR using the SYBR Green PCR Kit (Tiangen, Beijing, China). Expression levels of target genes were normalized to β-actin and differences were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used were as follows:

METTL3 forward primer: 5′-CATCCGTCTTGCCATCTCTACGC-3′,
reverse primer: 5′-GCAGACAGCTTGGAGTGGTCAG-3′;
METTL14 forward primer: 5′-TCGACCGAAGTCACCTCCTC-3′,
reverse primer: 5′-AGGAGTAAAGCCGCCTCTGT-3′;
FTO forward primer: 5′-GACACTTGGCTTCCTTACCTGACC-3′,
reverse primer: 5′-ACCTCCTTATGCAGCTCCTCTGG-3′;
ALKBH5 forward primer: 5-′GCAAGGTGAAGAGCGGCATCC-3′,
reverse primer: 5′-GTCCACCGTGTGCTCGTTGTAC-3′;
MafA forward primer: 5′-GCTTCAGCAAGGAGGAGGTCAT-3′,
reverse primer: 5′-TCTCGCTCTCCAGAATGTGCCG-3′;
β-Actin forward primer: 5′-CGTGAAAAGATGACCCAGATCA-3′,
reverse primer: 5′-CACAGCCTGGATGGCTACGT-3′.

Western Blot Analysis

NIT-1 and β-TC-6 cells were washed twice with cold PBS and lysed in 100 μl of modified RIPA buffer (Beyotime, Shanghai, China). Proteins were separated using SDS-PAGE and immediately transferred to nitrocellulose membranes. The membranes were incubated with the primary antibody overnight and then with the appropriate secondary antibodies for 2 h. The antibodies used were as follows: anti-METTL3 (Cat. No.: ab195352, 1:1000; Abcam, Cambridge, UK), anti-METTL14 (Cat. No.: ab220030, 1:1000; Abcam, Cambridge, UK), anti-RAGE (Cat. No.: 42544, 1:800; CST, Massachusetts, USA), anti-Glo-1 (Cat. No.: NP-006699.2, 1:500; ABclonal, Wuhan, China), anti-MafA (Cat. No.: 79737, 1:1000; CST, Massachusetts, USA), and anti-β-actin (Cat. No.: T0022, 1:3000; Affinity, Melbourne, Australia). The target proteins on the blots were detected using a Tanon 5200 visualizer. The results were assessed by densitometry using ImageJ software.

Glucose-Stimulated Insulin Secretion

As described in our previous study, cultured cells were washed with Krebs buffer (128.8 mM of NaCl, 4.8 mM of KCl, 1.2 mM of $MgSO_4$, 1.2 mM of KH_2PO_4, 1.2 mM of $CaCl_2$, and 10 mM of HEPES, pH 7.4) containing 0.2% bovine serum albumin. NIT-1 cells were incubated in Krebs buffer containing 2.8 mM of glucose for 30 min, and basal insulin secretion was measured (22–24). Stimulated insulin secretion was measured after incubating NIT-1 cells in Krebs buffer with 25 mM of glucose for 60 min. An aliquot of the buffer was collected, and insulin release was measured using an ELISA kit (Abcam, Cambridge, UK). The glucose-stimulated insulin secretion (GSIS) index was calculated by dividing the insulin secreted in cells exposed to 25 mM of glucose by the insulin secreted in cells exposed to 2.8 mM of glucose (24).

Methylated RNA Immunoprecipitation Coupled With qPCR Assay

Methylated RNA immunoprecipitation coupled with qPCR (MeRIP-qPCR) was performed using the MeRIP kit (Bersinbio, Guangzhou, China), according to the manufacturer's instructions. Briefly, total RNA was extracted from NIT-1 cells using TRIzol reagent, and the extracted RNA was fragmented using ultrasound treatment. The processed fragments were approximately 300 bp. After fragmentation, 50 μl of each RNA sample (the input sample) was stored at −80°C and the remaining portion of each RNA sample was immunoprecipitated with an anti-m^6A antibody (Abcam, Cambridge, UK) or anti-IgG antibody. The RNA-antibody hybridization solution was incubated with Protein A/G magnetic beads for 1 h at 4°C in a vertical mixer. The beads were washed three times and digested with proteinase K at 55°C for 45 min. The supernatant was transferred to new RNase-free tubes, and the RNA was purified and subjected to qPCR. The MafA primer sequences were as follows:

Forward: 5′-CAGGAAAAGCGGTGCTGGAGG-3′,
Reverse: 5′-CGAAGCTCTGACCCCGGAAGG-3′.

RNA Stability Assay

NIT-1 cells were treated with 5 μg/ml actinomycin D (Sigma, Missouri, USA) to inhibit mRNA transcription. After incubation for the indicated times, the treated cells were collected, and total RNA was extracted using TRIzol reagent. MafA mRNA expression was measured by qPCR. β-Actin was used for normalization.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD). One-way analysis of variance followed by the Newman–Keuls test was used to compare differences among groups. Statistical significance was set at $p < 0.05$.

RESULTS

m^6A RNA Modification and METTL3 Expression Levels Were Reduced in MG-Treated Pancreatic β Cells

To explore the potential role of MG in the m^6A modification in pancreatic β cells, the m^6A content in total RNA was measured in MG-treated and untreated NIT-1 and β-TC-6 cells. As shown in **Figure 1A**, the m^6A levels in RNA were significantly reduced in NIT-1 (reduced by 36.8%) and β-TC-6 (reduced by 39.3%) cells after MG treatment, indicating an MG-induced decrease in m^6A modification in pancreatic β cells. We then evaluated the mRNA and protein expression of the m^6A methyltransferases METTL3 and METTL14 and the demethylases FTO and ALKBH5 in MG-treated and untreated NIT-1 and β-TC-6 cells. Following treatment with MG for 24 h, METTL3 mRNA levels were markedly downregulated in both NIT-1 cells (reduced by 43.5% versus the untreated control (Con), $p < 0.05$) and β-TC-6 cells (reduced by 35.5% versus Con, $p < 0.05$) (**Figures 1B, C**). In contrast, the mRNA expression levels of METTL14, FTO, and ALKBH5 did not change

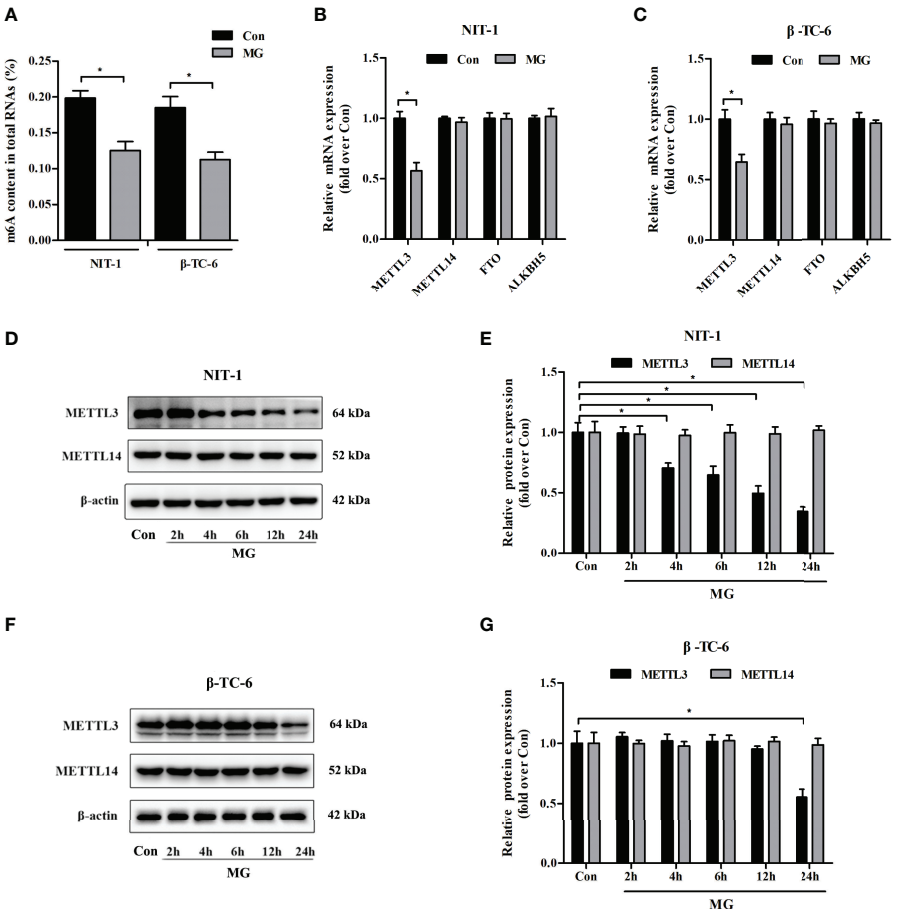

FIGURE 1 | Methylglyoxal (MG) treatment decreased m^{6}A RNA methylation and METTL3 expression levels in pancreatic β cells. **(A)** m^{6}A levels in total RNA from NIT-1 and β-TC-6 pancreatic β cells treated with 1 mM of MG (MG) for 24 h and untreated control cells (Con). **(B, C)** mRNA expression of the m^{6}A methyltransferases METTL3 and METTL14 and the demethylases FTO and ALKBH5 in NIT-1 **(B)** and β-TC-6 **(C)** cells treated with 1 mM of MG for 24 h as measured by qPCR. The mRNA level of each gene was normalized to β-actin. **(D–G)** Immunoblotting of METTL3 and METTL14 protein expression levels in NIT-1 **(D, E)** and β-TC-6 **(F, G)** cells treated with 1 mM of MG for different time periods. β-Actin was used as an internal control. Results are presented as the means ± SD of 3–4 independent experiments. *$p < 0.05$.

significantly (**Figures 1B, C**). Immunoblotting analysis showed that MG treatment decreased METTL3 protein expression in NIT-1 cells in a time-dependent manner (reduced by 29.4% at 4 h, 35.3% at 6 h, 50.4% at 12 h, and 65.2% at 24 h versus Con, $p < 0.05$; **Figure 1D**). In contrast, in MG-treated β-TC-6 cells, METTL3 protein expression was only reduced after 24 h of treatment (reduced by 44.5% versus Con, $p < 0.05$; **Figure 1F**). These findings suggest that MG may reduce m^{6}A levels in pancreatic β cells by decreasing METTL3 expression; NIT-1 cells are more sensitive than β-TC-6 cells to this MG treatment-induced decrease in METTL3 expression. Therefore, we selected NIT-1 cells to further characterize this effect.

Effects of METTL3 on Glucose-Stimulated Insulin Secretion From Pancreatic β Cells

To clarify the role of METTL3 in β-cell insulin secretion, we assessed the effects of METTL3 on GSIS from NIT-1 cells under normal culture conditions. We suppressed the expression of METTL3 with siRNA (**Figures 2A, B**) and found that the GSIS index was significantly reduced (by 22.2%, $p < 0.05$ versus si-NC, **Figure 2C**). To further investigate the biological function of METTL3, an adenovirus vector to overexpress METTL3 was transfected into NIT-1 cells (**Figures 2D, E**). Upregulation of METTL3 significantly reversed the MG-induced reduction in the GSIS index in NIT-1 cells (by 44.2%, $p < 0.05$; **Figure 2F**).

Effects of RAGE Knockdown on METTL3 Expression and m^{6}A RNA Levels in NIT-1 Cells

Advanced glycation end products (AGEs) exert biological effects *via* specific receptors; the most well-characterized is RAGE (25). Treatment of NIT-1 cells with MG, a major precursor of AGEs, enhanced RAGE expression (**Figures 3A, B**). To investigate whether the effects of MG on the m^{6}A modification are mediated by RAGE, we knocked down RAGE expression using a siRNA (**Figures 3A, B**). The MG-induced reductions in

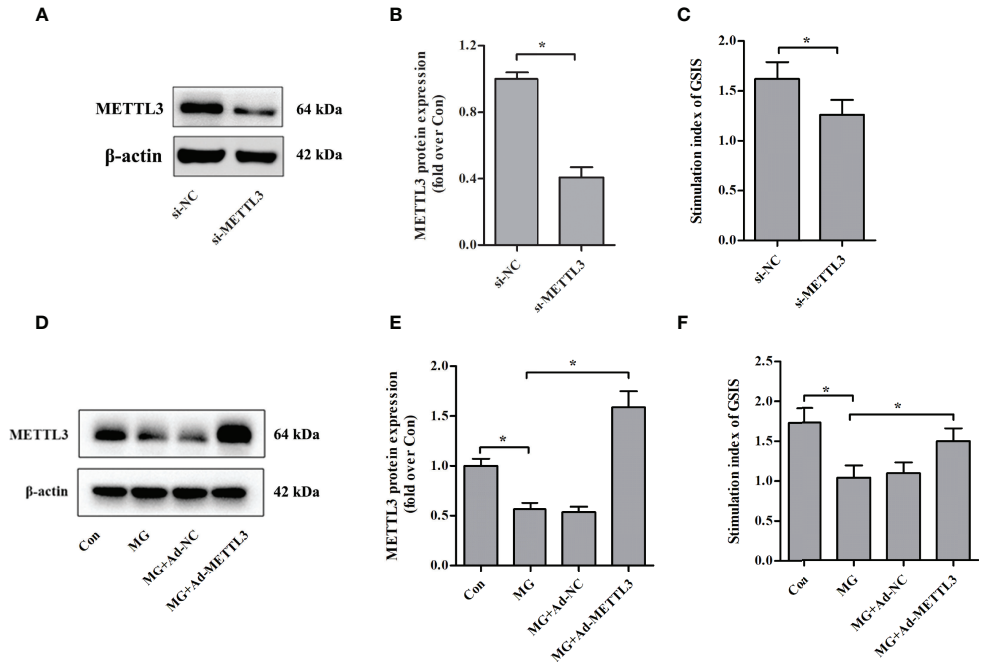

FIGURE 2 | Effects of METTL3 on GSIS from pancreatic β cells. **(A, B)** Immunoblot of METTL3 protein expression in NIT-1 cells transfected with METTL3 siRNA (si-METTL3) or a nonspecific control siRNA (si-NC), which was set to 1. **(C)** The GSIS index of NIT-1 cells transfected with si-METTL3 or si-NC. **(D, E)** Immunoblot of METTL3 protein expression in NIT-1 cells (MG), NIT-1 cells transfected with METTL3 expression adenovirus (MG+Ad-METTL3), and NIT-1 cells transfected with a nonspecific control adenovirus (MG+Ad-NC) that were treated with 1 mM of MG for 24 h. **(F)** The GSIS index in NIT-1 cells was transfected with Ad-METTL3 or Ad-NC and treated with 1 mM of MG for 24 h. Results are presented as the means ± SD of $n = 3$–4 independent experiments. *$p < 0.05$.

METTL3 expression (**Figures 3A, C**) and m^{6}A RNA modification levels (**Figure 3D**) were not reversed by knockdown of RAGE.

Effects of Glo-1 Knockdown on METTL3 Expression and m^{6}A Levels in NIT-1 Cells

Glo-1 is the main component of the glyoxalase system and is essential for MG detoxification in all mammalian cells (7). Similar to previous studies (26–29), MG treatment decreased Glo-1 expression in NIT-1 cells (**Figures 4A, B**). Interestingly, Glo-1 knockdown further reduced METTL3 expression (decreased by 50.2% versus MG treated, $p < 0.05$; **Figures 4A, C**) and m^{6}A RNA levels (decreased by 52.3% versus MG treated, $p < 0.05$; **Figure 4E**) in MG-treated NIT-1 cells. These effects were attributed to decreased MG degradation and an increased intracellular MG concentration (increased by 23.7% versus MG treated, $p < 0.05$; **Figure 4D**).

