RESEARCH ADVANCES IN GESTATIONAL DIABETES MELLITUS, NEONATAL DIABETES MELLITUS, AND METABOLIC DISORDERS

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RESEARCH ADVANCES IN GESTATIONAL DIABETES MELLITUS, NEONATAL DIABETES MELLITUS, AND METABOLIC DISORDERS

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The Depletion of Carbohydrate Metabolic Genes in the Gut Microbiome Contributes to the Transition From Central Obesity to Type 2 Diabetes

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Obesity, especially central obesity, is a strong risk factor for developing type 2 diabetes (T2D). However, the mechanism underlying the progression from central obesity to T2D remains unknown. Therefore, we analyzed the gut microbial profiles of central obese individuals with or without T2D from a Chinese population. Here we reported both the microbial compositional and gene functional alterations during the progression from central obesity to T2D. Several opportunistic pathogens were enriched in obese T2D patients. We also characterized thousands of genes involved in sugar and amino acid metabolism whose abundance were significantly depleted in obese T2D group. Moreover, the abundance of those genes was negatively associated with plasma glycemia level and percentage of individuals with impaired plasma glucose status. Therefore, our study indicates that the abundance of those depleted genes can be used as a potential biomarker to identify central obese individuals with high risks of developing T2D.

Keywords: central obesity, type 2 diabetes, transition, biomarker, gut microbiome, metagenomics

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INTRODUCTION

Obesity and type 2 diabetes (T2D) are metabolic disorders with increasing incidence rates both worldwide (1) and within China (2). The International Diabetes Federation estimated that the number of people with diabetes was 463.0 million in 2019 and is expected to rise to 578.4 and 700.2 million by 2035 and 2045, respectively (1). Obesity, particularly central (intra-abdominal) obesity, is a well-established strong risk factor for T2D (3). Nevertheless, all obese individuals do not develop T2D (4, 5). Currently, the causal factors driving or preventing the transition from obesity to T2D remain unknown. However, numerous factors have been reported to play important roles in developing T2D, including genetic background and personal lifestyle, many of which have also been linked to the gut microbiome (6, 7). Thus, it is worthwhile to identify the distinguishing microbiome markers driving the transition from obesity to T2D. Such markers will contribute to a better understanding of the pathophysiological mechanism of progressing from obesity to T2D.

Increasing evidence has demonstrated the close relationship between the microbiome and various human diseases, especially metabolic-related diseases (8, 9). There is general agreement that the loss of a butyrate producer microbiota is associated with T2D in different cohorts with different ethnicities (3, 9, 10). Furthermore, the loss of those butyrate producers may contribute to low-grade inflammation, dysregulated glucose metabolism, and insulin resistance; thus, it is associated with the progression of T2D (3, 11, 12). Given these common observations, the attempts to identify an association between a single microbe and T2D have yielded inconsistent results (13, 14). The discrepancies may be due to the differences in trial designs and the influence of confounders, such as medical treatment (15). It has been well reported that medical treatment, such as metformin, greatly impacts on the gut microbiome taxonomic composition and ecological diversity (11, 16).

Thus, in this study, to exclude the influences of those confounders, we performed 16S rRNA gene sequencing and metagenomic sequencing analysis of 60 central obesity non-diabetic participants and 183 central obesity patients who were newly diagnosed as T2D and hence medicine treatment-naïve. We aim to identify the potential compositional and/or functional features of the gut microbiota that contribute to the transition from central obesity to T2D.

MATERIALS AND METHODS

Study Cohort and Patient Characteristics

We collected the stool samples of 243 adults for this study. The sample collection was approved by the ethics committee of Suzhou Municipal Hospital. All volunteers signed a written informed consent before sample collection. The volunteers were classified into two groups (183 in the experimental group and 60 in the control group). The experimental group was the abdominal obesity patients who were newly diagnosed with type 2 diabetes mellitus (waist circumference, man ≥90 cm; woman ≥85 cm; fasting plasma glucose level >7 mmol/L) (the criteria of weight for Chinese adults is according to the instructions of the National Health Commission of the People's Republic of China, http://www.nhc.gov.cn/wjw/yingyang/201308/a233d450fdbc47c5ad4f08b7e394d1e8.shtml), so no treatment medicines had been taken. Abdominal obese individuals who did not have diabetes served as control.

The exclusion criteria for the two groups include (1) antibiotic consumption during the last 2 months, (2) family history of diabetes or other related diseases, (3) major gastrointestinal surgery and any known chronic disorders, (4) pregnancy at the time of sample collection, and (5) hypothyroidism.

Stool Samples Collection and DNA Extraction

The stool samples were collected using the stool collection kit, Flora Prep, provided by Admera Health Inc. (Suzhou, China). After collection, the samples were stored at 4°C and sent to the laboratory within 1 to 2 days. As instructed, genomic DNA was extracted from the stool samples using a Qiagen DNeasy PowerSoil HTP 96 kit (cat. no. 12955-4). The Qubit dsDNA HS assay kit was used to determine the concentration and quality of the purified DNA.

16S rRNA Gene Amplicon Preparation and Sequencing

Purified DNA (20–30 ng) was used to generate amplicons. The V3 and V4 hypervariable regions of prokaryotic 16S rRNA were selected for generating amplicons and following taxonomy analysis. These regions were amplified using forward primers containing the sequence "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing the sequence "GGACTACNVGGGTWTCTAATCC" (patent no. US 9745611B2). Finally, indexed adapters were added to the ends of the 16S rRNA amplicons to generate indexed libraries ready for downstream next-generation sequencing on an Illumina MiSeq platform.

The PCR reactions were performed in triplicates of 25-μl mixtures containing 2.5 μl TransStart Buffer, 2 μl of dNTPs, 1 μl of each primer, and 20 ng template DNA.

The DNA library concentration was determined using the Qubit 3.0 Fluorometer. The library was quantified to 10 nM. The DNA libraries were multiplexed and loaded on an Illumina MiSeq instrument with pair-end 250-bp (PE250) mode according to the instructions of the manufacturer (Illumina, San Diego, CA, USA). Image analysis and base calling were conducted by the control software embedded in the instrument. All the 16S rRNA gene sequencing experiments were conducted by GENEWIZ Inc. (Suzhou, China).

Metagenomic Sequencing

Metagenomic libraries were constructed according to the protocol of the manufacturer (Vazyme, VAHTS Universal DNA Library Prep Kit for Illumina, cat. ND607-01). For each sample, 200 ng of genomic DNA was randomly fragmented to <500 bp by sonication (Covaris S220). The fragments were treated with End Prep Enzyme Mix for end repairing, 5′ phosphorylation, and dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of adaptor-ligated DNA was then performed using VAHTS DNA Clean Beads (Vazyme, N411-01), and fragments of approximately 470 bp (with an approximate insert size of 350 bp) were recovered. Each sample was then amplified for eight cycles using Illumina P5 and P7 indexed primers, with both primers carrying sequences, which can anneal with the flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using VAHTS DNA Clean Beads, and the fragment size can be visualized using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and quantified by Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

Then, libraries with different indexes were multiplexed and loaded on an Illumina HiSeq X instrument according to the instructions of the manufacturer's (Illumina, San Diego, CA, USA). Sequencing was conducted using a paired-end 150-bp (PE150) mode. Image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6

(Illumina). All metagenomic sequencing experiments were conducted by GENEWIZ Inc. (Suzhou, China).

16S rRNA Gene Sequencing Data Processing

Paired-end reads were firstly assembled to filter reads smaller than 200 bp with N base. Then, chimeric reads were removed, and the resulting sequences were used for downstream operational taxonomic unit (OTU) clustering with the VSEARCH program (v1.9.6) (17). Sequences with similarities larger than 97% were classified as the same OTU. The annotations of taxonomic information were achieved from the Ribosomal Database Project classifier algorithm (http://sourceforge.net/projects/rdp-classifier) according to the SILVA_132 database (18).

Based on OUT taxonomic annotation information, alpha diversity indices, such as Shannon and Chao1, and rarefaction curves and rank–abundance graphs reflecting species richness and evenness were calculated by the methods implemented in Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 (19). Venn diagrams of the overlapping OTUs between two groups were drawn by using the package in R. The principal coordinate analysis (PCoA) and the nonmetric multidimensional scaling (NMDS) analysis are based on the Bray–Curtis distances.

Metagenomic Sequencing Data Processing

Raw shotgun sequencing reads were trimmed using cutadapt (v1.9.1) (20). Reads with low-quality and N bases and adapter-contaminated were removed. Then, human host contamination reads were removed by mapping the sequencing reads to the human genome (hg38) with BWA (v0.7.12) (21). Finally, the remaining reads were assembled *de novo* with MEGAHIT(v1.13) (22) using different k-mer to obtain separate assemblies in each individual. The best assemble scaffold with the largest N50 was selected for downstream gene prediction analysis.

To identity the composition of microbial communities from metagenomic shotgun sequencing data, taxonomic assignment was performed using MetaPhlAn (v1.7.7) (23) with default parameters.

Gene Catalog Construction and Functional Annotations

The assembled genes of each sample were predicted using Prodigal (v3.02) (24). CD-HIT was used to cluster genes derived from all samples with a default identity of 0.95 and coverage of 0.9 (25). In order to analyze the relative abundance of unigenes in each sample, paired-end clean reads were mapped to unigenes using SOAPAligner (version 2.2.1) (26). Gene abundance was calculated based on the number of aligned reads and normalized to gene length, combined with grouping information. Venn diagrams were drawn based on the number of unique genes shared by two groups.

Diamond (version v0.8.15.77) (27) was used to search the protein sequences of the unigenes with the NR database, CAZy database, eggNOG database, and Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The statistical significance threshold of the sequence alignment was set to 1e-5, and the sequence alignment length was set as no less than 60% of the reference gene protein length. Finally, the matched result with best hit scores was selected for annotation.

Statistical Analysis of 16S rRNA Gene Sequencing and Metagenomic Sequencing Data

The Pearson coefficient was calculated by R stats package corr function based on the genus level abundance from 16S rRNA gene sequencing and metagenomic sequencing in the same sample. PCoA and NMDS analysis were performed using APE package in R, based on the Brary–Curtis matrix of either taxonomic abundance or selected unigene abundance. Additionally, Vegan package in R was used for ANOSIM analysis to analyze the differences between and within groups. Lefse analysis (http://huttenhower.sph.harvard.edu/galaxy) was used to characterize the differentially enriched microbes (linear discriminant analysis, LDA >2; *P*-value <0.05) in each group.

The differentially significantly enriched or depleted genes in each group were identified with the following criteria: (1) at least twofold changes between the Ob&H and Ob&T2D groups and (2) false discovery rate should be less than 0.1. The identified enriched or depleted genes in the Ob&T2D group were submitted to KEGG enrichment analysis, and the significantly enriched KEGG Ontology (KO) (*q*-value <0.05) was summarized in **Supplementary Table S3**.

Random Forest Model Prediction

Random forest (28) analysis provided in the R package random forest was used to build the prediction model to identify the potential diagnostic biomarker genes between the Ob&H and Ob&T2D groups. The gene abundance profiles were calculated from metagenomic sequencing results. The top 300 depleted genes in the Ob&T2D group were filtered as prediction input variables. The important genes that contributed to prediction were identified *via* a 10-fold cross-validation procedure. The area under curve (AUC) index and receiver operating characteristic (ROC) analysis were used to predict the efficiency of the possible cutoff values of the tests.

RESULTS

Overview of the Characteristics of the Cohort

We recruited 60 individuals with central obesity (male/female, 27:33) with normal fasting glucose level ("central obese healthy," Ob&H, average fasting blood glucose level: 5.2 ± 0.7 mmol/L), 183 individuals with central obesity (male/female, 72:111) who were newly diagnosed as T2D and thus diabetes-treatment-naïve ("central obese T2D," Ob&T2D, average fasting blood glucose level: 8.8 ± 2.5 mmol/L) (**Table 1**; **Figure 1A**). Similarly, the hemoglobin A1c (HbA1c) level in Ob&T2D (average: 7.6 ± 1.5%) is also significantly higher than in Ob&H individuals (average:

TABLE 1 | The overall characteristics of the participants in this study.

Characteristics	Ob&H (*n* = 60)	Ob&T2D (*n* = 183)	*P*-value (Wilcoxon rank-sum test)
Sex (M/F)	27/33	72/111	
Fast plasma glucose (mmol/L)	5.2 ± 0.7	8.8 ± 2.5****	<0.0001
HbA1c (%)	5.6 ± 0.6	7.6 ± 1.5****	<0.0001
Weight (kg)	81.1 ± 17.1	73.4 ± 12.7***	0.0003
Height (cm)	164.0 ± 7.9	160.8 ± 7.8**	0.0066
BMI	30 ± 5	28 ± 4**	0.0092
Waist circumference (cm)	102.9 ± 10.1	97.1 ± 7.7****	<0.0001
Hip circumference (cm)	102.6 ± 10.0	101.2 ± 7.8$^{n.s.}$	0.3413
Waist–hip ratio	1.01± 0.09	0.96 ± 0.07****	<0.0001

Plus–minus values are means ± SD. P-values were counted from Wilcoxon rank-sum tests.
M, male; F, female; HbA1c, hemoglobin A1c; BMI, body mass index; n.s., non-significant.
*****P < 0.0001, ***P < 0.001, **P < 0.01.*

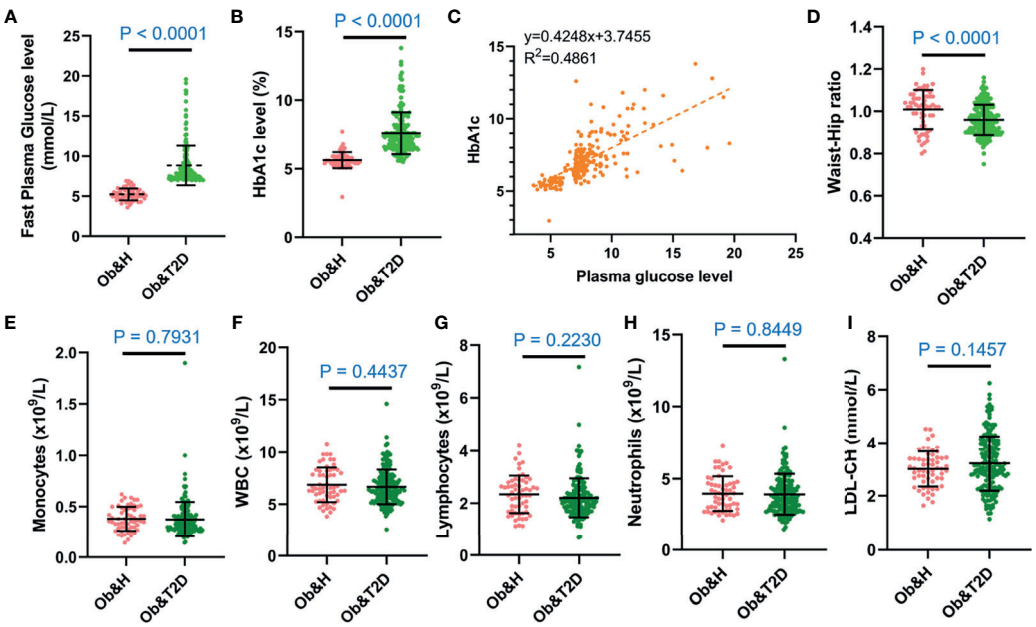


FIGURE 1 | The overall characteristics of the study participants. **(A, B)** Dot plots display the fasting plasma glucose **(A)** and HbA1c levels **(B)** of the study participants. **(C)** Correlation between capillary plasma glucose level and HbA1c level. **(D)** Waist-to-hip ratio of the study participants. **(E–I)** Inflammation-related indices of the study participants: monocytes **(E)**, white blood cell **(F)**, lymphocytes **(G)**, neutrophils **(H)**, and low-density lipoprotein **(I)**. Ob&H, obese and healthy; Ob&T2D, obese and type 2 diabetes. Data are shown as means ± SD. All *P*-values were from Wilcoxon rank-sum tests.

5.6 ± 0.6%) (**Table 1**; **Figure 1B**), in line with the positive correlation between HbA1c level with fasting blood glucose level ($R^2 = 0.46$) (**Figure 1C**). In addition to the expected alterations in blood glucose status, lower weight, height, body mass index, and waist circumference were observed in Ob&T2D, compared with the Ob&H group (**Table 1**; **Figure 1D**).

The other clinical characteristics and metabolic biomarkers in the two groups are summarized in **Table 1** and **Supplementary Table S1**. No significant differences in inflammation-related markers, including monocytes, white blood cells, lymphocytes, neutrophils, or low-density lipoprotein, were observed between the Ob&H and Ob&T2D groups (**Figures 1E–I**; **Table 1**).

Alterations in Gut Microbiota Composition Profiles in Ob&H and Ob&T2D Based on the 16S rRNA Gene Sequencing Data

Bacterial genomic DNA was isolated from the 243 stool samples for 16S rRNA (V3 and V4 regions) gene and metagenomic sequencing. The average number of raw paired reads per sample was 61,268 ± 16,703 reads for the 16S library of intestinal flora and 19,890,123 ± 2,854,537 reads from the metagenomic sequencing intestinal flora (**Supplementary Table S2**). Additionally, the rarefaction curves generated from the OTUs showed sufficient sequencing depth (**Supplementary Figure S1A**).

As shown in **Figure 2A**, 929 OTUs (87% of OTUs in Ob&T2D and 97% of the OTUs in Ob&H) were shared by both groups. Furthermore, 26 and 134 specific OTUs were observed in the Ob&H and Ob&T2D groups, respectively (**Figure 2A**). The 26 Ob&H group-specific OTUs were randomly distributed in the Ob&H participants, most of which were found only in one or two individuals. However, 31 of 134 (23%) Ob&T2D OTUs existed at least in five individuals, and eight (6%) of 134 were shared by at least 10 individuals (**Figures 2B, C**). Further taxonomy annotations of those eight OTUs revealed that they were from the genera *Ruminiclostridium*, *Pseudoalteromonas*, *Alloprevotella*, and *Bacteroides*, suggesting the preferential

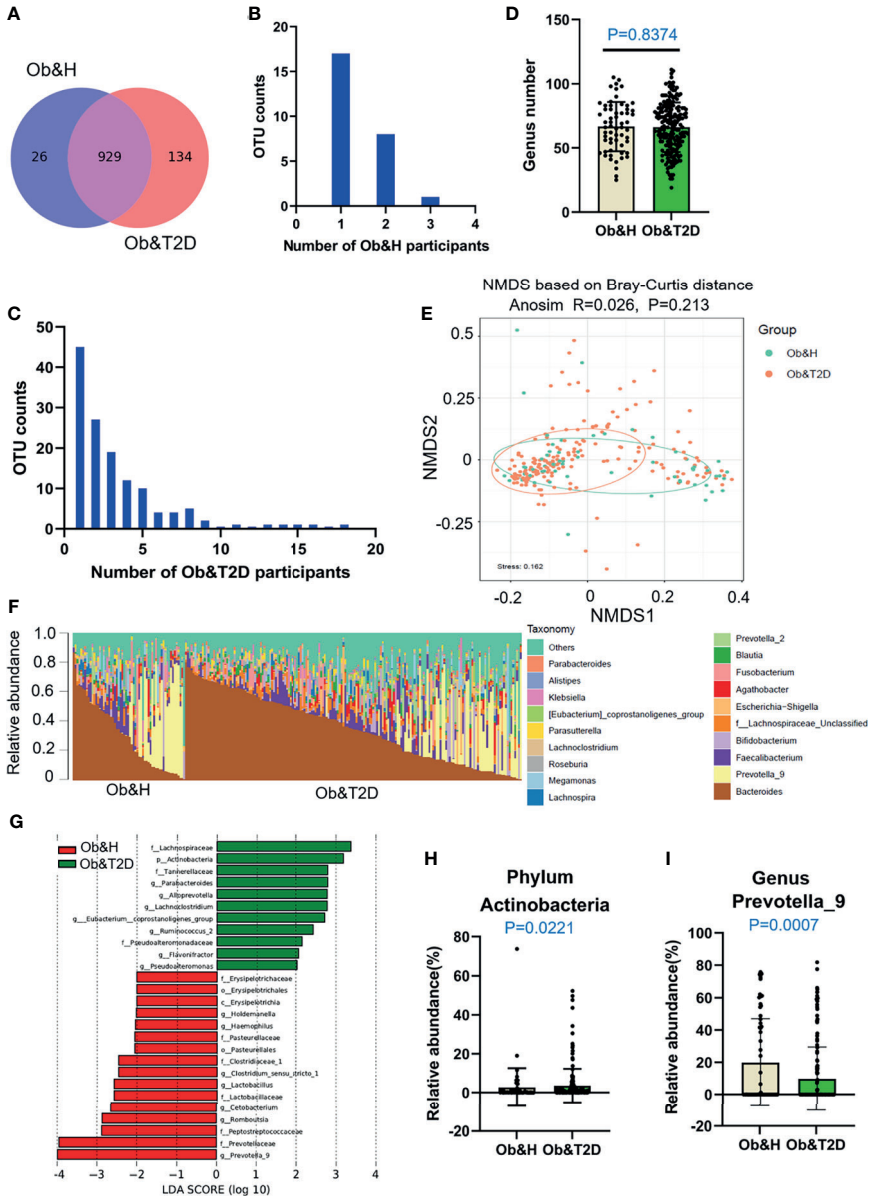


FIGURE 2 | Differential gut microbiota analysis of obese and healthy (Ob&H) and obese and type 2 diabetes (Ob&T2D) patients. **(A)** Venn diagram of the observed operational taxonomic units (OTUs) in Ob&H and Ob&T2D. **(B, C)** Frequency distributions of the 26 Ob&H- **(B)** and 134 Ob&T2D-specific OTUs **(C)** in the study participants. **(D)** Estimate of richness analysis between the two groups at the genus level. Data are shown as means ± SD. **(E)** Nonmetric multidimensional scaling analysis of the gut microbiota based on the Bray–Curtis distance for Ob&H and Ob&T2D, ANOSIM, $R = 0.026$, $P = 0.213$. **(F)** Genus-level taxonomic abundances for gut 16S rRNA gene sequencing data. The top 20 abundant genera are annotated in the panel legend. Samples are ordered according to decreasing relative abundance of Bacteroides in the Ob&H and Ob&T2D groups. **(G)** Linear discriminant analysis (LDA) scores for the gut bacterial taxa differentially abundant between Ob&H and Ob&T2D. Red bars with LDA score greater than 2 indicate that the taxa were enriched in Ob&H, and green bars with LDA score greater than 2 indicate that the taxa were enriched in Ob&T2D. **(H, I)** Relative abundance of phylum *Actinobacteria* **(H)** and genus *Prevotella_9* **(I)** in Ob&H and Ob&T2D. Data are shown as means ± SD. All *P*-values are from Wilcoxon rank-sum tests.

colonization of these kinds of gut microbiota in the Ob&T2D individuals. Nevertheless, species richness at the genus level was comparable between the Ob&H and Ob&T2D groups (P = 0.8374) (**Figure 2D**). Furthermore, the NMDS analysis (**Figure 2E**) and the PCoA (**Supplementary Figure S1B**) of the microbial communities based on the Bray–Curtis distances indicated similar gut microbiota communities from these two cohorts (ANOSIM, R = 0.026, P = 0.213). The Shannon index (**Supplementary Figure S2A**) and Chao1 (**Supplementary Figure S2B**), which account for the evenness and abundance of species, of the Ob&H group showed no significant changes compared with the Ob&T2D group. Furthermore, the phylogenetic diversity (PD) index, which measures the degree of evolutionary divergence, was also comparable between the two groups (**Supplementary Figure S2C**). This agrees with the previous finding that the alpha diversity was not significantly reduced between obese individuals with or without T2D (29). Since obesity is a strong risk factor for developing T2D (3) and the participants were newly diagnosed as T2D in our Ob&T2D cohort, they are expected to share similar microbiome features with regards to alpha diversity.

Although there is no significant difference in alpha diversity between the two groups, the bacterial community profiles at the genus level showed different patterns among individuals (**Figure 2F**). The LDA distribution diagram analysis (LDA score >2) showed a clear alteration in the microbiota characterized by higher *Actinobacteria* levels in Ob&T2D individuals (from 2.73 ± 9.88% in Ob&H to 3.58 ± 8.69% in Ob&T2D, Wilcoxon rank-sum test p-value = 0.0221) (**Figures 2G, H**). The genera *Parabacteroides*, *Alloprevotella*, *Lachnoclostridium*, *Eubacterium_coprostanoligenes*, *Ruminococcus 2*, *Flavonifractor*, and *Pseudoalteromonas* were markedly enriched (LDA score >2) in the Ob&T2D group, while the genera *Prevotella 9*, *Romboutsia*, *Cetobacterium*, *Lactobacillus*, *Clostrium_sensu_stricto*, *Haemophilus*, and *Holdemanella* were more abundant (LDA score >2) in the Ob&H group (**Figures 2G, I**), indicating that these kinds of differential genera might be involved in the gastrointestinal status transition from central obesity to central obese diabetes. The other abundant phyla, such as *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* (**Supplementary Figure S3**), and genera, such as *Bacteroides*, *Faecalibacterium*, and *Bifidobacterium* (**Supplementary Figure S4**), were comparable between the groups. The abovementioned analyses altogether suggested that the dysbiosis of gut microecology may contribute to the development of T2D from obesity.

Metagenomic Sequencing Revealed an Accumulation of Opportunistic Pathogens in Ob&T2D Patients

To investigate the potential effect of the gut microbiome on gastrointestinal symptoms in Ob&T2D patients and to address whether gut microbial changes at the genera level, species level, or both are associated with progressing from central obesity to T2D, metagenomic sequencing was applied to these fecal samples. As expected, the relative genus abundances from metagenomic sequencing were highly correlated with those from 16S rRNA gene sequencing (**Figure 3A**). The genus numbers were comparable between the two groups, consistent with the 16S rRNA gene sequencing data (**Figure 2B** and **Figure 3B**). The PCoA analysis based on the Bray–Curtis distance matrix revealed small differences in microbial composition between the Ob&H and Ob&T2D groups at the genus level (ANOSIM, R = 0.107, P = 0.001) (**Figure 3C**). Next, we compared the bacterial profile differences between the Ob&H and Ob&T2D groups. Consistent with 16S rRNA gene sequencing data at the phylum and genus levels, *Actinobacteria* was markedly enriched in the Ob&T2D group, and the genus *Prevotella* was more abundant in the Ob&H group (**Figure 3D**). A total of 22 species from various genera showed differential enrichment between the two groups. Seven species (for example, *Prevotella_copri*, *Fusobacterium_mortiferum*, and *Bacteroides_coprocola*) from five genera were enriched in the Ob&H group, and 15 species from 12 genera were enriched in the Ob&T2D group (**Table 2**). In contrast to the Ob&H group, many of the Ob&T2D-enriched species were opportunistic pathogens, such as *Eggerthella lenta*, *Clostridium hathewayi*, *Clostridium bolteae*, and *Parvimonas micros* (**Figure 3D**; **Table 2**), which have previously been reported to cause or be correlated with human abdominal infectious diseases (30, 31). Although the decreased abundance of butyrate producer microbiota has been reported to be associated with the development of T2D (9, 10), we did not find any significant difference in several representative butyrate producer microbiota (*Eubacterium rectale*, *Roseburia intestinalis*, *Roseburia inulinivorans*, *Eubacterium hallii*, and *Faecalibacterium prausnitzii*) abundance between the Ob&H and Ob&T2D groups (**Supplementary Figure S5**). This suggested that those butyrate producers may have already been depleted in centrally obese patients.

Interestingly, in our study, although the relative abundance of each microbiota varies greatly among individuals (**Figure 3E**), their functional compositions were consistent with each other, regardless of the Ob&H or Ob&T2D groups (**Figure 3F**), which was also observed in other study populations (29). The genes involved in replication, recombination and repair, carbohydrate transport and metabolism, cell wall/membrane/envelope biogenesis, inorganic ion transport and metabolism, and amino acid transport and metabolism represented the most abundant five eggNOG-annotated categories in the two groups (**Figure 3F**). This result implied that the genic pathway composition is shaped by gut microbiota communities rather than the microbiota population itself, which matters in the gastrointestinal ecosystem.