Loss of METTL3 Attenuated the Expression of MafA

Similar to the results observed in specific β cells in the islets of METTL3/14 knockout mice (18), we found that MafA protein and mRNA expression levels were markedly downregulated in NIT-1 cells after METTL3 knockdown (decreased by 44.6% and 57.0%, respectively, versus si-NC, $p < 0.05$; **Figures 5A–D**). We conducted rescue experiments and observed that overexpression of METTL3 reversed the decreases in MafA protein and mRNA expression in MG-treated NIT-1 cells (increased by 38.5% and 39.1%, respectively, versus MG, $p < 0.05$; **Figures 5E-G**). MeRIP-qPCR confirmed that the m^{6}A levels in MafA mRNA were decreased by METTL3 silencing in NIT-1 cells under normal culture conditions (decreased by 34.6% versus si-NC, $p < 0.05$; **Figure 5H**). The m^{6}A levels in MafA mRNA were increased by upregulation of METTL3 in MG-treated NIT-1 cells (increased by 81.2% versus MG, $p < 0.05$; **Figure 5I**). We conducted an RNA stability assay to investigate the relationship between m^{6}A and MafA mRNA stability. As shown in **Figure 5J**, MafA mRNA levels were decreased in METTL3-silenced NIT-1 cells after ActD treatment, indicating METTL3 knockdown led to reduced stability of MafA mRNA. MafA mRNA decay induced by MG treatment in NIT-1 cells was significantly ameliorated by transfection with Ad-METTL3 (**Figure 5K**). These results indicate that METTL3 regulates MafA expression in an m^{6}A-dependent manner.

A Change in MafA Expression Was Associated With METTL3-Regulated GSIS From NIT-1 Cells

To test whether METTL3 regulates GSIS from NIT-1 cells through MafA, rescue experiments were conducted by transfecting NIT-1 cells transfected with both si-METTL3 and either pcDNA or pcDNA MafA (**Figures 6A–C**). Overexpression of MafA (pcDNA MafA) greatly improved the decrease in GSIS triggered by METTL3 silencing in NIT-1 cells (increased by 36.7% versus si-METTL3 + pcDNA, $p < 0.05$). NIT-1 cells were

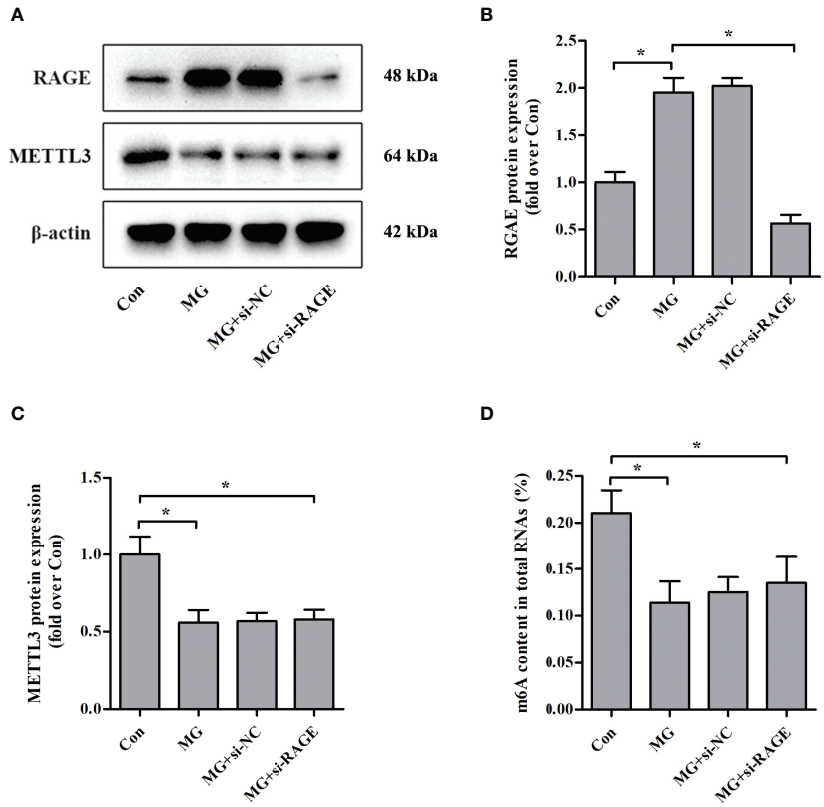

FIGURE 3 | Effects of RAGE knockdown on METTL3 expression and m^{6}A RNA methylation levels in NIT-1 cells. **(A–C)** Immunoblotting of RAGE and METTL3 protein expression in NIT-1 cells transfected with RAGE siRNA (si-RAGE) or a nonspecific control siRNA (si-NC) and treated with 1 mM of MG for 24 h. **(D)** m^{6}A levels in total RNA from NIT-1 cells treated as described for **(A–C)**. Results are presented as the means ± SD of n = 3–4 independent experiments. *$p < 0.05$.

transfected with Ad-METTL3 + si-NC or Ad-METTL3 + si-MafA and the GSIS index was measured (**Figures 6D–F**). The reversal of the MG-induced GSIS decrease caused by METTL3 overexpression in NIT-1 cells was abrogated by knockdown of MafA (decreased by 42.4% compared to MG + Ad-METTL3, $p < 0.05$).

DISCUSSION

Accumulation of MG in plasma has been implicated in the development of both DM and diabetic complications (5–7). We previously showed that plasma MG levels are markedly enhanced in patients with newly diagnosed T2DM, indicating that MG accumulation plays an important role in the onset of DM and not merely its complications (2). In fact, MG levels are increased and insulin content and GSIS were reduced in pancreatic islets isolated from a rat model with chronic MG infusion-induced T2DM, suggesting that MG accumulation leads to pancreatic β-cell dysfunction in T2DM (8). Therefore, the current study was designed to explore the regulatory mechanisms of MG β-cell dysfunction.

Although increasing evidence suggests that m^{6}A plays a role in many pathological processes in eukaryotic cells, studies on its roles in controlling pancreatic β-cell maturity and physiological function have just begun (14). We reported here, for the first time, that MG treatment significantly decreased m^{6}A levels in NIT-1 and β-TC-6 cells. Although MG treatment had no effect on the expression of METTL14, FTO, and ALKBH5, it obviously reduced METTL3 mRNA and protein expression. METTL3 and METTL14 form stable heterodimers and maintain high levels of m^{6}A (30). METTL3 may be more important for regulating pancreatic β-cell function than METTL14 because the increase in blood glucose is higher in β-cell METTL3 knockout mice than in β-cell METTL14 knockout mice (19, 20). Therefore, the decrease in m^{6}A levels in β cells was attributed to MG-induced downregulation of METTL3 expression. Silencing of METTL3 impaired GSIS from NIT-1 cells under normal culture conditions, whereas upregulation of METTL3 in NIT-1 cells ameliorated the MG-induced decrease in GSIS. These data suggest that METTL3 plays a significant role in MG-induced reductions in pancreatic β-cell m^{6}A levels and GSIS.

AGE-RAGE interaction stimulates the generation of reactive oxygen species and inflammation mechanisms that enhance AGE-induced cell and tissue injury (25). MG can increase AGE accumulation and RAGE expression, resulting in human endothelial cell injury (31, 32). RAGE-deficient mice have characteristics that antagonize the decrease in insulin

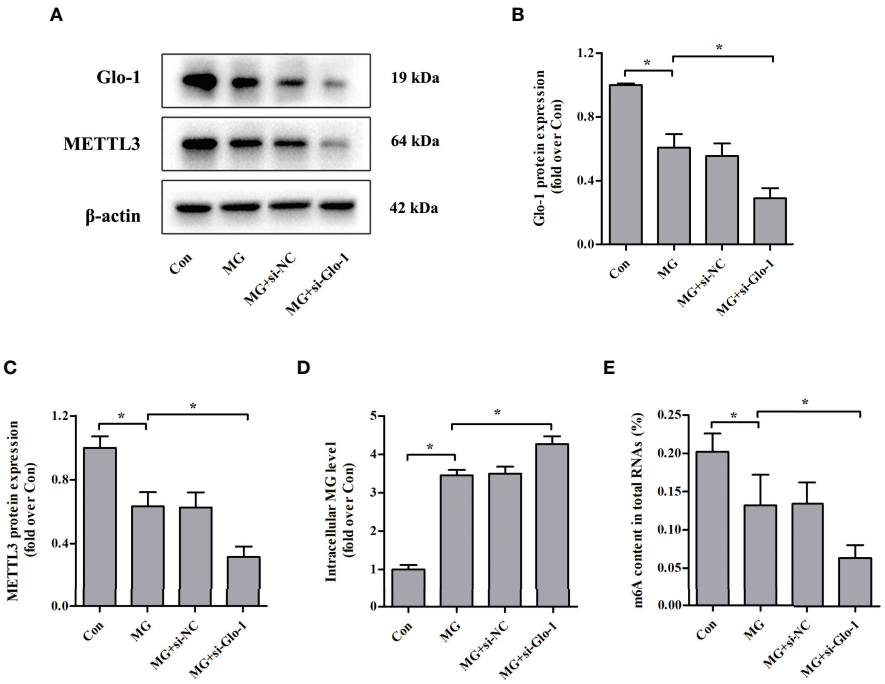

FIGURE 4 | Effects of Glo-1 knockdown on METTL3 expression and m^{6}A RNA methylation levels in NIT-1 cells. **(A–C)** Immunoblotting of Glo-1 and METTL3 protein expression in NIT-1 cells transfected with Glo-1 (si-Glo-1) or a nonspecific control siRNA (si-NC) and treated with 1 mM of MG for 24 h. **(D)** Intracellular MG levels in NIT-1 cells were treated as described in **(A–C)**. **(E)** m^{6}A levels in total RNA from NIT-1 cells treated as described in **(A–C)**. Results are presented as the means ± SD of n = 3–4 independent experiments. *$p < 0.05$.

sensitivity caused by MG administration (33). Thus, we investigated whether the MG-induced changes in m^{6}A levels and METTL3 expression were associated with RAGE. However, RAGE silencing failed to reverse the MG-induced reductions in METTL3 expression and m^{6}A levels in RNA, indicating that these m^{6}A changes are not closely related to the RAGE pathway.

As described in our previous studies (34) and others (9), incubation of pancreatic β cells with MG dramatically increased intracellular MG content. Further experiments were performed to determine whether the intracellular accumulation of MG in NIT-1 cells is involved in the m^{6}A-related changes caused by MG treatment. The glyoxalase system detoxifies most cellular MG, and Glo-1 converts MG into a nontoxic hemithioacetal metabolite using glutathione (28, 29). Upregulation of Glo-1 reduced hyperglycemia-induced carbonyl stress, AGE accumulation, and oxidative stress in diabetic rats (35). Similar to previous studies (26–29), Glo-1 expression was markedly suppressed in NIT-1 cells following MG treatment, which promoted intracellular accumulation of MG. The results showed that Glo-1 silencing increased the intracellular concentration of MG and further reduced METTL3 expression and m^{6}A RNA levels. Taken together, our findings suggest that the decreases in METTL3 expression and m^{6}A content in NIT-1 cells after MG exposure may be attributed, at least in part, to the increase in intracellular MG accumulation.

In a rat model of MG-induced T2DM, MafA expression was reduced in pancreatic tissue (8, 36). We also confirmed that MafA mRNA and protein expression were decreased in MG-treated NIT-1 cells. In the present study, METTL3 silencing markedly decreased the half-life of MafA mRNA and protein levels in NIT-1 cells, indicating that MafA might be a direct target of METTL3. Moreover, upregulation of METTL3 reversed the MG-induced reduction in MafA expression. The results of the MeRIP-qPCR assay suggested that m^{6}A levels in MafA mRNA were increased by METTL3 overexpression in MG-treated NIT-1 cells. MafA mRNA decay in NIT-1 cells induced by MG exposure was significantly ameliorated by upregulation of METTL3. Therefore, we conclude that MafA is a critical transcription factor regulated by METTL3 during MG-induced pancreatic β-cell damage.

MafA is not only a key activator of insulin transcription but also a master regulator of genes involved in maintaining β-cell function (37). Knockdown of MafA with siRNA led to impaired insulin secretion in EndoC-βH1 cells (a human-derived β-cell line) and human islets (38). Matsuoka et al. generated transgenic db/db mice that specifically overexpress MafA in islet β cells and found that these mice had significantly lower plasma glucose levels, higher plasma insulin levels, and augmented islet β-cell mass (39). This is consistent with our observations that MafA overexpression reversed the β-cell GSIS impairment caused by METTL3 silencing. MafA silencing significantly abolished the protective effects of METTL3 upregulation against GSIS reduction in MG-treated NIT-1 cells. Taken together, these data indicated that changes in MafA expression are associated with METTL3-regulated GSIS in NIT-1 cells.

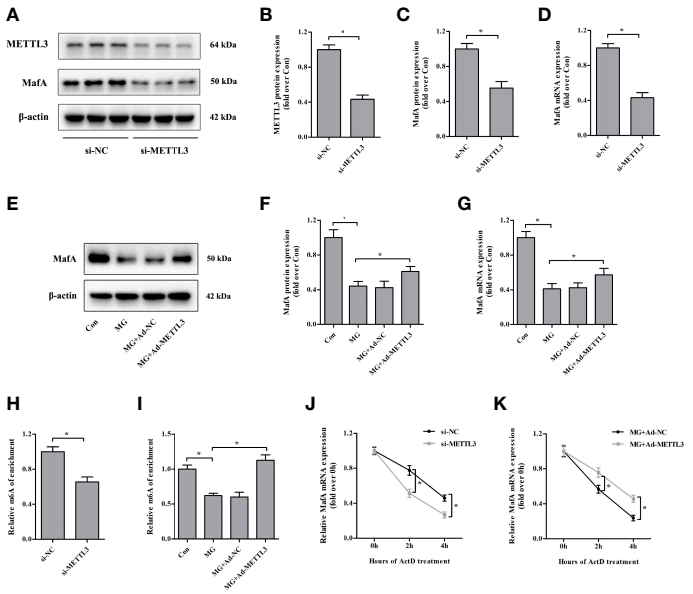

FIGURE 5 | Loss of METTL3 attenuates the expression of MafA. **(A–D)** METTL3 and MafA protein and mRNA levels were measured by immunoblotting and qPCR, respectively, in NIT-1 cells transfected with METTL3 siRNA (si-METTL3) or a nonspecific control siRNA (si-NC), which was set to 1. **(E–G)** MafA protein and mRNA expression levels in NIT-1 cells transfected with Ad-METTL3 or a nonspecific control adenovirus (Ad-NC) and treated with 1 mM of MG for 24 h were measured by immunoblotting and qPCR, respectively. **(H, I)** m^6A MafA mRNA levels as detected by MeRIP-qPCR in NIT-1 cells transfected with si-METTL3 or si-NC, which was set to 1 **(H)**, or Ad-METTL3 or Ad-NC and treated with 1 mM of MG for 24 h **(I)**. **(J, K)** MafA mRNA as measured by qPCR in NIT-1 cells transfected with si-METTL3, si-NC, Ad-METTL3, or Ad-NC and treated with ActD to block transcription. Results are presented as the means ± SD of $n = 3–4$ independent experiments. *$p < 0.05$.

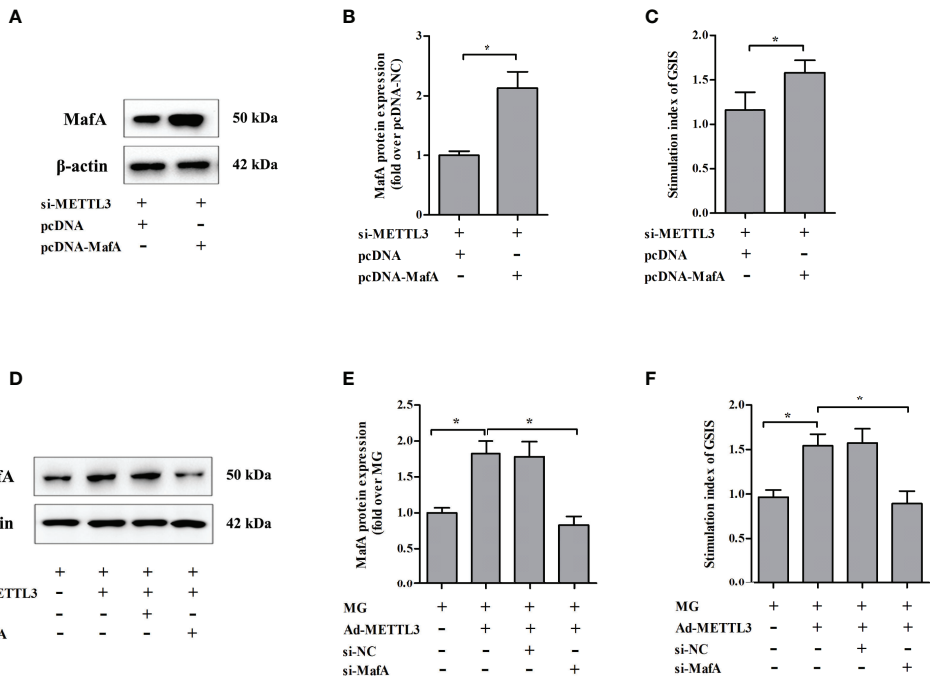

FIGURE 6 | Changes in MafA expression were associated with METTL3-regulated GSIS from NIT-1 cells. **(A)** Immunoblot of MafA expression in NIT-1 cells transfected with si-METTL3 + pcDNA or si-METTL3 + pcDNA MafA. **(B)** Quantification of the immunoblot in **(A)**. **(C)** The GSIS index of the cells described in **(A)**. **(D)** Immunoblot of MafA expression in NIT-1 cells transfected with Ad-METTL3 + si-NC or Ad-METTL3 + si-MafA and treated with 1 mM MG for 24 h. **(E)** Quantification of the immunoblot in **(D)**. **(F)** The GSIS index of the cells described in **(D)**. Results are presented as means ± SD of $n = 3-4$ independent experiments. *$p < 0.05$.

Our study provides new insights into the cause of islet β-cell dysfunction in patients with diabetes. The decrease in pancreatic β-cell m^6A levels could be partly attributed to MG accumulation in pancreatic islets during DM development. Increasing METTL3 expression in islet β cells may be a novel method for ameliorating MG-induced diabetic β-cell dysfunction. However, further animal and clinical studies are required to confirm this finding.

CONCLUSION

In summary, the present study demonstrated, for the first time, the connection between METTL3-regulated m^6A RNA levels and MG-induced pancreatic β-cell insulin secretion dysfunction. We found that treatment with MG reduced the m^6A levels in β cells by decreasing METTL3 expression. Upregulation of METTL3 ameliorated MG-induced impairment of insulin secretion in pancreatic β cells by regulating MafA expression.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

QH and S-JW designed the experiment and supervised the project. YC, X-MY, S-MZ, YS, and X-JM performed the experiments. X-MY, YW, and S-JW analyzed the data and drafted the manuscript. Y-JX, Y-JX, and QH were responsible for critical reading, editing, and revising the manuscript. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

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Associations between rs3480 and rs16835198 gene polymorphisms of *FNDC5* with type 2 diabetes mellitus susceptibility: a meta-analysis

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Background: FNDC5 is a novel and important player in energy regulation related to glucose metabolism and insulin levels. Thus, it may affect the incidence of type 2 diabetes mellitus (T2DM). Nevertheless, the association between FNDC5 single nucleotide polymorphisms (SNPs) and susceptibility to T2DM remains unclear. The aim of this meta-analysis was to explore whether the SNPs, rs3480 and rs16835198, are associated with the risk of T2DM.

Methods: Studies published before February 1st, 2022 were screened to identify the included studies. R software was also applied for calculation of odds ratio (OR), 95% confidence interval (95% CI), heterogeneity, and sensitivity analysis.

Results: Seven studies for rs3480 (involving 5475 patients with T2DM and 4855 healthy controls) and five studies for rs16835198 (involving 4217 patients with T2DM and 4019 healthy controls) were included in this meta-analysis. The results revealed a statistically significant association of rs3480 with T2DM under homozygote (GG vs AA: OR = 1.76, 95% CI = 1.31–2.37, P = 0.0002, I^2 = 59%) genetic model. However, there was no statistically significant correlation between rs16835198 and susceptibility to T2DM under allelic (G vs T: OR = 1.33, 95% CI = 0.94–1.89, P = 0.11, I^2 = 84%),

heterozygote (GT vs TT: OR = 1.17, 95% CI = 0.80–1.69, P = 0.42, I^2 = 71%), homozygote (GG vs TT: OR = 1.35, 95% CI = 0.95–1.94, P = 0.10, I^2 = 62%), recessive (GG+GT vs TT: OR = 1.25, 95% CI = 0.88–1.79, P = 0.22, I^2 = 72%), and dominant (GG vs GT+GG: OR = 1.20, 95% CI = 0.96–1.50, P = 0.11, I^2 = 46%) genetic models.

Conclusions: The present meta-analysis revealed that rs3480 in FNDC5 is significantly associated with susceptibility to T2DM, while rs16835198 does not show such an association.

KEYWORDS

polymorphism, type 2 diabetes mellitus, susceptibility, meta-analysis, FNDC5

1 Introduction

The number of patients with type 2 diabetes mellitus (T2DM) is increasing worldwide, and T2DM has become one of the most serious medical and health issues worldwide (1) . According to the International Diabetes Federation (IDF), the number of diabetes cases worldwide will reach 600 million by 2035 (2). Diabetes may be accompanied by a variety of complications, such as stroke, blindness, kidney failure, and myocardial infarction (3). Furthermore, it should be noted that even in individuals with mild hyperglycemia (prediabetes), such complication had been observed (4–6). These complications are the main cause of death and disability in patients with diabetes (4, 7). T2DM not only seriously affects the quality of life of patients, but also brings heavy economic burden to societies and families. Therefore, early detection of T2DM could have important clinical significance, studying the etiology and pathogenesis of T2DM is of great significance to the survival and development of human beings.

Irisin, a novel intriguing myokine, was recently reported and described by Bostrom et al. Irisin is released upon cleavage of the plasma membrane protein fibronectin type III domain containing protein 5 (FNDC5), whose gene expression is suggested to be driven by muscle-specific transgenic overexpression of the exercise-responsive transcriptional co-activator peroxisome proliferator-activated receptor (PPAR)-γ co-activator-1α (PGC-1α) (5, 8). In an animal model of obesity and T2DM, irisin intervention increases mitochondrial uncoupling, mitochondrial oxidative metabolism, and fatty acid oxidation in skeletal muscle (6, 9). Clinical studies have discovered that there is an association between irisin levels and metabolic disturbance. Its serum concentration is reduced in patients with T2DM, obesity, metabolic syndrome, and nonalcoholic fatty liver disease (7, 8, 10, 11). Furthermore, young male athletes possess higher irisin levels than middle-aged obese women (9, 12). Therefore, FNDC5 is considered an attractive target for metabolic disease.

The incidence of T2DM is closely related to genetic and environmental factors (10, 13). Searching for pathogenic genes involved in T2DM and revealing the pathogenesis of T2DM at the molecular level can provide help for early detection of individuals at high risk of T2DM and prevention of complications. Single nucleotide polymorphism (SNP) refers to polymorphisms in the DNA sequence caused by variations in a single nucleotide at the genomic level. SNPs are commonly inherited in humans, accounting for more than 90% of all known polymorphisms.

Several studies have evaluated the association of SNPs in FNDC5 with susceptibility to T2DM. However, the results are inconsistent. Therefore, the role of these FNDC5 SNPs in the risk of T2DM remains unclear. Here, we conducted a meta-analysis based on the available data to determine whether FNDC5 rs3480 (G>A) and rs16835198 (G>T) SNPs are associated with susceptibility to T2DM.

2 Methods

2.1 Guideline selection

In order to ensure the transparency and accuracy of the reporting medical research, the present meta-analysis was conducted following the PRISMA guidelines, as they are appropriate for systematic reviews and meta-analyses (14, 15).

2.2 Literature search

PubMed, Embase, Cochrane, China National Knowledge Infrastructure, and Chinese BioMedical Literature databases were

used to retrieve literatures systematically. The language of the studies was limited to Chinese and English. The search strategy involved the use of the following terms: "FNDC5," "fibronectin type III domain containing protein 5," "type 2 Diabetes mellitus," "T2DM," "single nucleotide polymorphism," and "SNP." Systematic retrieval was conducted until February 1st, 2022.

2.3 Inclusion criteria

The inclusion criteria were as follows: (1) case-control study on the correlation between the SNPS, rs3480 and rs16835198, and T2DM risk; (2) the diagnosis of T2DM conforms to WHO diagnostic criteria; (3) the study population in the study is consistent with Hardy-Weinberg Equilibrium (HWE); (4) the literature provides genotypic and/or allelic frequencies of the rs3480 and rs16835198 SNPs.

Studies were excluded if one of the following exclusion criteria was fulfilled: (1) no control group; (2) comments, review, abstracts, letters, conference presentations, and studies on animal models; (3) lack of genotypic and/or allelic frequencies of the rs3480 and rs16835198 SNPs. In case of duplicate publications, the study with the largest sample size was included.

2.4 Data extraction and quality assessment

Two authors (Yang and Ni) read the titles of the articles independently and assessed the quality of the included articles. In case of any disagreement, a decision was made after discussion. The two authors extracted the following data from all included articles: first author, year of publication, country of participants, number of cases and controls, genotypic distribution in cases and controls, genotyping methods, and HWE. An external referee was invited in case of disagreements not resolved by both investigators.

We applied the Newcastle Ottawa scale (NOS) to evaluate the quality of eligible studies from different aspects: (1) adequate definition of case; (2) representativeness of the cases; (3) selection of controls; (4) definition of controls; (5) comparability of cases and controls; (6) ascertainment of exposure; (7) same method of ascertainment for cases and controls; (8) non-response rate. The NOS has a score range of 0 to 9, and ≥7 was considered of high quality (14, 16).

2.5 Statistical analysis

We employed R (version 4.0.3) software and meta package for statistical analyses. To evaluate the strength of correlation between rs3480, rs16835198, and T2DM under five genetic models, odds ratios (ORs) and 95% confidence interval (CIs) were calculated. Statistical significance was set at $P < 0.05$. Q test and I^2 statistic were used to assess heterogeneity among the included studies. The heterogeneity was obvious if the *P* value of the Q test < 0.1 or $I^2 \geq 50\%$ (16, 17).

The random-effect model was used when significant heterogeneity was present, otherwise, the Mantel-Haenszel fixed-effect model was used. Actually, considering the clinical heterogeneity among the observational studies (e.g, sex, age, adjusted confounders, and so on), it would be more proper to use random-effects model first, even not statistical heterogeneity was observed (18, 19), therefore, we used random-effects model to calculate all the genetic models. Sensitivity analysis, test the stability of results, was conducted using R software (4.0.3) and meta package. The publication bias was assessed by *Egger*'s test (17, 20).

3 Results

3.1 Characteristics of the included studies

Literature search was carried out according to the PRISMA flow chart shown in Figure 1. A total of 27 potentially relevant articles were found after the retrieval process. 15 articles were selected for further analyses after exclusion of all duplicate articles identified by screening through the titles and abstracts. Another 12 articles were subsequently excluded after careful reading of the abstracts and titles. 9 articles were finally included in the present meta-analysis (21–29). Table 1 shows the qualities of all included studies as determined by NOS evaluation (30). Detailed information of the 9 included articles is presented in Table 2.

3.2 Quantitative analysis

3.2.1 Association between rs3480 and susceptibility to T2DM

Seven studies involving 5475 patients with T2DM and 4855 healthy controls were included in the meta-analysis to explore the potential association between rs3480 and susceptibility to T2DM.

The present meta-analysis discovered that the correlations between an FNDC5 rs3480 (G/A) and susceptibility to T2DM in homozygote (GG vs AA: OR = 1.76, 95% CI = 1.31–2.37, $P = 0.0002$, $I^2 = 59\%$) genetic model was statistically significant. In contrast, no statistical significance was found for correlations between rs3480 and T2DM susceptibility in allelic (G vs A: OR = 1.21, 95% CI = 0.98-1.50, $P = 0.08$, $I^2 = 82\%$), heterozygote (GA *vs* AA: OR = 1.14, 95% CI = 0.86–1.52, $P = 0.35$, $I^2 = 65\%$), recessive (GG vs GA+AA: OR = 1.12, 95% C = 0.91-1.37, $P = 0.28$, $I^2 = 68\%$), and dominant (GG+GA vs AA: OR = 1.17, 95% CI = 0.98–1.39, $P = 0.09$, $I^2 = 23\%$) genetic models. Our results suggested that people carrying the G allele in rs3480 had higher

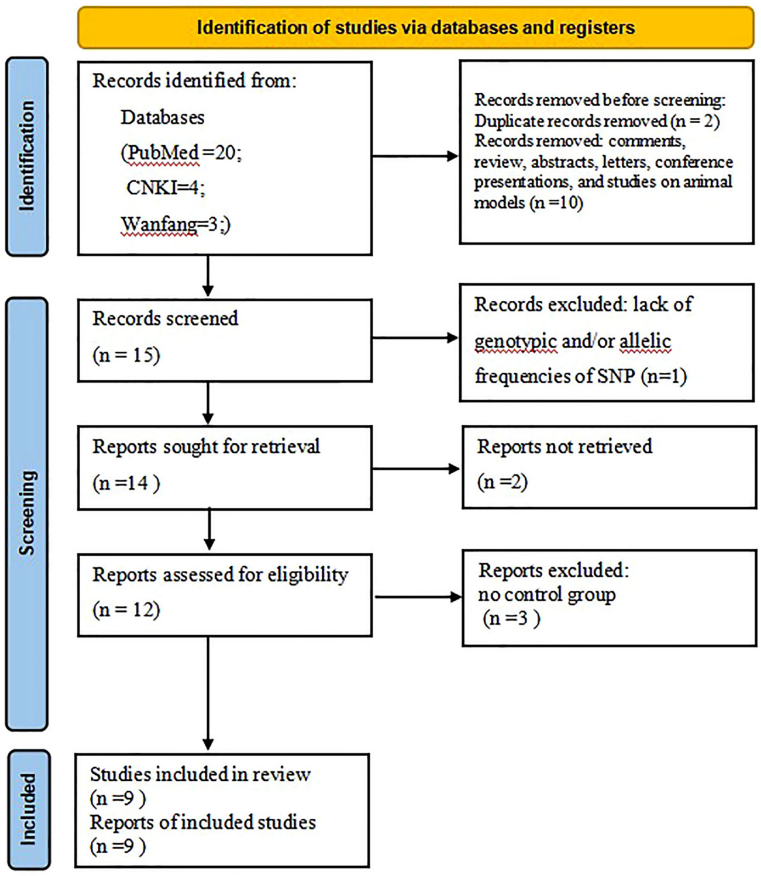

FIGURE 1
The Flow chart showing the study selection process.

susceptibility to T2DM. The forest plots are presented in Figure 2.

There was obvious heterogeneity in the allelic, heterozygote, homozygote, and recessive models; therefore, subgroup analysis was performed. As shown in Figure 3, rs3480 possessed a significant association with susceptibility to T2DM in Chinese individuals under the homozygote (GG vs AA: OR = 2.30, 95% CI = 1.18-4.49, P = 0.01, I^2 = 62%) models. Sensitivity analyses were further applied for different genetic models. As shown in Figure 4, no significant heterogeneity was observed in any of the genetic models after excluding each study.