The Depletion of Gut Microbial Carbohydrate Metabolism Is Associated With the Development of T2D From Central Obesity

To further compare the functional bacteria gene alteration during the development of Ob&T2D from obesity, we analyzed gut microbial functions at the gene level across the two groups in our study cohort. A total of 2,850,117 genes were predicted in

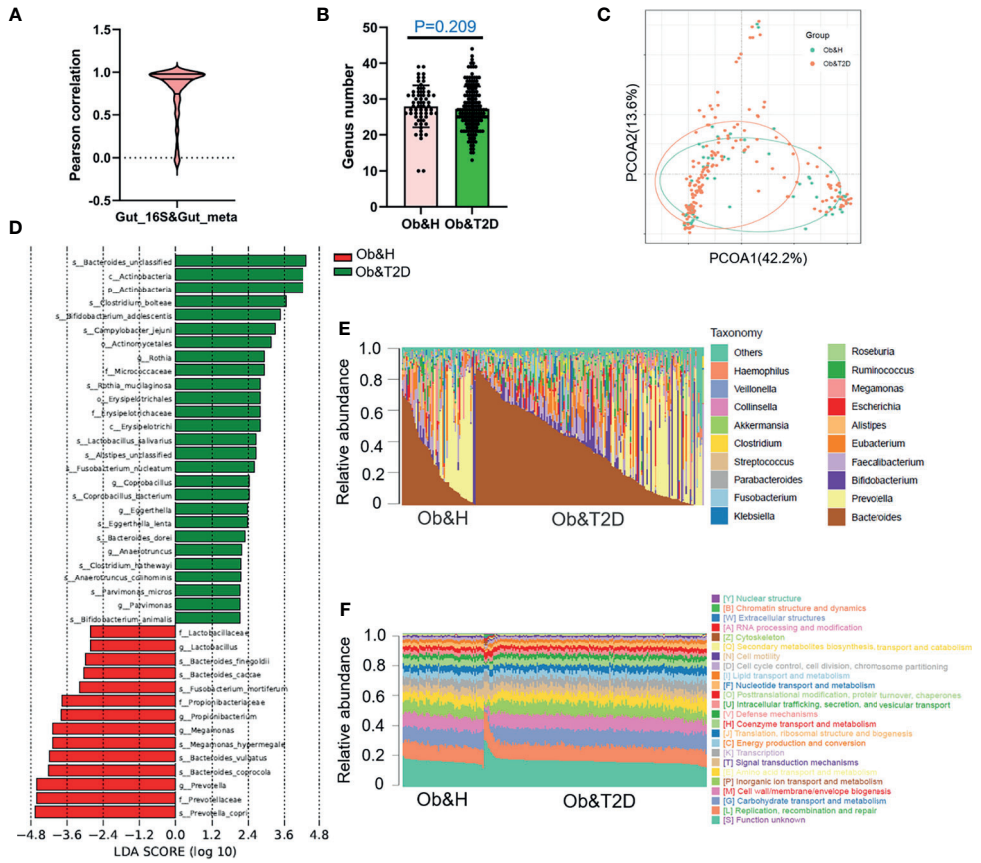


FIGURE 3 | Gut microbiota divergence analysis in obese and healthy (Ob&H) and obese and type 2 diabetes (Ob&T2D) based on metagenomic sequencing data. **(A)** Violin plot displaying Pearson correlation between gut 16S rRNA gene sequencing and metagenomic sequencing data. **(B)** Estimate of richness analysis between the two groups at the genus level. Data are shown as means ± SD. **(C)** Principal coordinate analysis (PCoA) of the microbiota based on the Bray–Curtis distance metrics for Ob&H and Ob&T2D. ANOSIM, $R = 0.107$, $P = 0.001$. **(D)** Genus-level taxonomic abundances for gut metagenomic sequencing data. The top 20 abundant genera are annotated in the panel legend. The samples are ordered according to decreasing relative abundance of *Bacteroides* in the Ob&H and Ob&T2D groups. **(E)** Predicted eggNOG orthologous functional annotations of gut microbial unique genes from metagenomic data. **(F)** The linear discriminant analysis (LDA) scores for the gut bacterial taxa are differentially abundant between Ob&H and Ob&T2D. Red bars with LDA score greater than 2 indicate that the taxa were enriched in Ob&H, and green bars with LDA score greater than 2 indicate that the taxa were enriched in Ob&T2D.

this study, with an average of 243,400 and 245,900 genes predicted in the Ob&H and Ob&T2D groups, respectively (**Figures 4A, B**). Notably, nearly one million unique genes were specifically detected in the Ob&T2D group, in line with previous findings that 134 OTUs were specifically detected in the Ob&T2D group (**Figure 2A**). Furthermore, we identified 3,744 differentially enriched (fold change ≥2, q-value <0.1) unique genes between two groups, the vast majority [3,002 (80.2%) of the total of 3,744 genes] of which were evidently depleted in Ob&T2D, and only a small subset [742 (19.8%) of the total of 3,744 genes] of the genes was significantly enriched in Ob&T2D (**Figure 4C** and **Supplementary Table S3**). Meanwhile, the depletion fold (average fold change = 22.6) was significantly higher than that of enrichment (average fold change = 2.8) (**Figure 4D**), resulting in an overall depletion of the gut micro-ecologic gene abundances during the development of T2D from obesity. Next, the KO enrichment analysis of 3,002 depleted and 742 enriched genes in Ob&T2D revealed that several sugar and amino acid metabolic pathways were significantly enriched (q-value <0.05) in the 3,002 depleted genes in the Ob&T2D subgroup (**Figure 4E** and **Supplementary Table S4**). Those depleted pathways included, for example, the metabolism of galactose, sphingolipid, glycan, starch and sucrose, amino sugar and nucleotide sugar, and cysteine and methionine (**Figure 4E** and **Supplementary Table S4**). However, no significantly enriched KO was identified in the 742 enriched genes in the Ob&T2D cohort (**Supplementary Table S4**). Our study together revealed a profound genic composition alteration during the transition from central obesity to T2D. Our results also suggested that the dysfunction in several sugar- and amino acid-related metabolism pathways (especially those of galactose, glycan, starch, and sucrose metabolism) in the gut microbial communities may be highly associated with this transition process.

TABLE 2 | List of Ob&T2D- and Ob&H-enriched species from metagenomic sequencing data.

Species	Enriched group	LDA	*P*-value
Ob&T2D-enriched			
s:Bacteroides_dorei	Ob&T2D	2.29	0.04
s:Clostridium_hathewayi	Ob&T2D	2.17	0.04
s:Rothia_mucilaginosa	Ob&T2D	2.78	0.02
s:Parvimonas_micros	Ob&T2D	2.13	0.03
s:Campylobacter_jejuni	Ob&T2D	3.31	0.04
s:Clostridium_bolteae	Ob&T2D	3.65	0.01
s:Coprobacillus_bacterium	Ob&T2D	2.42	0.01
s:Fusobacterium_nucleatum	Ob&T2D	2.61	0.03
s:Bifidobacterium_animalis	Ob&T2D	2.12	0.01
s:Bifidobacterium_adolescentis	Ob&T2D	3.46	0.01
s:Anaerotruncus_colihominis	Ob&T2D	2.15	0.01
s:Eggerthella_lenta	Ob&T2D	2.38	0.01
s:Lactobacillus_salivarius	Ob&T2D	2.66	0.02
s:Alistipes_unclassified	Ob&T2D	2.66	0.04
s:Bacteroides_unclassified	Ob&T2D	4.36	0.00
Ob&H-enriched			
s:Prevotella_copri	Ob&H	4.66	0.00
s:Bacteroides_finegoldii	Ob&H	2.98	0.04
s:Megamonas_hypermegale	Ob&H	4.08	0.01
s:Bacteroides_coprocola	Ob&H	4.20	0.00
s:Bacteroides_vulgatus	Ob&H	4.18	0.02
s:Fusobacterium_mortiferum	Ob&H	3.17	0.02
s:Bacteroides_caccae	Ob&H	3.03	0.04

Gut Microbial Gene Profiles-Based Screening of Central Obese Individuals With a High Risk of Developing T2D

Next, we employed the gut microbiota gene profile-based method to identify the potential diagnostic biomarkers for T2D status in obese populations. Firstly, we performed PCoA analysis based on the abundances of the 96 depleted genes involved in significantly enriched KEGG pathways (galactose metabolism, sphingolipid metabolism, glycan degradation, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, and cysteine and methionine metabolism) (**Figures 4E, F**; **Supplementary Table S5**). The results showed that the two groups were indistinguishable based on the 96 depleted gene abundances as mentioned above (**Figure 4G**), and the differences within groups were larger than that of between groups (ANOSIM, $R = -0.0744$, $P = 0.999$) (**Figure 4H**). This may be explained because the abundance differences of these 96 depleted genes between Ob&H and Ob&T2D were not large enough to set them apart. To further test this hypothesis, we conducted a series of PCoA, NMDS, and ANOSIM analyses based on the abundance of the top *N*-ranked ($N = 100$, 200, 300, 400, 500, and 742 for enriched genes and $N = 100$, 200, 300, 400, 500, and 1,000 for depleted genes, ranked by the changing folds of gene abundance between the Ob&T2D and Ob&H groups) enriched or depleted genes in the Ob&T2D subgroup. As expected, the PCoA results based on the abundance of the top *N*-ranked enriched genes in Ob&T2D, whose changing folds are much smaller than those of depleted genes (**Figure 4D**), showed no distinguishing effects between the two groups (**Supplementary Figure S6**). Interestingly, Ob&T2D and Ob&H can be obviously separated into two different clusters based on the abundance of the top 300 depleted genes in the Ob&T2D subgroup (ANOSIM $R = 0.6298$, $P = 0.001$) (**Figures 5A, B**). A similar separation was achieved when we performed the same analyses based on the abundance of the top 100, 200, 400, and 500 genes, but not the top 1,000 depleted genes, and the maximum differences between groups were achieved when using the top 300 depleted genes for ANOSIM analysis (**Figure 5C**; **Supplementary Figure S7**), supporting the hypothesis that the top depleted gene sets are better biomarkers than the enriched ones in distinguishing Ob&H and Ob&T2D. We then divided all the samples into four stages, stage I to stage IV, with decreasing abundances of these 300 depleted genes (**Figure 5D**). Interestingly, we found that their fasting plasma glucose level negatively correlated, and the percentage of people with impaired fasting glucose (between 6.1 and 7.0 mmol/L) was positively correlated with the abundance of those 300 depleted genes (**Figures 5E, F**). These results supported that the gut microbiota gene profiles can be used as potential diagnostic biomarkers to predict the central obese individuals with high risks of developing T2D.

We then performed KEGG enrichment analysis by using these top 300 depleted genes in the Ob&T2D group. Similarly, genes that were involved in glycan degradation, sphingolipid metabolism, and galactose metabolism were also enriched as observed in the 3,002 depleted genes (**Supplementary Table S6**). In addition, numerous other pathways, including nucleotide excision repair and glycosaminoglycan degradation, were also enriched (q-value <0.05) (**Supplementary Table S6**). A further unique gene annotation revealed that 60% (180 of 300) of the most significantly depleted genes are from genus *Prevotella*, in line with the depletion of genus *Prevotella* in the Ob&T2D group (**Figures 2G**, **3D**). Several species, such as *Prevotella* sp. *CAG:520* (42 of 300), *Prevotella stercorea* (37 of 300), and *Prevotella pectinovora* (nine of 300), were also enriched (**Supplementary Table S3**). Therefore, the disturbance of those

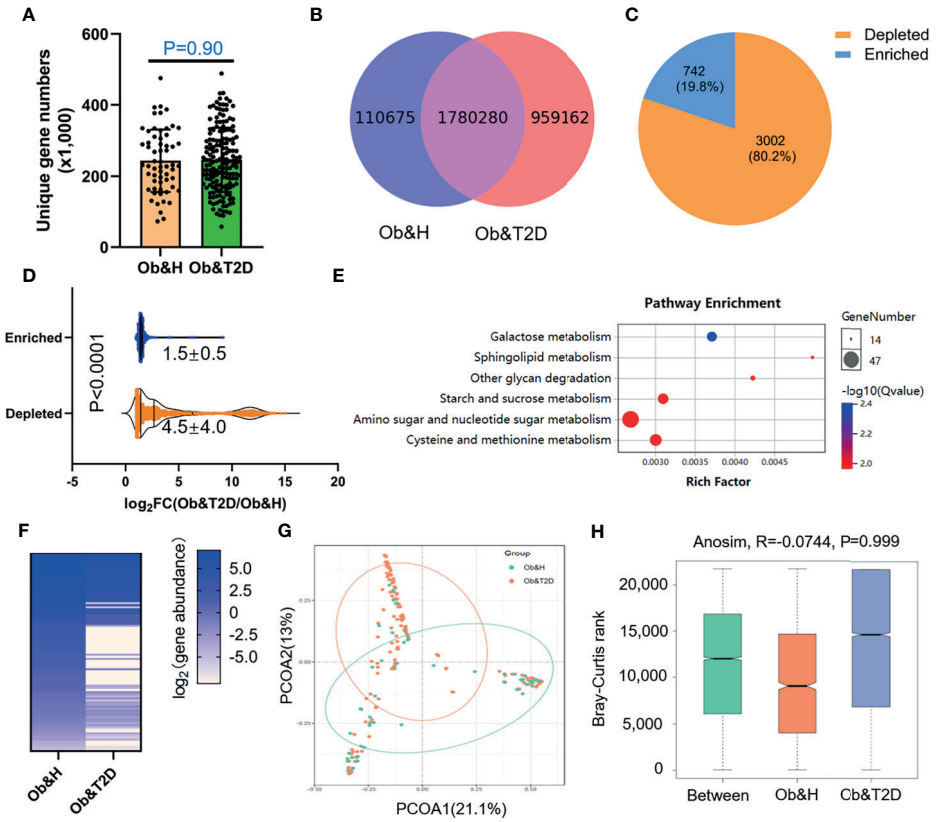


FIGURE 4 | Differential gut microbial gene functions annotation on Kyoto Encyclopedia of Genes and Genomes (KEGG) in obese and healthy (Ob&H) and obese and type 2 diabetes (Ob&T2D). **(A)** Box plots show the number of observed gut microbial unique genes in Ob&H and Ob&T2D. Data are shown as means ± SD. The *P*-value is from Wilcoxon rank-sum tests. **(B)** Venn diagrams demonstrate the number of unique genes shared between Ob&H and Ob&T2D. **(C)** The pie chart displays the number of enriched or depleted genes (filtering criteria: *q*-value <0.1, at least twofold change) in Ob&T2D compared with Ob&H. **(D)** Violin plots display the changing fold of enriched or depleted genes. Data are shown as means ± SD. The *P*-value is from Wilcoxon rank-sum tests. **(E)** Representative gene ontology enrichment (*q*-value <0.05) analysis of 3,002 depleted genes in Ob&T2D. **(F)** Heat map depicting the $\log_2$ (gene abundance) of 96 representative genes involved in the enriched KEGG pathways in **(E)**. **(G)** Principal coordinate analysis based on the Bray–Curtis distance of 96 depleted gene abundance between Ob&H and Ob&T2D. **(H)** Comparison of the enriched KEGG orthologous between Ob&H and Ob&T2D. ANOSIM, $R = -0.0744$, $P = 0.999$.

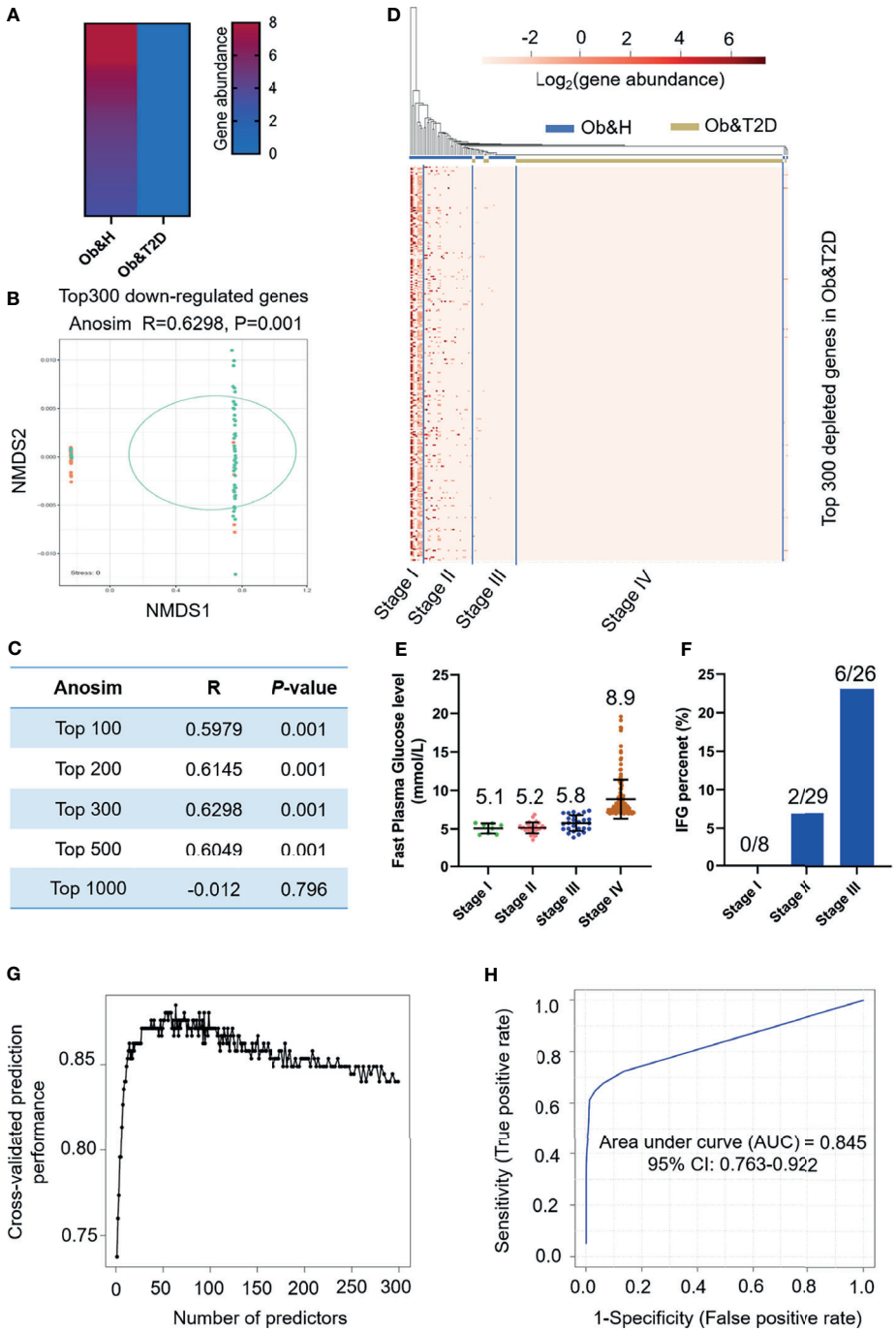
pathways in the representative species may collectively play a role in the progression from central obesity to T2D.

To further narrow the potential diagnostic biomarkers to predict Ob&T2D status, we developed a random forest classifier model based on the abundance profiles of the top 300 depleted genes. The optimal model utilized 63 marker genes which provided the best discriminatory power (**Figure 5G**). These genes in the optimal model were mostly from genus *Bacteroides* (15 of 63) and *Prevotella* (13 of 63) (**Supplementary Table S7**). The ROC curve was obtained based on the 63 optimal markers, achieving an AUC value of 0.845 with 95% CI of 0.763–0.922 (**Figure 5H**). Therefore, it is concluded that the prediction model showed a high discriminatory power to predict Ob&T2D status.

DISCUSSIONS

This study examined the gut microbial profiles of central obese individuals with or without T2D to dissect the potential gut microbial features associated with progressing from central obesity to T2D. Here we have found an alteration in the gut microbiota, especially at the gene functional level, in individuals with diabetes-treatment-naïve obese T2D compared with obese individuals. Furthermore, we identified a group of genes from the gut microbiota community involved in carbohydrate-related metabolism that can be used as potential diagnostic biomarkers to predict T2D status among the obese population.

As expected, our results showed that the microbial profiles were quite similar between the central obese individuals with or without T2D in alpha (Shannon index and Chao1) and beta diversity indices (PD), which is in line with the previous finding (29). Our further observation on the colonization of opportunistic pathogens, such as *Eggerthilla lenta*, *Clostridium hathewayi*, and *Clostridium bolteae*, in the gut of T2D is also in good agreement with previous studies (12). We extended those previous findings by showing that it is independent of central obesity or medical treatment, two major confounding factors



Anosim	R	*P*-value
Top 100	0.5979	0.001
Top 200	0.6145	0.001
Top 300	0.6298	0.001
Top 500	0.6049	0.001
Top 1000	-0.012	0.796

FIGURE 5 | Distinguishing obese and type 2 diabetes (Ob&T2D) from obese and healthy (Ob&H) based on differentially enriched gut microbial genes. **(A)** Heat map depicting the gene abundance of the top 300 [rank by $\log_2$ (fold change)] Ob&T2D-depleted genes in both groups. **(B)** Nonmetric multidimensional scaling based on the Bray–Curtis distance of the top 300 depleted gene abundance in Ob&T2D. ANOSIM, $R = 0.6298$, $P = 0.001$. **(C)** Listing of ANOSIM analysis R, *P*, and stress values based on the abundance of top *N* ($N = 100$, 200, 300, 500, and 1,000) depleted genes in Ob&T2D. **(D)** Heat map displaying the gene abundance of the top 300 [rank by $\log_2$ (fold change)] Ob&T2D-depleted genes in each participant. The samples were grouped into four stages (stages I–IV) according to their gene abundance. **(E)** Dot plots presenting the fasting plasma glucose levels in the individuals of each stage. The number of each stage is as follows: stage I ($n = 8$), stage II ($n = 29$), stage III ($n = 26$), and stage IV ($n = 159$). **(F)** Percentage of people with impaired fasting plasma glucose levels (6.1 mmol/L<fasting plasma glucose level<7 mmol/L). **(G)** Classification performance of a random forest model using the abundance of the top 300 depleted genes as assessed by R random forest package. **(H)** Receiver operating characteristic curve displaying the classification for Ob&H and Ob&T2D using gene abundance profiles. AUC, area under curve.

affecting the gut microbiota (11, 16). Although several other kinds of opportunistic pathogens, such as *Escherichia coli*, were also reported to accumulate in the gut, inducing obesity and insulin resistance (12), our analyses revealed that the opportunistic pathogens colonized in the gut of Ob&T2D vary in each participant, and the types of colonized species are also different from previously reported species (10, 12, 32, 33). This may be due to the particular types of pathogens that our volunteers encountered. However, it is conceivable that the dysbiotic gut microbiome may causatively contribute to T2D development. The mechanism underlying the opportunistic pathogen colonization in the gut environment upon onset of T2D from central obesity remains unclear. Thus, further studies are needed to dissect its pathophysiology.

Our results also revealed profound genic alterations between the two groups. Thousands of genes involved in the metabolism of galactose, sphingolipid, glycan, starch, and sucrose were depleted in the central obese individuals with T2D. This finding suggests that the capabilities of sugar metabolism in gut microbiota are greatly impaired in central obese T2D individuals. It has been widely reported that short-chain fatty acids (SCFAs), which are the primary metabolites produced by gut bacterial fermentation of dietary fiber in the gastrointestinal tract, play an essential role in regulating intestinal homeostasis, and their anti-inflammatory activities have also been well documented (34, 35). Different kinds of carbohydrates are sources for SCFA biogenesis (36); therefore, we proposed that gut microbial sugar metabolism dysfunction may lead to a shortage of SCFA production, resulting in low-grade inflammation in the gut microenvironment. Low sugar usage efficiency by gut bacteria, in turn, also strengthens the burden of the human host to absorb and digest the remaining sugar, and accumulating evidence has demonstrated that high-sugar diets promote the development of T2D by inducing weight gain and insulin resistance (37, 38). In this scenario, our study provided a potential prebiotics development or transplantation strategy to prevent and/or delay T2D development from central obesity.

Furthermore, we identified hundreds of genes whose abundance is correlated with glycemic level (**Figure 5** and **Supplementary Figure S8**). Thus, these can be used as potential gene markers to predict central obese individuals with a high risk of developing T2D. Several of these genes are enriched in the metabolism of different carbohydrates and nucleotide excision repair. Nevertheless, the biological functions and their potential roles in T2D development of other genes remain to be elucidated. These differentially enriched marker genes can also be the potential targets for probiotic bacterial engineering to prevent or manage T2D.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the China National GeneBank Sequence Archieve (CNSA)(https://db.cngb.org/cnsa/), accession number CNP0002048.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

HW, YH, RJ, MH, WC, and YJ conceived the project and designed the experiments. RJ, LQ, MH, XY, QL, and HY collected the experimental samples. RJ, MH, YH, and HW performed the 16S rRNA gene sequencing and metagenomic sequencing and bioinformatic data analysis with input from LY, YC, XW, WC, and YJ. RJ, MH, HW, and YH wrote the manuscript with input from all authors. 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.2021.747646/full#supplementary-material.

Supplementary Figure 1 | Gut microbiota analysis in Ob&H and Ob&T2D according to the 16S rRNA gene sequencing data. **(A)** Rarefaction curves of gut 16S rRNA gene sequencing reads. **(B)** Principal coordinate analysis (PCoA) of the gut microbiota based on the Bray-Curtis distance for Ob&H and Ob&T2D.

Supplementary Figure 2 | Alpha and beta diversity indices of gut microbiota genus for Ob&H and Ob&T2D. Estimate of Shannon index **(A)**, Chao1 **(B)** and phylogenetic diversity index **(C)**. Data are shown as means ± SD. All P values are from Wilcoxon rank-sum tests. Ob&H: n = 60, Ob&T2D: n = 183.

Supplementary Figure 3 | Relative abundance of indicated gut microbial phylum in Ob&H and Ob&T2D. **(A-C)**, Relative abundance of phylum Bacteroidetes **(A)**, Firmicutes **(B)** and Proteobacteria **(C)** in Ob&H and Ob&T2D gut microbiota. Data are shown as means ± SD. All P-values are from Wilcoxon rank-sum tests.

Supplementary Figure 4 | Relative abundance of indicated gut microbial genera in Ob&H and Ob&T2D. **(A-C)**, Relative abundance of genus Bacteroides **(A)**, Faecalibacterium **(B)** and Bifidobacterium **(C)** in Ob&H and Ob&T2D gut microbiota. Data are shown as means ± SD. All P-values are from Wilcoxon rank-sum tests.

Supplementary Figure 5 | Relative abundance of representative butyrate producer microbiota in Ob&H and Ob&T2D. **(A–E)** Relative abundance of specie Eubacterium rectale **(A)**, Eubacterium hallii **(B)**, Faecalibacterium prausnitzii **(C)**, Roseburia intestinalis **(D)** and Roseburia inulinivorans **(E)** in Ob&H and Ob&T2D. All P values are from Wilcoxon rank- sum tests.

Supplementary Figure 6 | NMDS analysis based on the Bray-Curtis distance of top-ranked depleted gene abundance in Ob&T2D. **(A–D)**, NMDS analysis based on the Bray-Curtis distance of top N (N = 100, 200, 500 and 1000) depleted genes abundance in Ob&T2D. Anosim R and P values are shown as indicated.

Supplementary Figure 7 | NMDS analysis based on the Bray-Curtis distance of top-ranked enriched gene abundance in Ob&T2D. **(A–D)**, NMDS analysis based on the Bray-Curtis distance of top N (N = 100, 200, 300 and 500) enriched genes abundance in Ob&T2D. Anosim R and P values are shown as indicated.

Supplementary Figure 8 | Redundancy analysis(RDA) illustrating the relationship between clinal characteristics and microbial gene community. RDA analysis based on the abundance of top 300 depleted genes in Ob&T2D illustrating the relationship between clinical indices and microbial gene community. Glu: glucose level; HbA1c: Hemoglobin A1c; Mono: monocyte; ANC, neutrophils; WBC, white blood cell; LYMPH, ymphocyte.

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Preference of Women for Gestational Diabetes Screening Method According to Tolerance of Tests and Population Characteristics

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Aims: To determine the preferred method of screening for gestational diabetes mellitus (GDM).

Methods: 1804 women from a prospective study (NCT02036619) received a glucose challenge test (GCT) and 75g oral glucose tolerance test (OGTT) between 24-28 weeks. Tolerance of screening tests and preference for screening strategy (two-step screening strategy with GCT compared to one-step screening strategy with OGTT) were evaluated by a self-designed questionnaire at the time of the GCT and OGTT.

Results: Compared to women who preferred one-step screening [26.2% (472)], women who preferred two-step screening [46.3% (834)] were less often from a minor ethnic background [6.0% (50) *vs*. 10.7% (50), p=0.003], had less often a previous history of GDM [7.3% (29) *vs*. 13.8% (32), p=0.008], were less often overweight or obese [respectively 23.1% (50) *vs*. 24.8% (116), p<0.001 and 7.9% (66) *vs*. 18.2% (85), p<0.001], were less insulin resistant in early pregnancy (HOMA-IR 8.9 (6.4-12.3) *vs*. 9.9 (7.2-14.2), p<0.001], and pregnancy outcomes were similar except for fewer labor inductions and emergency cesarean sections [respectively 26.6% (198) *vs*. 32.5% (137), p=0.031 and 8.2% (68) *vs*. 13.0% (61), p=0.005]. Women who preferred two-step screening had more often complaints of the OGTT compared to women who preferred one-step screening [50.4% (420) *vs*. 40.3% (190), p<0.001].

Conclusions: A two-step GDM screening involving a GCT and subsequent OGTT is the preferred GDM screening strategy. Women with a more adverse metabolic profile preferred one-step screening with OGTT while women preferring two-step screening had a better metabolic profile and more discomfort of the OGTT. The preference for the GDM screening method is in line with the recommended Flemish modified two-step screening method, in which women at higher risk for GDM are recommended a one-step screening strategy with an OGTT, while women without these risk factors, are offered a two-step screening strategy with GCT.

Clinical Trial Registration: NCT02036619 https://clinicaltrials.gov/ct2/show/NCT02036619

Keywords: gestational diabetes mellitus, preference for screening method, tolerance, glucose challenge test, two-step screening, one-step screening, oral glucose tolerance test

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as diabetes diagnosed in the second or third trimester of pregnancy, given that overt diabetes early in pregnancy has been excluded (1). Adverse pregnancy outcomes, such as large-for-gestational age (LGA) infants, preeclampsia and cesarean sections, can be reduced by treatment of GDM between 24-28 weeks of pregnancy (2, 3). Women with a history of GDM have a seven-fold increased risk of developing type 2 diabetes mellitus (T2DM) compared to normal glucose tolerant women (NGT). These women also have a higher risk of developing a metabolic syndrome and cardiovascular events later in life compared to NGT women (4–7).

A universal one-step approach for GDM screening is recommended by the 'International Association of Diabetes and Pregnancy Study Groups' (IADPSG) with the 75g oral glucose tolerance test (OGTT) between 24-28 weeks of pregnancy (8). The World Health Organization (WHO) adopted these recommendations in 2013, whereby the IADPSG guidelines are now commonly referred to as the 2013 WHO criteria (9). However, using this one-step approach leads to an increased workload and possible unnecessary medicalization of care. Therefore, many European countries still use selective screening for GDM based on risk factors or recommend a two-step screening strategy with a glucose challenge test (GCT) (10). In addition, evidence is lacking that treatment of GDM based on the IADPSG screening strategy improves pregnancy outcomes compared to other screening strategies (11, 12). Moreover, a GCT is generally better tolerated than an OGTT and has the advantage that it can be performed in a non-fasting state (12). A modified two-step screening strategy with GCT combined with risk-factors was proposed based on the BEDIP-N study in Flanders. This allows women at higher risk for GDM (women with a history of GDM, obesity and/or impaired fasting glycaemia in early pregnancy) to receive a one-step screening strategy with an OGTT, while women without these risk factors, are offered a two-step screening strategy with GCT (13). However, data are lacking on which GDM screening method and which screening test pregnant women prefer. We aimed therefore to determine which GDM screening method (a two-step screening strategy with a GCT or a one-step screening approach with a 75g OGTT) participants preferred. In addition, we specifically aimed to determine the preference of GDM screening method according to the tolerance for the different screening tests and in relation to the population characteristics.

SUBJECTS AND METHODS

Study Design and Setting

This article is a sub-analysis of the BEDIP-N cohort (NCT02036619). The BEDIP-N study was a multicentric prospective cohort study that has previously been described in detail (10, 14–17). The BEDIP-N study was approved by the Institutional Review Boards of all participating centers and all investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008. Before inclusion to the study, participants provided informed consent. Participants were enrolled between 6 and 14 weeks of pregnancy, when fasting plasma glucose (FPG) was measured. Women with impaired fasting glucose or diabetes in early pregnancy according to the American Diabetes Association (ADA) criteria, were excluded. Women without (pre)diabetes received universal screening for GDM between 24-28 weeks of pregnancy with both a non-fasting 50g GCT and a 75g 2-hour OGTT. Results of the GCT were blinded for participants and health care provides, so all participants received an OGTT irrespective of the GCT result. The diagnosis of GDM was based on the IADPSG/2013 WHO criteria (9, 14, 15). For treatment of GDM, the ADA-recommended glycemic targets were used (9). If targets were not reached within two weeks after the start of lifestyle measures, insulin was started. Women with GDM received an invitation for a postpartum 75g OGTT 6 to 16 weeks after delivery. The ADA criteria were used to define diabetes and glucose intolerance [impaired fasting glycemia (IFG) and/or impaired glucose tolerance (IGT)] (9, 14).