3.2.2 Association between rs16835198 and T2DM risk

A total of five studies involving 4217 patients with T2DM and 4019 healthy controls were finally included to assess the potential correlation between rs16835198 and susceptibility to T2DM. Figure 5 showed that there was no association between rs16835198 and susceptibility to T2DM under allelic (G vs T: OR = 1.33, 95% CI = 0.94–1.89, P = 0.11, I^2 = 84%), heterozygote (GT vs TT: OR = 1.17, 95% CI = 0.80–1.69, P = 0.42, I^2 = 71%), homozygote (GG vs TT: OR = 1.35, 95% CI = 0.95–1.94, P = 0.10, I^2 = 62%), recessive (GG+GT vs TT: OR = 1.25, 95% CI = 0.88–1.79, P = 0.22, I^2 = 72%), and dominant (GG vs GT+GG: OR = 1.20, 95% CI = 0.96–1.50, P = 0.11, I^2 = 46%) genetic models. Figure 6 exhibited the results of sensitivity analyses for the included studies, there was no heterogeneity for all the genetics models after excluding each study.

No obvious asymmetry was observed in the Egger's test for any comparison, which suggested that the findings were unlikely to be influenced by publication bias. The results of rs3480 and rs16835198 were summarized in Table 3.

4 Discussion

T2DM is a complex polygenic metabolic disease caused by the interaction of genetic and environmental factors. Unhealthy

TABLE 1 The newcastle-ottawa quality assessment scale.

Author (year)	Selection				Comparability	Exposure			
	Adequate definition of case	Representativeness of the cases	Selection of controls	Definition of controls	Comparability of cases and controls	Ascertainment of exposure	Same method of ascertainment for cases and controls	Nonresponse rate	Total score
Tanisawa (29)	*	*		*	**	*	*	*	8
Brondani (23)	*	*	*	*	**	*	*	*	9
Gao (24)	*	*		*	*	*	*	*	7
Tang (21)	*	*		*	**	*	*	*	8
Al-Daghri (25)	*	*		*	**	*	*	*	8
Allah (26)	*	*		*	**	*	*	*	8
Khidr (22)	*	*		*	**	*	*	*	8
Pan (27)		*		*	*		*	*	5
Zabibah (28)	*	*		*	**	*	*	*	8

*Represents one scores following the Newcastle-Ottawa. Quality Assessment Scale.
**Represents two scores following the Newcastle-Ottawa Quality Assessment Scale.

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TABLE 2 Characteristics of included studies.

Author(year)	Country	Case/Control	Genotype distribution						Genotyping methods	HWE
			Case			Control				
			rs3480							
			GG	AG	AA	GG	AG	AA		
Brondani (23)	Southern Brazilian	942/434	178	359	405	79	185	170	TaqMan assay	>0.05
Gao (24)	China	281/286	23	110	148	22	114	150	Mass array	>0.05
Tang (21)	China	3397/3405	267	1275	1855	225	1306	1874	Mass array	>0.05
Al-Daghri (25)	Saudi	376/410	78	181	117	88	186	136	TaqMan assay	>0.05
Allah (26)	Egypt	71/70	24	35	12	10	28	32	TaqMan assay	>0.05
Pan (27)	China	358/200	30	133	195	13	76	111	Mass array	>0.05
Zabibah (28)	Iraq	50/50	7	25	18	4	18	28	PCR-RFLP	>0.05
			rs16835198							
			GG	GT	TT	GG	GT	TT		
Tanisawa (29)	Japan	82/81	32	35	15	19	50	12	TaqMan assay	>0.05
Gao (24)	China	280/286	83	150	47	81	131	74	Mass array	>0.05
Tang (21)	China	3397/3402	929	1661	807	899	1735	768	Mass array	>0.05
Khidr (22)	Egypt	100/50	54	37	9	18	21	11	TaqMan assay	>0.05
Pan (27)	China	358/200	97	178	83	50	96	54	TaqMan assay	>0.05

lifestyles increase the risk of T2DM, but not all individuals with unhealthy lifestyle habits develop the disease. Therefore, genetic factors play a very important role in the onset and progression of T2DM, which needs to be further studied. SNPs are polymorphisms of the DNA sequence caused by variations in a single nucleotide at the genomic level. They are commonly inherited in humans, accounting for more than 90% of all known polymorphisms.

In 2007, Sladek et al. used Genome Wide Association Study (GWAS) to identify diabetes susceptibility genes in the French population. Several research groups further identified and confirmed SNPs associated with diabetes susceptibility. Thus, the association between SNPs and T2DM susceptibility is being gradually revealed (31–33). FNDC5 is a precursor of irisin and can significantly disrupt metabolism. In an obese mouse model, overexpression of FNDC5 enhances energy expenditure, lipolysis, and insulin sensitivity, and improves hyperlipidemia, hyperglycemia, and hyperinsulinemia (15). A high-fat diet increases the mRNA and protein levels of FNDC5 in muscle tissue of obese mice (34). Moreover, FNDC5 protein levels are increased in muscle tissue after endurance training.

Multiple SNPs significantly associated with metabolic disease susceptibility in different populations have been found in the FNDC5 gene. Rs16835198 was found to be significantly associated with insulin sensitivity and obesity in the German and Egyptian populations, respectively (35). The results showed a significant association of the rs16835198 G allele with fasting insulin levels and body mass index in 6822 Chinese Han individuals (21). The G allele of rs3480 has been associated with elevated hemoglobin a1c levels in Brazilian women with T2DM (23). In addition, the rs3480 GG genotype has been associated with a reduced risk of obesity and a lower body weight index in the Saudi population. Therefore, SNPs in FNDC5 are critical for regulating metabolic homeostasis (36). Our meta-analysis showed that rs3480 is associated with susceptibility to T2DM, and that people carrying the G allele have a higher susceptibility to T2DM. Previous studies have shown that miR-135-5p preferentially binds to the G allele of rs3480 after upregulation, thus enhancing the attenuating effect of miR-135-5p on FNDC5 and reducing the FNDC5 mRNA levels, which results in a weakened regulatory effect of FNDC5 on metabolic diseases (37). In addition, miR-135-5p is upregulated in serum and renal tissue of patients with diabetic nephropathy (38). Taken together, these results suggest that the G allele of rs3480 is detrimental to FNDC5 expression, which may explain the association with T2DM.

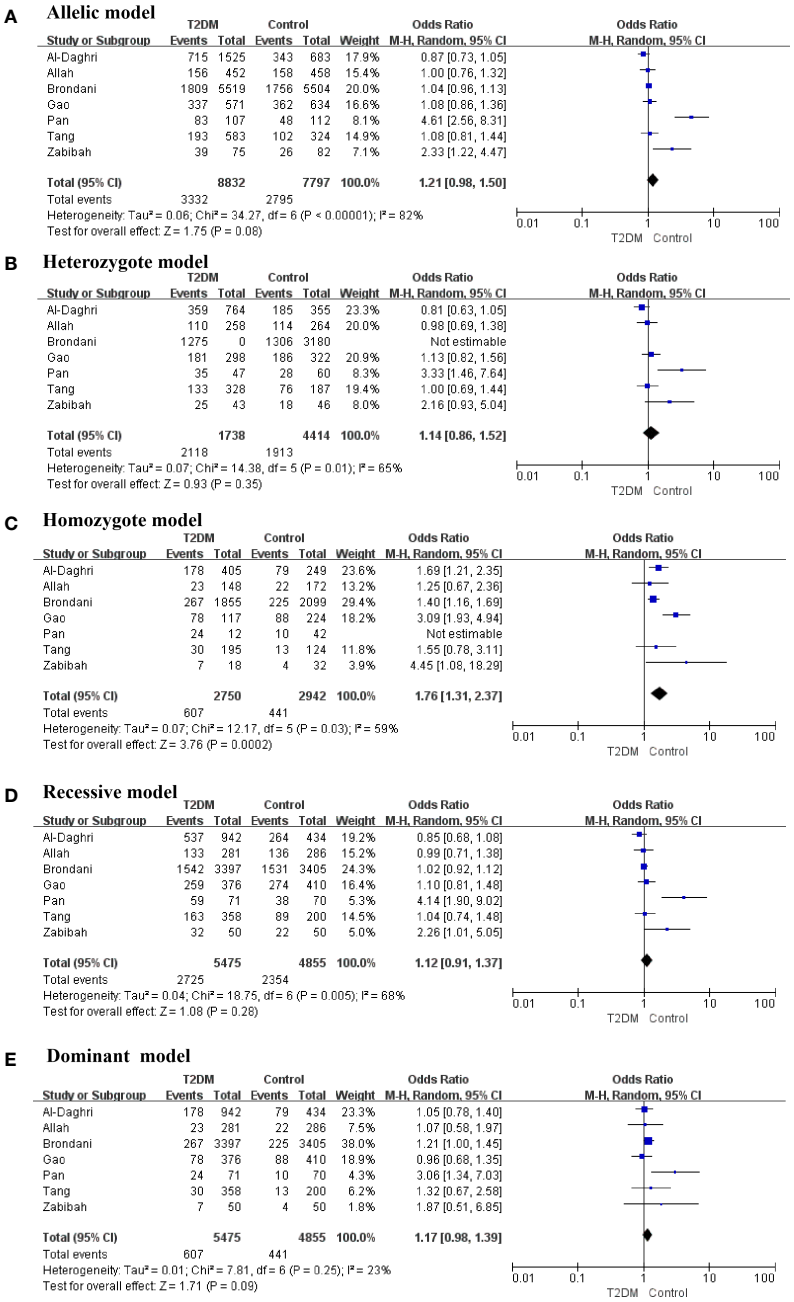

FIGURE 2
Forest plots of the polymorphism of rs3480 and the T2DM risk under five genetic models **(A–E)**.

In addition, our meta-analysis showed that rs16835198 is not associated with susceptibility to T2DM. Rs16835198 is located on the 3' region of the FNDC5 gene, which is unlikely to affect the amino acid sequence of the protein products (39). Rs16835198 may not be significantly related to FNDC5 expression changes; therefore, rs16835198 is not strongly associated with susceptibility to T2DM. However, the number of articles included in this study is very limited and further exploration is needed.

This meta-analysis has some limitations. First, it included nine articles with large and heterogeneous populations, including three studies on Chinese Han individuals, two on Egyptian populations, and four on individuals from Southern Brazil, Saudi Arabia, Iraq, and Japan each. The differences

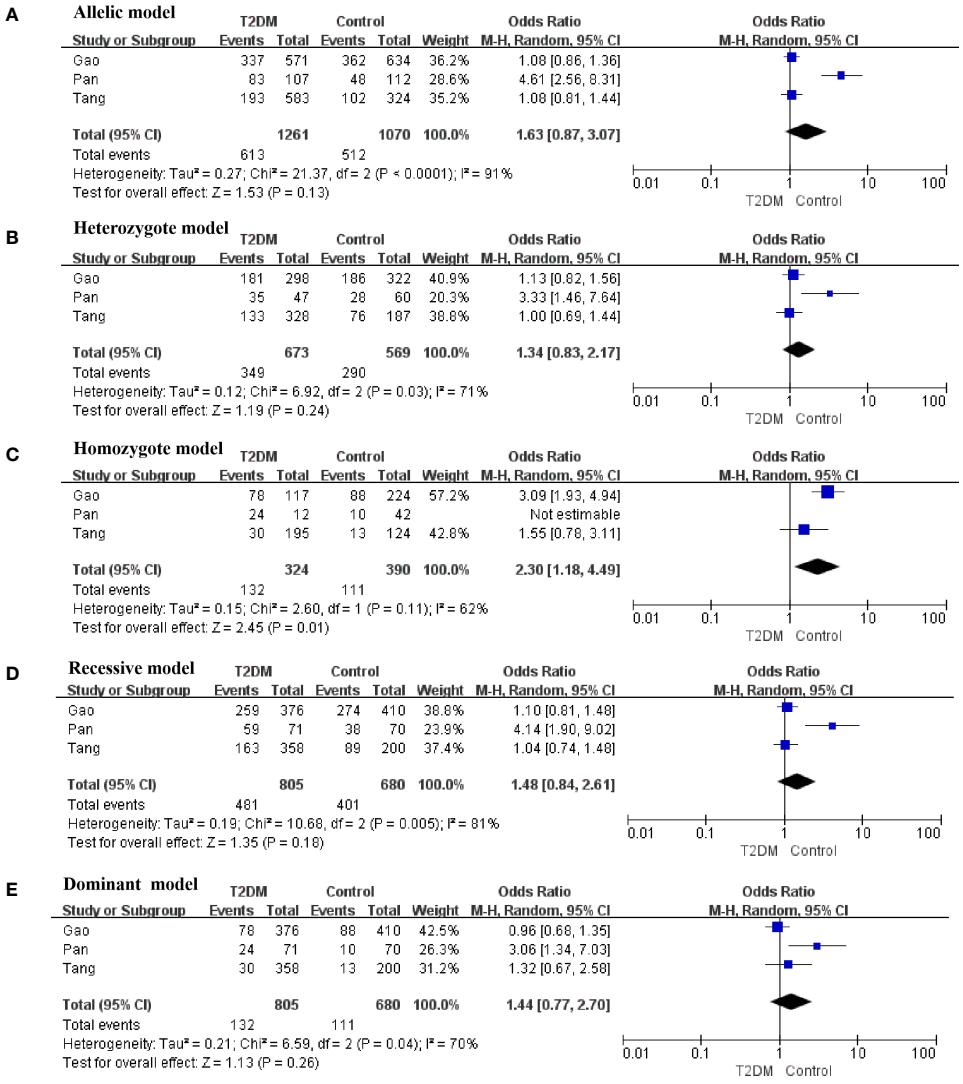

FIGURE 3
Forest plots of the polymorphism of rs3480 and the T2DM risk under five genetic models in Chinese. **(A)** allelic, **(B)** heterozygote, **(C)** homozygote, **(D)** recessive, and **(E)** dominant genetic models.

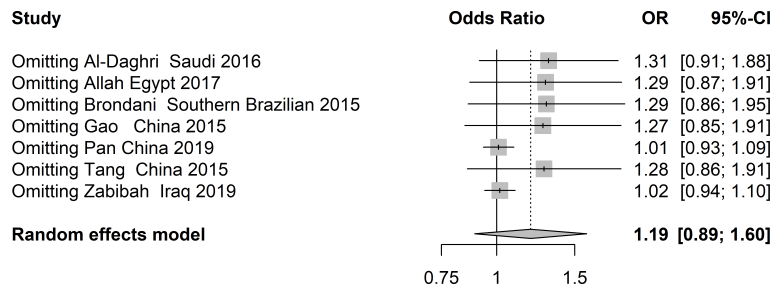

FIGURE 4
Sensitivity analysis of studies included in analysis of rs3480 and T2DM risk.