Study Visits and Measurements

Baseline characteristics and obstetrical history were collected at first visit (14). At first visit and at the time of the OGTT, anthropometric measurements were obtained and several self-administered questionnaires were completed (14). The tolerance for the GCT and OGTT was evaluated by a self-designed

questionnaire evaluating any discomfort or complaint with the test such as bad taste, nausea, vomiting, dizziness or abdominal pain. In addition, at the time of the OGTT the questionnaire also evaluated whether women considered it cumbersome to have to be fasting for the test, which screening test they would prefer (non-fasting GCT or the fasting OGTT) and whether they preferred a two-step screening strategy with GCT or a one-step screening approach with 75g OGTT. The CES-D questionnaire to evaluate symptoms of depression was completed at time of the OGTT (before the diagnosis of GDM was communicated), and at the postpartum OGTT for women with GDM (with a score ≥16 being suggestive for clinical depression (18). At first visit and at the time of the OGTT, a food questionnaire was used to question servings per week of different important food categories and beverages (19). Less healthy consumption was assigned 0 or -1 points. By summing up the points for all 14 food groups, the diet score could range from -12 to 15. At the time of the OGTT, the International Physical Activity Questionnaire (IPAQ) questionnaire (validated for the Belgian population) assessed physical activity (14, 20). Results of the IPAQ were reported in categories (low, moderate or high activity levels) as previously reported (21). In early postpartum, participants completed the SF-36 health survey (22)

Blood pressure (BP) was measured twice, with 5 minutes between each measurement using an automatic BP monitor. A BMI ≥ 25 kg/m² was defined as overweight, a BMI ≥ 30 kg/m² was defined as obese based on the BMI at first prenatal visit. During the first perinatal visit, a fasting blood test was taken to measure FPG, insulin, lipid profile (total cholesterol, HDL and LDL cholesterol, triglycerides) and HbA1c. The homeostasis model assessment of insulin resistance (HOMA-IR) and beta-cell function (HOMA-B) was measured in early pregnancy (23). During the OGTT, glucose and insulin were measured fasting, at 30min, 60min and 120min. The results of glucose and insulin levels during the OGTT were used to calculate the Matsuda index, which is a measure of whole body insulin sensitivity (24). Furthermore, a fasting lipid profile, HbA1c and different indices of beta-cell function [HOMA-B, the insulinogenic index divided by HOMA-IR and the insulin secretion-sensitivity index-2 (ISSI-2)] were also measured at time of the OGTT (14, 23, 25–27).

Pregnancy and Delivery Outcome Data

The following pregnancy outcome data were collected: gestational age, preeclampsia (*de novo* BP ≥140/90mmHg > 20 weeks with proteinuria or signs of end-organ dysfunction), gestational hypertension (*de novo* BP ≥140/90mmHg > 20 weeks), type of labor and type of delivery, birth weight, macrosomia (>4 kg), birth weight ≥4.5 kg, LGA defined as birth weight >90 percentile according to standardized Flemish birth charts adjusted for sex of the baby and parity (28), small-for-gestational age (SGA) defined as birth weight <10 percentile according to standardized Flemish birth charts adjusted for sex of the baby and parity (28), preterm delivery (<37 completed weeks), 10min Apgar score, shoulder dystocia, neonatal respiratory distress syndrome, neonatal jaundice, congenital anomalies and admission on the neonatal intensive care unit (NICU) (14). Irrespective of the need for intravenous administration of glucose and admission on the NICU, a glycemic value <2.2mmol/l was considered as a neonatal hypoglycemia across all centers. Admission to the NICU was decided by the neonatologist in line with normal routine in each center. The difference in weight between first prenatal visit and the time of the OGTT was calculated as early weight gain. The total gestational weight gain was calculated as the difference in weight between first prenatal visit and the delivery. Excessive total gestational weight gain was defined according to the 2009 Institute of Medicine (IOM) guidelines (29).

Statistical Analysis

Descriptive statistics were presented as frequencies and percentages for categorical variables and means with standard deviations or medians with interquartile range for continuous variables. Categorical variables were analyzed using the Chi-square test or the Fisher exact test in case of low (<5) cell frequencies, whereas continuous variables were analyzed using the Kruskal-Wallis test for not normally distributed variables or One-way ANOVA test for normally distributed variables. A p-value <0.05 was considered significant. Analyzes were performed by statistician A. Laenen by using SAS software.

RESULTS

Preference for the GDM Screening Method

1803 women received both a GCT and an OGTT in the BEDIP-N study. Of all women, 46.3% (834) preferred two-step screening with a GCT, 26.2% (472) preferred a one-step screening strategy with an OGTT and 27.6% (497) had no clear preference. The most preferred screening test was a GCT, (by 54.8% (989) of all participants), while only 6.2% (112) preferred an OGTT and 39% (703) had no clear preference.

Tolerance of Screening Tests

Women who preferred a two-step screening strategy tolerated the GCT in general significantly better than the OGTT compared to women who preferred a one-step screening approach and compared to women without clear preference (**Table 1**). In addition, women who preferred a two-step screening indicated that it was more cumbersome to be fasting for the OGTT compared to women who preferred a one-step screening strategy or had no clear preference and they reported more complaints of the OGTT (**Table 1**). The most common complaint during an OGTT was nausea (in each group more than half of all women reported nausea). There were no significant differences in the type of complaints for the OGTT between both groups, except that more women who preferred one-step screening reported abdominal pain compared to women who preferred two-step screening [8.4% (16) *vs.* 3.1% (13), p=0.004] (**Table 1**).

Women who preferred a GCT test had less complaints of the GCT compared to women with a preference for the OGTT [18.6% (180) *vs.* 33.9% (37), p<0.001]. Significantly more women

TABLE 1 | Comparison of tolerance for screening tests between women who prefer two-step screening compared to women who prefer one-step screening with OGTT or without clear preference.

	Preference two-step screening	Preference one-step OGTT	No preference	Pairwise comparisons		
	N = 834 (46.3%)	N = 472 (26.2%)	N = 497 (27.6%)	1 *vs*2	1 *vs* 3	2 *vs* 3
% Any discomfort of GCT:				**0.003**	0.290	0.086
No	82.0 (667)	75.0 (342)	79.7 (388)			
Yes	18.0 (146)	25.0 (114)	20.3 (99)			
% Bad taste				0.621	0.494	0.844
No	74.7 (109)	71.9 (82)	70.7 (70)			
Yes	25.3 (37)	28.1 (32)	29.3 (29)			
% Nausea				0.626	0.168	0.080
No	54.8 (80)	51.7 (59)	63.6 (63)			
Yes	45.2 (66)	48.2 (55)	36.4 (36)			
% Dizziness or feeling faint				0.916	0.870	0.802
No	61.6 (90)	62.3 (71)	60.6 (60)			
Yes	38.4 (56)	37.7 (43)	39.4 (39)			
% Vomiting				0.278	0.721	0.480
No	97.3 (142)	99.1 (113)	98.0 (97)			
Yes	2.7 (4)	0.9 (1)	2.0 (2)			
% Abdominal pain GCT				0.759	0.628	0.861
No	97.9 (143)	97.4 (111)	97.0 (96)			
Yes	2.0 (3)	2.6 (3)	3.0 (3)			
% Any discomfort of OGTT:				**<.001**	**<.001**	0.084
No	49.6 (413)	59.7 (281)	65.0 (322)			
Yes	50.4 (420)	40.3 (190)	34.9 (173)			
% Bad taste				0.196	0.121	**0.016**
No	70.0 (294)	64.7 (123)	76.3 (132)			
Yes	30.0 (126)	35.3 (67)	23.7 (41)			
% Nausea				0.746	0.301	0.536
No	43.3 (182)	44.7 (85)	48.0 (83)			
Yes	56.7 (238)	55.3 (105)	52.0 (90)			
% Dizziness or feeling faint				0.260	0.463	0.759
No	49.3 (207)	54.2 (103)	52.6 (91)			
Yes	50.7 (213)	45.8 (87)	47.4 (82)			
% Vomiting				0.802	0.855	0.960
No	95.7 (402)	95.3 (181)	95.4 (165)			
Yes	4.3 (18)	4.7 (9)	4.6 (8)			
% Abdominal pain				**0.004**	0.067	0.454
No	96.9 (407)	91.6 (174)	93.6 (162)			
Yes	3.1 (13)	8.4 (16)	6.4 (11)			
% Cumbersome to be fasting				**<.001**	**<.001**	**<.001**
No	43.0 (358)	64.3 (303)	78.4 (388)			
Yes	57.0 (474)	35.7 (168)	21.6 (107)			

OGTT, oral glucose tolerance test; GCT, glucose challenge test; Categorical variables are presented as frequencies %(n); Differences are considered significant at p-value < 0.05. Bold value means that this is significant, meaning that the p-value < 0.05.

who preferred a GCT found it cumbersome to be fasting for the OGTT compared to women who preferred an OGTT or had no clear preference [respectively 58.7% (579) *vs*. 34.2% (38), p<0.001 and 58.7% (579) *vs*. 18.7% (131), p<0.001] (**Table 2**).

Characteristics of Women According to the Preference of GDM Screening Method

Compared to women who preferred one-step screening, women who preferred two-step screening, had less often a minor ethnic background, had less often a low income, had less often a first degree family history of GDM or a previous history of GDM [7.3% (29) *vs*. 13.8% (32), p=0.008], had a lower BMI [23.9 ± 4.0 *vs*. 25.4 ± 5.3, p<0.001), were less often overweight or obese [respectively 23.1% (50) *vs*. 24.8% (116), p<0.001 and 7.9% (66) *vs*. 18.2% (85), p<0.001], and were less insulin resistant in early pregnancy (HOMA-IR 8.9 (6.4-12.3) *vs*. 9.9 (7.2-14.2), p<0.001] (**Table 3**). There was no difference in the multiparity rate between both groups.

Characteristics of Women According to the Preference of Screening Test

Compared to women who preferred an OGTT, women who preferred a GCT had less often a minor ethnic background, had less often a previous history of GDM, had a lower BMI [24.1 ± 4.2 *vs*. 25.7 ± 6.1, p=0.023], and were less often overweight or obese [respectively 22.3% (219) *vs*. 25.0% (28), p=0.005 and 10.1% (99) *vs*. 19.6% (22), p=0.005]. At 24-28 weeks of pregnancy, significantly more women needed treatment with insulin for GDM in the group who preferred an OGTT compared to the group who preferred a GCT [5.4% (6) *vs*. 1.1% (11), p=0.005] (**Table 4**). There was no difference in the multiparity rate between both groups.

TABLE 2 | Comparison of tolerance for screening tests between women who prefer a GCT compared to women who prefer an OGTT or without clear preference.

	Preference OGTT	Preference GCT	No preference	Pairwise comparisons		
	N = 112 (6.21%)	N = 989 (54.82%)	N = 703 (38.97%)	1 *vs* 2	1 *vs* 3	2 *vs* 3
% Any discomfort of GCT:				**<.001**	**0.002**	0.259
No	66.1 (72)	81.4 (787)	79.1 (539)			
Yes	33.9 (37)	18.6 (180)	20.8 (142)			
% Bad taste				0.102	**0.002**	**0.004**
No	89.2 (33)	77.2 (139)	62.7 (89)			
Yes	10.8 (4)	22.8 (41)	37.3 (53)			
% Nausea				0.233	0.172	0.748
No	45.9 (17)	56.7 (102)	58.4 (83)			
Yes	54.0 (20)	43.3 (78)	41.5 (59)			
% Dizziness or feeling faint				0.666	0.706	0.190
No	62.2 (23)	58.3 (105)	65.5 (93)			
Yes	37.8 (14)	41.7 (75)	34.5 (49)			
% Vomiting				0.167	0.143	0.852
No	94.6 (35)	98.3 (177)	98.6 (140)			
Yes	5.4 (2)	1.7 (3)	1.4 (2)			
% Abdominal pain				0.282	0.279	0.947
No	94.6 (35)	97.8 (176)	97.9 (139)			
Yes	5.4 (2)	2.2 (4)	2.1 (3)			
% Any discomfort of OGTT:				0.872	**0.002**	**<.001**
No	50.9 (56)	50.1 (495)	66.5 (466)			
Yes	49.1 (54)	49.9 (493)	33.5 (235)			
% Bad taste				0.949	0.790	0.530
No	70.4 (38)	70.8 (349)	68.5 (161)			
Yes	29.6 (16)	29.2 (144)	31.5 (74)			
% Nausea				0.562	0.165	0.107
No	38.9 (21)	43.0 (212)	49.4 (116)			
Yes	61.1 (33)	57.0 (281)	50.6 (119)			
% Vomit OGTT test				0.334	0.184	0.502
No	92.6 (50)	95.5 (471)	96.6 (227)			
Yes	7.4 (4)	4.5 (22)	3.4 (8)			
% Dizziness or feeling faint				0.382	0.840	0.230
No	55.6 (30)	49.3 (243)	54.0 (127)			
Yes	44.4 (24)	50.7 (250)	46.0 (108)			
% Abdominal pain				**0.001**	0.132	**0.042**
No	87.0 (47)	96.5 (476)	93.2 (219)			
Yes	13.0 (7)	3.4 (17)	6.8 (16)			
% Cumbersome to be fasting				**<.001**	**<.001**	**<.001**
No	67.8 (73)	41.3 (408)	81.3 (570)			
Yes	34.2 (38)	58.7 (579)	18.7 (131)			

OGTT, oral glucose tolerance test; GCT, glucose challenge test; Categorical variables are presented as frequencies %(n); Differences are considered significant at p-value<0.05. Bold value means that this is significant, meaning that the p-value < 0.05.

Pregnancy Outcomes

Pregnancy outcomes were similar between women who preferred a one-step or two-step screening strategy, except for a lower rate of labor inductions and emergency cesarean sections (CS) in the group who preferred a two-step screening [respectively 26.6% (198) *vs*. 32.5% (137), p=0.031 and 8.2% (68) *vs*. 13.0% (61), p=0.005] (**Table 3**).

Women who preferred a GCT had less often emergency CS and less often neonatal jaundice [respectively: 9.3% (92) *vs*. 15.3% (92), p=0.046 and 9.3% (91) *vs*. 16.2% (18), p=0.021] compared to women who preferred an OGTT (**Table 4**).

Postpartum Outcomes

Women who preferred an OGTT had a better diet score postpartum compared to women who preferred a GCT. There was no difference in rate of glucose intolerance postpartum between the different groups (**Tables 3**, **4**).

DISCUSSION

We found that the majority of pregnant women preferred a two-step screening strategy with a GCT for GDM. In addition, we show that the preference of GDM screening method differed by metabolic risk profile of participants and tolerance for the screening tests. Women with a more adverse metabolic profile preferred a one-step screening approach with OGTT while women preferring a two-step screening strategy had a better metabolic profile and more discomfort of the OGTT.

Several international societies such as the IADPSG and WHO recommend a one-step screening approach for GDM with a 75g OGTT (8, 9). However, this leads to an important increase in the number of women diagnosed with GDM and important increase in workload. Moreover, this could also lead to increased medicalization of care with more labor inductions and CS. Evidence is lacking that treatment of GDM based on the one-

TABLE 3 | Comparison of characteristics and pregnancy outcomes between women who prefer two-step screening compared to women who prefer one-step screening with OGTT or without clear preference.

	Preference two-step screening N = 834 (46.3%)	Preference one-step OGTT N = 472 (26.2%)	No preference N = 497 (27.6%)	Pairwise comparisons		
				1 *vs* 2	1 *vs* 3	2 *vs* 3
General						
Age (years)	30.6 ± 3.8	30.8 ± 4.0	31.0 ± 4.4	0.180	0.071	0.717
% Minor ethnicities	6.0 (50)	10.7 (50)	12 (59)	**0.003**	**<.001**	0.523
% multiparity	47.4 (395)	47.9 (226)	46.7 (232)	0.857	0.809	0.708
% Highest education:				0.105	**<.001**	**0.002**
primary school	0.4 (3)	1.1 (5)	2.7 (13)			
till 15 years	2.5 (21)	3.9 (18)	7.6 (37)			
high school	14.1 (117)	17.5 (80)	22.4 (109)			
bachelor	43.5 (360)	41.0 (188)	39.2 (191)			
master	39.5 (327)	36.5 (167)	28.1 (137)			
% paid job	94.3 (784)	91.7 (431)	88.4 (436)	0.065	**<.001**	0.091
% low monthly net income family <1500 euro	1.8 (15)	5.0 (23)	6.6 (32)	**0.003**	**<.001**	0.276
% 1500-5000 euro	90.8 (739)	89.6 (415)	89.7 (435)			
% >5000 euro	7.4 (60)	5.4 (25)	3.7 (18)			
Low income (<1500 euro)						
% No	98.2 (799)	95.0 (440)	93.4 (453)	**0.003**	**<.001**	0.331
% Yes	1.8 (15)	5.0 (23)	6.6 (32)			
%living without partner	15.0 (124)	22.5 (106)	17.8 (88)	**<.001**	0.176	0.064
% smoking before pregnancy	29.1 (242)	31.3 (147)	28.2 (139)	0.415	0.719	0.295
% smoking during pregnancy	3.7 (31)	3.0 (14)	3.8 (19)	0.474	0.917	0.459
% First degree family history of diabetes	12.0 (97)	13.0 (60)	13.2 (64)	0.588	0.502	0.915
% First degree family history of GDM	3.8 (29)	6.4 (28)	3.4 (16)	**0.039**	0.773	**0.041**
% History of GDM*	7.3 (29)	13.8 (32)	5.5 (13)	**0.008**	0.392	**0.002**
%History of impaired glucose intolerance	1.8 (13)	1.0 (4)	1.1 (5)	0.267	0.366	0.820
6-14 weeks visit						
Week first visit with FPG	11.8 ± 1.8	11.9 ± 1.7	12.0 ± 1.8	**0.027**	**0.002**	0.454
BMI (Kg/m²)	23.9 ± 4.0	25.4 ± 5.3	25.4 ± 4.7	**<.001**	**<.001**	0.426
% Overweight	23.1 (50)	24.8 (116)	29.3 (145)	**<.001**	**<.001**	0.430
% Obesity	7.9 (66)	18.2 (85)	15.3 (76)			
Waist circumference (cm)	85.5 ± 10.0	88.5 ± 12.6	88.4 ± 11.6	**<.001**	**<.001**	0.943
% Waist ≥80cm	71.9 (586)	76.4 (346)	78.1 (367)	**<.001**	**0.003**	0.627
Weight gain (first visit till OGTT) (Kg)	7.2 ± 2.9	6.9 ± 3.6	7.1 ± 3.6	0.534	0.796	0.424
Systolic blood pressure (mmHg)	114.6 ± 10.3	115.7 ± 10.8	115.1 ± 11.0	0.093	0.377	0.483
Diastolic blood pressure (mmHg)	70.4 ± 7.8	70.8 ± .8.3	70.5 ± 8.6	0.401	0.949	0.463
Total Score lifestyle						
Physical activity	1.0 (0.0-2.0)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.470	0.432	0.190
Diet	2.0 (0.0-4.0)	2.0 (0.0-5.0)	2.0 (0.0-4.0)	0.658	0.347	0.240
Fasting glycaemia (mg/dl)	82.0 (78.0-85.0)	82.0 (78.0-86.0)	82.0 (78.0-85.0)	0.438	0.675	0.752
HOMA-IR	8.9 (6.4-12.3)	9.9 (7.2-14.2)	9.4 (6.6-13.5)	**<.001**	**0.042**	0.257
HOMA-B	879.7 (663.1-1218.5)	948.0 (665.1-1361.2)	928.3 (673.2-1344.6)	**0.038**	0.062	0.879
HbA1c (mmol/mol and %)	31.0 (29.0-32.0)	31.0 (29.0-33.0)	31.0 (29.0-32.0)	0.054	0.433	0.274
	5.0 (4.8-5.1)	5.0 (4.8-5.2)	5.0 (4.8-5.1)			
Fasting TG (mg/dl)	88.0 (70.0-109.5)	89.0 (73.0-114.0)	90.0 (71.0-117.0)	**0.032**	0.087	0.703
24-28 weeks visit						
BMI (Kg/m²)	26.4 ± 4.1	27.9 ± 5.2	27.9 ± 4.6	**<.001**	**<.001**	0.331
% Overweight	38.5 (310)	39.5 (182)	44.0 (213)	**<.001**	**<.001**	0.482
% Obesity	17.6 (142)	28.6 (132)	26.0 (126)			
Systolic blood pressure (mmHg)	112.7 ± 9.9	114.4 ± 10.6	113.5 ± 10.3	**0.014**	0.215	0.257
Diastolic blood pressure (mmHg)	67.0 ± 7.8	67.6 ± 8.3	67.5 ± 8.1	0.252	0.235	0.968
Total score lifestyle						
Physical activity	1.0 (0.0-2.0)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.243	0.416	0.080
Diet	2.0 (0.0-4.0)	2.0 (0.0-4.0)	2.0 (-1.0-4.0)	0.260	0.059	0.540
IPAQ low	15.5 (126)	18.6 (84)	16.9 (80)	0.155	0.509	0.497
METs category:				0.095	0.791	0.335
% Low	15.5 (126)	18.6 (84)	16.9 (80)			
% Moderate	46.1 (375)	48.7 (220)	45.8 (217)			
% High	38.4 (313)	32.7 (148)	37.3 (177)			
% clinical depression (≥16 on CES-D questionnaire)	16.2 (135)	15.8 (74)	14.8 (73)	0.840	0.489	0.666
Glucose 60 min on GCT (mg/dl)	117.9 ± 27.8	121.0 ± 25.9	123.9 ± 27.7	**0.032**	**<.001**	0.139
Fasting glycaemia (mg/dl)	78.0 (74.0-82.0)	79.0 (75.0-83.0)	78.0 (74.0-83.0)	**0.005**	0.083	0.341

(Continued)

TABLE 3 | Continued

	Preference two-step screening N = 834 (46.3%)	Preference one-step OGTT N = 472 (26.2%)	No preference N = 497 (27.6%)	Pairwise comparisons		
				1 *vs* 2	1 *vs* 3	2 *vs* 3
1-hour glucose OGTT (mg/dl)	126.0 (108.0-146.0)	128.5 (109.0-149.0)	131.0 (111.5-151.0)	0.191	**0.011**	0.311
2-hour glucose OGTT (mg/dl)	111.0 (95.0-129.0)	110.0 (95.0-130.0)	113.5 (95.0-132.0)	0.825	0.376	0.360
HbA1c	30.0 (29.0-32.0)	31.0 (29.0-32.0)	30.0 (29.0-32.0)	**<.001**	**0.031**	0.106
(mmol/mol and %)	4.9 (4.8-5.1)	5.0 (4.8-5.1)	4.9 (4.8-5.1)			
Matsuda insulin sensitivity	0.6 (0.4-0.8)	0.5 (0.4-0.7)	0.5 (0.4-0.8)	**0.006**	0.079	0.343
HOMA-IR	11.6 (8.5-16.9)	13.4 (9.5-18.8)	12.6 (9.0-17.9)	**0.009**	0.069	0.716
HOMA-B	1528.8 (1096.4-2259.0)	1588.1 (1139.3-2256.0)	1594.8 (1118.6-2248.0)	0.867	0.977	0.866
ISSI-2	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.061	0.528	0.265
Insulinogenic index/HOMA-IR	0.3 (0.2-0.5)	0.3 (0.2-0.5)	0.3 (0.2-0.4)	0.196	**0.003**	0.171
Fasting TG (mg/dl)	160.0 (128.0-202.0)	165.0 (133.0-206.0)	164.0 (132.0-207.0)	0.345	0.327	0.962
% Need for treatment with insulin (total)	1.0 (8)	3.4 (16)	2.2 (11)	**0.002**	0.092	0.330
% short acting insulin	0.2 (2)	0.9 (4)	0.4 (2)	**0.006**	0.179	0.616
% long acting insulin	0.6 (5)	1.1 (5)	1.0 (5)			
% short and long-acting insulin	0.1 (1)	1.5 (7)	0.8 (4)			
Delivery						
Total Weight gain (first visit till delivery) (Kg)	12.0 ± 4.5	11.2 ± 5.7	11.7 ± 5.3	**0.024**	0.242	0.408
% excessive weight gain	27.7 (205)	27.8 (113)	31.9 (138)	0.282	0.316	0.186
Gestational age (weeks)	39.3 ± 1.6	39.2 ± 1.5	39.2 ± 1.7	0.565	0.511	0.988
% Preeclampsia	1.2 (10)	2.3 (11)	2.0 (10)	0.114	0.238	0.722
% Gestational hypertension	3.4 (28)	5.3 (25)	4.6 (23)	0.083	0.242	0.615
% Preterm delivery	4.9 (41)	5.3 (25)	7.1 (35)	0.755	0.106	0.264
% Induction labor	26.6 (198)	32.5 (137)	33.2 (148)	**0.031**	**0.015**	0.841
% Forceps or vacuum	12.4 (103)	11.5 (54)	12.5 (62)	0.089	0.239	0.803
% Cesarean sections (total)	19.5 (162)	23.1 (108)	21.3 (105)	0.127	0.440	0.496
% Planned CS	11.3 (94)	10.0 (47)	9.7 (48)	0.089	0.239	0.803
% Emergency CS (during labor)	8.2 (68)	13.0 (61)	11.5 (57)	**0.005**	**0.043**	0.480
Weight baby (g)	3391.7 ± 498.1	3393.0 ± 482.4	3375.2 ± 541.7	0.908	0.675	0.827
% Macrosomia (>4Kg)	9.3 (77)	8.8 (41)	9.1 (45)	0.746	0.917	0.842
% Weight baby ≥4.5Kg	1.4 (9)	1.1 (5)	1.4 (7)	0.979	0.592	0.626
% LGA	12.9 (107)	12.6 (59)	12.1 (60)	0.894	0.693	0.818
% SGA	5.2 (43)	4.9 (23)	4.5 (22)	0.844	0.556	0.729
% Apgar 10min <7	1.3 (11)	0.0 (0)	1.0 (5)	**0.010**	0.796	0.062
%Shoulder dystocia	0.8 (7)	1.1 (5)	1.2 (6)	0.685	0.508	0.831
% Congenital anomaly	4.2 (35)	4.5 (21)	3.8 (19)	0.887	0.776	0.632
% Respiratory Distress syndrome	1.2 (10)	0.4 (2)	1.0 (5)	0.230	0.796	0.453
% Neonatal hypoglycemia <40mg/dl	4.6 (26)	5.6 (17)	7.8 (26)	0.543	0.050	0.261
% Neonatal jaundice	17.5 (100)	21.0 (72)	18.8 (68)	0.186	0.609	0.462
% NICU admission	10.0 (83)	9.2 (43)	10.9 (54)	0.616	0.602	0.364
Days on NICU	8.1 ± 13.4	8.4 ± 13.3	8.6 ± 13.7	0.757	0.538	0.442
Postpartum						
% Postpartum OGTT	9.0 (92)	12.5 (59)	12.5 (62)	**0.045**	**0.043**	0.991
% glucose intolerance				0.122	0.082	0.084
None	80.0 (60)	88.1 (52)	80.6 (50)			
IFG	10.7 (8)	5.1 (3)	1.6 (1)			
IGT	9.3 (7)	3.4 (2)	16.1 (10)			
IFG+IGT	0.0 (0)	3.4 (2)	1.61 (1)			
% breastfeeding	85.5 (65)	81.0 (47)	80.0 (48)	0.487	0.393	0.887
Lifestyle score:						
Physical activity	1.0 (0.0-1.0)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.432	0.170	0.379
Diet	5.0 (1.0-7.0)	2.0 (-1.0-4.0)	2.0 (0.0-5.0)	**0.048**	0.202	0.366
Energy	62.5 (50.0-75.0)	62.5 (50.0-75.0)	62.5 (50.0-75.0)	0.652	0.883	0.776
Emotional Wellbeing	70.0 (65.0-75.0)	70.0 (65.075.0)	70.0 (65.0-75.0)	0.793	0.326	0.519
Social functioning	87.5 (75.0-100.0)	87.5 (75.0-100.0)	87.5 (75.0-100.0)	0.697	0.842	0.866
Pain	90.0 (77.5-100.0)	90.0 (77.5-100.0)	90.0 (77.5-100.0)	0.750	0.482	0.390
General Health	75.0 (65.0-85.0)	75.0 (65.0-85.0)	75.0 (60.0-85.0)	**0.046**	0.217	0.583
Health Transition	50.0 (50.0-50.0)	50.0 (50.0-50.0)	50.0 (50.0-50.0)	0.513	0.882	0.626

*OGTT, oral glucose tolerance test; GDM, gestational diabetes mellitus; BMI, Body Mass Index; HDL, high-density lipoprotein; LDL, low-density-lipoprotein; TG, triglycerides; MET, metabolic equivalent of task; LGA, large-for-gestational age infant; SGA, small-for-gestational age infant; NICU, neonatal intensive care unit; IFG, impaired fasting glycemia; IGT, impaired glucose tolerance; SF-36, 36-Item Short Form Health Survey; CES-D, Center for Epidemiologic Studies – Depression. Overweight: BMI ≥25-29.9 Kg/m²; Obesity: BMI ≥30 Kg/m². Questionnaires in the postpartum period were only administered by women with GDM who attended the OGTT. Categorical variables are presented as frequencies %(n); continuous variables are presented as mean ± SD if normally distributed and as median ± IQR if not normally distributed; Differences are considered significant at p-value<0.05. *A history of GDM and a history of a macrosomic baby were calculated on the number of women with a previous pregnancy.*

Bold value means that this is significant, meaning that the p-value < 0.05.

TABLE 4 | Comparison of characteristics and pregnancy outcomes between women who prefer a GCT compared to women who prefer an OGTT or without clear preference.

	Preference OGTT N = 112 (6.21%)	Preference GCT N = 989 (54.82%)	No preference N = 703 (38.97%)	Pairwise comparisons		
				1 *vs* 2	1 *vs* 3	2 *vs* 3
General						
Mean age (years)	31.0 ± 3.8	30.7 ± 4.0	30.8 ± 4.1	0.464	0.587	0.600
% Minor ethnicities	12.6 (14)	6.8 (67)	11.3 (79)	**0.027**	0.691	**0.001**
% multiparity	51.8 (58)	47.1 (466)	47.2 (332)	0.349	0.370	0.965
% Highest education:				0.099	0.396	**<.001**
primary school	1.8 (2)	0.6 (6)	1.7 (12)			
till 15 years	3.6 (4)	2.8 (27)	6.6 (45)			
high school	20.0 (22)	14.7 (144)	20.4 (140)			
bachelor	47.3 (52)	43.2 (423)	38.5 (264)			
master	27.3 (30)	38.6 (378)	32.8 (225)			
% paid job	91.0 (101)	93.0 (915)	90.6 (634)	0.441	0.888	0.072
% low monthly net income family <1500 euro	3.6 (4)	2.4 (23)	6.1 (42)	0.547	0.516	**<.001**
% 1500-5000 euro	91.0 (101)	90.6 (876)	89.6 (614)			
% >5000 euro	5.4 (6)	7.0 (68)	4.2 (29)			
Low income (<1500 euro)						
% No	96.4 (107)	97.6 (944)	93.9 (643)	0.514	0.382	**<.001**
% Yes	3.6 (4)	2.4 (23)	6.1 (42)			
% living without partner	15.2 (17)	15.9 (156)	20.6 (144)	0.849	0.184	**0.013**
% smoking before pregnancy	25.9 (29)	31.3 (309)	27.4 (191)	0.237	0.739	0.082
% smoking during pregnancy	2.7 (3)	4.0 (40)	3.1 (22)	0.613	1.000	0.360
% First degree family history of diabetes	13.8 (15)	11.3 (108)	14.5 (100)	0.448	0.844	0.057
% First degree family history of GDM	5.7 (6)	4.1 (37)	4.5 (30)	0.443	0.617	0.645
% History of GDM*	21.4 (12)	8.2 (39)	7.1 (24)	**0.002**	**<.001**	0.562
% history of PCOS	11.6 (13)	6.9 (68)	7.0 (49)	0.071	0.088	0.934
6-14 weeks visit						
Week first visit with FPG	11.9 ± 1.6	11.8 ± 1.8	12.0 ± 1.7	0.269	0.906	**0.041**
BMI (Kg/m²)	25.7 ± 6.1	24.1 ± 4.2	25.4 ± 4.8	**0.023**	0.752	**<.001**
% Overweight	25.0 (28)	22.3 (219)	29.4 (205)	**0.005**	0.680	**<.001**
% Obesity	19.6 (22)	10,1 (99)	15.2 (106)			
Waist circumference (cm)	88.4 ± 14.4	86.0 ± 10.4	88.4 ± 11.7	0.166	0.741	**<.001**
% Waist ≥80cm	74.3 (81)	74.0 (702)	76.9 (515)	0.155	0.837	**<.001**
Weight gain (first visit till OGTT) (Kg)	7.0 ± 4.0	7.1 ± 3.0	7.0 ± 3.6	0.237	0.251	0.884
Systolic blood pressure (mmHg)	115.6 ± 10.6	114.6 ± 10.7	115.5 ± 10.5	0.315	0.929	**0.049**
Diastolic blood pressure (mmHg)	71.3 ± 8.4	70.2 ± 8.0	70.9 ± 8.4	0.280	0.695	0.149
Total Score lifestyle						
Physical activity	1.0 (0.0-2.0)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.069	0.167	0.487
Diet	2.0 (1.0-5.0)	2.0 (0.0-4.0)	1.0 (0.0-4.0)	0.050	**0.007**	0.137
Fasting glycaemia (mg/dl)	83.0 (78.0-86.0)	81.0 (78.0-85.0)	82.0 (78.0-86.0)	0.146	0.436	0.191
Fasting insulin (pmol/l)	50.1 (34.0-70.7)	44.6 (33.0-61.3)	47.5 (34.4-68.2)	**0.043**	0.515	**0.006**
HOMA-IR	10.1 (6.9-14.4)	8.9 (6.5-12.5)	9.6 (6.7-14.2)	**0.038**	0.526	**0.004**
HOMA-B	953.4 (715.1-1307.4)	890.3 (648.0-1239.8)	936.0 (687.0-1353.6)	0.210	0.946	**0.020**
HbA1c (mmol/mol and %)	31.0 (29.0-33.0)	31.0 (29.0-32.0)	31.0 (29.0-33.0)	0.272	0.763	0.082
	5.0 (4.8-5.2)	5.0 (4.8-5.1)	5.0 (4.8-5.2)			
Fasting TG (mg/dl)	89.0 (74.0-118.0)	89.0 (71.0-111.0)	89.0 (71.0-114.0)	0.187	0.297	0.597
24-28 weeks visit						
BMI (Kg/m²)	28.3 ± 6.0	26.6 ± 4.2	27.8 ± 4.7	**0.007**	0.726	**<.001**
% Overweight	33.6 (37)	38.8 (372)	42.9 (293)	**<.001**	**0.001**	**<.001**
% Obesity	34.5 (38)	19.5 (187)	25.8 (176)			
Systolic blood pressure (mmHg)	115.0 ± 12.7	112.9 ± 9.8	113.7 ± 10.4	0.174	0.431	0.217
Diastolic blood pressure (mmHg)	67.6 ± 9.4	67.2 ± 7.8	67.4 ± 8.0	0.822	0.931	0.487
Total score lifestyle						
Physical activity	1.0(0.0-2.0)	1.0(0.0-2.0)	1.0(0.0-2.0)	0.322	0.717	0.212
Diet	3.0(1.0-5.0)	2.0(0.0-4.0)	2.0(-1.0-4.0)	**0.033**	**0.003**	0.056
IPAQ low	17.0 (18)	16.2 (156)	17.3 (116)	0.833	0.938	0.555
METs category:				0.975	0.737	0.284
% Low	17.0 (18)	16.2 (156)	17.3 (116)			
% Moderate	48.1 (51)	48.2 (465)	44.3 (297)			
% High	34.9 (37)	35.6 (343)	38.4 (258)			
% clinical depression (≥16 on CES-D questionnaire)	11.6 (13)	16.8 (166)	14.7 (103)	0.156	0.380	0.246
Glucose 60 min on GCT (mg/dl)	122.6 ± 24.5	119.0 ± 27.1	122.2 ± 28.4	0.123	0.613	0.059

(Continued)

TABLE 4 | Continued

	Preference OGTT N = 112 (6.21%)	Preference GCT N = 989 (54.82%)	No preference N = 703 (38.97%)	Pairwise comparisons		
				1 *vs* 2	1 *vs* 3	2 *vs* 3
Fasting glycaemia (mg/dl)	79.0 (75.0-85.0)	78.0 (74.0-82.0)	79.0 (75.0-83.0)	**0.042**	0.448	**0.006**
1-hour glucose OGTT (mg/dl)	132.0 (110.0-154.0)	127.0 (108.0-146.0)	129.0 (110.0-150.0)	0.211	0.640	0.108
2-hour glucose OGTT (mg/dl)	116.0 (98.0-136.0)	111.5 (94.0-129.5)	111.0 (95.0-130.0)	0.125	0.115	0.843
HbA1c	31.0 (29.0-33.0)	30.0 (29.0-32.0)	30.0 (29.0-32.0)	0.066	0.446	**0.019**
(mmol/mol and %)	5.0 (4.8-5.1)	4.9 (4.8-5.1)	4.9 (4.8-5.1)			
Matsuda insulin sensitivity	0.5(0.3-0.8)	0.6 (0.4-0.8)	0.5(0.4-0.7)	0.803	0.410	0.159
HOMA-IR	13.7 (9.0-21.1)	11.8 (8.6-16.8)	13.0 (9.1-18.1)	0.140	0.772	0.050
HOMA-B	1728.4 (1107.8-2268.0)	1548.0 (1123.7-2259.0)	1568.6 (1122.9-2241.0)	0.723	0.627	0.888
ISSI-2	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.338	0.137	0.188
Insulinogenic index/HOMA-IR	0.3 (0.2-0.4)	0.3 (0.2-0.5)	0.3 (0.2-0.4)	0.487	0.897	0.255
Fasting Total cholesterol (mg/dl)	248.0 (221.5-270.0)	243.0 (220.0-273.0)	241.0 (216.0-273.0)	0.901	0.529	0.121
Fasting HDL (mg/dl)	75.0 (64.5-85.5)	74.0 (64.0-87.0)	74.0 (64.0-86.0)	0.835	0.957	0.704
Fasting LDL (mg/dl)	135.0 (112.0-153.0)	134.0 (113.0-161.0)	131.0 (109.0-159.0)	0.551	0.740	0.059
Fasting TG (mg/dl)	165.0 (141.0-216.5)	160.0 (127.0-204.0)	165.0 (133.0-202.0)	0.057	0.161	0.276
Increase (difference) in TG between first and second visit (mg/dl)	74.0 (52.0-112.0)	70.0 (43.0-101.0)	71.0 (47.0-99.0)	0.143	0.239	0.462
% Need for treatment with insulin (total)	5.4 (6)	1.1 (11)	2.6 (18)	**0.005**	0.125	**0.035**
% short acting insulin	0.9 (1)	0.6 (6)	1.1 (8)	**<.001**	0.153	0.088
% long acting insulin	1.8 (2)	0.3 (3)	0.4 (3)			
% short and long-acting insulin	2.7 (3)	0.2 (2)	1.0 (7)			
Delivery						
Total Weight gain (first visit till delivery) (Kg)	11.7 ± 4.3	11.9 ± 4.6	11.3 ± 5.8	0.496	0.675	**0.026**
% excessive weight gain	30.4 (28)	27.6 (241)	30.7 (188)	0.840	0.942	0.251
Gestational age (weeks)	39.1 ± 1.7	39.3 ± 1.6	39.2 ± 1.6	0.626	0.967	0.273
% Preeclampsia	0.9 (1)	1.4 (14)	2.3 (16)	1.000	0.716	0.196
% Gestational hypertension	4.5 (5)	3.3 (33)	5.4 (38)	0.515	0.706	**0.037**
% Preterm delivery	6.3 (7)	5.2 (51)	6.2 (43)	0.618	0.953	0.393
% Induction labor	26.0 (26)	27.7 (246)	33.9 (212)	0.713	0.120	**0.011**
% Forceps or vacuum	14.4 (16)	11.9 (117)	12.2 (85)	0.167	0.532	0.444
% Cesarean sections (total)	25.2 (28)	19.9 (196)	21.5 (150)	0.167	0.532	0.444
% Planned CS	9.9 (11)	10.6 (104)	10.6 (74)	0.167	0.532	0.444
% Emergency CS (during labor)	15.3 (17)	9.3 (92)	10.9 (76)	**0.046**	0.174	0.297
% Postpartum blood loss				0.655	0.847	0.798
≥500ml	21.1 (23)	20.9 (204)	20.4 (141)			
≥1000ml	3.7 (4)	2.3 (22)	2.7 (19)			
Weight baby (g)	3358.5 ± 510.9	3394.2 ± 509.5	3382.7 ± 503.0	0.460	0.619	0.575
% Macrosomia (>4Kg)	9.0 (10)	9.5 (93)	8.6 (60)	0.877	0.893	0.556
% Weight baby ≥4.5Kg	0.9 (1)	1.4 (14)	0.9 (6)	1.000	1.000	0.365
% LGA	11.7 (13)	13.2 (130)	12.0 (84)	0.654	0.923	0.471
% SGA	4.5 (5)	4.8 (47)	5.3 (37)	0.895	0.721	0.624
% Apgar 10min <7	0.0 (0)	1.1 (11)	0.7 (5)	0.615	1.000	0.457
%Shoulder dystocia	0.0 (0)	1.5 (15)	0.4 (3)	0.387	1.000	**0.032**
% Congenital anomaly	4.5 (5)	4.4 (43)	3.7 (26)	1.000	0.603	0.535
% Respiratory Distress syndrome	0.0 (0)	1.1 (11)	0.9 (6)	0.616	1.000	0.632
% Neonatal hypoglycemia <40mg/dl	2.8 (2)	5.8 (39)	6.1 (28)	0.416	0.407	0.799
% Neonatal jaundice	17.9 (15)	18.9 (129)	18.9 (96)	0.820	0.815	0.983
% NICU admission	16.2 (18)	9.3 (91)	10.4 (72)	**0.021**	0.069	0.457
Days on NICU	7.2 ± 9.5	7.9 ± 13.1	8.9 ± 14.7	0.817	0.671	0.679
Postpartum						
% Postpartum OGTT	15.2 (17)	9.2 (91)	12.5 (88)	**0.044**	0.435	**0.029**
% glucose intolerance				0.416	0.514	0.437
IFG	5.9 (1)	8.8 (8)	3.4 (3)			
IGT	0.0 (0)	11.0 (10)	10.2 (9)			
IFG+IGT	0.0 (0)	2.2 (2)	1.1 (1)			
% breastfeeding	81.2 (13)	84.6 (77)	80.5 (70)	0.734	0.941	0.465
Lifestyle score:						
Physical activity	1.0 (0.0-1.0)	1.0 (0.0-2.0)	1.0 (0.0-2.0)	0.432	0.170	0.379
Diet	5.0 (1.0-7.0)	2.0 (-1.0-4.0)	2.0 (0.0-5.0)	**0.048**	0.202	0.366
SF36						
Physical functioning	85.7 (80.0-95.0)	90.0 (83.3-100.0)	90.0 (80.0-100.0)	0.062	0.089	0.982
Role physical	81.2 (62.5-100.0)	87.5 (65.6-100.0)	87.5 (68.7-100.0)	0.806	0.999	0.566
Role Emotional	100.0 (66.7-100.0)	100.0 (75.0-100.0)	100.0 (66.7-100.0)	0.685	0.760	0.854

(Continued)

TABLE 4 | Continued

	Preference OGTT N = 112 (6.21%)	Preference GCT N = 989 (54.82%)	No preference N = 703 (38.97%)	Pairwise comparisons		
				1 *vs* 2	1 *vs* 3	2 *vs* 3
Energy	62.5 (56.2-75.0)	62.5 (50.0-75.0)	62.5 (50.0-75.0)	0.078	0.419	0.056
Emotional Wellbeing	70.0 (65.0-75.0)	70.0 (65.0-75.0)	70.0 (65.0-75.0)	0.218	0.255	0.903
Social functioning	100.0 (75.0-100.0)	87.5 (75.0-100.0)	87.5 (75.0-100.0)	0.335	0.845	0.109
Pain	80.0 (67.5-100.0)	90.0 (77.5-100.0)	90.0 (77.5-100.0)	0.093	**0.028**	0.330
General Health	75.0 (65.0-85.0)	75.0 (65.0-85.0)	75.0 (65.0-85.0)	0.640	0.857	0.641
Health Transition	50.0 (50.0-50.0)	50.0 (50.0-50.0)	50.0 (50.0-50.0)	0.541	0.925	0.266
METs category:				1.000	0.489	0.065
% Low	13.3 (2)	17.0 (15)	7.6 (6)			
% Moderate	53.3 (8)	51.1 (45)	45.6 (36)			
% High	33.3 (5)	31.8 (28)	46.8 (37)			
% clinical depression (≥16 on CES-D questionnaire)	11.8 (2)	17.4 (16)	15.9 (14)	0.566	0.663	0.790

*OGTT, oral glucose tolerance test; GCT, glucose challenge test; GDM, gestational diabetes mellitus; BMI, Body Mass Index; HDL, high-density lipoprotein; LDL, low-density-lipoprotein; TG, triglycerides; MET, metabolic equivalent of task; LGA, large-for-gestational age infant; SGA, small-for-gestational age infant; NICU, neonatal intensive care unit; IFG, impaired fasting glycemia; IGT, impaired glucose tolerance; SF-36, 36-Item Short Form Health Survey; CES-D, Center for Epidemiologic Studies – Depression. Overweight: BMI ≥25-29.9 Kg/m²; Obesity: BMI ≥30 Kg/m². Questionnaires in the postpartum period were only administered by women with GDM who attended the OGTT. Categorical variables are presented as frequencies %(n); continuous variables are presented as mean ± SD if normally distributed and as median ± IQR if not normally distributed; Differences are considered significant at p-value<0.05. *A history of GDM and a history of a macrosomic baby were calculated on the number of women with a previous pregnancy.*

Bold value means that this is significant, meaning that the p-value < 0.05.

step IADPSG screening approach improves pregnancy outcomes compared to other screening strategies. Recently, two large RCT's from the US showed that a one-step screening strategy with the IADPSG criteria leads to a 2-3 fold increase in GDM prevalence compared to screening with a two-step approach with GCT but without improvement of pregnancy outcomes (11). In addition, the OGTT is often considered a cumbersome test during pregnancy. In our study, nearly half of all women indicated that it was difficult to come fasting. When choosing a GDM screening approach, it is therefore also important to take into account the preference of pregnant women for the GDM screening method and tolerance of the screening tests. To our knowledge, our cohort is the first study to systematically assess the preference of pregnant women for GDM screening method and tests. Our results show that nearly half of all women preferred a two-step screening strategy over a one-step screening approach with OGTT. Women who preferred a two-step screening strategy tolerated the GCT in general better than the OGTT compared to women who preferred a one-step screening approach or women without clear preference. More women preferred therefore a GCT as screening test. This is in line with other studies reporting difficulties with an OGTT in pregnancy, in which vomiting is often a reason for failure of the test (30). A recent RCT from the US showed that a 75g OGTT was better tolerated than a 100g OGTT for the diagnosis of GDM (11). However, when using a two-step screening strategy with GCT, a 100g OGTT is only needed in about 20% of all pregnant women. In line with normal clinical practice, adverse events of the screening tests would therefore occur in only 4% of women using a two-step screening strategy with GCT compared to 13% in women undergoing the one-step IADPSG approach with OGTT (11).

Women who preferred a GCT or two-step screening strategy had a better metabolic profile (were less often obese and less insulin resistant) and had less risk factors for GDM compared to women who preferred an OGTT or one-step screening approach. We have previously demonstrated that women with a higher risk-profile, such as women with a previous history of GDM and higher BMI have the highest risk to develop GDM and would therefore benefit from a one-step approach with OGTT (10). The Flemish consensus on screening for GDM was revised in 2019 based on the BEDIP-N study. A modified two-step screening strategy for GDM with GCT and also based on risk-factors, was proposed to limit the number of missed cases with GDM and at the same time avoid an OGTT in about 50% of all pregnant women (16). Based on this modified two-step screening strategy, women at higher risk for GDM (women with a history of GDM, obesity and/or impaired fasting glycaemia in early pregnancy), are recommended a one-step screening strategy with an OGTT, while women without these risk factors, are offered a two-step screening strategy with GCT (13). With current study we show now that this screening approach also fits with the preference of women for GDM screening method according to their metabolic risk profile and tolerance of the tests. In our study, the preference of GDM screening method was not different in women who had been pregnant before and had already experienced screening for GDM. However, most women with a previous history of GDM preferred a one-step screening strategy with OGTT. This is probably due to the fact that these women perceive themselves to be at high risk for a recurrent diagnosis of GDM and will therefore more often need an OGTT (irrespective of screening approach).

Pregnancy outcomes were in general similar irrespective of the preference of the GDM screening method, expect for lower rates of labor inductions and emergency CS in women who preferred a two-step screening strategy. This is probably due to the lower metabolic risk of women who preferred two-step screening. In addition, research has shown that a higher

income and a higher education leads to less inductions and emergency CS (31, 32). There was no difference in the rate of glucose intolerance postpartum between both groups. Women who preferred an OGTT had a higher diet score, suggesting a healthier diet in early postpartum. This might be due to the fact that they perceive themselves at higher risk to develop diabetes postpartum.

Strengths of our study are the large prospective cohort with detailed data on clinical characteristics and obstetrical outcomes. In addition, women were blinded for the result of the GCT, so that they could not be biased by this result and their preference for a GCT or OGTT. Moreover, the tolerance and preference of GDM screening method was systematically recorded at the time of the GCT and OGTT. A limitation of the study is that the cohort consisted mostly of a Caucasian population with a rather low background risk for GDM. In addition, we did not perform extensive interviews to assess the tolerance of tests and reasons for the preference of GDM screening method.

In conclusion, our study showed that most women preferred a two-step screening strategy with GCT for GDM. In addition, we show that the preference of GDM screening method differed by metabolic risk profile of participants and tolerance of tests.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics committee of UZ Leuven, Leuven, Belgium. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KB, PC, and CMa conceived the project. CMo prepared the data and ALa did the statistical analysis. LR did the literature review. LR and KB wrote the first draft of the manuscript. All authors contributed to the study design, including data collection, data interpretation and manuscript revision. The corresponding author LR had full access to all the data in the study and had final responsibility for the contents of the article and the decision to submit for publication. 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.2021.781384/full#supplementary-material

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COVID-19 Delta Variant-of-Concern: A Real Concern for Pregnant Women With Gestational Diabetes Mellitus

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The emergence of the COVID-19 Delta variant-of-concern (VOC), a novel variant of SARS-CoV-2, has threatened the total health systems throughout the world. This highly contagious strain is spreading at a higher exponential rate than any other variants of COVID-19 by infecting and subsequently killing hundreds of thousands of people globally. Among the most sensitive groups, pregnant women are at high risk of increased hospitalization, pneumonia, respiratory support, and admission to intensive care units during the Delta period. Pregnant people with gestational diabetes mellitus (GDM) are at increased chances of Delta VOC infection. GDM patients are nine and three times more likely to be infected by Delta VOC than those pregnant patients suffering from diabetes and cardiovascular diseases and hypertension, respectively. Additionally, they are more vulnerable to Delta VOC infection than wild-type and Alpha COVID-19 VOC ones. Thus, this review critically sheds light on the current scenario of the vulnerability of pregnant mothers, especially those with GDM, to Delta VOC infection.

Keywords: gestational diabetes mellitus, COVID-19, SARS-CoV-2, Delta VOC, pregnant woman

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INTRODUCTION

The world economy and healthcare systems have collapsed due to the ongoing COVID-19 pandemic (1). The adverse effects of the pandemic on pregnancy have been reported to affect maternal and perinatal health, resulting in higher morbidity and mortality (2–7). Additionally, the emergence of the new SARS-CoV-2 Delta strain, regarded as a variant-of-concern (VOC), has worsened the wellbeing of pregnant women and their babies (8). Pregnant people with comorbidities of diabetes, cardiovascular diseases, and hypertension are vulnerable to COVID-19 infections as much as twice (9). Among the most common pregnancy complications is gestational diabetes mellitus (GDM), defined as an increase in blood sugar (glucose) level during pregnancy due to insulin resistance or insufficient insulin production. This is associated with poor maternal health (depression, preeclampsia, and high risk of cesarean section) and neonatal outcomes (large gestational age, hypoglycemia, high risk of macrosomia, and type 2 diabetes mellitus at adulthood) (10). Here, we have critically reviewed the susceptibility of pregnant women with GDM to Delta VOC infection to inform pregnant mothers, their caretakers, and policymakers to minimize associations of medical complications during pregnancy. In addition, this timely mini

review will be a resource providing information on healthcare for the layperson to understand the sensitive complications of SARS-CoV-2 infections during pregnancy.

REVIEW PROTOCOL

Search Strategy

PubMed, Scopus, and Google Scholar were the sources of literature searching up to December 2019–September 2021, whereas the Centers for Disease Control and Prevention (CDC), European Centre for Disease Prevention and Control (ECDC), and the WHO provided up-to-date data, statistics, and health-related information about COVID-19. Search terms included COVID-19 or SARS-CoV-2 or 2019-nCoV or novel coronavirus 2019, COVID-19 variant-of-concern, Delta VOC, COVID-19 Delta VOC, COVID-19 variants, gestational diabetes, and evolutionary genomics.

Selection Criteria

The references of selected original articles, reviews, perspectives, meta-analyses, reports, and guidelines were manually searched. For the data mining and literature review, only those articles, published reports, statistics, or preprints that had been subjected to only COVID-19 and gestational diabetes were taken into consideration. All the authors enlisted herein mutually agreed upon the handpicked articles or reports for analysis during the preparation of the manuscript. Prominence was given to contemplation of information for the average person as well as for general medical readers.

SARS-COV-2 DELTA VARIANT-OF-CONCERN AND GESTATIONAL DIABETES MELLITUS

SARS-CoV-2 has continuously been evolving as a new variant since it was first identified in China through genetic mutations occurring during the replication of the genome. A variant, by definition, contains one or more mutations that separate it from other variants of the SARS-CoV-2 viruses. During the COVID-19 pandemic, different variants of SARS-CoV-2 have already been documented globally (11). Based on potential consequences of transmissibility, severity, and immunity of SARS-CoV-2 on epidemiological situation, variants were classified into i) variant-being-monitored (VBM), ii) variant-of-interest (VOI), iii) VOC, and iv) variant-of-high-consequence (VOHC) (12). The COVID-19 Delta VOC (B.1.617.2), a double mutated variant (E484Q and L452R) of wild-type SARS-CoV-2 (Wuhan strain), was first identified in India in December 2020 (13). In existing time, it is spreading throughout the globe at an alarming rate, faster than previous variants of SAR-CoV-2, and becoming the dominant strain in more than a dozen of countries; it is associated with high transmissibility (40%–60% more than Alpha variant and twice than Wuhan strain) and an increased risk of hospitalization (14, 15). The Delta variant is now responsible for more than 83% and 90% of COVID-19 cases being reported in the United States and the United Kingdom, respectively (15–17). In the European Union/European Economic Area (EU/EEA), in accordance with ECDC, 90% of new COVID-19 infections will be owing to the Delta variant by the end of August 2021 (16). Among the vulnerable groups of COVID-19 infections, pregnant women are more likely to get severe illness and symptoms of COVID-19 along with the possible requirement of admission to intensive care units since they are immunocompromised while Delta is highly contagious and infectious (18, 19). New surveillance by UK Obstetric Surveillance System reported an increase in the rate of hospitalization of pregnant women (54.2%) amid the spread of the Delta variant with severe illness than its previous waves (8). Although the association of disease severity with the Delta VOC infections during the period of pregnancy has not been well studied like wild-type COVID-19 strain and is until now under investigation, certain studies on the Delta-dominant period during pregnancy reported severe infections with worse pregnancy outcomes in contrast to the wild-type infections (8, 20). Pregnant women with preexisting medical complications like diabetes, hypertension, asthma, and cardiovascular diseases are more sensitive to COVID-19 infections, posing worse scenarios during pregnancy (8, 19, 20). Because GDM develops during pregnancy with an estimation of 1–28% depending on geographical locations, population characteristics, and diagnostics (10), it is imperative to know the possible risks and complications associated with COVID-19 infections, especially with the predominant Delta VOC to ensure maternal and neonatal sound health. A recent national and prospective observational cohort study in the United Kingdom consisting of 3,371 pregnant women revealed that infection severity increased during the Delta period (n = 171, 45%) compared with the wild-type (n = 1,435, 24.4%) and Alpha periods (n = 1,765, 35.8%) with increased risks of pneumonia (n = 171, 36.8%) and requiring more respiratory support (n = 171, 33.3%) and admission to intensive care units (n = 171, 15.2%). During the Delta period, the majority of pregnant people admitted to the hospital were aged ≥35 years (22.9%), and most of the pregnant women across all three periods were overweight or obese. The number of women admitted in the Alpha (n = 247, 14.0%) and Delta (n = 23, 13.5%) periods with one or more comorbidities was higher compared with that in the wild-type period (n = 169, 11.8%) (8). The study also showed that pregnant women having GDM are more vulnerable to the Delta VOC infection (n = 171, 11.1%) than wild-type variant (n = 1,435, 10.2%) and Alpha VOC (n = 1,765, 10.4%) (**Figure 1**). Additionally, GDM patients are among those groups that are most sensitive to Delta infections (n = 171, 11.1%), which is in contrast to pregnant people who have comorbidities like diabetes (n = 171, 1.2%), hypertension (n = 171, 2.9%), asthma (n = 171, 8.8%), and cardiovascular diseases (n = 171, 1.2%) (**Figure 2**) (8). During the Delta period, GDM patients are nine and three times more vulnerable to the Delta VOC infection than those with diabetes and cardiovascular diseases, and hypertension, respectively (**Figure 2**). A retrospective cohort study from India concluded that pregnant women with GDM during the Delta VOC dominant second wave had higher rates of infection than those in the first wave (20). Thus, Delta VOC provides an increased risk of infection to

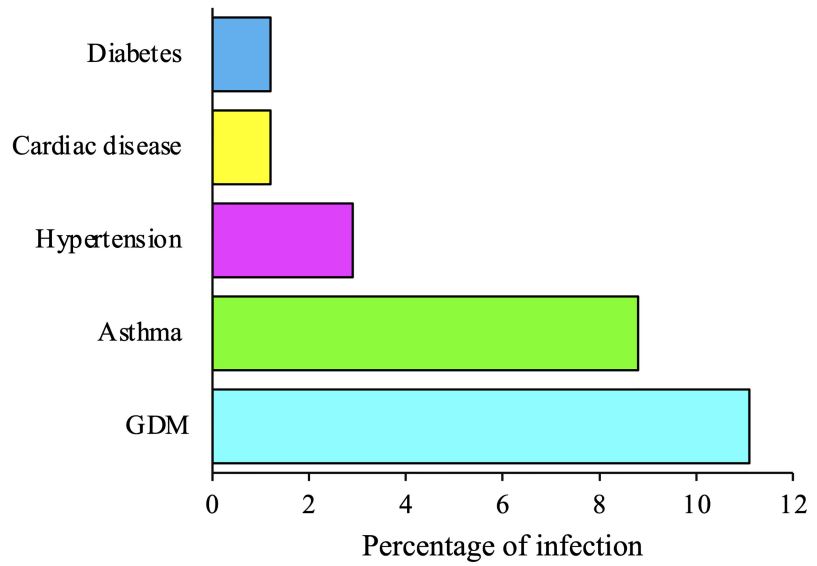

FIGURE 1 | COVID-19 infection of pregnant women with gestational diabetes mellitus (GDM) and comorbidities during Delta period. Schematic representation of GDM infections during COVID-19 Delta variant-of-concern (VOC) period along with comorbidities like diabetes, cardiovascular disease, hypertension, and asthma.

pregnancy, and pregnant mothers with GDM are more prone to Delta infections. However, more robust estimates of the maternal complications during pregnancy arising due to the Delta VOC infections are urgently needed to figure out health hazards associated with pregnancy.