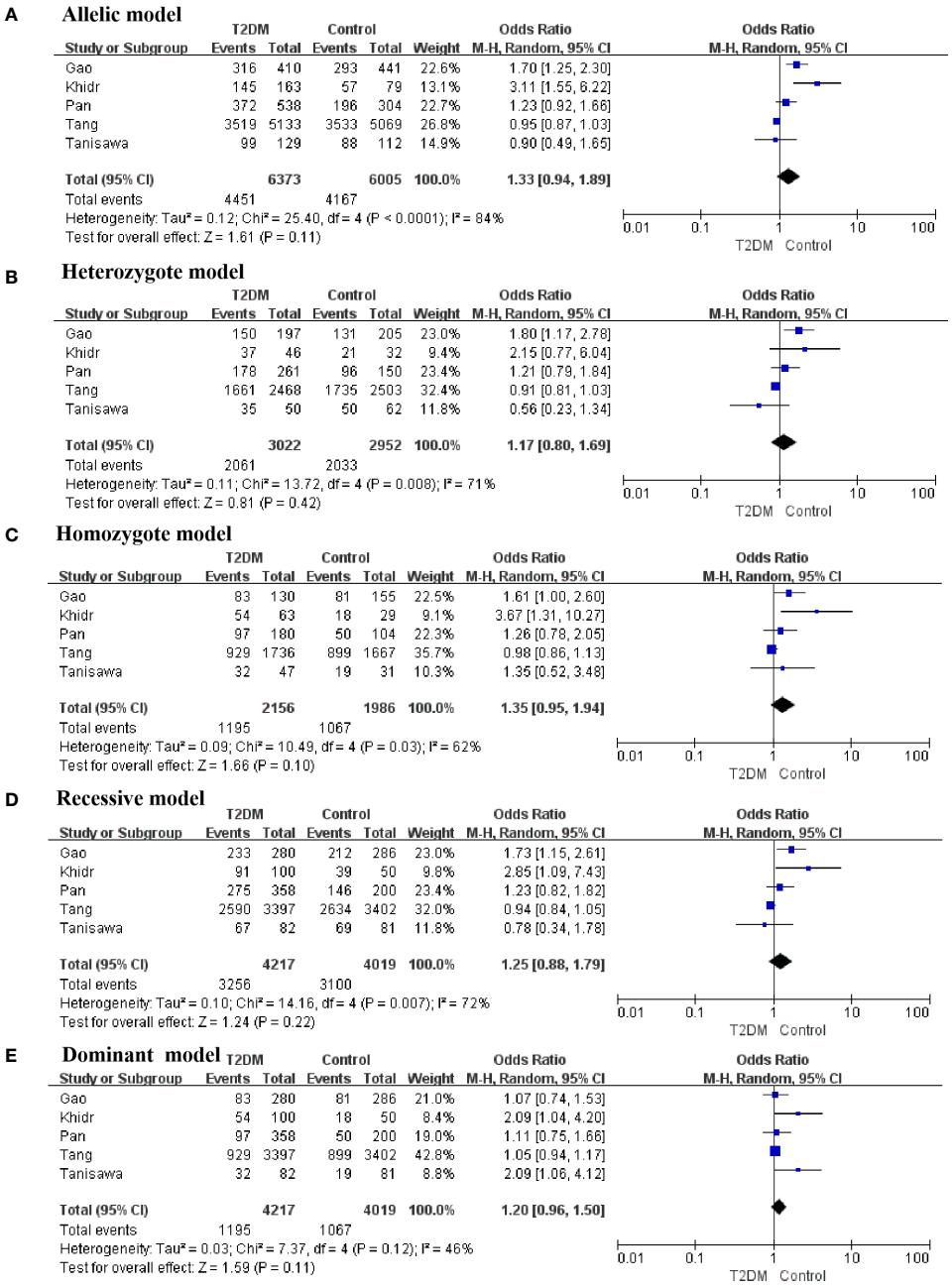

FIGURE 5
Forest plots of the polymorphism of rs16835198 and the T2DM risk under five genetic models. **(A)** allelic, **(B)** heterozygote, **(C)** homozygote, **(D)** recessive, and **(E)** dominant genetic models.

among races may have affected the results. The best approach would have been to conduct subgroup analysis for each race, but the literature volume of the corresponding subgroups was not sufficiently large. Therefore, a comprehensive analysis can only be conducted after the inclusion of more articles. Second, only English and Chinese articles were included in this meta-analysis, and data presented in other languages were not included.

In conclusion, we found that the rs3480 G allele in FNDC5 may confer moderate risk for T2DM. Further investigation of these SNPs may improve our understanding of the occurrence

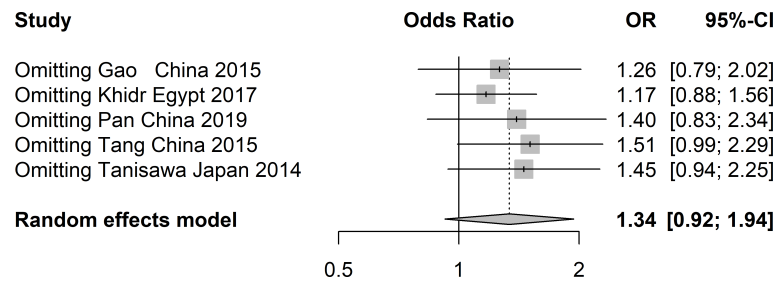

FIGURE 6
Sensitivity analysis of studies included in analysis of rs16835198 and T2DM risk.

TABLE 3 Summary OR and 95% CI of rs3480, rs16835198 polymorphisms and T2DM.

SNP	Genetic models	n	OR	95% CI	*P*value	I^2	*P* publication bias(Egger)
rs3480							
Allelic model	G *vs* A	7	1.21	0.98-1.50	0.08	82%	0.146
Heterozygote model	GA *vs* AA	7	1.14	0.86-1.52	0.35	65%	0.624
Homozygote model	GG *vs* AA	7	**1.76**	**1.31-2.37**	**0.0002***	59%	0.229
Dominant model	GG+GA *vs* AA	7	1.17	0.98–1.39	0.09	23%	0.402
Recessive model	GG *vs* GA+AA	7	1.12	0.91-1.37	0.28	68%	0.586
rs16835198							
Allelic model	G *vs* T	5	1.33	0.94-1.89	0.11	84%	0.620
Heterozygote model	GT *vs* TT	5	1.17	0.80-1.69	0.42	71%	0.098
Homozygote model	GG *vs* TT	5	1.35	0.95-1.94	0.10	62%	0.610
Dominant model	GG+GT *vs* TT	5	1.20	0.96-1.50	0.11	46%	0.384
Recessive model	GG *vs* GT+GG	5	1.25	0.88-1.79	0.22	72%	0.810

Bold values represents significant association.

and progression of T2DM. We are aware that the present meta-analysis results were derived from a limited sample size. Therefore, future analyses with larger sample sizes and including more studies are required to define the associations between rs3480 and T2DM risk.

Data availability statement

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

Author contributions

XYa and LN searched literature and collected data. JS and XYu analyzed the data. DL supervised the project. XYa wrote the original manuscript. DL reviewed and revise the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest

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

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Circadian rhythms and pancreas physiology: A review

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Type 2 diabetes mellitus, obesity and metabolic syndrome are becoming more prevalent worldwide and will present an increasingly challenging burden on healthcare systems. These interlinked metabolic abnormalities predispose affected individuals to a plethora of complications and comorbidities. Furthermore, diabetes is estimated by the World Health Organization to have caused 1.5 million deaths in 2019, with this figure projected to rise in coming years. This highlights the need for further research into the management of metabolic diseases and their complications. Studies on circadian rhythms, referring to physiological and behavioral changes which repeat approximately every 24 hours, may provide important insight into managing metabolic disease. Epidemiological studies show that populations who are at risk of circadian disruption such as night shift workers and regular long-haul flyers are also at an elevated risk of metabolic abnormalities such as insulin resistance and obesity. Aberrant expression of circadian genes appears to contribute to the dysregulation of metabolic functions such as insulin secretion, glucose homeostasis and energy expenditure. The potential clinical implications of these findings have been highlighted in animal studies and pilot studies in humans giving rise to the development of circadian interventions strategies including chronotherapy (time-specific therapy), time-restricted feeding, and circadian molecule stabilizers/analogues. Research into these areas will provide insights into the future of circadian medicine in metabolic diseases. In this review, we discuss the physiology of metabolism and the role of circadian timing in regulating these metabolic functions. Also, we review the clinical aspects of circadian physiology and the impact that ongoing and future research may have on the management of metabolic disease.

KEYWORDS

circadian rhythm, pancreas, insulin, metabolism, obesity

Introduction

Diabetes mellitus is estimated to affect 415 million adults worldwide, approximately 90% of whom have type 2 diabetes (1). Diabetes can lead to microvascular (e.g. retinopathy, nephropathy and neuropathy) and macrovascular complications which substantially increase the risk of developing cardiovascular

disease and can substantially decrease the quality of life of these individuals (2). Managing diabetes and the associated complications will introduce more strain on healthcare systems worldwide as this disease becomes more prevalent (3). This highlights the need for both determining the importance of risk factors, which may modulate susceptibility to disease, and management of the metabolic syndrome (referring to metabolic abnormalities listed in Figure 1, which together increase the risk of cardiovascular diseases and type 2 diabetes), obesity and type 2 diabetes (T2DM).

Many physiological processes, including energy expenditure and glucose homeostasis are regulated by circadian rhythms (4, 5), which are 24-hour daily cycles of physiological and behavioral patterns (6). The circadian rhythm apparatus consists of a central master clock located in the suprachiasmatic nuclei (SCN) within the hypothalamus, which synchronizes peripheral oscillators located in various tissues such as the liver, pancreas, adipose tissue and skeletal muscle. The SCN is entrained by light (7, 8), which allows synchronization with the external environment i.e. the 24-hour light-dark cycle governed by the Earth's rotation. This directs the central and peripheral clocks to adapt to changes in light, optimizing physiological processes to these daily cycles. Through a number of regulatory mechanisms (e.g. endocrine, neurological, thermal), the SCN coordinates responses with the peripheral clocks, which have their own phases, to ensure synchronized daily rhythms are maintained (9). In addition, peripheral rhythms can also be modulated, for example, by nutrient sensing (i.e. from food intake), hormonal cues and temperature. Although the SCN acts as the master pacemaker in the human body, it is evident that circadian oscillations are observable in almost every cell of the body and these rhythms may persist in isolation from the SCN (10).

Disruption of circadian rhythms exacerbates metabolic diseases that include T2DM, obesity and metabolic syndrome in both animal models and humans (11–15) (Figure 1). Experimental and epidemiological studies show that night shift-workers are more likely to develop metabolic abnormalities, predisposing these individuals to developing T2DM compared to daytime workers (16–22). This elevated risk of developing T2DM is also seen in populations with social jetlag, a condition characterized by disruption to an individual's sleeping pattern, and thus circadian rhythms, due to social engagements, leading to individuals feeling "jet lagged" or tired (20, 21, 23).

Many central and peripheral hormones that influence metabolism exhibit circadian rhythmicity. This review will focus on the current understanding of how circadian rhythms can influence pancreatic physiology and the consequent effects on metabolism and how this knowledge may be used to enhance clinical management of T2DM, obesity and metabolic syndrome. While we will focus on the pancreas in this review, other metabolic tissues e.g. liver, skeletal muscle and adipose tissue are also altered by circadian rhythms (Figure 2). While

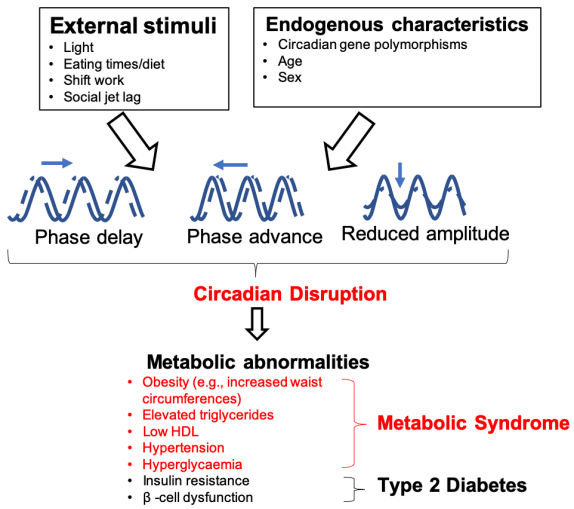

FIGURE 1
Influences on, and consequences of, circadian rhythm disruption. Both endogenous and external factors can predispose individuals to circadian disruption. This can cause dysfunction of peripheral oscillators, which are involved in the regulation of metabolic functions such as body weight homeostasis, glucose metabolism and β-cell function. Individuals who experience circadian disruption, which may be through phase delays/advances or by changes in amplitude (difference between peak and trough of the rhythms) are at an elevated risk of developing metabolic abnormalities, which can lead to metabolic syndrome and T2DM.

providing background information on blood glucose homeostasis and insulin resistance to those less familiar with these concepts Section 2: (Pancreatic physiology), we will also highlight key ways circadian rhythms are disrupted and how they interact to increase the risk of obesity and T2DM (Figure 3) discussed in Section 3 (Circadian rhythms in the pancreas), 4 (Molecular circadian rhythms in the pancreas) and 5 (Modulation of circadian rhythm).

Pancreatic physiology

The pancreas is a multifunctional organ which regulates metabolism and digestion through several endocrine and exocrine mechanisms (24). These tightly regulated, interrelated mechanisms are necessary for blood glucose homeostasis, lipolysis, and food intake (4, 5). Histologically, the pancreas contains clusters of exocrine cells known as acini which surround a network of interconnected ducts (25). The acini secrete inactive forms of pancreatic enzymes known as zymogens which subsequently enter the gut and become active digestive enzymes, including lipase, amylase and proteases. The endocrine cells of the pancreas are arranged in clusters known as the islets of Langerhans which contain α, β, γ and δ cells (26). Each of these cell populations within the islets secrete different hormones, as discussed later. Together, these pancreatic hormones regulate blood glucose homeostasis, food intake and insulin responses and are therefore integral to understanding T2DM and metabolic syndrome.

Introduction to blood glucose homeostasis

Blood glucose homeostasis is a highly regulated process, strongly influenced by both local (i.e. pancreas) and distal (i.e. liver, intestine, brain) signals (Figure 4). In this section, we discuss the role different hormones have on regulating blood glucose.

In normal pancreatic physiology, the β cells of the islets of Langerhans express glucose transporter type-2 (GLUT-2) molecules which detect changes in blood glucose levels, having high capacity and low affinity for glucose (27). Glucose enters the β cells through these high-capacity transporters and subsequently enters the glycolytic pathway and mitochondrial metabolism, which increases the cytoplasmic concentration of ATP leading to the gating of ATP-sensitive potassium channels (K_{ATP}). The subsequent plasma membrane depolarization opens voltage-dependent calcium channels (VDCC) and allows an influx of calcium into the cell. The increased intracellular concentration of calcium causes the fusion of insulin granules with the cell membrane and the secretion of insulin.

Insulin exerts its effect on cells by binding to insulin receptors on the cell surface. These insulin receptors are homodimers, consisting of two α and two β subunits. Insulin binds to the extracellular α subunits, leading to autophosphorylation of the β subunits that are tyrosine receptor kinases (RTKs). These RTKs phosphorylate insulin receptor substrate (IRS) that activates downstream pathways mediating the cellular effects (28). Insulin acts on several body tissues including the liver, adipose tissue and skeletal muscle to

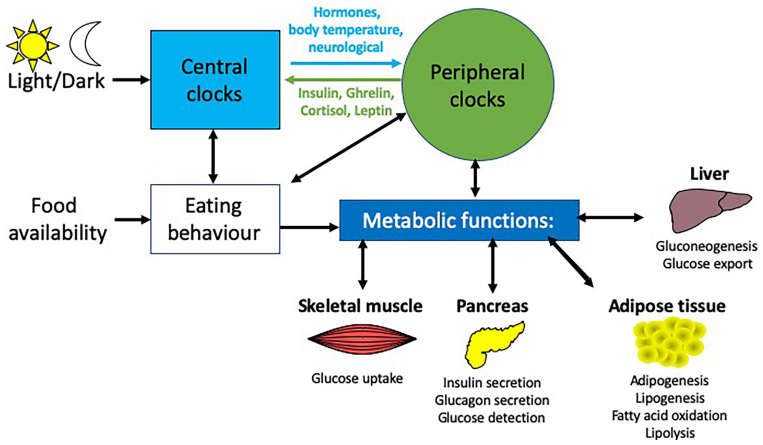

FIGURE 2
Circadian influences on different metabolic tissues. Light is the main entrainment factor for the SCN, the master pacemaker of the circadian system, which, through a number of signals e,g. hormones and neurotransmitters, synchronizes the circadian rhythms of peripheral tissues to light exposure. Crosstalk between these peripheral tissues and the brain enable feedback to modulate these rhythms e.g.the hormones insulin, ghrelin, leptin and cortisol provide feedback to the arcuate nucleus in the brain. There are many peripheral tissues, which regulate metabolic functions, including the liver, pancreas, skeletal muscle and adipose tissue, each of which exhibit their own rhythmicity. Together, these peripheral rhythms regulate many metabolic functions, including glucose homeostasis, insulin secretion and fatty acid metabolism.