MANAGEMENT OF GESTATIONAL DIABETES MELLITUS DURING THE DELTA VARIANT-OF-CONCERN PERIOD

Pregnant women are more vulnerable to becoming severely ill from COVID-19 than non-pregnant women of a similar age in accordance with the WHO (17). Approximately 1 in 10 women admitted to the hospital with symptoms of COVID-19 require intensive care, and 1 in 5 pregnant women give birth prematurely (19). Since pregnant women with GDM have higher chances of infection during the Delta period, avoiding exposure to COVID-19 is the best way to prevent the disease. Vousden et al. conducted a study at the national level in the United Kingdom covering the wild-type, Alpha, and Delta periods and showed that over 99% of pregnant women admitted to the hospital with symptomatic COVID-19 were not unimmunized, which provides good news for pregnant women to be vaccinated against COVID-19 (8). Because the admissions of pregnant women to the hospital with COVID-19 are increasing and pregnant women appear to be more severely affected by the Delta variant of the disease, it is urgent to prioritize vaccination to pregnant women (8). Pregnant women with/without GDM or

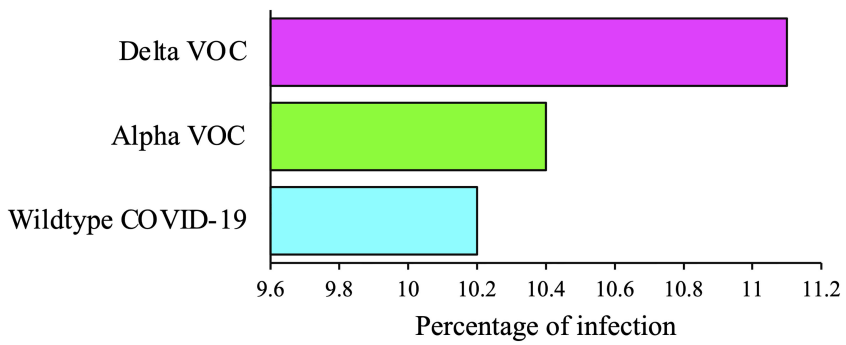

FIGURE 2 | Gestational diabetes mellitus (GDM) with COVID-19 infections during wild-type, Alpha, and Delta periods. Comparison of COVID-19 infection rates in percentage among pregnant women with GDM during wild-type, Alpha VOC, and Delta VOC periods.

comorbidities should wear masks, practice good hand hygiene, and maintain social distance to protect pregnant themselves from infection (21). Importantly, wearing masks and maintaining physical distance have been shown to be effective to reduce the disease spread because the primary route of the viral transmission is through respiratory particles, and they transmit from both symptomatic and asymptomatic individuals. Thus, lowering disease spread requires limiting contacts of infected individuals *via* social distancing, and reducing the probability of transmission per contact *via* wearing masks (22). Pregnant women should regularly check blood glucose, avoid crowded places or mass gatherings, consult with clinicians regularly, and practice healthy life programming during the Delta period (23). Due to COVID-19 pandemic-controlling measures like lockdown, pregnant people with GDM or any other medical complications might have limited access to healthcare, medication, healthy diet, and healthy lifestyle; hence, self-care, in this situation, is the most prioritized measure to be adopted for these patients (24–28). During the COVID-19 pandemic, therapeutic approaches like telemedicine and digital care have been practiced globally, and these have become more effective, fruitful, promising, and successful in optimizing pregnancy care during the COVID-19 pandemic (23, 24, 26). Indeed, the tele-/digital medicine/care helps to avoid physical contact among those who are infected, uninfected, or clinicians in many situations like during quarantine or self-isolation or lockdown, thereby maintaining social distance to lower the spread of COVID-19. Although the remote monitoring of COVID-19 patients *via* telemedicine or other e-health technologies has improved the healthcare system, the deployment of e-devices and digital applications utilized to enhance screening and monitoring disease progression require accessibility for commoners and practitioners.

FUTURE RESEARCH GUIDANCE

Although the Delta variant is spreading globally at a faster rate than any other previous variants of SARS-CoV-2, to our knowledge, until now there have been no national statistics or research surveillance studies conducted that address medical complications associated with Delta infections, especially in pregnant women with GDM. Since GDM patients have increased risks of pre-eclampsia, depression, and requirement of cesarean section, and the newborns are at an increased risk of becoming overweight and jaundice and developing type 2 diabetes, or even having a chance of stillbirth (29), it is urgent to conduct studies about pregnancy-associated complications following a Delta infection.

CONCLUDING REMARKS

Delta-dominant period involves more severe infection and poor pregnancy outcomes than the other strains of SARS-CoV-2. In this scenario, a clear instructional piece of advice has been provided for the care of the mother and baby. Pregnant mothers, regardless of whether they have GDM or any preexisting medical complications, need to focus on self-care and be vaccinated against SARS-CoV-2 as early as possible. A policy should be made and implemented by prioritizing pregnant women under a quick vaccination program. Additional data are required from surveillances and cohort studies on SARS-CoV-2 infections during pregnancy for better understanding and patients' counseling.

AUTHOR CONTRIBUTIONS

MM conceptually designed this study and wrote the manuscript. MK reviewed the overall manuscript by making critical comments and valuable inputs. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Fasting Plasma Glucose Mediates the Prospective Effect of Maternal Metal Level on Birth Outcomes: A Retrospective and Longitudinal Population-Based Cohort Study

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Objective: Previously, we found that the presence of maternal serum metals before the 24th week of gestation prospectively increased fasting plasma glucose (FPG) at 24–28 weeks. We further explored the prospective association between levels of metals and neonatal outcomes and assessed the mediating effects of FPG on these relationships.

Methods: A total of 7,644 pregnant women were included in a retrospective cohort study, and the relationships between metals [manganese (Mn), copper (Cu), lead (Pb), zinc (Zn), and magnesium (Mg)] and birth outcomes were explored. Quantile and linear regressions were performed to detect the shifts and associations between metals and neonatal size distribution focused on the 10th, 50th, and 90th percentiles. Mediation analysis was performed to assess the mediating effect of FPG on metals and birth outcomes.

Results: After adjustment, a 50% increase in Mn and Zn levels was related to a 0.136-cm (95% CI: 0.067–0.205) and 0.120-cm (95% CI: 0.046–0.193) increase in head circumference, respectively. Based on head circumference distribution, the magnitude of the association with Mn was smaller at the upper tail, while the magnitude of correlation with Zn was greater at the upper tail. A 50% increase in Mn and Zn levels was related to a 0.135-cm (95% CI: 0.058–0.212) and 0.095-cm (95% CI: 0.013–0.178) increase in chest circumference, respectively. The magnitude of the association with Mn increased with increasing chest circumference, while the magnitude of correlation with Zn decreased with increasing chest circumference. FPG explained 10.00% and 17.65% of the associations of Mn with head and chest circumference. A positive indirect effect of Zn associated with head circumference (0.004, 95% CI: 0.002–0.006) and chest circumference (0.005, 95% CI: 0.003–0.008) through FPG was also observed, and the estimated proportion of the mediating effect was 13.79% and 26.32%, respectively.

Conclusion: Maternal serum Mn and Zn levels before the 24th week of gestation may prospectively increase the circumference of the neonatal head and chest. FPG at 24–28 weeks had positive mediating effects on these relationships. Further research is needed to identify a balance between maternal blood glucose and birth size.

Keywords: metal, fasting plasma glucose, birth outcome, mediating effect, cohort study

INTRODUCTION

Environmental exposure including metals in pregnant women has been associated with health effects in both the mother and her offspring (1, 2). Such metals can enter the maternal body through air, water, food, and other forms of exposure and, thus, have an impact on health (3). Moreover, the metals can have greater health implications on the offspring through the mother as fetal organs and systems are in a critical period of plasticity and sensitivity to the environment (4). Metal exposure has been related to fetal growth in the short term, and it could also result in various health problems in the long term, such as neurodevelopment and cardiovascular disease (5, 6).

Pregnant women are in a critically important state for susceptibility to metal effects because of hemodynamics, hormone changes, and immature immune systems (7), and metals could further affect fetal growth after passing through the placental barrier (8). Studies have found that prenatal exposure to certain ranges of manganese (Mn) (9), copper (Cu) (10), lead (Pb) (11), zinc (Zn) (12), and magnesium (Mg) (13) is linked to adverse birth outcomes, including small-for-gestational-age (SGA) births, low birth weight, and preterm delivery. Similarly, low-level prenatal Pb exposure, as well as elevated Mn and Zn levels, might increase the risk of preterm birth and SGA (14). Moreover, increasing concentrations of Mn have been linked to decreasing birth weight (15). However, the associations between these metals and birth outcomes have been inconsistent. Increasing placental Mn could slightly increase neonatal head circumference in females (16). Pb has been shown to elevate the risk of SGA in infants (11) and was inversely associated with birth weight, birth length, and head circumference in observational studies (17). Furthermore, Mg supplementation during pregnancy might increase birth weight (13). Moreover, prenatal exposure to Pb and Mn has been assessed as showing no relation to birth weight and length (8). Additionally, there seems to be an optimal range of exposure to some metals; for instance, both low and high levels of Mn are associated with lower birth weight and smaller head circumference (18). Meanwhile, an optimum level of Zn and Cu in pregnant women could reduce the risk of low birth weight (19).

Fasting plasma glucose (FPG) is predominantly produced following the decomposition of liver glycogen, while basal insulin secretion can inhibit the output of liver glycogen and prevent higher FPG. Therefore, the level of fasting blood glucose can objectively reflect the secretion level of basal insulin in patients. Elevated metal levels can accumulate in pancreatic tissue where they may contribute to insulin resistance or damage islet β cells and impair insulin secretion (20). Mn has been shown to inhibit glucose-stimulated insulin secretion in β cells (21), while higher urinary Zn or Pb could increase FPG of Chinese adults (22). In addition, serum Mg levels have been negatively associated with fasting insulin, and higher copper concentrations could increase the risk of glucose dysregulation during pregnancy (23, 24). Our previous study (25) also found that metals including Mn, Cu, Pb, Zn, and Mg in early pregnancy were prospectively related to later FPG in pregnant women. Moreover, high FPG levels have been linked to macrosomia and large-for-gestational-age infants (26). It has also been revealed that correlations between postprandial blood glucose and primary outcomes are weaker than for FPG (27). Nevertheless, we found no relevant studies that clarify the relationship among metals, FPG, and birth outcomes, especially in a longitudinal direction of complete pregnancy.

Therefore, after combining our previous study with those of others, we hypothesized that earlier metal exposure in pregnant women was prospectively associated with final birth outcome, and later FPG mediated their relationship. Here, we tested this hypothesis in a retrospective and longitudinal population-based cohort study to explore i) whether maternal early metal exposure including Mn, Cu, Pb, Zn, and Mg before 24 weeks was related to birth outcomes and ii) whether a maternal FPG of 75 g oral glucose tolerance test (OGTT) at 24–28 weeks mediated the relationship between metal exposure and birth outcomes.

METHODS

Study Population

The retrospective cohort study was conducted between January 2017 and December 2018 in Foshan, China. Pregnant women were recruited at the first obstetric clinic visit in the Southern Medical University Affiliated Foshan Women and Children Hospital. The study was approved by the Human Subjects Committee of our hospital. The inclusion criteria were as follows: 1) singleton pregnancy, 2) no diabetes prior to pregnancy, and 3) gestational age in the first trimester (<14 weeks). The exclusion criteria were as follows: 1) no available data on metal detection before 24 weeks of pregnancy (<24 weeks), 2) missing data of OGTT at 24–28 weeks of gestation, and 3) stillbirth or without delivery data in our hospital. Of 11,845 women who initially met the above inclusion criteria, 7,646 were ultimately included for the study (**Figure S1**).

Data Collection and Definition of Variables

Medical data were collected from hospital computerized databases or clinical charts. The information of maternal

demographic characteristics included maternal age, employment status, maternity insurance, family history of diabetes, family history of hypertension, smoking habit, and drinking habit. The clinical history information included parity, and prenatal visit records consisted of gestational age and BMI at the first prenatal examination in the first trimester; gestational age for metal detection; serum levels of the metals Mn, Cu, Pb, Zn, and Mg; OGTT results at 24–28 weeks; diagnosis of gestational diabetes mellitus (GDM) and hypertensive disorders of pregnancy (HDP); and weight gain during the whole pregnancy. The delivery records (e.g., gestational age at delivery, neonatal sex, birth weight, birth length, head and chest circumference) were retrieved. All identifying patient information was anonymized to protect patient identity.

The diagnostic criteria for GDM referred to the criteria of the International Association for Diabetes in Pregnancy Study Group (IADPSG): pregnant women were given OGTT at 24–28 weeks. The results were classified based on one of the following cutoff points: fasting plasma glucose (FPG, OGTT0) ≥5.1 mmol/L or 1-h PG (1-h postprandial plasma glucose, OGTT1) ≥10.0 mmol/L or 2-h PG (2-h postprandial plasma glucose, OGTT2) ≥8.5 mmol/L (28). First-trimester BMI (kg/m^2) was divided into categories according to Chinese BMI criteria for general adults: BMI < 18.5 kg/m^2, 18.5 kg/m^2 ≤ BMI < 24 kg/m^2, 24 kg/m^2 ≤ BMI < 28 kg/m^2, and BMI ≥ 28 kg/m^2 (29). Hypertensive disorders of pregnancy (HDP) included gestational hypertension, preeclampsia, eclampsia, superimposed preeclampsia on chronic hypertension, and chronic hypertension in pregnancy (30).

Measurement of Serum Metal Concentration

Metal detection was performed according to the methods described in our previous article (25). Maternal serum metal concentration was assessed only once before 24 weeks of pregnancy in our hospital. Peripheral venous blood samples (2 ml) were drawn in the obstetric clinic and then transported to the Department of Laboratory Medicine of the hospital within 1 h for serum metal detection, using the polarography method (AS-9000 C, AWSA, Wuhan, China). The detection limit of the polarographic channel was ≤1 × 10^{-8} mol/L. All of the metal measurements were above the limit. The coefficient of variation (CV) of detection was ≤1%, and the relative error (B) of detection was ≤1%.

Statistical Analysis

Baseline characteristics were described as mean ± SD or number (%). In the analysis of birth outcomes, maternal serum metal levels were categorized into three parts according to tertiles. Comparisons for birth outcomes among three groups of metals used ANOVA, and the pairwise comparisons were conducted using Dunnett with a control category of the 1st tertile. The analyses were conducted using SPSS 24.0 software.

Quantile regression was performed to detect the distribution shifts of neonatal size (e.g., head circumference) and to explore the associations with metal levels primarily occurring at the tails of neonatal size. In particular, the 10th, 50th, and 90th percentiles of neonatal size were focused on. The metal linear regression was established for each percentile and the mean of neonatal size. Metal level was log-transformed based on a 1.5 logarithm function, assuring that 1-unit change on the transformed scale was close to or within the IQR. One-unit difference on the natural log-transformed scale corresponded to a much wider range than the IQR on the original range (24). The models were adjusted for the following factors: maternal age, employment status, parity, maternity insurance, family history of diabetes, family history of hypertension, HDP, GDM, BMI, gestational age for metal detection, weight gain during pregnancy, infant sex, and gestational age at delivery. These analyses were performed in R 3.5.2, using the packages quantreg and forestplot in quantile regression.

In our previous study (25), we found significant associations between Mn, Cu, Pb, Zn, and Mg and OGTT0. Thus, we aimed to further explore the mediating effect of OGTT0 on metals and birth outcomes. Mediation analysis, one model of the structural equation modeling, was performed using the robust maximum-likelihood estimation. Standardized coefficients of direct, indirect, and total effects were estimated, and the 95% CI was measured using the bootstrap method with 2,000 resamplings. The model was adjusted for the following factors: maternal age, employment status, parity, maternity insurance, family history of diabetes, family history of hypertension, HDP, BMI, gestational age for metal detection, weight gain during pregnancy, infant sex, and gestational age at delivery. The mediation proportion of indirect effect was calculated as follows: Estimated mediated = Indirect effect/Total effect × 100%. Total effect = Direct effect + Indirect effect. The data were analyzed using Mplus 7.4 (Muthén and Muthén). A two-sided *P*-value <0.05 was considered to indicate statistical significance.

RESULTS

Baseline Characteristics

Tables 1 and **S1** show the characteristics of our study population. The mean maternal age was 30.13 years. Of the pregnant participants, 55.45% were employed and 67.51% of those had maternity insurance. None of the participants had smoking or drinking habits. Only 0.47% of women had a family history of diabetes. However, the incidence of GDM was 15.93%. The mean BMI in the first trimester was 21.38 kg/m^2, and the mean weight gain during the whole of pregnancy was 12.70 kg. The mean gestational age at delivery was 38.47 weeks. The mean birth weight, birth length, head circumference, and chest circumference of the newborns were 3,155.13 g, 49.12 cm, 32.89 cm, and 32.70 cm, respectively.

Distribution of Metals and the Association With Birth Outcomes

The mean gestational age of metal detection was 16.56 weeks (**Table 1**). **Table S2** shows the geometric mean and percentiles of

TABLE 1 | The baseline characteristics of the study population in the cohort study.

Characteristics	Total (*n* = 7,646)
Maternal characteristics	
Maternal age (mean ± SD, years)	30.13 ± 4.70
Age < 30 (*n*, %)	3,749 (49.03)
30 ≤ age < 35 (*n*, %)	2,556 (33.43)
Age ≥ 35 (*n*, %)	1,341 (17.54)
Employment status (*n*, %)	
Unemployed	3,406 (44.55)
Employed	4,240 (55.45)
Parity (*n*, %)	
0	3,685 (48.20)
1	3,726 (48.73)
≥2	235 (3.07)
Maternity insurance (*n*, %)	5,162 (67.51)
Family history of diabetes (*n*, %)	36 (0.47)
Family history of hypertension (*n*, %)	31 (0.41)
Smoking habit (*n*, %)	0
Drinking habit (*n*, %)	0
GDM (*n*, %)	1,218 (15.93)
HDP (*n*, %)	130 (1.70)
First prenatal examination in the first trimester	
Gestational age (mean ± SD, weeks)	11.13 ± 1.55
BMI (mean ± SD, kg/m^2)	21.38 ± 3.09
BMI < 18.5 (*n*, %)	1,212 (15.85)
18.5 ≤ BMI < 24 (*n*, %)	5,107 (66.79)
24 ≤ BMI < 28 (*n*, %)	1,067 (13.96)
BMI ≥ 28 (*n*, %)	260 (3.40)
Gestational age for metal detection (mean ± SD, weeks)	16.56 ± 2.94
OGTT (mean ± SD, mmol/L)	
OGTT0	4.27 ± 0.38
OGTT1	7.82 ± 1.65
OGTT2	6.80 ± 1.40
Weight gain during pregnancy (kg)	12.70 ± 4.02
Birth characteristics	
Female (*n*, %)	3,595 (47.02)
Gestational age at delivery (mean ± SD, weeks)	38.47 ± 1.45
Birth weight (mean ± SD, g)	3,155.13 ± 436.44
Birth length (mean ± SD, cm)	49.12 ± 2.03
Head circumference (mean ± SD, cm)	32.89 ± 1.50
Chest circumference (mean ± SD, cm)	32.70 ± 1.81

HDP, hypertensive disorders of pregnancy; GDM, gestational diabetes mellitus; SD, standard deviation; BMI, body mass index; OGTT, oral glucose tolerance test; OGTT0, fasting plasma glucose; OGTT1, 1-h postprandial plasma glucose; OGTT2, 2-h postprandial plasma glucose.

the levels of five metals. Results from metals classified into tertiles indicated that head circumference in the second and third tertiles of Mn or Zn was significantly larger than that in the first tertile ($P < 0.05$) (**Table 2**). Chest circumference in the second and third tertiles of Mn was also significantly larger than that in the first tertile ($P < 0.05$). No significant difference of gestational age, birth weight, or birth length was observed among the three tertiles of all five metals.

In the further analysis, we explored the associations between metals and the distribution shifts of head and chest circumference. As shown in **Figure 1**, a 50% increase in Mn and Zn levels was related to a 0.136-cm (95% CI: 0.067–0.205) and 0.120-cm (95% CI: 0.046–0.193) increase in mean head circumference after adjustment, respectively. Based on the 10th, 50th, and 90th percentiles of head circumference distribution, the magnitude of the association with Mn was smaller at the upper tail, while the magnitude of correlation with Zn was greater at the upper tail. However, no significant association was found between Pb, Cu, and Mg levels and head circumference distribution.

The correlations of metals with chest circumference were similar to those of head circumference (**Figure 2**). After adjustment, a 50% increase in Mn and Zn levels was related to a 0.135-cm (95% CI: 0.058–0.212) and 0.095-cm (95% CI: 0.013–0.178) increase in mean chest circumference, respectively. The magnitude of the association with Mn was increased with the increase of the 10th, 50th, and 90th percentiles of chest circumference distribution, while the magnitude of correlation with Zn was decreased with the increase of the distribution shifts. There was also no relationship between Pb, Cu, and Mg concentrations and chest circumference distribution.

Mediating Effects of OGTT0 on Metals and Head and Chest Circumference

Because of the significant correlations of Mn/Zn and head/chest circumference, mediation analysis was performed to explore the

TABLE 2 | Association between metal concentrations and birth outcomes based on three different metal levels.

Metals	GA (weeks)	*P*-value[a]	BW (g)	*P*-value[a]	BL (cm)	*P*-value[a]	HC (cm)	*P*-value[a]	CC (cm)	*P*-value[a]
Mn (μmol/L)		0.977		0.801		0.504		**<0.001****		**0.014***
1st tertile (≤0.78)	38.56 ± 1.41	Ref	3,150.07 ± 432.22	Ref	49.08 ± 1.97	Ref	32.79 ± 1.45	Ref	32.62 ± 1.75	Ref
2nd tertile (0.79–0.86)	38.57 ± 1.50	0.996	3,158.07 ± 440.76	0.762	49.13 ± 2.09	0.574	32.94 ± 1.52	**0.001***	32.73 ± 1.84	0.052
3rd tertile (≥0.87)	38.57 ± 1.44	0.968	3,155.89 ± 436.44	0.823	49.14 ± 2.03	0.440	32.93 ± 1.52	**0.002***	32.76 ± 1.894	**0.011***
Cu (μmol/L)		0.081		0.564		0.383		0.665		0.105
1st tertile (≤18.00)	38.62 ± 1.39	Ref	3,158.68 ± 435.14	Ref	49.16 ± 1.98	Ref	32.90 ± 1.48	Ref	32.75 ± 1.79	Ref
2nd tertile (18.01–23.70)	38.55 ± 1.51	0.170	3,147.66 ± 438.10	0.511	49.10 ± 2.08	0.409	32.90 ± 1.50	0.998	32.70 ± 1.83	0.522
3rd tertile (≥23.71)	38.53 ± 1.44	0.062	3,158.06 ± 436.16	0.987	49.09 ± 2.04	0.357	32.86 ± 1.52	0.632	32.64 ± 1.81	0.063
Pb (μg/L)		0.953		0.481		0.421		0.572		0.381
1st tertile (≤19.60)	38.57 ± 1.44	Ref	3,151.82 ± 432.40	Ref	49.14 ± 2.00	Ref	32.91 ± 1.50	Ref	32.74 ± 1.80	Ref
2nd tertile (19.61–34.00)	38.56 ± 1.46	0.939	3,149.96 ± 434.40	0.983	49.14 ± 2.01	1.000	32.89 ± 1.49	0.919	32.68 ± 1.81	0.410
3rd tertile (≥34.01)	38.57 ± 1.45	0.997	3,163.60 ± 442.05	0.526	49.07 ± 2.09	0.421	32.89 ± 1.51	0.478	32.68 ± 1.82	0.350
Zn (μmol/L)		0.923		0.990		0.874		**0.006***		0.117
1st tertile (≤107.80)	38.56 ± 1.41	Ref	3,152.55 ± 430.64	Ref	49.10 ± 1.97	Ref	32.81 ± 1.44	Ref	32.64 ± 1.75	Ref
2nd tertile (107.81–120.00)	38.58 ± 1.49	0.891	3,154.71 ± 437.72	1.000	49.13 ± 2.03	0.838	32.93 ± 1.51	**0.007***	32.72 ± 1.83	0.177
3rd tertile (≥120.01)	38.57 ± 1.45	0.973	3,256.12 ± 441.13	0.988	49.12 ± 2.03	0.900	32.92 ± 1.54	**0.016***	32.70 ± 1.81	0.101
Mg (mmol/L)		0.809		0.976		0.304		**0.045***		**0.026***
1st tertile (≤1.31)	38.56 ± 1.41	Ref	3,154.41 ± 425.44	Ref	49.11 ± 1.98	Ref	32.90 ± 1.47	Ref	32.72 ± 1.77	Ref
2nd tertile (1.32–1.50)	38.58 ± 1.44	0.860	3,154.32 ± 443.57	1.000	49.17 ± 2.00	0.455	32.93 ± 1.48	0.694	32.75 ± 1.80	0.762
3rd tertile (≥1.51)	38.56 ± 1.49	0.977	3,156.71 ± 440.54	0.974	49.08 ± 2.11	0.873	32.83 ± 1.55	0.150	32.62 ± 1.87	0.086

GA, gestational age; BW, birth weight; BL, birth length; HC, head circumference; CC, chest circumference; Mn, manganese; Cu, copper; Pb, lead; Zn, zinc; Mg, magnesium.
**P < 0.05, **P < 0.001. Ref, reference; Bold value, P < 0.05.*
[a]*One-way ANOVA and pairwise comparisons using Dunnett with a control category of the 1st tertile.*

mediating effects of OGTT0 (**Figure 3**). After controlling for the influence of covariates, the results showed that OGTT0 level explained 10.00% (indirect effect: 0.004, 95% CI: 0.003–0.007, $P < 0.001$) and 17.65% (indirect effect: 0.006, 95% CI: 0.004–0.009, $P < 0.001$) for the associations of serum Mn concentration with head and chest circumference. A positive indirect effect of Zn association with head circumference (0.004, 95% CI: 0.002–0.006, $P < 0.001$) and chest circumference (0.005, 95% CI: 0.003–0.008, $P < 0.001$) through OGTT0 level was also observed, and the estimated proportions of the mediating effect were 13.79% and 26.32%, respectively. In conclusion, there were positive correlations between maternal serum Mn and Zn levels and neonatal head and chest circumference, and these positive correlations were mediated by OGTT0 glucose level.

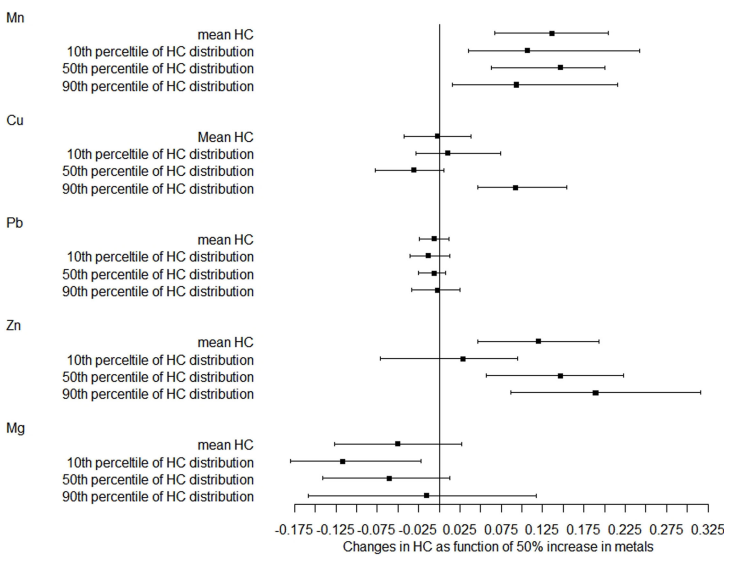

FIGURE 1 | Distribution changes in head circumference as a function of 50% increase in metals. The models were adjusted for maternal age, employment status, parity, maternity insurance, family history of diabetes, family history of hypertension, HDP, GDM, BMI, gestational age for metal detection, weight gain during pregnancy, infant sex, and gestational age at delivery. Mn, manganese; Cu, copper; Pb, lead; Zn, zinc; Mg, magnesium; HC, head circumference.

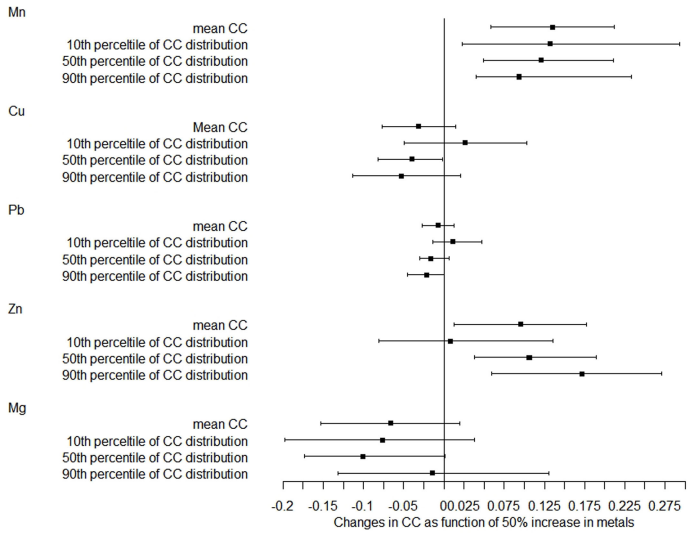

FIGURE 2 | Distribution changes in chest circumference as a function of 50% increase in metals. The models were adjusted for maternal age, employment status, parity, maternity insurance, family history of diabetes, family history of hypertension, HDP, GDM, BMI, gestational age for metal detection, weight gain during pregnancy, infant sex, and gestational age at delivery. Mn, manganese; Cu, copper; Pb, lead; Zn, zinc; Mg, magnesium; CC, chest circumference.

DISCUSSION

The current study showed that maternal serum Mn and Zn levels before 24 weeks of gestation could prospectively increase neonatal head and chest circumference. For the head circumference, the magnitude of the association with Mn was smaller at the upper tail, while the magnitude of correlation with Zn was greater at the upper tail. For the chest circumference, the magnitude of the association with Mn was greater at the upper tail, while the magnitude of correlation with Zn was smaller at the upper tail. In addition, FPG at 24–28 weeks mediated the positive effect of Mn or Zn exposure on head or chest circumference. No relationships between Mn and Zn and other birth outcomes including gestational age, birth weight, and birth length were found. No correlation between Pb, Cu, and Mg and birth outcomes was observed.

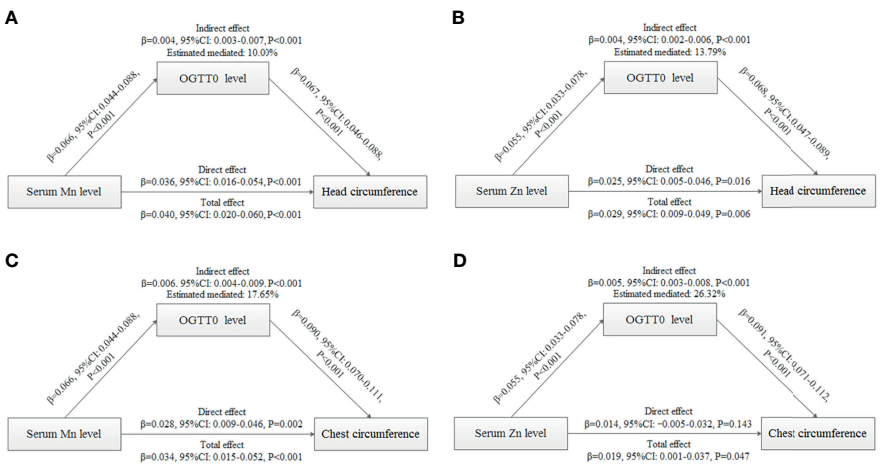

FIGURE 3 | Mediation analysis with standardized coefficients of metals, OGTT0, and birth outcomes. **(A)** Analysis of serum Mn level, OGTT0 level, and head circumference. **(B)** Analysis of serum Zn level, OGTT0 level, and head circumference. **(C)** Analysis of serum Mn level, OGTT0 level, and chest circumference. **(D)** Analysis of serum Zn level, OGTT0 level, and chest circumference. The model was adjusted for maternal age, employment status, parity, maternity insurance, family history of diabetes, family history of hypertension, HDP, BMI, gestational age for metal detection, weight gain during pregnancy, infant sex, and gestational age at delivery. All models were found to be statistically significant ($P < 0.001$).