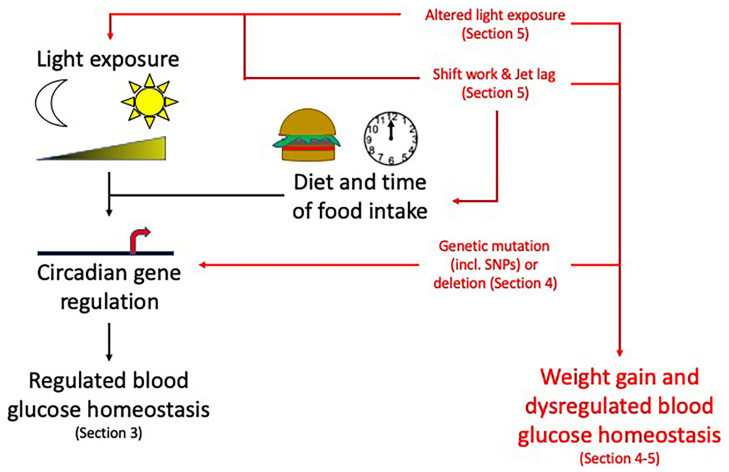

FIGURE 3
Circadian rhythms and their disruption: Topics to be discussed in this review. Circadian rhythms, influenced by light/dark cycles and diet and time of food intake, control many pancreas functions. Disruption to these circadian influences (altered light exposure, shift work/jet lag, genetic mutations) can lead to weight gain and dysregulated blood glucose homeostasis increasing the risk of developing obesity and T2DM. For information related to these factors altering the circadian rhythms please see the specific sections mentioned above within the review. Section 3: Circadian rhythms in the pancreas, Section 4: Molecular circadian rhythms in the pancreas and Section 5: Modulation of circadian rhythm.

allow the entry of glucose into these cells to undergo glycolysis and mitochondrial metabolism or anabolic processes such as glycogenesis or lipogenesis (29). Importantly, insulin inhibits hepatic gluconeogenesis and glucose secretion, regulating blood glucose levels. The liver is also sensitive to decreased blood concentrations of insulin, and this stimulates glucose synthesis and secretion. Insulin receptors are expressed throughout different regions of the brain (30). In the arcuate nucleus of the hypothalamus (ARC), insulin modulates anorexigenic and orexigenic neuronal activity. Anorexigenic neurons (pro-

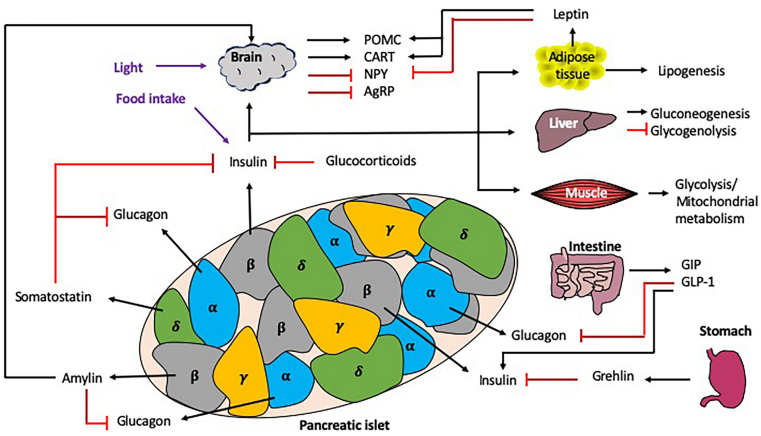

FIGURE 4
Interactions regulating blood glucose homeostasis. Blood glucose homeostasis is controlled by many signals both locally within the pancreas, and more distally, from the brain, liver, muscle, intestine, stomach and adipose tissue. Within the pancreatic islets, the α cells secrete glucagon, the β cells secrete insulin (and amylin), γcells secrete pancreatic polypeptide and the δ cells secrete somatostatin. In response to high glucose e.g. from dietary intake, the islet ββcells secrete insulin, which is detected by multiple tissues in the periphery, leading to the synthesis or induction of many molecules/pathways e.g. lipogenesis and gluconeogenesis, as well as the inhibition of others e.g. glycogenolysis. Importantly, there are many mechanisms of regulation to control the secretion of insulin both locally (e.g. glucagon and somatostatin) and more distally, e.g. intestine/stomach *via* hormones (e.g. grehlin and glucocorticoids) and incretins (GIP/GLP-1). Exogenous circadian modulation factors such as light and food intake (shown in purple) can also regulate blood glucose homeostasis, as can endogenous factors such as glucocorticoids, which also show rhythmicity. Black arrows indicate induction, red lines indicate inhibition. .

opiomelanocortin (POMC) and cocaine–amphetamine-regulated-transcript (CART)) are stimulated by insulin whilst orexigenic neurons [neuropeptide Y (NPY) and agouti-related peptide (AgRP)] are inhibited (31). These actions simultaneously decrease food intake and increase energy expenditure. Leptin, a hormone released by adipose cells and also controlled in a circadian manner (32), exerts a similar effect by stimulating POMC and CART, whilst inhibiting NPY neurons (33). Together leptin and insulin act as signals of adiposity which allow the body to regulate adipose tissue mass (34).

Glucagon, another pancreatic hormone, is secreted by α cells in the pancreatic islets in response to decreased blood glucose levels (35). It opposes the action of insulin in glucose control by stimulating glucose synthesis and secretion. Glucagon also stimulates ketogenesis and lipolysis in the liver. Glucagon levels in the hepatic portal vein are detected by the liver and this signal is relayed centrally *via* the vagal afferents to the ARC to reduce meal sizes by stimulating postprandial satiety (36). Additionally, glucagon is able to cross the blood-brain barrier (BBB) and has been shown to activate GPCR pathways in the ARC in animal models (37). This implies that glucagon may have a direct effect on the CNS to regulate food intake.

The hormone amylin is co-secreted with insulin by the pancreatic β cells, reducing food intake by inhibiting orexigenic neurons in the ARC (38). Amylin also activates the area postrema (AP) in the medulla oblongata of the brainstem to slow gastric emptying, inhibit gastrointestinal secretions and inhibit the postprandial secretion of glucagon (39).

Somatostatin, a hormone secreted by pancreatic δ cells, regulates digestion, food intake and glucose metabolism through endocrine, exocrine and neurological mechanisms. This hormone inhibits the secretion of the insulin and glucagon as well as prolactin, thyroid stimulating hormone, gastrin and secretin. In the gut, somatostatin inhibits digestive secretions including pancreatic enzymes, gastric acid and bile.

Corticosteroids, produced in the adrenals, regulate a variety of physiological processes including stress responses, immune responses and inflammation, blood glucose homeostasis and electrolyte balance (40). The secretion of these hormones is also regulated by the circadian clock and follows a 24-hour cycle (41, 42). The two main classes of corticosteroids are mineralocorticoids and glucocorticoids (43). Whereas mineralocorticoids, such as aldosterone, regulate fluid and electrolyte balances by modulating the activity of the renal tubules (44), glucocorticoids have anti-inflammatory effects and also regulate carbohydrate, protein and lipid metabolism (45). Cortisol is the main endogenous glucocorticoid in human physiology and is also released as part of the stress response, which in the presence of hypoglycemia, increases blood glucose levels, in both stress and hypoglycaemia, by stimulating gluconeogenesis (46).

Incretins are peptide hormones that are secreted by gut cells postprandially to regulate blood glucose levels and nutrient absorption (47). The two main incretins are glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP) (48, 49), and they decrease blood glucose levels by facilitating the secretion of insulin from pancreatic β cells (50). GLP-1 also inhibits the secretion of glucagon by pancreatic α cells. In addition, incretins also slow the rate of gastric emptying to regulate the rate of nutrient absorption (51). Both GLP-1 and GIP are inactivated by dipeptidyl peptidase-4 (DPP-4) (47). Several GLP-1 analogues and DPP-4 inhibitors are used clinically in the management of T2DM (52). Preliminary reports indicate that GIP analogues may also be effective in the management of T2DM, although further investigation is needed to elucidate the clinical efficacy of these drugs (53–56).

Ghrelin initiates appetite by stimulating orexigenic NPY neurons and inhibiting POMC neurons in the ARC, and is also secreted by gastrointestinal cells, located predominantly in the stomach. Ghrelin also raises blood glucose levels by inhibiting glucose-stimulated insulin secretion (GSIS) and impairing glucose tolerance (57, 58).

An introduction to insulin resistance

Insulin resistance, a core component in the pathophysiology of T2DM, is associated with the metabolic syndrome (MS) and obesity (59), and influenced by many factors (Figure 5). Insulin resistance occurs in the presence of chronic energy excess, which leads to accumulation of ectopic lipids in hepatic and skeletal muscle tissue, impairing insulin signaling in these tissues, resulting in hyperglycemia. Although insulin resistance and obesity are strong risk factors for T2DM, these factors alone are not sufficient to produce hyperglycemia (60). β cell dysfunction in the islets of Langerhans is also required to produce T2DM, although the degree of β cell function and insulin resistance varies between individuals. β cell dysfunction results from an inability to detect elevated glucose levels to stimulate an appropriate secretion of insulin (59), which exacerbates hyperglycemia.

The mechanisms of β cell dysfunction are not fully understood. A number of factors are believed to contribute to this phenomenon, including proinflammatory cytokines which are associated with obesity and induce mitochondrial stress in β cells (61). Macrophage infiltration into adipose tissue is considered to be the main source of cytokines in obese individuals, with both macrophage infiltration into the adipose tissue and cytokine secretion, shown to be modulated by circadian rhythms (62). Thus, their immune rhythms may also play an important role in β cell dysfunction. Chronic exposure to hyperglycemia causes oxidative stress which damages the organelles such as the mitochondria and endoplasmic reticula,

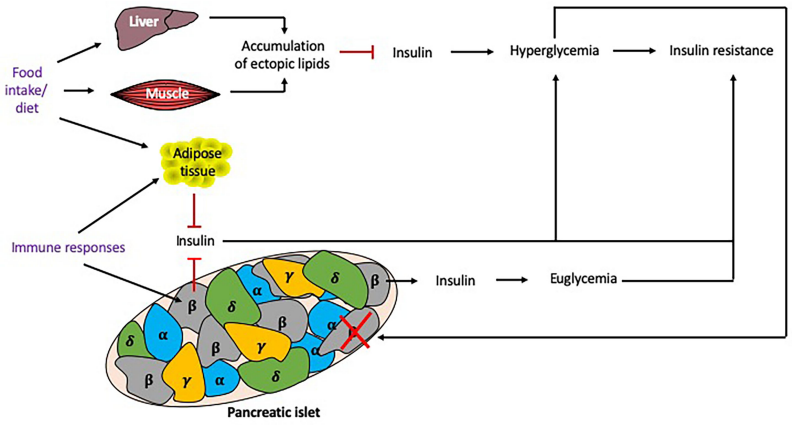

FIGURE 5
Factors influencing insulin resistance. Multiple factors may contribute to the development of insulin resistance. Excess food intake and the type of diet eaten can promote the accumulation of lipids in tissues, reducing insulin signaling and causing hyperglycemia and insulin resistance. Chronic hyperglycemia induces β cell stress, leading to β cells that fail to secrete sufficient insulin to maintain euglycemia, or exhausted/ destroyed β cells, which do not secrete insulin, further promoting hyperglycemia. Genetics play an important role as some individuals may develop insulin resistance but remain euglycemic. Immune responses, particularly cytokines secreted from macrophages, can also promote adiposity and insulin resistance. Responses to food intake and immune responses (shown in purple) can be altered by the time of day and thus circadian rhythms may alter these responses. Black arrows indicate induction, red lines indicate inhibition.

leading to the apoptosis of these cells. Inflammation and oxidative stress contribute to the accumulation of reactive oxygen species in β cells, which hinders the mitochondrial electron transport chain and further damages organelles, thus exacerbating β cell dysfunction.

Although obesity and insulin resistance are risk factors for developing T2DM, not everyone who has these risk factors will progress to developing this condition (63). Indeed, some insulin resistant, obese individuals remain euglycemic because their β cells compensate by secreting more insulin. Genetic factors are determinants of whether or not β cell dysfunction develops in these individuals and there are several genetic variants which may protect or predispose to T2DM (64, 65).

Dietary factors are key risk factors for developing insulin resistance and β cell dysfunction (66). For example diets containing high amounts of saturated fats cause increased levels of circulating fatty acids, which is a risk factor for developing insulin resistance (67). Fatty acids compete with glucose for uptake and metabolism by tissues. Therefore, hyperglycemia will further increase free fatty acid concentrations in the blood, leading to a glucolipotoxic state which is toxic to β cells (59).

Circadian rhythms in the pancreas

Blood glucose homeostasis and insulin resistance are strongly influenced by both local (i.e. pancreas) and distal (i.e. liver, intestine, brain) circadian rhythms, impacting multiple cell types and the secretion of many hormones (Figures 4, 5).

It is clear that the secretion of insulin and glucagon, insulin sensitivity and glucose tolerance all display circadian rhythmicity (68–73), which can be disrupted in individuals with T2DM and their first degree relatives (74). Glucagon secretion is also controlled in a rhythmic manner; however, the circadian rhythms in both β and α cells are in different phases allowing them to respond accordingly to the local changes in glucose and insulin concentrations respectively (75). Bilateral thermic SCN ablation in rats has demonstrated the role of the central clock in glucose metabolism as in these rats, the diurnal patterns of glucose levels and insulin and glucagon secretion became arrythmic (70, 76). Furthermore, this SCN ablation caused desynchrony between peripheral clocks, indicating that the SCN master pacemaker maintains synchronization under normal physiological conditions (77, 78).

In addition to this, cortisol is secreted in a rhythmic, diurnal manner, with peak levels occurring shortly after waking in the morning (79, 80). Circadian disruption and misalignment are associated with aberrant cortisol secretion patterns (81), while a flattened diurnal cortisol curve and a diminished cortisol awakening response have both been associated with T2DM (82–84).

Both GIP and GLP-1 display circadian rhythmicity in humans and disruptions of these secretory patterns have been associated with obesity and T2DM (85–87). Furthermore, it has been postulated that GLP-1 is a key component of peripheral

metabolic clocks, which entrains pancreatic, hepatic and gut clocks to daily patterns of nutrient intake (88, 89).

Ghrelin secretion oscillates in a circadian pattern which is reciprocally correlated to insulin secretion patterns (90). Immunolabelling studies show that ghrelin-responsive neurons in brain centers, including the ARC, receive direct synaptic input from the SCN, indicating that the downstream effects of ghrelin are regulated by the circadian timing system (91).

Thus, the regulation of blood glucose homeostasis is strongly influenced by circadian rhythms, both directly in the pancreas and through influences in other peripheral tissues i.e. the intestine, brain and liver. The successful coordination of these rhythms between the different tissues is paramount for maintaining good health. Preclinical animal model as well as human studies have been performed to investigate the role of the circadian molecular clock in regulating glucose homeostasis, insulin sensitivity and energy expenditure as discussed in more detail next.

Molecular circadian rhythms in the pancreas

Circadian rhythms are coordinated by tightly regulated central and peripheral clocks which respond to environmental and behavioral cues such as light, food intake and sleep-wake cycles (92). At the molecular level (Figure 6), Brain and Muscle Aryl hydrocarbon receptor nuclear translocator (*Bmal*) 1 and 2, Circadian Locomotor Output Cycles protein Kaput (*Clock*), Cryptochrome (*Cry*) 1 and 2, Period (*Per*) 1-3 genes regulate circadian rhythms *via* transcriptional-translational feedback loops (92). CLOCK and BMAL1 form heterodimers which bind to E-box sequences (CANNTG, where N is any nucleotide) to promote the transcription of *Per* and *Cry* genes. After translation, PER and CRY proteins form heterodimers in the cytoplasm and subsequently translocate into the nucleus to inhibit CLOCK : BMAL1 complexes from promoting further transcription. This cyclical regulation of transcription is achieved through modulating clock-specific and ubiquitous histone modifying factors. For example, CLOCK contains a histone-acetyltransferase (HAT) domain and also recruits histone 3 (H3) methyltransferase MLL1 and JARID1a, which inhibits histone deacetylase 1 (HDAC1) promoting CLOCK : BMAL1 activation (93–95), while PER1, recruits the SIN3A/HDAC1 complex which prevents CLOCK : BMAL1 complexes from binding to promoter regions (96).

CLOCK : BMAL1 complexes also promote the transcription of the nuclear receptors REV-ERBα/β and retinoic acid receptor-related orphan receptors (RORs) α/β. ROR proteins encourage the transcription of BMAL1 whereas REV-ERB proteins inhibit transcription (97, 98). These opposing factors compete for the ROR Response Element (RORE) binding sites (AGGTCA preceded by a 5 base pair A/T rich sequence). ROR also promotes the transcription of Nuclear factor, interleukin 3 regulated (*Nfil3*), which suppresses the transcription of *Per* and *Cry* genes (99) and more recently has also been shown to influence intestinal lipid uptake and obesity (100). Thus, the circadian clock genes modulate susceptibility to metabolic disease, as shown in both mouse and human genetic studies.

Mouse circadian genetic studies

Studies of gene-deficient mice have identified that deletion, or mutation, of any core circadian gene can lead to altered glucose homeostasis or weight gain. In this section, we discuss the impact these genes individually have on metabolic circadian functions.

Studies on homozygous Clock mutant mice, characterized by lengthened circadian periods due to a deletion of exon 19 and amino acid 51 in the C-terminal activation domain of *clock*, show that circadian feedback loops have a considerable role in regulating glucose metabolism (101, 102). These mice demonstrate hyperphagia, dyslipidemia, hyperglycemia and hyperinsulinemia, all of which are associated with type 2 diabetes, obesity and metabolic syndrome. Interestingly, the *clock* mutation in these mice also reduces islet size and β cell proliferation (13), indicating an important role for circadian rhythms in both islet and β cell development. This suggests circadian rhythm modulation may promote β cell development and expansion, thus limiting metabolic dysfunction.

Mice deficient in *bmal1* displayed impaired adipogenesis and hepatic carbohydrate metabolism (11, 12). Global *bmal1* deficiency resulted in a blunted response to hypoglycemia, due to reduced hepatic gluconeogenesis, whereas liver-specific *bmal1*-deficiency resulted in impaired glucose tolerance (11, 103). Pancreas-specific *bmal1* gene knock out (KO) models develop hyperglycaemia and hypoinsulinemia, both of which are characteristic of diabetes development (13). Together, these suggest fundamental roles for intrinsic circadian responses in individual cell types. Understanding the roles of individual cell types in modulating these rhythms, and how that impacts on crosstalk with other cell types, will be fundamental in developing targeted therapeutic strategies to be explored.

Per and *cry* are downstream target genes of Clock/Bmal1. Homozygous *per2* knockdown mice had lower total triacylglycerol and non-esterified fatty acids compared with their wild-type (WT; i.e. *per2*-sufficient) counterparts; however there was no difference in the expression of other clock genes in white adipose tissue (WAT) (104). In this study, Grimaldi and colleagues found that Per2 is likely to regulate lipid metabolism through a PPARγ2-dependent mechanism (104). Per3 is also involved in lipid metabolism and *Per3*-deficient mice are more prone to weight gain, when exposed to a high-fat diet (HFD), compared to their WT counterparts (105).

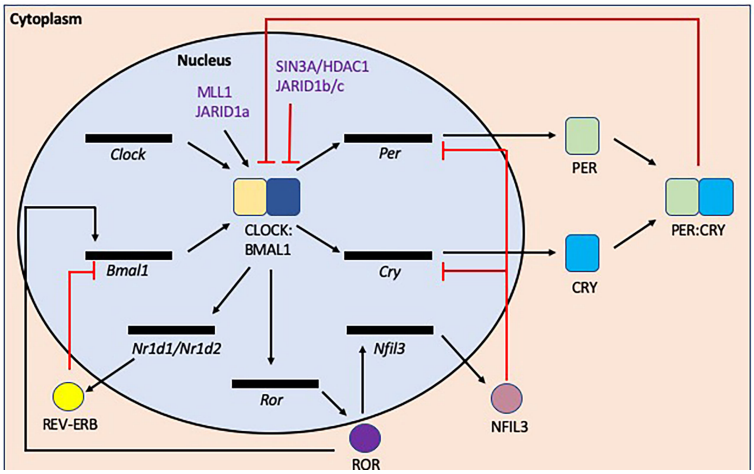

FIGURE 6
Transcription/translation feedback loops that modulate circadian rhythms at the molecular level. The induction of circadian rhythms relies on oscillations in gene expression and repression. In the initiation of the circadian rhythms, *Bmal1* and *Clock* are transcribed/translated and then form a heterodimer. This CLOCK : BMAL1 heterodimer initiates the transcription of a number of genes including *Per*, *Cry* and *Nr1d1/Nr1d2* (*rev-erbα/β*) genes, all which negatively repress the circadian initiators BMAL1 and/or CLOCK. In addition, CLOCK : BMAL1also activates *Ror* transcription, promoting transcription of *Bmal1*, while also inducing *Nfil3* transcription/translation, which inhibits *Per* and *Cry* gene transcription. There are also epigenetic modulation factors e.g. (de)acetylation or (de)methylation that also regulate the circadian rhythm as shown in purple.

Murine *cry1* and *cry2* gene deletions are associated with disruptions in the circadian rhythmicity of insulin and glucagon secretion, which in turn are associated with insulin resistance, hypertension and impaired glucose tolerance and dyslipidemia (106, 107). Similarly, agonists of Cry1 and Cry2 inhibit hepatic gluconeogenesis *in vitro* (108). In contrast, liver-specific expression of adenoviral encoded *cry1*, at a time when Cry1 is endogenously low, appeared to protect the mice from these metabolic risk factors and increased insulin sensitivity, whilst decreasing blood glucose (109). These differences may relate to cell-specific roles, differences in mice studied (food-restricted or not), timing and methodology of alteration (i.e. lifelong gene deficiency or induced expression following adenovirus delivery) or alterations in other circadian gene regulation or expression.

Rev-erbα plays an important role in regulating insulin and glucagon secretion and pancreatic β cell proliferation (110). Downregulation of *rev-erbα* using siRNA in mouse pancreatic islet cells reduces glucose-stimulated insulin secretion (GSIS) in mouse models (111). The authors of this study found that exogenous leptin treatment enhanced *rev-erbα* expression *in vitro* and *in vivo*, whilst a HFD further downregulated *rev-erbα* expression. Mouse models lacking *rorα* and *rev-erbα* have lower high-density lipoprotein and decreased adiposity compared to their WT counterparts (97, 112). Solt and colleagues showed that Rev-erb agonists can decrease fat mass and total serum cholesterol in diet-induced obese mice (113). Similarly, Rorα inverse agonists are effective at preventing hyperglycaemia in mouse models of type 2 diabetes (114).

Thus, studies of mice have greatly helped to identify key mechanisms and cell-specific contributions that aid in the modulation of circadian rhythms, leading to altered susceptibility to obesity and diabetes development. It is clear that this is a growing field and more understanding is required of how cell intrinsic clocks impact on other cell types and how other environmental factors may alter peripheral oscillations, leading to altered susceptibility to obesity and diabetes development. Further knowledge of how we can modulate the circadian rhythms in humans will be vital, but animal models may be very helpful for developing preclinical therapies for translation into humans.

Human genetic studies

There are single nucleotide polymorphisms (SNPs) in humans that have been associated with the risk of developing metabolic dysbiosis, obesity and T2DM (Table 1). *Clock* SNPs can predispose individuals to developing obesity, metabolic syndrome and T2DM by altering the metabolism of fatty acids, as well as the monosaturated fatty acid content of red blood cells (115–118). Similarly, a SNP in NPAS2, a paralog of CLOCK, which can also bind to BMAL1 (126), has also been linked to risk factors (e.g. hypertension) for developing metabolic syndrome (119). SNPs in *Bmal1* have also been associated with hypertension, hyperglycemia, T2DM and gestational diabetes (120, 121). In addition, SNPs in CLOCK : BMAL1-repressing

TABLE 1 SNPs in circadian rhythm-related genes associated with metabolic dysfunction, obesity and T2DM in humans.

Gene and location	SNP [allele(s)]	Study Population	Association	Reference
Clock (4q12)	rs1554483 (G) rs4580704 (C) rs68437222 (C) rs6850524 (G) rs4864548 (A) rs1554483-rs4864548 (GA)	Lean (n=715) and overweight/obese (n=391) individuals of self-reported European descent in Buenos Aires, Argentina.	Up to 1.8-fold risk of developing overweight/obesity	(115)
	rs4580704 (CC) rs1801260 (C)	1100 American individuals of European descent	Increased risk of developing metabolic syndrome components	(116)
	rs4864548-rs3736544-rs1801260 (CAT)	537 individuals from 89 British families (all white European)	Associated with presence of metabolic syndrome	(117)
	rs4580704 (CC)	7098 individuals with T2DM or with 3 or more cardiovascular risk factors (all European)	Increased fasting glucose, and increased development of T2DM. Increased risk of cardiovascular disease in individuals with T2DM.	(118)
Npas2 (2q11)	rs11541353 (C)	517 Finnish individuals	Associated with hypertension	(119)
Bmal1 (11p15)	rs7950226-rs11022775 (haplotype AC)	1304 individuals from 424 British families with T2DM of European descent	T2DM	(120)
	rs6486121-rs3789327-rs969485 (CCA)		Hypertension	
	rs7950226 (A) rs11022775 (C) rs7950226-rs11022775 (GC) rs7950226-rs11022775 (AC)	185 women with Gestational diabetes and 161 controls (Greek population)	Increased risk of developing gestational diabetes	(121)
Cry2 (11p11)	rs11605924 (A)	21 GWAS cohorts including up to 46,186 non-diabetic individuals, with a further follow up of 25 loci in 76,558 additional individuals of white European descent from United States or Europe	Higher fasting glucose levels	(122)
Per2 (2q37)	10870 (A)	517 Finnish individuals	Increased risk of raised plasma glucose	(119)
	rs2304672 (G) rs4663302 (T)	454 overweight/obese Spanish individuals	Increased snacking, higher stress when dieting, more likely to eat when bored Higher waist circumference and waist to hip ratio	(123)

(Continued)

TABLE 1 Continued

Gene and location	SNP [allele(s)]	Study Population	Association	Reference
Mtnr1b (11q14)	rs1387153 (T)	2151 non-diabetic (encompassing lean and obese) French subjects with European ancestry. Replication analysis conducted in 5,518 middle-aged non-diabetic Danish individuals, 3,886 and 1,453 non-diabetic French individuals from 2 cohorts and 5,237 young (16 years of age) Finnish individuals	Increased fasting blood glucose, increased risk of developing hyperglycemia and T2DM	(124)
	rs10830963 (G)	10 GWAS study cohorts and 13 case-control studies (18,236 cases, 64,453 controls) of European descent	Increased fasting blood glucose levels, reduced beta cell functions and an increased risk of developing T2DM	(125)

genes, such as *Cry* and *Per* genes, have also been implicated in metabolic disease. Both *Cry2* and *Per2* SNPs have been associated with impaired glucose tolerance (119, 122), while *Per2* SNPs have also been associated with binge eating and stress related to dieting, leading to increased weight gain (123). These core circadian rhythm-inducing genes, modulate the rhythmic expression of many other genes in the body. One example is the rhythmic secretion of melatonin, which in humans increases in the evening and decreases in the daytime, aiding in regulating our sleep/wake cycles (127). Interestingly, two SNPs in one of the melatonin receptors, the melatonin receptor 1B gene (*Mtnr1b*), have been associated with higher fasting glucose concentrations, reduced β cell function (as measured by homeostasis model assessment (HOMA)) and an increased risk of developing T2DM (124, 125). This SNP appears to influence the dynamics of melatonin secretion, which may modulate the susceptibility to developing T2DM (128). This suggests important roles for both the SNPs involved in the molecular circadian clock, but also their downstream genes in modulating susceptibility to metabolic syndrome, obesity and T2DM.

Information on the expression of clock genes in human pancreatic islets is limited, but circadian genes are expressed in human islets (129). In individuals with T2DM, *Cry2*, *Per2* and *Per3* expression was reduced in the islets compared to islet donors without T2DM (130). Additionally, *in vitro*, islets cultured in glucolipotoxic conditions (16.7mmol/L glucose per 1mmol/L palmitate) for 48 hours downregulated the expression of *Per3* in the pancreatic islets of individuals without T2DM (130). The aforementioned studies highlight the importance of circadian clock genes in regulating metabolic functions such as glucose tolerance and β cell function; however, many of these studies did not investigate the expression of these genes at the protein level. Although Stamenkovic and colleagues correlated mRNA expression to corresponding protein concentrations in human islets, post-transcriptional factors such as miRNA and post-translational modifications were not examined in this study (130). Future studies that address these interactions and mechanisms of regulation may provide additional insights into the relationship between the circadian clock and metabolic physiology. Additional studies, particularly in non-white European populations, with increased numbers of participants are also greatly needed.

Modulation of circadian rhythm

Misalignment between peripheral and central clocks is associated with insulin resistance, metabolic abnormalities and cardiovascular disease (17, 131, 132). This desynchrony can be achieved experimentally through a forced desynchronization (FD) protocol which involves altering behavioral patterns, such as feeding and sleep-wake cycles, so that they are substantially longer or shorter than 24 hours, whilst ensuring that the subjects are only exposed to dim light during their wake times (133). The aim of this is to desynchronize endogenous circadian rhythms from external influences e.g. food intake, light exposure as outlined in Figure 7. Buxton and colleagues demonstrated that a FD protocol (sleep restriction and circadian disruption) increased plasma glucose levels in human studies (134). Although the mechanism for this is unclear, a study which utilized human islet amyloid polypeptide (HIP) transgenic rats showed that circadian disruption accelerated the β cell loss and dysfunction in this model of T2DM (135). Furthermore, sleep deprivation studies demonstrated disrupted rhythmicity of insulin and glucagon levels, as well as insulin sensitivity and glucose tolerance (136–138). In these studies, circadian rhythm cycles have clearly influenced susceptibility to metabolic disease. In this section, we break down the different environmental cues that can significantly alter circadian rhythms in animal models and humans.