To date, several studies have explored the association between Mn exposure and birth outcome. However, only a few articles report a prospective and positive relationship between early maternal Mn level and neonatal birth size and the mediating effect of FPG on them. Maternal blood Mn has previously been shown to be positively related to neonatal head circumference, but not to birth weight and birth length (31), which is consistent with our findings. However, our present results were contrary to some other published studies, as follows: maternal Mn level in erythrocytes in the second trimester has been demonstrated to be negatively linked to birth weight and head and chest circumferences (32). This might have contributed to a slightly higher exposure to environmental Mn among pregnant women living in this region (geometric mean Mn in our study: 0.81 μmol/L vs. 0.72 μmol/L in theirs). Furthermore, elevated umbilical cord blood Mn may decrease birth weight, but was not related to head circumference and birth length (15). Moreover, maternal serum Mn level was not associated with birth weight, but was negatively linked to head circumference (33). Although some studies have also found a positive correlation between metals and birth size, the results were different from ours. A previous report described that maternal Mn was related to higher birth length (34). Also, maternal hair but not blood Mn concentrations showed a positive linear association with infant chest circumference (35). This may be because the amount of Mn might vary in different parts of the body, leading to different correlations between Mn and birth outcomes. In addition, considering that the rapid increase in fetal body weight during late pregnancy is associated with an accelerated growth of fetal peripheral muscle and fat deposition, neonatal birth weight might be more affected by the intrauterine environment, including nutritional supplementation from the mother during pregnancy, than neonatal head and chest circumference (36). Neonatal head and chest growth might be more susceptible to metal exposure in early pregnancy than birth weight and length; however, birth weight and length might be more susceptible to the combined effects of maternal nutrition and maternal and paternal height and genes in late pregnancy. We cautiously assumed that the combined effects in late pregnancy might be stronger than the effect of Mn in early pregnancy and, thus, masked the effect of metals. In future studies, it will be important to further explore the relationship between neonatal birth size and metals by integrating nutrition, genes, and other factors during pregnancy.

The results of the current study further showed that maternal Mn might affect the birth outcome by influencing FPG during pregnancy. Mn, both an essential element and a potential toxicant, is a component of several enzymes and an activator for enzymes. Deficiency of Mn might affect growth and development of the body and glucose metabolism, and excessive intake of Mn could cause poisoning (37). Mn is suggested to inhibit glucose-stimulated insulin secretion in β cells by impairing mitochondrial function (21). One population-based study has shown a positive relationship of urinary Mn with hyperglycemia risk in Chinese coke oven workers (38). Meanwhile, our previous study found that early maternal serum Mn could increase FPG at 24–28 weeks (25).

Larger offspring size has been attributed mainly to higher pregnancy glucose levels. We made a cautious hypothesis that the long-term effects of fetal glucose exposure could affect fetal β-cell capacity, especially exposure to higher FPG, and thus, higher maternal FPG might indirectly affect neonatal birth outcomes. Therefore, FPG might mediate the effect of Mn level on birth outcome. However, there are currently no metal requirement guidelines for pregnant women. The Mn level of our study population was slightly higher than that found in other populations, and serum Mn levels in most pregnant women in our study were below the lower limit of the reference range (0.15–0.22 μmol/L) for healthy adults (39). Mn requirements for pregnant women greatly increase as the fetus grows (40); however, this high demand might impair the function of the islets and cause elevated blood glucose. Further research is needed to investigate the balance between maternal blood glucose and neonatal size.

Zn is a component of zinc-containing metalloenzymes involved in the activity of more than 80 enzymes. Zn is also an important immunomodulator and growth cofactor and plays an important role in anti-oxidation, anti-apoptosis, and anti-inflammation. About 90% of Zn in the body is distributed in muscles and bone (19). Blood Zn concentration is about 0.1~0.15 mmol/L in the general population (41), and our study population showed similar levels. Previous studies have reported that higher Zn levels in early pregnancy could increase head circumference (34), and prenatal Zn has been associated with greater chest circumference (42), which are consistent with our findings. Zn supplementation is also beneficial to head growth rate in infants (43). However, one report also states that maternal Zn level was inversely related to birth weight (12). It could be argued that the discordant results are owing to the different levels of original Zn exposure and different study populations with different underlying diseases affecting fetal growth. Head circumference in early life is an important indicator of brain development. Furthermore, it could affect neuronal growth and brain function (19). Brain development is incredibly complex and takes place in two major stages: the first 20 weeks involve fetal organogenesis and neurogenesis, and during weeks 20–40, there is continued neuronal growth, neural migration, and maturation. Brain development is very rapid, from 32 to 39 weeks when the brain can come to weigh 30% as much as an adult (44). In our study, metal exposure before 24 weeks of gestation had significant implications for the two stages of fetal brain development. Rapid brain growth increases vulnerability to unfavorable environmental conditions. In our study, although the head circumference increased slightly with the increase of Zn concentration, it also had some clinical significance. Large population studies are needed to explore the relationship between small differences in neonatal head circumference and brain function. In addition, Zn and Mn were related to birth head and chest circumference but had no relationship with birth weight and length. This might be associated with neonatal birth size as Zn has the same effect as described for

Mn above. Zn is also an essential trace element for insulin synthesis, storage, and release (45). However, excess levels of Zn during embryogenesis could disturb biochemical processes and could be teratogenic or ultimately fatal (19). We found that Zn prospectively increased late second-trimester FPG in our previous study (25) and Zn could further increase the head and chest circumference through higher levels of FPG. The relationship of Zn among the three (Zn, FPG, and birth outcome) might be similar to that of Mn. These findings require further epidemiological and molecular studies in both the fetal stage and the childhood stage.

Our current study found no association between Pb and birth outcomes, which is consistent with a previous study (15). However, Pb has been reported to decrease birth weight and head circumference following fetal lead exposure during pregnancy and lactation (46, 47). The normal range of Pb in the general population is <50 μg/L (48), and the Pb levels of our participants were within the normal range. Therefore, Pb level in the normal range might not be associated with birth outcomes, but higher levels above the normal range might have toxic effects on neonatal birth size.

Excess Cu is harmful to human health, but it is rarely excessive in the general population (49), and the demand for Cu is significantly increased in pregnant women (19). Cu deficiency during pregnancy might result in oxidative stress that can reduce fetal growth (19). Serum Cu <350 μg/L (5.51 μmol/L) is linked to conditions of Cu deficiency (50). The minimum concentration in our study population was 7.60 μmol/L. Therefore, we speculated with caution that a significant association between maternal Cu exposure and adverse birth outcomes might not be observed at normal concentrations. The underlying mechanisms need further exploration.

The reference range for serum Mg in adults is 0.75 mmol/L (5% CI: 0.45, 1.05) (51); our study population had a slightly higher level. There is an increasing need for Mg throughout pregnancy, and Mg supplementation might have neuroprotective effects on preterm infants (52). However, limited studies have reported the association between Mg and birth size. Most prenatal Mg supplementation is given in the third trimester, in order to protect neurologic function in preterm infants. Although we found that Mg in early pregnancy was not associated with birth outcome, an appropriate Mg range should be established for pregnant women. Further studies are needed to determine whether Mg in early pregnancy is prospectively related to neurodevelopment of newborns in the long term.

The current study was the first study to show that metal exposure in early pregnancy could affect FPG in the late second trimester and, thus, affect neonatal head and chest circumference. This information may promote new regimens for the personalized control and management of birth size during pregnancy. The study sample size was also relatively large, which increased the accuracy of the findings and made them more applicable to other populations. However, some limitations in the study should be noted. First, despite the large sample size, none of the pregnant women in our study had a smoking or drinking habit. This limits the value of the study. Smoking and drinking habits were investigated in each pregnant woman during prenatal visits as they are risk factors for adverse pregnancy and birth outcomes, not just for the birth size of the newborn. This could be a good habit of women in southern China, although it is impossible to rule out a few cases of concealment. Second, our research was a single-center study; multicenter studies are needed in the future to make the results more precise and reliable. Third, data related to trace element supplementation during pregnancy in this study were not available, which might be a confounding factor affecting the results. We are currently conducting a prospective birth cohort study, aiming to collect more detailed and accurate data. Fourth, the study only examined maternal metal exposure in early pregnancy before 24 weeks, not in the third trimester, placenta, or umbilical cord blood. Metal requirements for pregnant women might vary in different trimesters, and the placental barrier might support different metal effects between mother and offspring. Further epidemiological and molecular studies should be conducted.

In conclusion, our study suggests that maternal serum Mn and Zn levels in early pregnancy may prospectively increase neonatal head and chest circumference. FPG in the late second trimester could positively mediate the association of Mn and Zn exposure with head or chest circumference. However, maternal Mn and Zn levels in early pregnancy were not associated with birth weight and birth length. Further studies should explore the relationship between neonatal birth size and metals by integrating maternal nutrition, living habits, the genes of both parents, and other factors throughout pregnancy. In addition, further research is needed to find a balance between maternal blood glucose and neonatal birth size; the corresponding epidemiological and molecular studies need to explore and reveal the molecular mechanisms.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Southern Medical University Affiliated Foshan Women and Children Hospital (FSFYMEC-2019-024). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

ZZ, DS, FW and ZL conceived and designed the study. ZZ, DY, GC, PL, LW, JY, DL, JR, DF, XG, HW and XG carried out data

collection. ZZ carried out the statistical analyses and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Supplementary Figure 1 | Follow charts of study population.

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Putrescine as a Novel Biomarker of Maternal Serum in First Trimester for the Prediction of Gestational Diabetes Mellitus: A Nested Case-Control Study

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Aims: Early identification of gestational diabetes mellitus (GDM) aims to reduce the risk of adverse maternal and perinatal outcomes. Currently, no acknowledged biomarker has proven clinically useful for the accurate prediction of GDM. In this study, we tested whether serum putrescine level changed in the first trimester and could improve the prediction of GDM.

Methods: This study is a nested case-control study conducted in Beijing Obstetrics and Gynecology Hospital. We examined serum putrescine at 8-12 weeks pregnancy in 47 women with GDM and 47 age- and body mass index (BMI)-matched normoglycaemic women. Anthropometric, clinical and laboratory variables were obtained during the same period. The receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to assess the discrimination and calibration of the prediction models.

Results: Serum putrescine in the first trimester was significantly higher in women who later developed GDM. When using putrescine alone to predict the risk of GDM, the AUC of the nomogram was 0.904 (sensitivity of 100% and specificity of 83%, 95% CI=0.832–0.976, P<0.001). When combined with traditional risk factors (prepregnant BMI and fasting blood glucose), the AUC was 0.951 (sensitivity of 89.4% and specificity of 91.5%, 95% CI=0.906-0.995, P<0.001).

Conclusion: This study revealed that GDM women had an elevated level of serum putrescine in the first trimester. Circulating putrescine may serve as a valuable predictive biomarker for GDM.

Keywords: putrescine, gestational diabetes mellitus, biomarker, prediction, receiver operating characteristic (ROC)

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INTRODUCTION

Gestational diabetes mellitus (GDM), a common gestational disorder, has been defined as glucose intolerance with onset or first detection during the second or third trimester (1). GDM has short and long-term adverse effects on the health of the mother and their offspring. Women with GDM are more likely to have preeclampsia, caesarean section and are likely to have a higher rate of developing type 2 diabetes mellitus (T2DM) and cardiovascular disease in the postpartum period than those without GDM (2–4). For neonates, GDM increases the risk of macrosomia, hypoglycaemia, shoulder dystocia, and respiratory distress syndrome (5). Additionally, the offspring of GDM women are more likely to develop obesity and abnormal blood glycaemia in later life (6). Currently, the diagnosis of GDM is based on the oral glucose tolerance test (OGTT) which is generally performed in the second trimester of the pregnancy. Although there is no consensus about screening algorithms and diagnostic criteria for GDM before 24 weeks of gestation, risk stratification in the first trimester could be beneficial to reduce adverse complications associated with GDM due to timely and targeted lifestyle interventions such as physical exercise and dietary changes (7). Thus, establishing a simple, practical prediction model of GDM according to the identified risk factors in the first trimester is of great clinical significance. However, the present predictive biomarker and model of GDM did not show a pronounced and reliable predictive value (8).

Putrescine is one of the predominant polyamine in mammalian cells and is pervasive in a wide range of organisms because it is necessary for cell growth and proliferation (9). Disturbance of the gut microbiota can cause an imbalance in polyamine metabolism, which may be related to the pathological development of metabolic diseases (10, 11). In our former study, we had reported that women with GDM already had different components of gut microbiota than normoglycaemic women in the first trimester (12). Through the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation and pathway enrichment analysis, we correlated multiple small molecular metabolites with gut microbiota that enriched in GDM. We found that seven intestinal bacteria enriched in GDM were significantly correlated with the metabolism of putrescine. Furthermore, putrescine has recently been shown to associate with the disrupted tight junction (TJ) integrity in the colon (13). It could decrease the intestinal mucosal barrier function and increase bacterial translocation, contributing to the onset of systemic inflammation-associated metabolic disorders. A recent case-control study found that serum putrescine levels in patients with T2DM were significantly higher than those in the control group, and abnormally elevated putrescine levels were related to insulin resistance and glycosylated haemoglobin levels (14). However, the relationship between serum putrescine levels and GDM has not yet been reported.

To the best of our knowledge, no study has evaluated the relationship between putrescine and GDM risk. Based on the findings in the GDM gut microbiota and the relationship between putrescine and the intestinal barrier, we hypothesized that serum putrescine levels might be associated with the development of GDM. If so, putrescine may serve as an effective predictive biomarker for GDM.

RESEARCH DESIGN AND METHODS

Patient Cohorts

The participants in this nested case-control study were from a prospective cohort study in the Beijing Obstetrics and Gynecology Hospital, Capital Medical University. All pregnant women who intended to give birth in this hospital were enrolled in the cohort study at 8–12 weeks of gestation and followed until delivery.

To evaluate the relationship between serum putrescine and GDM, we selected eligible subjects from the recruited pregnant women above. Women of 18 to 44 years of age and with a singleton pregnancy were recruited and only participants with complete clinical information were included in the analysis. Subjects were excluded if they had pre-existing chronic medical conditions, including hypertension, T2DM, and heart or kidney diseases. Pregnant women diagnosed with diabetes or impaired glucose tolerance in the first trimester were also excluded. GDM was diagnosed at gestational week 24 to week 28 according to American Diabetes Association (ADA) criteria (15), and a 75-g OGTT was performed to screen for GDM. Women with GDM were enrolled when any of the following criteria were met: fasting plasma glucose (FBG) ≥ 5.1 mmol/L, plasma glucose at 1 h ≥ 10 mmol/L, or plasma glucose at 2 h ≥8.5 mmol/L. Normoglycaemic women were matched for age (± 3 years) to each case of GDM women in the same cohort. We also matched the pre-pregnancy BMI according to the classification of Institute of Medicine (IOM) (16). The study was approved by the Ethics Committee of Beijing Obstetrics and Gynecology Hospital (2017-KY-015-01). Written informed consent was obtained from every participant. All procedures were conducted according to the guidelines laid down in the Declaration of Helsinki.

Clinical Measurements and Covariates

Anthropometric measurements of participants were completed by trained medical staff at recruitment using a standardized protocol. Clinical data were collected by medical record review. Pre-pregnancy body weight was self-reported. A family history of diabetes was defined as a first-degree relative with T2DM. Smoking was defined as either ongoing smoking or former smoking and never smoking (No). Drinking was categorized as never/occasional (No) and regular. The FBG and lipid profiles, including cholesterol (CHOL), triglyceride (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL), were determined as described in a previous study (17).

Putrescine Examination

Blood samples were collected from participants following an overnight fast at 8–12 weeks, and serum specimens were isolated

and stored at -80°C for further examination. The serum putrescine levels were examined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS, Thermo Scientific, USA). First, 100 μL of human serum was briefly added to a 0.5 mL glass centrifuge tube. After centrifugation at 14000 r/min for 5 min, the serum sample was dried under nitrogen at 50°C. Then, 60 μL of n-butyl alcohol and 12 mol/L HCI (95:5 v/v) were added and vortexed for 30 seconds in a seal. After incubation at 65°C for 15 min for derivatization, the derivatized solution was centrifuged, and dried under nitrogen at 50°C again. The residue was reconstituted by adding 100 μL of acetonitrile and water (4:1, v/v), vortexed for 30 seconds, centrifuged at 14000 r/min for 5 min and injected at 20 μL for LC-MS/MS analysis.

An API 4500 Qtrap (Agilent 1260 LC equipped with an ESI ion source) was employed for the analysis. Putrescine was separated on an FFAP elastic quartz capillary column (30 m× 0.25 mm× 0.25 μm) from interfering substances in the matrix. Mobile phases consisted of 80% acetonitrile (containing 0.1% formic acid). The pump flow rate setting is variable: 140 μL/min × 0.2 min, 30 μL/min × 1. 3 min and 300 μL/min × 0.5 min. The automatic injection device was set to inject 20 μL each time, and the sampling needle was flushed before injection. The chromatographic column temperature programme: initial temperature 60°C, 0-8 min increased to 180°C, retention for 2 min; carrier gas: high-purity nitrogen (purity 99.999%); carrier gas flow rate 0.89 ml/min; inlet temperature: 200°C. Data were acquired in multiple reaction monitoring (MRM) mode.

Sample Size and Statistics

The sample size was calculated using MedCalc v20.0.3. The total sample size was 94 when set the true proportion as 0.70 and the null hypothesis proportion as 0.50, and hypothesize α=0.01, β=0.20. Data were analysed using the SPSS 22.0 software. Data with normal distributions are shown as the mean ± standard deviation, and nonnormal distributed data are shown as the median (interquartile range), respectively. T-tests and Wilcoxon tests were used to analyse the differences in continuous variables between the GDM group and the control group. Serum putrescine concentrations were also compared by t-test. Categorical variables, including serum putrescine levels (categorized into quartiles), were evaluated using the X^2 test or Fishers's exact test. Although women in the control group were matched for age and prepregnancy BMI to each case of GDM women in the same cohort, the prepregnancy BMI was still higher in the GDM group. Thus, we adjusted the prepregnancy BMI when comparing the serum level of putrescine between the two groups. Binary logistic regression for the association between GDM and serum putrescine was carried out with adjustment for potentially confounding variables. The results are represented by the odds ratio (OR) and 95% confidence interval (CI). To further specify the clinical significance of putrescine, we built 3 multivariate predictive models using putrescine with or without traditional risk factors (i.e., maternal age, prepregnancy BMI, history of family diabetes, FBG and lipid profile) and the traditional risk factors only to evaluate the risk of GDM. The receiver operating characteristic (ROC) curve analysis was performed, and the area under the curve (AUC) was calculated to evaluate the accuracy, sensitivity and specificity of the model. The differences were considered statistically significant when P<0.05.

RESULTS

Clinical and Laboratory Characteristics

This study comprised 47 GDM women and 47 normoglycaemic controls. Although we matched the two groups according to age and BMI, the prepregnancy BMI was significantly higher in the GDM group than in the control group. As shown in **Table 1**, there were no statistically significant differences in other clinical characteristics (P>0.05). However, the lipid and glucose parameters in the first trimester were significantly different between the two groups. Serum lipid parameters, including CHOL, TG, HDL, and FBG were significantly higher in the GDM group (P<0.05).

Serum Putrescine Levels Between GDM and Normoglycemic Women

The serum putrescine concentration was significantly higher in women with GDM than in women compared to the controls in the first trimester. After adjusted the prepregnancy BMI, the difference was still significant (P<0.001, **Figure 1**). When stratified by quartile, those in the upper quartile of putrescine concentration were at increased risk of GDM compared to those with the lowest putrescine concentration (**Table 2**).

TABLE 1 | Anthropometric, clinical and laboratory variables in women with GDM and controls.

	GDM (N = 47)	Control (N = 47)	P
Age	33.0 ± 3.57	32.1 ± 2.88	0.161
Height	162.04 ± 4.98	163.82 ± 5.78	0.118
Pre-pregnancy weight	58.65 ± 7.84	55.60 ± 7.03	0.053
Pre-pregnancy BMI	22.32 ± 2.69	20.68 ± 2.01	0.001**
Gravidity	1.98 ± 1.14	1.74 ± 0.96	0.278
Parity	1.38 ± 0.53	1.32 ± 0.47	0.532
Family history of diabetes			0.284
Yes	11 (23.4%)	6 (12.8%)	
No	36 (76.6%)	41 (87.2%)	
Smoking			1
Yes	2 (4.3%)	1 (2.1%)	
No	45 (95.7%)	46 (97.9%)	
Drinking			1
Yes	5 (10.6%)	4 (8.5%)	
No	42 (89.4%)	43 (91.5%)	
Education background			1
University or above	38 (80.9%)	39 (83.0%)	
Junior college or below	9 (19.1%)	8 (17.0%)	
Laboratory parameter			
FBG (mmol/L)	4.86 ± 0.49	4.64 ± 0.36	0.015*
CHOL (mmol/L)	4.46 ± 0.73	4.12 ± 0.64	0.019*
TG (mmol/L)	1.26 (0.91-1.63)	1.02 (0.85-1.26)	0.023*
HDL (mmol/L)	1.48 ± 0.30	1.49 ± 0.29	0.920
LDL (mmol/L)	2.33 ± 0.60	2.08 ± 0.47	0.026*
Putrescine (μmol/L)	52.10 (48.00-55.12)	33.16 (20.09-39.74)	<0.001***

*P < 0.05. **P < 0.01. ***P < 0.001.

GDM, gestational diabetes mellitus; BMI, body mass index; FPG, fasting plasma glucose; CHOL, cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein.

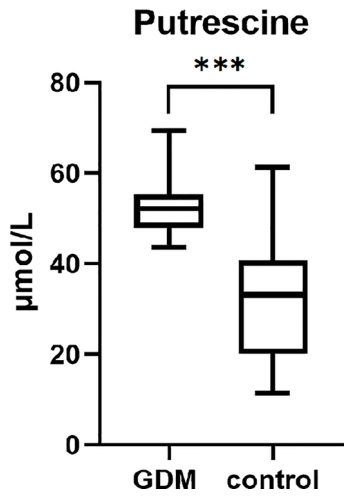

FIGURE 1 | The serum putrescien in the first trimester of women with GDM and controls. (***P < 0.001).

Traditional Risk Factors for GDM Explored by Logistic Regression

Regarding clinical risk factors (**Table 3**), univariable logistic regression showed that only prepregnancy BMI was positively correlated with GDM risk (P<0.05). However, a family history of diabetes and smoking may increase the risk of GDM, with an ORs of 2.088 (95% CI = 0.701-6.215) and 2.044 (95% CI = 0.179-23.348) respectively. Regarding laboratory risk factors (**Table 3**), FBG, CHOL, TG and LDL in the first trimester were significantly and positively correlated with GDM risk (P<0.05).

According to the univariable logistic regression, we used prepregnancy weight, prepregnancy BMI, family history of diabetes, smoking, FBG, CHOL, TG and LDL to build a traditional risk prediction model. An ROC curve was constructed using the model with an AUC of 0.761 (sensitivity of 53.2% and specificity of 93.6%, 95% CI=0.665–0.858, P<0.001, **Figure 2A**).

The Prediction Effect of Serum Putrescine for GDM

Univariable logistic analyses indicated that serum putrescine concentration was significantly and positively associated with a higher risk of GDM (OR=1.231, 95% CI=1.130–1.341, P<0.001). After adjustment for maternal age, prepregnancy BMI, history of family diabetes, FBG and lipid profile, the results did not change (OR=1.274, 95% CI=1.148–1.413, P<0.001).

To evaluate the performance of serum putrescine as predictive biomarker for GDM, ROC curves were constructed using the putrescine with an AUC of 0.904 (sensitivity of 100% and specificity of 83%, 95% CI=0.832–0.976, P<0.001, **Figure 2B**). When putrescine was combined with traditional risk factors, the final risk prediction model included prepregnancy BMI, FBG and putrescine (**Table 4**), which achieved an AUC of 0.951 (sensitivity of 89.4% and specificity of 91.5%, 95% CI=0.906-0.995, P<0.001, **Figure 2C**).

TABLE 2 | The quartile stratification comparison of putrescine.

	GDM (N = 47)	Control (N = 47)	X^2	P
Putrescine (μmol/L)			50.80	<0.001***
<33.36	0	24		
33.36-45.07	7	16		
45.08-52.70	19	4		
>52.70	21	3		

****P < 0.001.*
GDM, gestational diabetes mellitus.

DISCUSSION

Our study demonstrated that serum putrescine levels in the first trimester were significantly higher in women who later developed GDM. Measurement of serum putrescine concentration showed excellent diagnostic value for predicting the risk of GDM. We also built a final model that included putrescine and traditional clinical risk factors (prepregnancy BMI and FBG). And this model showed a considerable degree of discrimination and achieved an AUC of 0.951.

There is evidence that some women with GDM already have abnormal glucose metabolism in the first trimester (18). However, the diagnosis of GDM is often made during the second trimester, which presents a limited time for an intervention. Detection of women at risk earlier during pregnancy would be important to enable early lifestyle modification or even drug treatment to improve the perinatal outcomes of these women. Under those circumstances, using new risk prediction models to improve the existing selective screening algorithms and therefore to effectively reduce the number of inconvenient and belated oral glucose tolerance tests is meritorious.

There are many traditional risk factors for GDM, including advanced maternal age, overweight or obesity, ethnicity, family history of diabetes, history of GDM in a previous pregnancy, high FBG, glycated haemoglobin A1c (HbA1c) and lipid profile. However, the predictive performance of these risk factors is criticized as having limited diagnostic accuracy (8, 19, 20). The AUC values of these traditional clinical variables ranged from 0.6-0.8, and most of these previous studies used different GDM diagnosis criteria (8, 21, 22). Therefore, their clinical benefit when applied to the most recent GDM definition has not yet been well investigated. Recently, a number of new markers have been evaluated for use in predicting GDM with variable success, such as protein biomarkers (23), adiponectin (24) and leptin (25), pentraxin 3 (26), trace elements (27), RNA (28), single-nucleotide polymorphisms (29), and other combined metabolite models (30–33). The AUC values of some of these models can reach above 0.8. However, interindividual variability, sample collection and transportation, confounding variables and cost limit the application of these new markers in clinical.

Metabolomics is a rapidly developing science that aims to quantitatively describe the dynamic changes of many metabolites in organisms. Metabolomics has the capacity to recognize early

TABLE 3 | Univariate logistic regression for GDM.

	B	P	OR (95% CI)
Age	0.089	0.169	1.093 (0.963-1.240)
Height	-0.061	0.120	0.941 (0.871-1.016)
Pre-pregnancy weight	0.055	0.056	1.056 (0.999-1.117)
Pre-pregnancy BMI	0.288	0.003**	1.333 (1.106-1.608)
gravidity	0.213	0.287	1.237 (0.836-1.829)
parity	0.257	0.536	1.293 (0.573-2.919)
family history of diabetes	0.736	0.186	2.088 (0.701-6.215)
smoking	0.715	0.565	2.044 (0.179-23.348)
Drinking	0.247	0.726	1.280 (0.321-5.096)
Education background	0.144	0.789	1.155 (0.403-3.306)
Laboratory parameter			
FBG	1.393	0.022*	4.027 (1.221-13.283)
CHOL	0.710	0.025*	2.035 (1.092-3.793)
TG	1.002	0.022*	2.723 (1.154-6.427)
HDL	-0.070	0.921	0.932 (0.233-3.733)
LDL	0.880	0.030*	2.412 (1.089-5.343)
Putrescine	0.208	<0.001***	1.231 (1.130-1.341)

**P < 0.05. **P < 0.01. ***P < 0.001.*

GDM, gestational diabetes mellitus; BMI, body mass index; FPG, fasting plasma glucose; CHOL, cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein.

TABLE 4 | Multivariate logistic regression for GDM.

	B	P	OR (95% CI)
Putrescine	0.242	<0.001***	1.274 (1.148-1.413)
Pre-pregnancy BMI	0.441	0.009**	1.555 (1.118-2.162)
FBG	2.102	0.051	8.183 (0.990-67.642)
constant	-30.123	<0.001***	0

***P < 0.01. ***P < 0.001*

GDM, gestational diabetes mellitus; BMI, body mass index; FPG, fasting plasma glucose.

deregulations and disruptions in metabolism associated with diseases or disorders. To investigate physiological processes and to develop early diagnostics, metabolomics is one of the most promising technologies (34, 35). Plasma metabolomics is a powerful technology for the rapid and profound analysis of all kinds of metabolic diseases. The amount of information obtained by this approach is still not fully understood. The GDM prediction model from the UK Pregnancies Better Eating and Activity Trial (UPBEAT), which included multiple serum metabolites, reached an AUC of 0.78 (30). Other metabolite models that included adiponectin reached an AUC of 0.79-0.85 (24, 33). A study using untargeted and targeted metabolomic protocols to analyse plasma and urine samples of pregnant women with and without GDM reported that the combination of 11 metabolites from blood samples and 5 metabolites from urine samples improved the AUC prediction accuracy to 0.99 (31). However, this study only investigated 14 GDM women and 18 non-GDM women. The multivariable predictive model based on a small sample size may have limited promotion value. In addition, the association between metabolites and the risk of a certain disease may considerably vary among different ethnicities and the heterogeneity of GDM. Alternately, different GDM diagnosis criteria across studies and the diversity of different

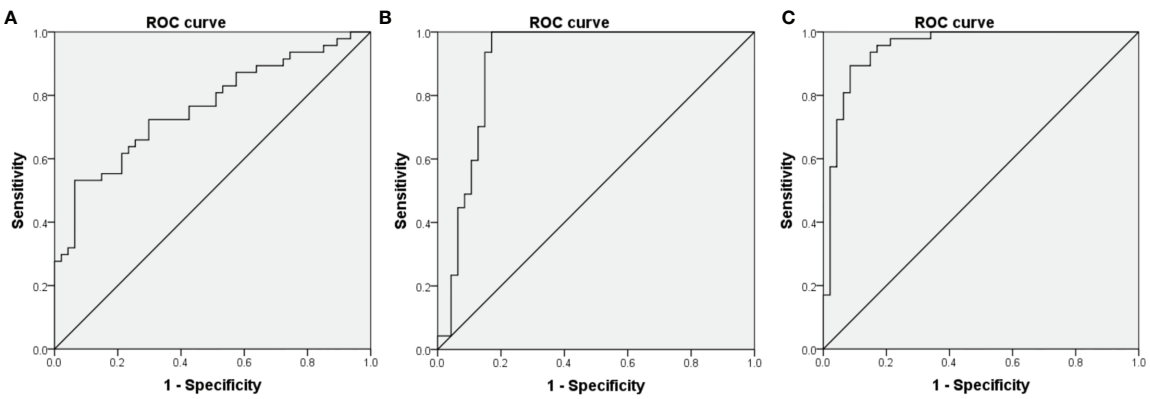

FIGURE 2 | Receiver operating characteristic (ROC) curves for logistic regression models utilizing clinical risk factors **(A)**, serum putrescine **(B)** serum putrescine and clinical risk factors **(C)**.

laboratory instruments may also influence the effects of prediction and extrapolation. Although combined clinical risk factors and metabolite biomarkers improve the prediction of GDM, selective screening based on the presence of one or more risk factors has shown to have limited diagnostic accuracy (36, 37). In light of these previous studies, the model in this study included only one metabolite, which can reach an AUC of 0.95 and a sensitivity of 89.4% is encouraging.