Altered light cycles

In mice, electrophysiological monitoring has shown that exposure to constant light reduced the amplitude (the difference between peak and trough) of SCN rhythmicity (139). This resulted in increased food intake, whilst energy expenditure was decreased. These mice also showed a

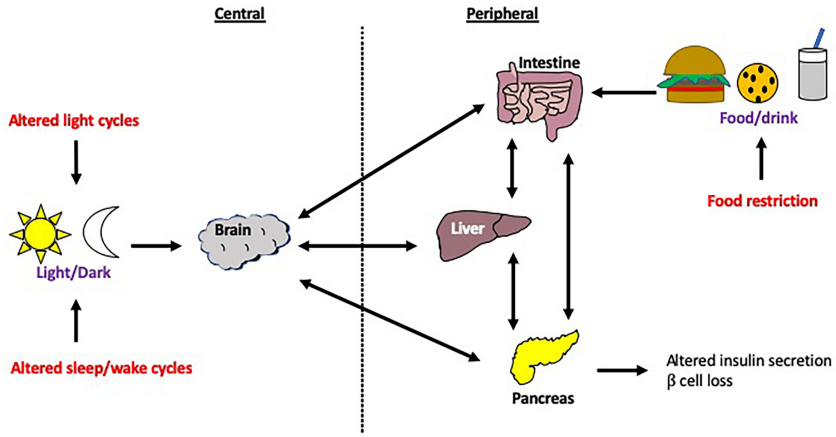

FIGURE 7
Modulation of host circadian rhythms. Circadian rhythms can be modulated through a number of interventions, including changes to the central clock *via* changes in the light/dark cycle or sleep/wake cycle, as well as changes to peripheral clocks e.g. food restriction. Disruption to the central clock can lead to disconnection from the peripheral clocks and vice versa. These changes to the circadian rhythm are often experienced by those with jet lag or in overnight shift workers. .

complete loss of rhythmicity in insulin sensitivity, energy expenditure and food intake. In humans, light intensity has been shown to regulate postprandial glucose levels and triglycerides (140). In this study, healthy lean men and obese men with T2DM were exposed in the morning to 5 hours of either bright light (4000 lux) or dim light (10 lux), with a 600kcal meal given 1 hour after the start of the light exposure. While no changes were seen in the fasting or postprandial glucose levels of healthy lean men between dim or bright light exposure, obese men with T2DM had improved fasting and postprandial glucose levels, when exposed to bright light. In addition, healthy lean men exposed to bright light had higher fasting and postprandial plasma triglycerides, while in obese men with T2DM bright light only increased postprandial plasma triglycerides and did not change fasting triglyceride concentrations. Similar results were also seen in a study of individuals with insulin-resistance exposed to either bright day – dim evening light or dim day – bright evening light conditions (141). Thus, the light exposure can significantly impact our glucose homeostasis.

Altered diets and time of food intake

A high-fat diet also disrupts central and peripheral clocks in mouse models, including hypothalamic, adipose and hepatic clocks (142). The timing of food intake also affects circadian clocks. When kept in a 12-hour light/12-hour dark cycle (12:12h LD), mice consume the majority of their food during the dark phase when food is available *ad libitum* (143). In contrast, during timed food restriction, mice fed a HFD only during the light phase gained more weight than those fed during the dark phase (144). Similarly, mice fed a HFD restricted to the dark phase are less likely to develop metabolic abnormalities such as obesity and glucose intolerance than mice fed the same HFD *ab libitum* (145). Time-restricted feeding, where food is provided for a specific duration only, is also effective at preventing obesity and metabolic syndrome in circadian gene-deficient mice (whole body *cry1/2*-deficient mice and liver-specific *bmal1* and *rev-erbα/β*-deficient mice*)* (146). Feeding restricted to the light phase also caused a desynchrony of peripheral clocks in the pancreas, liver, heart and kidneys by up to 12 hours, which did not affect the SCN (147). Mice exposed to calorific restriction in 12:12h LD cycles will become partly diurnal, as opposed to purely nocturnal and this is attributed to changes in peripheral clocks and the SCN (148, 149).

As in the animal studies, the timing of eating also influences risk of diabetes in humans. For example, a randomized crossover study showed that a later dinner was associated with impaired glucose tolerance in a subset of MTNR1B (melatonin receptor 1B) risk allele carriers (150). The postulated role of melatonin in this process agreed with a previous study showing that exogenous melatonin could also cause impaired glucose tolerance (151). Conversely, feeding restricted to 9 hours improved glycemic control in men with type 2 diabetes (152). Similarly, time-restricted eating (TRE) also improved metabolic parameters such as weight, visceral fat, atherogenic lipids and blood pressure in individuals with metabolic syndrome (153, 154); however, these benefits of TRE were observed in small sample sizes (n=15-20) and are currently under investigation larger cohorts (155, 156). Maintaining TRE after weight is potentially a challenge and further research would also be necessary.

In a FD protocol study involving 5 male and 5 female adults, increased blood glucose levels were coupled with a paradoxical rise in insulin secretion during the misalignment phase (where eating/sleeping is 12 hours out of synchrony with the normal schedule) (131). Another study which combined a FD protocol, with restricted sleeping hours (6.5 hours in a 28-hour day), showed a similar rise in glucose levels in the individuals, coupled with increased insulin secretion (134). A possible explanation for this may be reduced insulin sensitivity secondary to circadian misalignment. This is supported by a 12-hour rapid shift work protocol, which utilized the hyperinsulinemic-euglycemic clamp and showed that circadian misalignment is associated with decreased insulin sensitivity (157).

The timing of nutrient intake also alters the circadian rhythmicity of the gut bacterial composition in mice (158, 159). For example, bacterial species belonging to the phylum Firmicutes thrive postprandially in response to dietary glycan intake, whilst the phyla Bacteroidetes and Verrucomicrobia usually peak in numbers during fasting periods (158–160). As mentioned, the gut microbiota can oscillate, altering important metabolic functions in mice. In line with this, a recent study in humans identified a gut bacterial signature, encompassing 13 taxa with disrupted rhythmicity, which, in conjunction with BMI, could be used to predict individuals who would later develop T2DM (161). Thus, host-microbial rhythms may act as a biomarker for disease development. Interestingly, common gastric bypass procedures such as Roux-en-Y gastric bypass, which enable individuals to lose weight, are associated with altered microbial composition (162). It would be interesting to assess whether these procedures also alter host and microbial rhythmicity.

Shift work

As mentioned previously, circadian misalignment, and by extension shift-work, is a risk factor for developing metabolic syndrome, obesity and type 2 diabetes (18). Circadian misalignment as a risk factor for metabolic abnormalities has been corroborated in a real-life study that compared day-shift and night-shift workers (16). In this study, night-shift workers were found to have increased postprandial glucose and insulin levels as well as elevated triacylglyerol levels, compared to day-shift workers. A meta-analysis of 12 observational studies revealed that shift-work is associated with a 9% increase in the chance of developing T2DM compared to people who have not been exposed to shift work (19). Importantly, people who have rotating shift work are more at risk than those employed in constant shift work (22). This is likely due to exposure to both light and food intake at times-of-day different to when the body naturally anticipates these changes. Furthermore, a rat study using a simulated shift-work protocol (using rotating running wheels) was consistent with the human data and caused the animals to develop abdominal obesity and blunted rhythmicity of glucose levels (163). In rats, disruption of 12:12h LD cycles, with continuous light exposure, accelerated β cell dysfunction and loss, whilst impairing GSIS (135, 164). Studies on β cell-specific *bmal1* knockout mice showed that Bmal1 plays an important role in adapting to circadian disruptions and preventing oxidative stress (14). Thus, a loss of function of this gene predisposes mice to β cell dysfunction and further to diabetes.

Jet lag

Jet lag is also associated with circadian misalignment and is characterized by a series of psychological and physiological symptoms such as low mood, impaired cognitive performance, loss of appetite, gastrointestinal disturbances and general malaise (165). Chronic jet lag may elevate the risk of developing cancer, cardiomyopathy and T2DM (166–168) and can be simulated in animal experiments by altering the duration of light or dark cycles (169). Studies utilizing this approach have found that there is extensive desynchrony between different body tissues and also variation in the time taken by different tissues to adjust to new light/dark cycles (170). Adrenal glucocorticoids (GCs) appear to play a key role in the re-entrainment process of circadian rhythms in jet lagged mice (171–173). Injection of metyrapone (MET; an inhibitor of corticosterone synthesis) prior to performing the jet lag protocol, was found to prolong re-entrainment when administered in the inactive phase and accelerate re-entrainment when given during the active phase (170). Since the SCN does not express GC receptors, GCs are unlikely to directly feedback to the SCN to regulate this process (173, 174). It has been hypothesized that dysregulation of the adrenal clock may cause aberrant secretion of other adrenal hormones such as aldosterone alongside GCs, which may feedback to the master pacemaker to regulate re-entrainment (175).

In jet-lagged mice, both the composition of the bacteria and metabolic functions were altered compared to control mice (158). These mice experienced impaired glucose tolerance and obesity caused by the bacteria as (a) these metabolic abnormalities were diminished following the eradication of the microbiota using antibiotics and (b) the transplantation of microbiota from these jet-lagged mice to germ-free mice, which lack all bacteria, transferred these metabolic abnormalities to the recipient mice. Together, these studies suggest that there is a functional relationship between host and bacterial rhythms which are important in modulating metabolic dysbiosis. Thus, alterations of the bacterial composition or oscillations could potentially be harnessed to prevent obesity, metabolic abnormalities or T2DM.

Social jet lag, defined as a temporal discrepancy between a person's sleep pattern on working days and non-working days

(23), has also been associated with diabetes susceptibility. Individuals with greater than 1 hour of social jet lag have a 75% greater chance of developing diabetes or prediabetes, compared to people with less than 1 hour of social jet lag (21).

Together, rodent and human studies have identified that the timing of food intake, nutrient content and light exposure are important stimuli in regulating the metabolic clock. These findings indicate that novel interventions such as time-specific therapy (chronotherapy) or interventions which target the circadian system such as synthetic circadian protein analogues may be beneficial in the future management of metabolic syndrome and T2DM (176).

Chronotherapy

Chronotherapy is the concept of administering drugs or other treatments at optimal times of the day in order to produce the most benefit (177). In clinical practice, statins are typically taken in the evening as their mechanism of action is to inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, an enzyme which peaks in concentration in the night (178). There are many other examples of chronotherapy already in existence and its importance in relation to diabetes management as discussed below.

Bromocriptine is a dopamine agonist which is used as an adjunct in the treatment of T2DM (179). Dopaminergic activity in the hypothalamus follows a circadian rhythm and drives hepatic gluconeogenesis and adipocyte lipolysis. Bromocriptine is thought to reduce this drive when given within 2 hours of waking to prevent hyperglycaemia and dyslipidaemia. Although bromocriptine demonstrated efficacy in glycemic control and has been approved by the Food and Drug administration (FDA) in T2DM, this drug is infrequently used in clinical practice (52, 180, 181).

Despite preclinical studies showing that metformin interacts with molecular components of the circadian system and has time-dependent effects on blood glucose (182–184), there have not been any clinical studies to investigate the timing of metformin administration. This may be especially important given oscillations in both host and bacterial rhythms, as metformin alters the bacterial composition and function to mediate therapeutic effects of the drug (185). As metformin is a first line therapeutic for T2DM, it would seem particularly important to maximise efficacy. Similarly, the short-acting sulfonylurea drug tolbutamide appears to have time-dependent effects on insulin secretion but its chronotherapeutic potential is yet to be investigated (186).

Targeting circadian rhythm proteins may also be important for preventing/reversing metabolic dysbiosis. REV-ERBα agonist SR9011 and REV-ERBβ agonist SR9009 reduced obesity and hyperglycaemia in mouse models (113). Both of these synthetic REV-ERB agonists induced increased energy expenditure in white adipose tissue. Furthermore, the naturally-derived compound nobiletin activates circadian molecules RORα and RORγ and prevents metabolic syndrome from developing in diet-induced obese mice (187). Synthetic CRY stabilizers have also been reported to have a protective effect against diabetes by improving glucose tolerance in mice (188). In these rodent studies, these drugs have a short half-life and are typically administered by injection at intervals shorter than 3 hours to maintain suitable bioavailability (189). It has been hypothesized that humans would eliminate the active metabolites of these drugs even more quickly, therefore requiring more frequent injections (189). In a clinical context, this may not be practical. Therefore, this pharmacological obstacle must first be addressed before studies in humans can be carried out effectively. Novel therapies for T2DM, obesity and metabolic syndrome may be identified if these and similar drugs can demonstrate similar effects in human studies.

Future directions

In today's industrialized world, only a minority of people have an internal sleep-wake cycle which is consistent with their social commitments (190). Therefore, it is unsurprising that social jetlag is common in the population (23). An individual's personal circumstances has a substantial influence on many of the exogenous factors which influence circadian rhythms including sleep-wake cycles, exposure to light, eating times and activity level (191). These environmental factors, together with endogenous characteristics such as age, genetics and chronotype (i.e. the time of day people are most alert/sleep), influence the degree of circadian desynchrony which an individual experiences (9).

In clinical practice, there is currently neither a standardized scoring system which precisely encompasses the exogenous determinants of circadian activity, nor is there a genetic screening program which identifies carriers of genetic variations predisposing people to circadian disruption. The future development of these risk stratification tools may influence clinical practice by allowing disease management to be tailored to an individual's circadian rhythmicity. For example, the timing of drug administration could be regulated in order to maximize efficacy, which may enable reduced drug concentrations to be used, thus limiting any toxicity or potential side effects. In order for this to be achieved, further epidemiological studies are necessary to quantify the relative risk of circadian disruption associated with different behavioral and genetic risk factors.

There are many exogenous factors which can alter host/microbial rhythmicity, which can then modulate susceptibility to metabolic dysfunction and T2DM. Recent evidence has shown arrhythmic bacterial signatures could be used as a biomarker to predict individuals who would later develop T2DM; thus, further

investigation into host/microbial rhythms, or lack thereof, as biomarkers for predicting metabolic dysfunction or the onset of diabetes should be conducted. Non-pharmacological interventions can also be used to target the circadian system in metabolic disease. Currently, trials investigating the effects of time-restricted feeding are taking place on large cohorts with type 2 diabetes and metabolic syndrome (155, 156). The results from these studies will provide insight into the potential for simple lifestyle changes that can modulate circadian rhythms as a therapy. Understanding the mechanism behind these changes will be vital.

Summary

Human epidemiological and genetic studies have highlighted the importance of circadian rhythms in metabolic diseases such as T2DM and metabolic syndrome. This has led to extensive research in animal models and humans, which have concluded that circadian dysregulation and misalignment is associated with the development of metabolic abnormalities. Clinical applications of this knowledge may include the optimization of existing antidiabetic therapies such as metformin. Circadian molecules such as nobiletin, REV-ERB agonists and CRY stabilizers have demonstrated efficacy in preclinical studies and may lead to the development of novel treatments for diseases linked to circadian dysregulation. However, limitations in current knowledge mean that further research is required before these interventions can be used clinically.

Author contributions

KC and JP wrote the manuscript. FW and JP edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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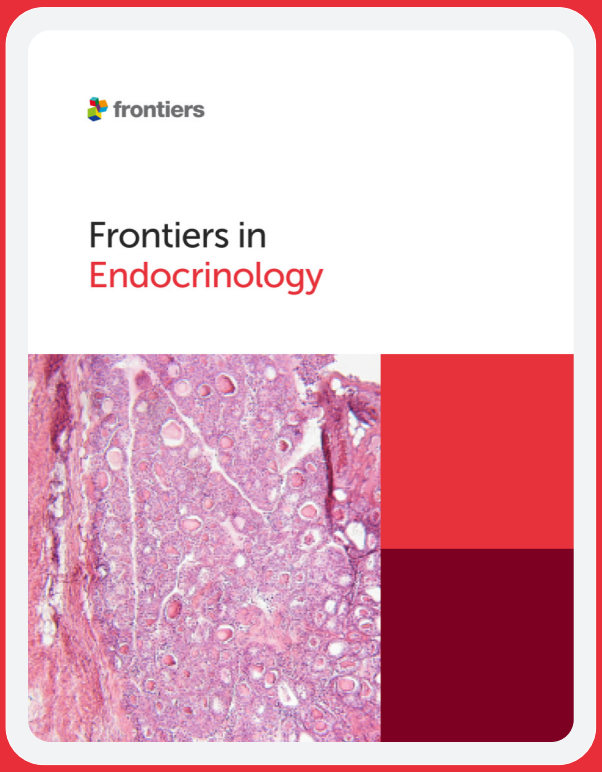

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