Some studies reported that elevated serum putrescine levels were associated with T2DM (14). However, there are few reports about putrescine metabolism in pregnant women with GDM. Putrescine is a metabolite of intestinal bacteria and is produced by collective biosynthetic pathways of the commensal microbiome (38). In recent years, the gut microbiota and its metabolites have shown a significant relationship with GDM. Our former study demonstrated that women who develop GDM exhibit distinct gut microbiota compositions in the first trimester, and the bacteria enriched in GDM have been found to be related to the metabolism of putrescine (12). A recent study conducted high-throughput sequencing of thousands of indicators, including secreted proteins, microbial metabolites, and drugs, and found for the first time that putrescine can destroy intestinal barrier function by disrupting TJ integrity and cause colon inflammation (13). Disruption of gut barrier integrity generates a "leaky gut", allowing the influx of bacterial ligands and inflammatory cytokines into the portal and systemic circulation through leaky gut to trigger systemic inflammation in a broad range of target organs, such as adipose tissues. GDM, similar to T2DM, is an inflammatory clinical entity with different mechanisms involved in its physiopathology. Impaired intestinal barrier structure and function are important to the low-grade inflammation (39) and have been validated as an important pathogenic process in T2DM (40, 41). Chronic systemic inflammation and adipose tissue inflammation have also been regarded to play a role in the progression of GDM (42, 43). This may suggest the pathogenic mechanism of putrescine, which impairs the intestinal barrier and triggers chronic inflammation, in GDM. Although the direct cause and effect relationship between putrescine and its pathological states has not yet been confirmed, reversing harmful intestinal microbiota metabolites may become an attractive and potent target for disease prevention and treatment.

Our current study revealed that women with GDM had an elevated level of serum putrescine in the first trimester. We first used serum putrescine levels in the first trimester to build a sensitive and reliable prediction model of GDM, which could help identify high-risk individuals at an early stage. However, this study was a single-center study, limited by the sample size and restricted ethnicity. Furthermore, the metabolome is influenced by patient intrinsic factors such as ethnicity, epigenetics, and genetic mutations, and extrinsic factors such as environment, stress, and diet. We did not conduct the diet survey and stress assessment, so we were unable to evaluate these extrinsic factors. This conclusion deserves to be verified in a large and multiethnic population cohort and the influencing factors of metabolome should be evaluated. The causality of putrescine and GDM may be connected by disruption of the intestinal barrier and systemic and local inflammation. The specific mechanism between putrescine and GDM requires to be explored in greater depth.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

GL and XM contributed to the study design and interpretation of the data. CL and YW contributed to the drafting and revision of the manuscript. CL and WZ coordinated and executed the statistical analysis. JW and YZ contributed to the collection of data. AW and WS contributed to the enrollment and follow-up in clinic. All authors reviewed and approved the final, submitted version.

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Association of Preadmission Metformin Use and Prognosis in Patients With Sepsis and Diabetes Mellitus: A Systematic Review and Meta-Analysis

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Background and Aim: A growing body of evidence suggests that preadmission metformin use could decrease the mortality of septic patients with diabetes mellitus (DM); however, the findings remain controversial. Therefore, this meta-analysis was conducted on available studies to confirm the relationship between preadmission metformin use and mortality in patients with sepsis and DM.

Methods: A comprehensive search of the PubMed, Embase, and Cochrane Library databases was performed for studies published before August 8, 2021. Observational studies assessing the correlation between metformin use and mortality in patients with sepsis and DM were considered eligible studies. We used the Newcastle–Ottawa Scale (NOS) to assess the outcome quality of each included article. Furthermore, the odds ratios (ORs) and 95% confidence intervals (*CI*s) were analyzed using the inverse variance method with random effects modeling.

Results: Eleven articles including 8195 patients were analyzed in this meta-analysis. All the included articles were scored as low risk of bias. Our results showed that preadmission metformin use had a lower mortality rate (OR, 0.74; 95% *CI*s, 0.62–0.88, $P < 0.01$) in patients with sepsis and DM. Surprisingly, there was no statistically significant difference in the levels of serum creatinine (weighted mean difference (WMD), 0.36; 95% *CI*s, –0.03–0.75; $P = 0.84$) and lactic acid (WMD, –0.16; 95% *CI*s, –0.49–0.18; $P = 0.07$) between preadmission metformin use and non-metformin use.

Conclusions: This study is the most comprehensive meta-analysis at present, which shows that preadmission metformin use may reduce mortality and not increase the levels of

serum creatinine and lactic acid in adult patients with sepsis and DM. Therefore, these data suggest that the potential efficacy of metformin could be assessed in future clinical studies.

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Keywords: metformin, sepsis, mortality, systematic review, meta-analysis

INTRODUCTION

Sepsis is a life-threatening systemic inflammation characterized by host immune dysfunction and multiple organ damage (1). An estimated 30 million sepsis cases and 5.3 million deaths occur globally every year, indicating that sepsis is an urgent medical and health problem (2). However, the exact mechanism of sepsis development is unclear. Inflammatory factors and mediators such as high mobility group protein and nuclear factor-kappa B (NF-κB) may play significant roles in the pathogenesis of sepsis (3, 4). Recently, studies demonstrated that continual and active inflammatory molecular responses need sufficient metabolic supply. Thus, adjustments targeting metabolic pathways are a novel potential therapeutic strategy (5). Metformin reduces the inflammatory response in the body, which may reduce mortality in septic patients with diabetes mellitus (DM).

Metformin, a classic oral antidiabetes drug, is extensively recommended as a first-line treatment of type 2 DM (6, 7). In addition to the well-known hypoglycemic effects of metformin, evidence shows that metformin plays an anti-inflammatory role by inhibiting the expression of inflammatory factors (8–12). Metformin may also inhibit the respiratory chain complex I of the electron transport chain to increase the adenosine monophosphate/adenosine triphosphate ratio, and consequently, induce adenosine 5′-monophosphate-activated protein kinase (AMPK) activation. AMPK interferes with inflammation and other molecular processes. Given the AMPK activation, metformin may be a potential therapeutic therapy for sepsis (13).

Recent studies demonstrated the positive effects of metformin in patients with sepsis. Doenyas-Barak et al. (14) and Jochmans et al. (15) suggested that pre-exposure of patients to metformin lowered mortality compared with control, although metformin was associated with higher lactate concentrations. Green et al. (16) reported that patients not taking metformin had 2.5 times more risk of dying within 28 days than those taking metformin. However, no statistical differences in mortality between metformin users and nonusers were detected in acute respiratory distress syndrome, DM, sepsis, and critically ill patients (17–19). As the effects of metformin on mortality are controversial, we investigated the effects of metformin on mortality in patients with sepsis and DM.

METHODS

This systematic review and meta-analysis protocol was registered on INPLASY (ID: INPLASY2021100113). This study was conducted according to the guidance of the meta-analysis of observational studies in the epidemiological guidelines. The PRISMA 2020 checklist is shown as **Supplemental Table 1**.

Search Strategy

We comprehensively searched the PubMed, Cochrane, and Embase databases for English language studies published before August 08, 2021. We used a combination of MeSH/Emtree, title, abstract, and keyword terms to search for relevant studies. The search terms were "metformin", "sepsis", and "critically ill". Endnote x9 software was used for literature screening. We reviewed the eligible articles to identify other potentially relevant studies. Literature retrieval was conducted by two researchers. The search process is shown in **Supplemental Table 2**.

Inclusion Criteria

Studies were included in this meta-analysis if: the patients with sepsis and DM who used metformin before admission were enrolled; the control group was DM complicated with sepsis but not treated with metformin; mortality in metformin and non-metformin users was measured; the patients were adults; the articles were observational studies; and the articles were published in English. Articles lacking relevant outcomes or patients with sepsis complicated with other illnesses were excluded. Commentaries, reviews, and studies for which full articles could not be retrieved were excluded.

Eligible Studies and Extracted Data

The relevant articles and eligible data were assessed and extracted by two authors, respectively. If a disagreement occurred, which was discussed and the consensus with a third author was reached. The following data were collected from each study: first author name, publication date, the type of studied design and center, number of patients, study period, sex and mean age between groups and primary outcome.

Risk of Bias Evaluation

Using the Newcastle–Ottawa Scale (NOS) for cohort studies, the risk of bias was assessed for each outcome in all included studies. According to the selection of cohort (up to 4 points), the comparability of cohort design and analysis (up to 2 points) and the adequacy of result measurement (up to 3 points), a maximum of 9 points will be obtained. Seven to nine points are considered high quality (low risk of bias) (20).

Statistical Analysis

The interesting outcomes were mortality in sepsis patients with or without metformin before admission. Furthermore, the meta-

analysis used the combined effects of each result. We calculated odds ratios (ORs) and 95% confidence intervals (*CI*s) for each result using a random effects model to explore the heterogeneity between studies.

The outcome of interest was the mortality, serum creatinine and lactic acid levels of septic patients with or without preadmission metformin use. The meta-analysis used the pooled effects of each outcome. The random effects models were used to evaluate ORs and 95% *CI*s of each outcome to investigate the heterogeneity between studies. Furthermore, we used the I^2 and *P* values to assess the heterogeneity. When I^2 was 51%–74%, the percentage of variation caused by heterogeneity rather than sampling error was considered medium, while it was high when I^2 was ≥ 75%. Begg's funnel plot (21) was used to assess possible publication bias. We visually assessed the asymmetry of the funnel plots. For Begg's funnel, $P < 0.1$ suggested that the scale of the study is very small. All statistical analyses were performed using Stata 14.0 (College Station, Texas, 77845, USA).

RESULTS

Study Selection

854 studies were determined and 16 studies were eliminated because of duplication. After assessing the full-text eligibility, only 32 articles that addressed reasons potentially associated with the original study question remained. Eleven studies (14–19, 21–25) enrolling 8195 patients were included in the meta-analysis. **Figure 1** shows the process of study selection.

Study Characteristics

The eligible studies were observational studies that included septic patients with DM who used preadmission metformin. Furthermore, three articles (16, 17, 21) showed 28 day mortality, four studies (18, 19, 22, 23) reported 30 day mortality, and four studies (14, 15, 24, 25) reported in-hospital mortality. The primary outcome data were extracted. If ORs and 95% *CI*s were missing, the data were calculated based on the original data reported from the original studies. **Table 1** presents the baseline information regarding the analyzed studies.

Risk of Bias Evaluation

Ten studies were observational studies, and one was a prospective cohort study. The risk of bias assessment scores for all studies was greater than or equal to six points, indicating a low risk of bias based on the NOS. **Supplemental Table 3** shows the details of the risk of bias for the eligible studies.

Effects of Metformin on Outcomes

The preadmission use of metformin in septic patients significantly lowered mortality when compared with the mortality in patients who were not treated with metformin (OR, 0.74; 95% *CI*s, 0.62–0.88; $P < 0.01$; **Figure 2**). No significant differences in serum creatinine concentrations (weighted mean difference (WMD), 0.36; 95% *CI*s, −0.03–0.75; $P = 0.84$; **Figure 3**) were detected between patients taking metformin and those not taking metformin. Similarly, no significant differences in serum lactic acid concentrations (WMD, −0.16; 95% *CI*s, −0.49–0.18; $P = 0.07$; **Figure 4**) were detected between patients taking metformin and those not taking metformin.

Sensitivity Analyses

The eligible studies were observational articles with a low risk of bias (**Supplemental Table 3**). We conducted a sensitivity analysis to evaluate the influence of any study on the ORs and 95% *CI*s by omitting an individual study at a time. The data indicated that the results of mortality and lactic acid were robust and reliable (**Supplemental Figures 1**, **2**).

Evaluation of Publication Bias

We performed the Funnel plots (**Supplemental Figures 3**, **4**) and Begg's funnel plot (**Supplemental Figures 5**, **6**) to assess the publication bias in the included articles between mortality and lactic acid in this analysis. No publication bias of them was found ($P = 0.291$ and $P = 0.291$, respectively).

DISCUSSION

This meta-analysis included 8195 patients and demonstrated that the mortality rate of preadmission metformin users was lower than that of non-metformin users in adults with DM and sepsis. Our findings suggest that metformin could have a therapeutic potential for septic patients with DM.

The relationship between metformin use and mortality in patients with infectious diabetes remains controversial. Therefore, some studies (17–19) demonstrated no significant differences between metformin use and non-metformin use in patients with sepsis. In contrast, the reports of Green et al. (16) and Doenyas Bara et al. (14) showed that preadmission metformin use in septic patients significantly lowered mortality compared with those not using metformin. Subsequently, a meta-analysis (26) only including five observational cohort studies (1282 patients) suggested that preadmission metformin use could decrease the 28-d mortality in septic patients with DM. As the sample size of the study (26) was too small, the conclusions should be confirmed in the large sample study, especially in clinical studies. The recent large sample study (19) showed that preadmission metformin use was not significantly related to the risk of sepsis and 30-d mortality of septic patients with DM. Comparatively, the latest study (22) indicated that preadmission metformin use was associated with a 39% decrease in 30-d mortality in septic patients with DM. Therefore, the conclusions still exist in controversy among the available articles. This meta-analysis provides this comprehensive evidence that metformin use reduces mortality in septic patients with DM.

The mechanism by which metformin reduces mortality in patients with sepsis is unclear. The 2016 definition of sepsis

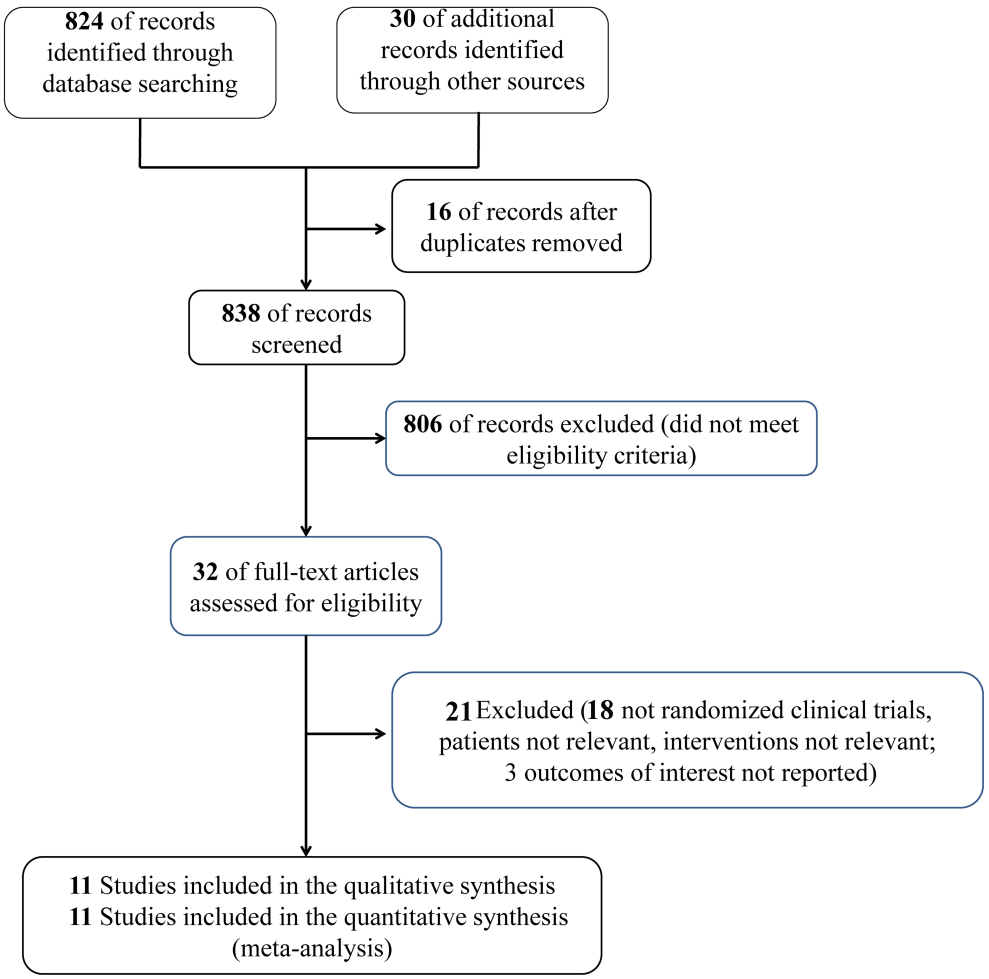

FIGURE 1 | Flowchart of the study selection process.

includes lactate concentration and measurement of creatinine concentration to determine the progress of organ failure (1). Furthermore, metformin has a low propensity for hyperlactatemia (27, 28). Previous studies (29, 30) showed that mortality due to lactic acidosis caused by metformin was lower than other forms of lactic acidosis. Intriguingly, a study showed that lactic acid could be considered an energy source and can provide energy, such as glucose, amino acids, and ketones for ischemic tissues (31). This may explain the results in the previous study (32) that the plasma concentration of lactic acid in surviving patients was higher than that in dying patients. Reciprocally, in our study, creatinine and lactate concentrations were not significantly different in patients with and without metformin (14–18, 21, 22). Additionally, increasing evidence was provided to show that metformin could ameliorate the autophagy and mitochondrial function of T-cells (33) and improve the systematic inflammation *via* decreased pro-inflammatory factors, such as NF-κB and tumor necrosis factor alpha (TNF-α) (34). Moreover, metformin reduces oxidative stress, enhances antioxidant defense (35), improves insulin resistance, and protects vascular endothelium (36). Notably, metformin upregulates the AMPK activation (13, 34), which is considered a potential therapeutic agent for sepsis-associated organ injury (37). Furthermore, a recent study suggested that the AMPK-dependent immunometabolism pathway disorder may conduce to an increased risk of sepsis (38). Finally, metformin may have antibacterial effects (39), improving the prognosis of sepsis in metformin users.

Meta-analysis is a comprehensive statistical analysis method for multiple studies on a subject. When the difference between the results of each study is greater than expected, there is statistical heterogeneity in the summary results of the meta-analysis. The high heterogeneity of the included studies of this study reflects the differences of these studies. Because of the low publication bias, we consider the heterogeneity was not significant. The heterogeneity may originate from differences in sample sizes, lactate concentrations, and the combined application of other antidiabetes drugs.

This meta-analysis has several strengths. Firstly, the sample size was large, making the results more convincing. Secondly, the

TABLE 1 | Characteristics of identified studies.

Study	Study design	M/S Centre	No. of MET group	No. of NM group	Female/Male in MET group	Female/Male in NM group	Mean age in MET group	Mean age in NM group	Study period	Primary Outcome
(24)	RC	Single	12	53	NA	NA	NA	NA	12/2005-6/2009	Incidence of LA
(16)	RC	Single	192	343	NA	NA	71	72	02/2007-10/2008	28d mortality
(25)	RC	Single	114	127	NA	NA	NA	NA	01/2011-07/2013	mortality
(23)	RC	Multi	73	182	NA	NA	NA	NA	01/2005-12/2011	30d mortality
(18)	PO	Multi	114	127	36/78	61/66	67	65.1	01/2011-07/2013	Hospital mortality
(14)	RC	Single	44	118	22/22	47/71	74	68	01/2011-06/2013	Hospital mortality
(21)	RC	Single	71	142	32/39	62/80	67	68	08/2008-09/2014	28d mortality
(15)	RC	Single	52	79	19/33	23/56	66	71	10/2010-12/2013	Hospital mortality
(17)	RC	Single	162	162	97/65	87/75	69	69	01/2007-12/2013	28d mortality
(19)	RC	Single	672	672	364/308	359/313	69.1	69.1	2011-2015	30d mortality
(22)	RC	Multi	476	1907	246/230	992/915	70.1	69.5	2001-2012	30d mortality

RC, retrospective cohort; PO, prospective observational study; M, multiple; S, single; MET, metformin; NM, non-metformin; NA, Not available; LA, lactic acidosis.

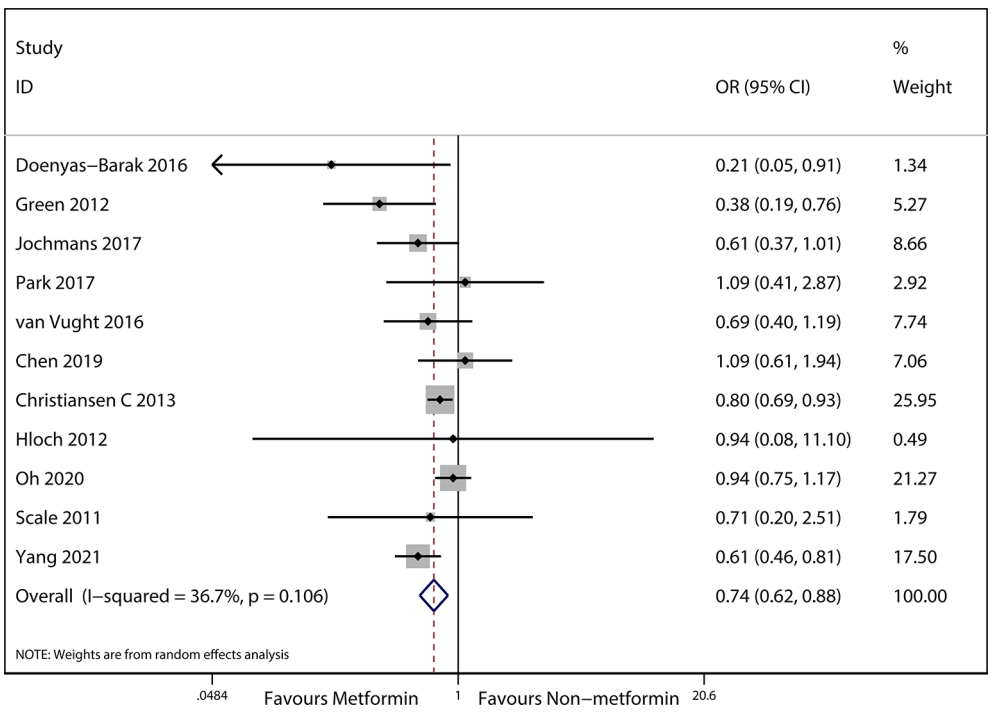

FIGURE 2 | Meta-analysis of the overall pooled ORs of studies investigating the mortality outcomes of patients with sepsis and DM. The Forest plot shows the significance of the association between metformin use and mortality in patients with sepsis and DM according to the random effects model.

risk of bias was low for all included studies. Finally, the random effects model with generic inverse variance was used, and the adjusted ORs and 95% *CI*s were extracted to compute the effect of metformin on mortality.

Our study has several limitations. Despite the comprehensive search, only eleven studies met the inclusion standards and ten were observational studies; therefore, the conclusion should be confirmed in future clinical studies.

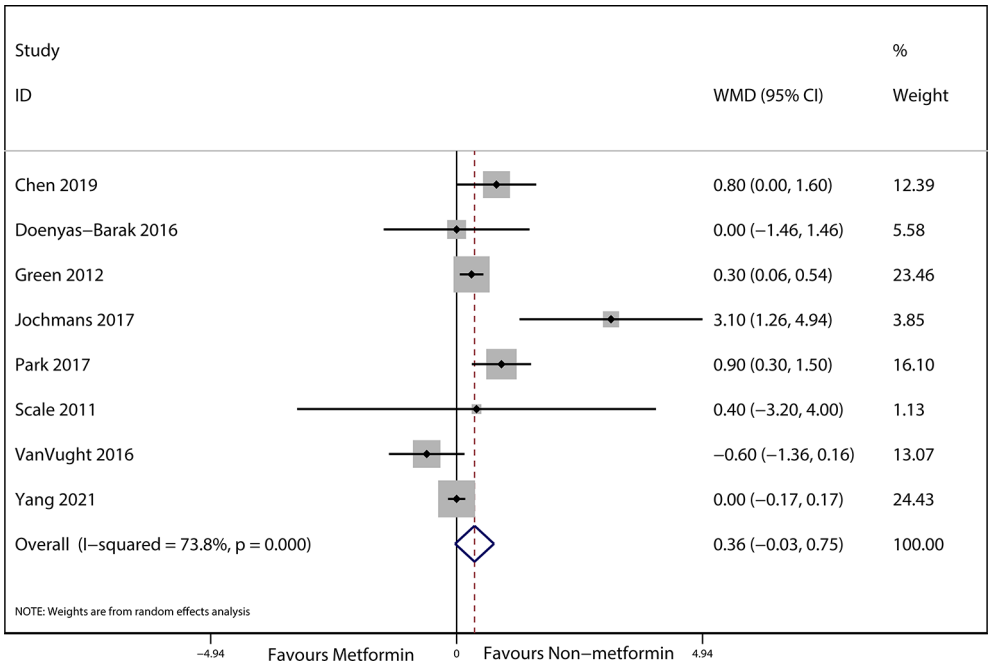

FIGURE 3 | Meta-analysis of the ORs of included articles researching the serum lactic acid of septic patients with DM. The outcome of Forest plot indicated the relation between preadmission metformin use and serum lactic acid in septic patients with DM based on the random effects model.

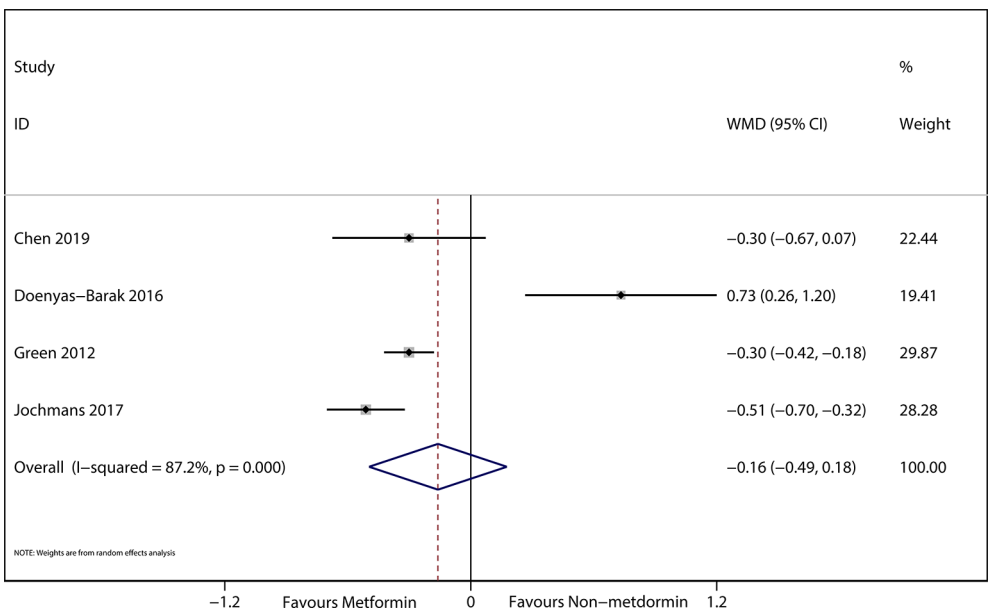

FIGURE 4 | The Forest plot suggested the association between preadmission metformin use and serum creatinine in septic patients with DM.

Furthermore, most original studies did not report the dose of metformin and whether other kinds of hypoglycemic agents were used. Hence, this study did not determine whether different doses of preadmission metformin use and other hypoglycemic agents would influence the effect of metformin on septic patients with DM. Thirdly, this study was constrained to studies published in English. Thus, publication bias cannot be excluded.

This meta-analysis suggests that we should continue perform the large sample observational and clinical study to confirm the effect of metformin on septic patients with DM, and thereby to searche the mechanism of metformin in reducing the mortality on septi patients with DM. Additionally, the does and time of metformin use in septic patients with DM should be researched in the future clinical studies.

CONCLUSION

This is the most comprehensive meta-analysis to investigate the association of metformin use with mortality in septic patients with DM. The findings suggest that preadmission metformin use may reduce the mortality of septic patients with DM, and without increasing the levels of serum creatinine and lactic acid. However, stronger support from more high-quality research is still needed.

AUTHOR CONTRIBUTIONS

All the authors contributed equally to the work presented in this article. YL, HZ, YjG, and XD conceived the idea of this study. YlG and YD contributed to the data extraction. YL and HZ computed and evaluated the pooled outcomes. YjG and XD contributed to the study protocol and wrote the article. YjG and XD revised the article. 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.2021.811776/full#supplementary-material

Supplementary Figure 1 | Funnel plot assessing the mortality after preadmission metformin use in septic patients with DM.

Supplementary Figure 2 | Funnel plot evaluating serum lactic acid after preadmission metformin use in patients with sepsis and DM.

Supplementary Figure 3 | Sensitivity analysis indicating that the included studies were conclusive and reliable regarding preadmission metformin use and the mortality of septic patients with DM.

Supplementary Figure 4 | Sensitivity analysis indicating that the included studies were conclusive and reliable regarding preadmission metformin use and the serum lactic acid of septic patients with DM.

Supplementary Figure 5 | Begg's funnel plot assessing the mortality of preadmission metformin use in patients with sepsis and DM.

Supplementary Figure 6 | Begg's funnel plot assessing the serum lactic acid of preadmission metformin use in patients with sepsis and DM.

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Upregulation of T Cell Receptor Signaling Pathway Components in Gestational Diabetes Mellitus Patients: Joint Analysis of mRNA and circRNA Expression Profiles

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Objective: Gestational diabetes mellitus (GDM) is one of the most common complications of pregnancy, and its pathogenesis is still unclear. Studies have shown that circular RNAs (circRNAs) can regulate blood glucose levels by targeting mRNAs, but the role of circRNAs in GDM is still unknown. Therefore, a joint microarray analysis of circRNAs and their target mRNAs in GDM patients and healthy pregnant women was carried out.

Methods: In this study, microarray analyses of mRNA and circRNA in 6 GDM patients and 6 healthy controls were conducted to identify the differentially expressed mRNA and circRNA in GDM patients, and some of the discovered mRNAs and circRNAs were further validated in additional 56 samples by quantitative realtime PCR (qRT-PCR) and droplet digital PCR (ddPCR).

Results: Gene ontology and pathway analyses showed that the differentially expressed genes were significantly enriched in T cell immune-related pathways. Cross matching of the differentially expressed mRNAs and circRNAs in the top 10 KEGG pathways identified 4 genes (*CBLB*, *ITPR3*, *NFKBIA*, and *ICAM1*) and 4 corresponding circRNAs (circ-CBLB, circ-ITPR3, circ-NFKBIA, and circ-ICAM1), and these candidates were subsequently verified in larger samples. These differentially expressed circRNAs and their linear transcript mRNAs were all related to the T cell receptor signaling pathway, and PCR results confirmed the initial microarray results. Moreover, circRNA/miRNA/mRNA interactions and circRNA-binding proteins were predicted, and circ-CBLB, circ-ITPR3,

and circ-ICAM1 may serve as GDM-related miRNA sponges and regulate the expression of CBLB, ITPR3, NFKBIA, and ICAM1 in cellular immune pathways.

Conclusion: Upregulation of T cell receptor signaling pathway components may represent the major pathological mechanism underlying GDM, thus providing a potential approach for the prevention and treatment of GDM.

Keywords: gestational diabetes mellitus, mRNA, circRNA, T cell receptor signaling pathway, microarray analysis

INTRODUCTION

Gestational diabetes mellitus (GDM) refers to varying degrees of impaired glucose tolerance that occur for the first time during pregnancy, and it is one of the most common complications of pregnancy (1). According to the latest global diabetes map released by the International Diabetes Federation, the worldwide prevalence of gestational hyperglycemia in 2019 was 15.8%, and GDM accounted for 83.6% of these cases (2). In China, with the gradual relaxation of the family planning policy, the continuous postponement of childbearing, and the increase in irregular lifestyles and incidences of overweight and obesity, the incidence of GDM has significantly increased in recent years. The latest epidemiological surveys show that the prevalence rate of GDM in China ranges from 17.6 to 18.3% (3, 4), which is considerably higher than the global average. In addition, the abnormal increase in blood glucose levels in GDM patients markedly increases the incidence of adverse pregnancy outcomes. A large number of studies have shown that GDM increases the risk of maternal miscarriage, cesarean section, postpartum hemorrhage, preeclampsia, neonatal macrosomia, infants larger than gestational age, and congenital malformations, and GDM also leads to an increased future risk of type 2 diabetes (T2D) and cardiovascular disease for mothers and offsprings (5).

Great efforts have been devoted to identifying the risk factors and pathological mechanisms underlying GDM (6). The well-known risk factors for GDM includes old age, race, T2D family history, past stillbirth, anemia, BMI >25, sedentary lifestyle, smoking, sugary drinks, and other dietary factors (7, 8). The pathological process of GDM is mainly related to increased peripheral insulin resistance, impaired glucose uptake, and the role of obesity-related inflammatory factors (1). At present, the focus of research on GDM has gradually shifted from metabolic disorder to placental dysfunction and immune system activation. However, the related genetic mechanism remains unclear (9, 10). In recent years, with advances in new-generation sequencing technologies, the number of studies predicting GDM biomarkers has been increasing, and most of these studies focus on the prediction of mRNAs, miRNAs, and lncRNAs (11), and few studies focus on the prediction of circRNAs (12). Nevertheless, the interactions of mRNAs and circRNAs from a transcriptomics perspective are still unknown.

Circular RNAs (circRNAs), covalently closed cyclic molecules characterized by a lack of 3' and 5' polar ends, are novel noncoding RNAs (ncRNAs) that have recently attracted considerable interest from researchers (13). In the past, circRNAs have been considered to be byproducts of RNA splicing errors and useless fragments, but now, they have been shown to be important regulators of gene expression in the pathophysiological processes of many diseases (14). Specifically, circRNAs can work as miRNA sponges, transcriptional regulators, protein-binding molecules, protein function enhancers, and protein scaffolds and can sometimes even be translated into functional proteins (7, 15). In addition, circRNAs are abundantly expressed in cells and tissues and have the characteristics of relative stability, long half-lives, tissue specificity, and high evolutionary conservation among species (16), suggesting that circRNAs might be new biomarkers for clinical applications.

Recently, an increasing number of studies have reported that circRNAs might play important roles in the regulation of blood glucose levels, for example, by regulating islet β cell proliferation and insulin secretion. Xu et al. showed that circRNA Cdr1as (also known as CIRS-7) has a strong effect on miR-7 sponge/inhibitor, and the Cdr1as/miR-7 pathway further promotes insulin production and secretion by regulating the insulin granule secretion target Myrip and enhancing the insulin transcription target Pax6 (17). A subsequent mouse study found that the expression of Cdr1as and another circRNA, circHIPK3, was significantly downregulated in the islets of diabetic db/db mice. Further analysis revealed that circHIPK3 acts by sequestering a group of microRNAs, namely, miR-124-3p and miR-338-3p, and by regulating the expression of key β-cell genes, especially Akt1 (18). A cohort study found that hsa_circ_0054633 was significantly overexpressed in the sera and placentas of GDM women in the second and third trimesters of pregnancy, and its expression level was positively correlated with postprandial blood glucose levels and glycosylated hemoglobin levels (19). Later, Zhao et al. found that hsa_circ_0054633 may regulate β cell metabolism and participate in the pathogenesis of GDM by affecting the cell cycle (20). Yang et al. found that hsa_circ_102893 was significantly downregulated in peripheral blood of GDM patients, which could bind to RNA binding protein EIF4A3 and regulate cell cycle and apoptosis process through TNF-α/NF-κB signaling pathway, thus affecting insulin secretion and β cell function in GDM patients (21, 22).

Inspired by these findings, we speculated that circRNAs might be potential regulators of GDM. In the present study, we used genome-wide microarrays to identify differentially expressed mRNAs and circRNAs between GDM patients and healthy controls. The joint analysis of circRNA and mRNA expression profiles may provide relevant information for understanding the

potential pathological mechanisms underlying GDM and developing new mechanism-based diagnostics and therapies.

MATERIALS AND METHODS

Participants and Sample Collection

The participants were recruited from the Affiliated People's Hospital of Ningbo University from September 2018 to November 2019. The recruited participants met the following inclusion criteria: 20–35 years old, Han nationality, and with BMI <30. GDM was diagnosed by the one-step criteria suggested by the WHO: fasting glucose level ≥5.1 mmol/L or 1 h 75 g oral glucose tolerance test (OGTT) ≥10.0 mmol/L or 2 h OGTT ≥8.5 mmol/L. The control group matched the GDM group through a 1:1 pattern according to age (± 2). Participants who met the following exclusion criteria were excluded from both groups: (1) history of diabetes, (2) history of taking hypoglycemic drugs, (3) recent fever, infection and other acute inflammatory periods, (4) pregnancy hypertension, polycystic ovary syndrome, (5) chronic diseases (thyroid dysfunction, cardio-cerebrovascular diseases, malignant tumors, etc.), (6) multiple pregnancies, and (7) the use of assisting reproductive technology. Finally, 68 pregnant women, 34 GDM patients and 34 healthy controls, were recruited in the current study. Six pairs of samples were used for microarray analysis and the remaining 28 pairs were used for further validation. The protocol of this study was approved by the Medical Ethics Committee of Ningbo University, and all participants signed the informed consent form.

RNA Preparation and Microarray Analysis

Aliquots of 2 ml of fresh peripheral blood were collected from each participant, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll separation solution (Bioss, USA). TRIzol pyrolysis solution (Omega, USA) was added, and then, the samples were stored in the freezer at −80°C before use. An HP total RNA extraction kit (Omega, USA) was used to extract the total RNA according to the manufacturer's instructions. The RNA quality was evaluated by a NanoDrop 2000 spectrophotometer on the basis of an optical density (OD) 260/280 ratio ≥1.8 and an OD260/230 ratio ≥2.0. RNA integrity was measured using an Agilent 2100 Bioanalyzer. The intensity of the 18S and 28S rRNA bands was examined by 1% formaldehyde denaturing gel electrophoresis. RNA samples with an RNA integrity number (RIN) of ≥7.0 and 28S/18S >1.5 were adjusted to the same concentration and then subjected to microarray analysis.

Microarray analysis, namely, RNA amplification, probe labeling, hybridization, and data extraction, was performed by CapitalBio Corporation. The CapitalBio mRNA Amplification and Labeling Kit (CapitalBio, China) and circRNA Amplification and Labeling Kit (CapitalBio, China) were used to amplify and transcribe mRNA, respectively, and the RNase R-enriched circRNA was used to generate fluorescent cRNA. The labeled cRNAs were hybridized onto the CapitalBio Technology Human mRNA Array V4 (CapitalBio, China) and CapitalBio Technology Human CircRNA Array v2.0 (CapitalBio, China). Subsequently, the arrays were scanned using the Agilent Scanner G2565CA (Agilent, USA). Acquired array images were analyzed with Agilent Feature Extraction (v10.7) software. Overall, 12 mRNA microarray chips (6 GDM patients and 6 healthy controls) and 12 circRNA microarray chips (6 GDM patients and 6 healthy controls) were analyzed in this study.

Statistical Analysis of the Microarray Data

Data summarization, quality control, quantile normalization, and analysis of differentially expressed mRNAs/circRNAs were performed with GeneSpring v13.0 (Agilent, USA). Individually, mRNAs and circRNAs with a fold change ≥2 or ≤0.5 and FDR <0.05 were considered significantly differentially expressed. Gene annotation and biological interpretation of the identified differentially expressed mRNAs/circRNAs were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.8. Biological functions, represented by the Gene Ontology terms (http://geneontology.org/) and the Kyoto Encyclopedia of Genes and Genomes pathways (http://genome.jp/kegg/), were considered significant at a Benjamini–Hochberg-corrected P <0.05.

Prediction of circRNA/miRNA/mRNA Interactions and circRNA-Binding Proteins

The miRanda software and TargetScan database (http://www.targetscan.org/) were used to predict target miRNAs for candidate mRNAs and circRNAs. Cytoscape 3.2.1 software was used to construct a circRNA–miRNA–mRNA coexpression network to predict the functions and interactions between RNAs. In addition, the CircInteractome database (https://circinteractome.nia.nih.gov/) was used to predict the candidate binding proteins of circRNAs.

Validation of Candidate mRNAs and circRNAs

Four differentially expressed mRNAs (CBLB, ITPR3, NFKBIA, and ICAM1) and four differentially expressed circRNAs (circ-CBLB, circ-ITPR3, circ-NFKBIA, and circ-ICAM1) selected from the T Cell Receptor Signaling Pathway were further validated by quantitative real-time PCR (qRT-PCR) and droplet digital PCR (ddPCR) in another 28 independent samples. qRT-PCR was performed with the LightCycler 480 SYBR Green I Master Mix (Roche, Germany), and ddPCR was performed by QX200™ ddPCR™ EvaGreenSupermix (Bio–Rad, USA) according to the manufacturer's instructions. After qRT-PCR, the relative mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method, with GAPDH as an internal control. The primers for candidate mRNAs and circRNAs are listed in **Supplemental Table 1**. The products of qPCR and ddPCR were further confirmed by electrophoresis.

Statistical Analysis

SPSS 22.0 statistical software was used for statistical analysis. For the baseline information and clinical data of the participants, the continuous normal data are expressed as the mean ± standard deviation (X ± S), and the difference between groups were

compared by two independent samples *t*-tests; the nonnormal continuous data are expressed as the median (upper and lower quartiles), and the Mann–Whitney rank test was used for intergroup comparison; the classified data are presented in the form of cases (%), and the intergroup comparisons were conducted by χ^2 test or Fisher exact probability method. The verification results of candidate circRNAs and mRNAs between the GDM group and the healthy pregnant group were expressed by $\bar{X} \pm S$ or median (upper and lower quartiles). Paired sample *t*-test or Wilcoxon signed rank test was used for inter-group comparison. For the microarray test results, the data are normalized and analyzed by Agilent GeneSpring software, and cluster analysis is carried out by Cluster 3.0 software. Statistical images were drawn by GraphPad Prism 7.0 and OriginPro 2019b drawing software. Statistical significance was defined as $P < 0.05$.

RESULTS

Characteristics of the Participants

In this study, 34 pairs of pregnant women in the GDM and control groups were selected. Of these pairs, samples from 6 pairs were randomly selected for mRNA/circRNA microarray analysis, and samples from the remaining 28 pairs were tested for follow-up verification. The epidemiological characteristics and clinical indicators of these 34 pairs of participants are shown in **Supplemental Table 2**. There was no significant difference in the general characteristic indices between the two groups, except for the significantly higher OGTT level in the GDM group; these results indicated that the baseline indices of the two groups were well matched.

Identification of the Differentially Expressed mRNAs and circRNAs

The current microarray data are available in the Gene Expression Omnibus (GEO, accession number GSE182737). The cluster analysis showed that both the expression profiles of mRNAs and circRNAs could distinguish the GDM group from the control group (**Supplemental Figures 1A**, **2A**). A total of 641 differentially expressed mRNAs were identified between the GDM group and the control group. Among these mRNAs, 269 were upregulated and 372 were downregulated (**Supplemental Figure 1B**). In addition, there were 7,950 differentially expressed circRNAs, of which 3,414 were upregulated and 4,536 were downregulated (**Supplemental Figure 2B**). Scatter plots and volcano maps were made according to their signal values and differential distributions (**Supplemental Figures 1C–D**, **2C–D**). The top 10 mRNAs and circRNAs that were the most significantly upregulated and downregulated are shown in **Supplemental Tables 3** and **4**.

Functional Annotation and Enrichment Analysis of the Differentially Expressed mRNAs/circRNAs

According to the GO analysis, the differentially expressed mRNAs and circRNAs were enriched in many similar GO terms as well as distinct GO terms (**Figure 1**). Compared with the profile of healthy controls, the GDM-associated differentially expressed mRNAs were primarily enriched in immune-related processes, such as immune response (GO:0006955), T cell aggregation (GO:0070489), and T cell activation (GO:0042110) (**Figure 1A**). Moreover, the GDM-associated differentially expressed circRNAs were enriched in the negative regulation of NF-kappaB signaling (GO:0043124), regulation of cellular amino acid metabolic process (GO:0006521), and T cell receptor signaling pathway (GO:0050852) (**Figure 1B**). Therefore, the majority of differentially expressed genes appear to be associated with the immune response mediated by T cells and receptor activity.

Similarly, KEGG pathway enrichment analysis showed that the significantly enriched pathways of differentially expressed mRNAs were immune-related pathways such as the T cell receptor signaling pathway (hsa04660), Th17 cell differentiation (hsa04659), primary immunodeficiency (hsa05340), and natural killer cell-mediated cytotoxicity (hsa04650) (**Figure 2A**). The pathways enriched by differentially expressed circRNAs were inflammatory and immune-related pathways such as proteasome (hsa03050), Epstein–Barr virus infection (hsa05169), human T-cell leukemia virus-1 infection (hsa05166), and IL-17 signaling pathways (hsa04657) (**Figure 2B**).

Further analysis indicated that the upregulated mRNAs were significantly enriched in pathways that were consistent with the overall result, which were immune-related pathways, especially T cell-related immunity (**Supplemental Figure 3A**). The pathways enriched by differentially expressed downregulated mRNAs were infection-related pathways, such as cytokine–cytokine receptor interaction (hsa04060), influenza A (hsa05164), hepatitis C (hsa05160), and human cytomegalovirus infection (hsa05163) (**Supplemental Figure 3B**).

Validation of Candidate mRNAs and circRNAs

Cross matching of the differentially expressed mRNAs and circRNAs in the top 10 KEGG pathways, 4 genes (*CBLB*, *ITPR3*, *NFKBIA*, and *ICAM1*) and 4 corresponding circRNAs (circ-CBLB, circ-ITPR3, circ-NFKBIA, and circ-ICAM1) were identified. These mRNAs and circRNAs are all listed in the T cell receptor signaling pathway. The results of the original microarray expression of these candidate mRNAs and circRNAs are shown in **Supplemental Table 5**.

In this study, the expression levels of candidate mRNAs and circRNAs were verified by qRT-PCR and ddPCR in the 56 samples. The results indicated that the above four mRNAs and three circRNAs (circ-CBLB, circ-ITPR3, and circ-ICAM1) were significantly highly expressed in the GDM group, which was consistent with our microarray results (**Figure 3**).

Prediction of circRNA/miRNA/mRNA Interactions and circRNA-Binding Proteins

A competing endogenous RNA (ceRNA) regulatory network was constructed by integrating the correlations and regulatory relationships among circRNAs, miRNAs, and mRNAs

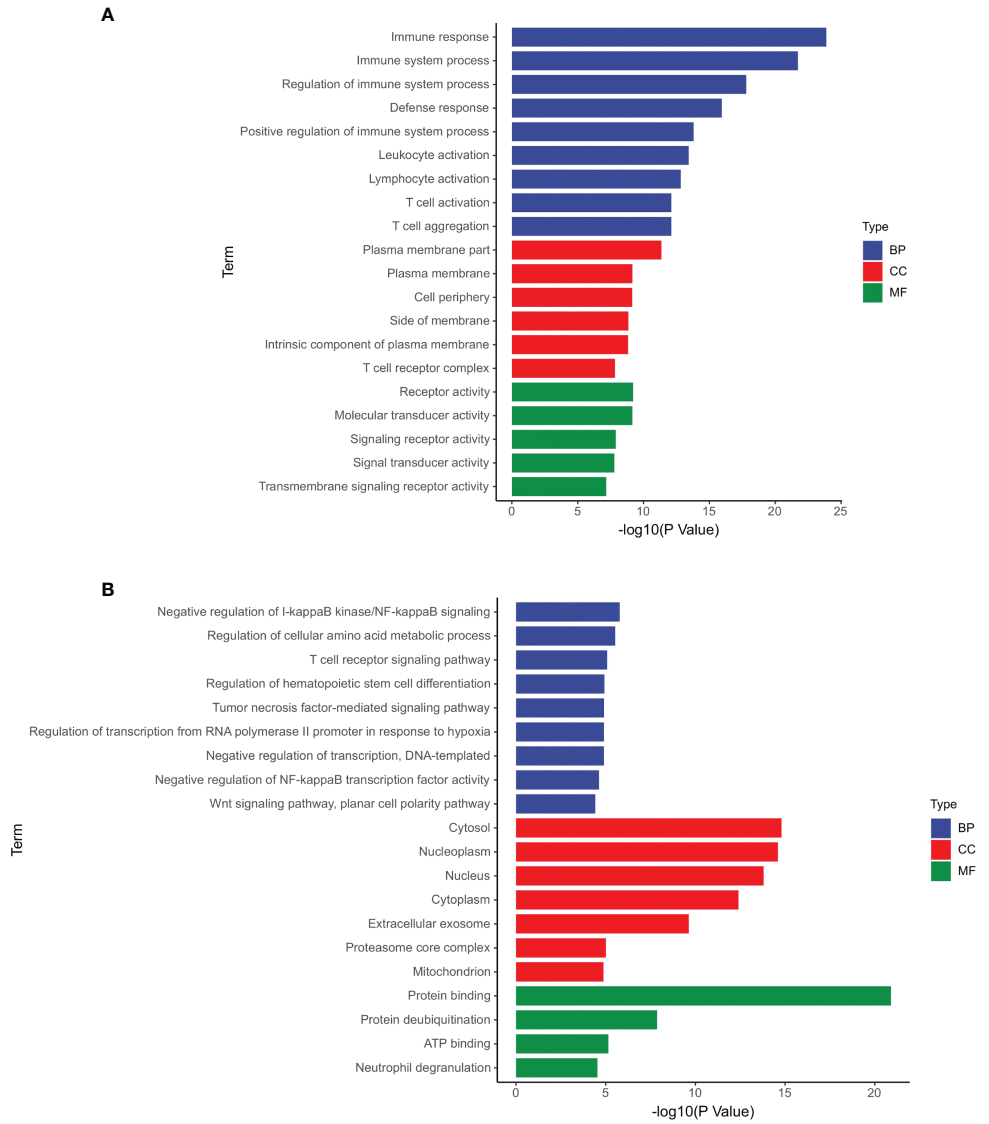

FIGURE 1 | GO terms of the differentially expressed mRNAs and circRNAs. **(A)** Differentially expressed mRNAs; **(B)** differentially expressed circRNAs.

(**Supplemental Figure 4**). There are two regulatory relationships of circRNA–miRNA and miRNA–mRNA in the network, thus forming a circRNA–miRNA–mRNA coexpression model. The network contains 4 significantly upregulated candidate circRNAs, 4 significantly upregulated candidate mRNAs, and 594 predicted miRNAs. By calculating the degrees of each node in the network, we found that the nodes with the most degrees in the three kinds of RNA were circ-ITPR3, CBLB, and hsa-miR-203a-3p. In addition, the circRNA–miRNA–mRNA coexpression model showed that the network predicted a total of 16 key miRNAs that may play an intermediate regulatory role.

The binding proteins of the differentially expressed circRNAs were also predicted. The results indicated that circ-ITPR3 had the most abundant RNA-binding protein sites, and the largest number of RNA-binding proteins bound to circ-ITPR3 was EIF4A3, with 58 sites, followed by FMRP, IGF2BP, AGO2, and HuR. In addition, EIF4A3 bound to the flanking regions of all three candidate circRNAs (**Supplemental Figure 5**).

DISCUSSION

GDM is the most common complication of pregnancy, affecting up to 20% of pregnancies; GDM significantly contributes to preeclampsia and depression, and requiring a caesarean section (23). Because complex pathways are implicated in the pathophysiology of GDM, there are still no specific means of predicting or preventing GDM progression. Gene expression microarrays have been widely used in diabetic studies because alterations in transcriptional profiles provide a robust and

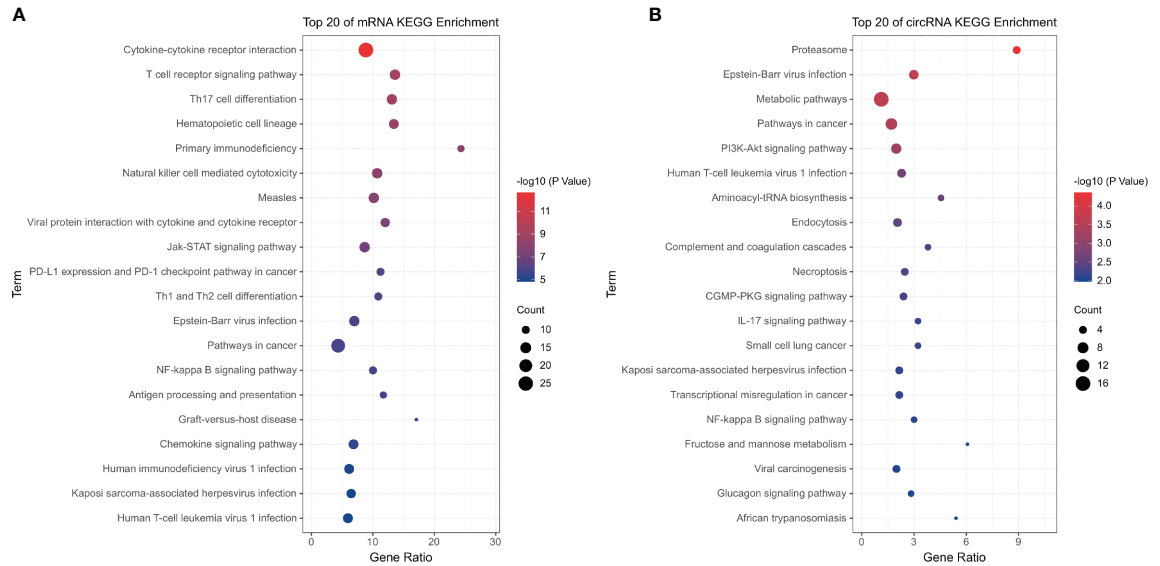

FIGURE 2 | KEGG pathway analysis of the differentially expressed mRNAs and circRNAs. **(A)** Differentially expressed mRNAs; **(B)** differentially expressed circRNAs.

sensitive way to better understand the pathogenesis of the disease. mRNA and ncRNA microarray analyses have previously been used to investigate the mechanisms underlying diabetic neuropathy (24), diabetic nephropathy (25), diabetic cardiomyopathy (26), and diabetic periodontitis (27). Similarly, microarray analyses have also been conducted in GDM. Genomic expression profiles of blood and placenta revealed significant immune-related pathways in Chinese women with gestational diabetes mellitus (28). The lncRNA microarray analysis revealed that lncRNAs ERMP1, TSPAN32, and MRPL38 form a coexpression network with TPH1, which is mainly involved in the tryptophan metabolism pathway, and lncRNA RPL13P5 forms a coexpression network with the TSC2 gene *via* the pi3k-akt and insulin signaling pathways (29). The miRNA expression profiles of plasma samples revealed that miR-574-5p and miR-3135b may serve as metabolic regulators of glucose and lipid levels in GDM (30).

CircRNA expression profiles were also measured in patients with GDM. One study analyzed the circRNA expression profiles in umbilical cord blood exosomes from normal subjects and gestational diabetes mellitus patients, and these results may delineate the roles of exosomal circRNAs in GDM development and fetal growth (31). However, the results cannot be used as predictive biomarkers for GDM since umbilical cord blood was used. Another study measured circRNA expression profiling in 6 paired women (with and without GDM) and showed that hsa_circRNA_102893 may be a potential novel and stable noninvasive biomarker for detecting GDM in early pregnancy (32). In addition, Yan et al. conducted transcriptome sequencing of placental tissues in Chinese pregnant women, and found 482 differentially expressed circRNAs in GDM patients. These circRNAs were significantly enriched in pathways related to glycometabolism and lipometabolism processes (33). However, these studies only screened the differentially expressed circRNAs, no further mRNA–circRNA joint analysis was conducted, and no potential pathways were further examined. Therefore, performing new transcriptional microarray analyses with commonly used human sample material, such as PBMCs, to identify the gene expression signatures and potential predictive biomarkers of GDM is necessary. The current study revealed that differentially expressed mRNAs/circRNAs were mainly enriched in immune-related pathways, such as the T cell receptor signaling pathway, Th17 cell differentiation, primary immunodeficiency, and NK cell-mediated cytotoxicity, strongly suggesting that GDM may involve immune responses, especially responses associated with T cell immune regulation. Furthermore, cross matching of the differentially expressed mRNAs and circRNAs in the top 10 KEGG pathways identified 4 genes and 4 corresponding circRNAs, which were all listed in the T cell receptor signaling pathway.

GDM has been associated with an impaired maternal immune response (34). During normal pregnancy, the antigenic substances expressed by the fetus can trigger maternal immune activation to a certain extent, and then, T cells and NK cells can help maintain homeostasis (35). When metabolism and immunity in the pregnancy environment are severely challenged, the mother will superimpose the dual effects of low-grade systemic inflammation and insulin resistance, thereby promoting the occurrence of GDM (36). Studies have shown that in GDM patients, the proportion of activated $CD4^+$ T cells significantly increased, while the percentage of $CD8^+$ T cells decreased, suggesting a state of superactivation and a deficiency of suppressive mechanisms (9). Moreover, the percentage of Th17 cells and the ratio of proinflammatory cells (Th17:Treg, Th17.1:Treg, and Th1:Treg) were increased in GDM patients,

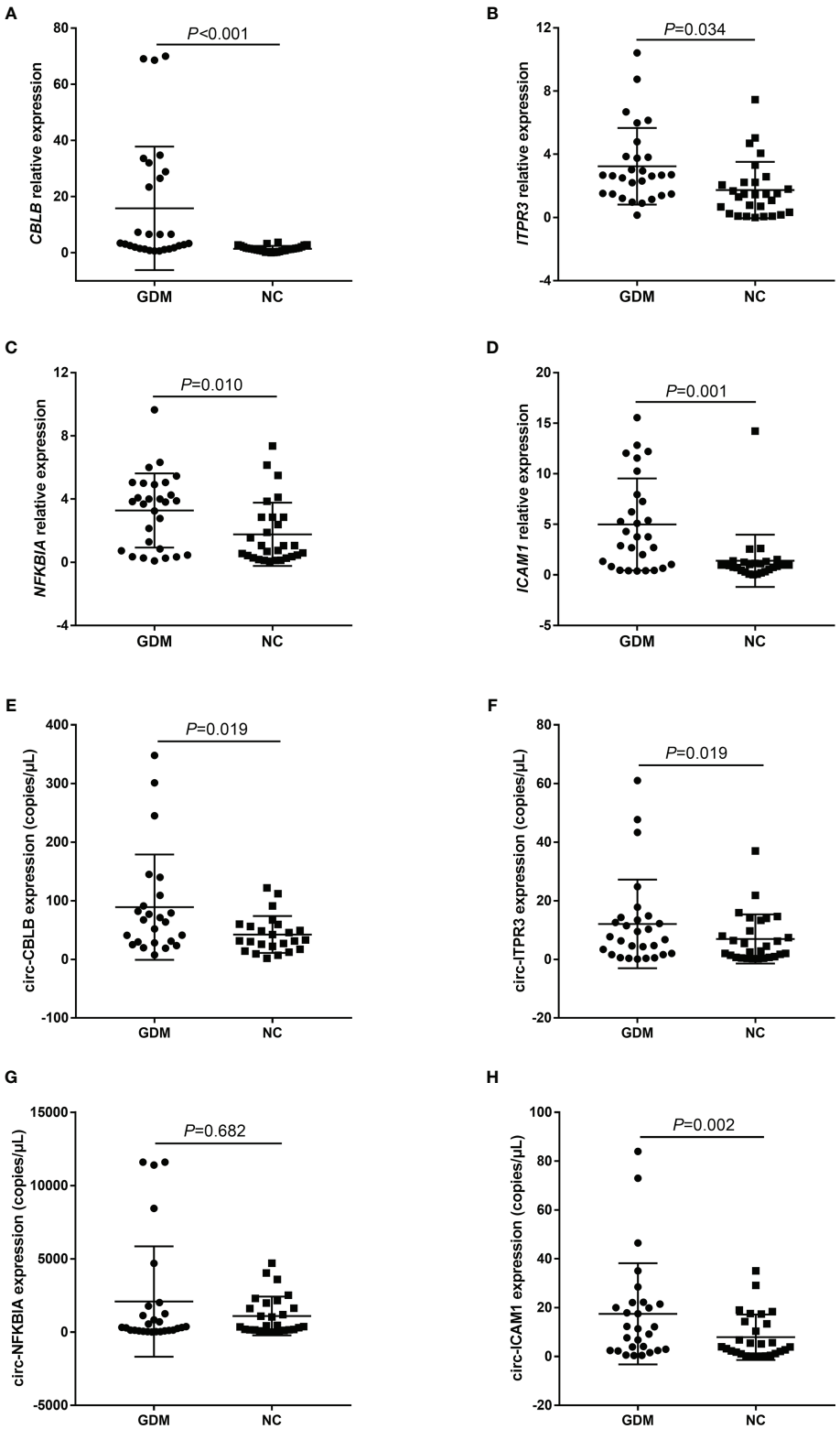

FIGURE 3 | Microarray data were validated by qRT-PCR and ddPCR. **(A–H)** represent the expression distribution of CBLB, ITPR3, NFKBIA, and ICAM1 and their corresponding candidate circRNAs, circ-CBLB, circ-ITPR3, circ-NFKBIA, and circ-ICAM1, in different samples of the GDM group and control group, respectively. NC, Normal Control.

which significantly promoted the secretion of proinflammatory factors, such as IL17 and TNF α (37). Abnormal elevation of TNF α can further interfere with insulin receptor tyrosine autophosphorylation and promote serine phosphorylation of insulin receptor substrate 1 (IRS1), thus destroying the insulin signal cascade (38, 39). Before our study, two independent microarray studies reported similar results (28, 40). A global placental gene expression study identified sixty-six genes participating in cell functions involving cell activation, immune response, organ development, and regulation of cell death that were differentially expressed in GDM placentas (40). Genomic expression profiles of blood and placenta reveal significant immune-related pathways, 'natural killer cell mediated cytotoxicity' in blood, and 'cytokine–cytokine receptor interaction' in placenta, in Chinese women with gestational diabetes mellitus (28). The current study not only revealed that upregulation of T cell receptor signaling pathway components might be the major pathological mechanism underlying GDM but also suggested that circRNAs circ-CBLB, circ-ITPR3, circ-ICAM1, and their related linear transcripts *CBLB*, *ITPR3*, *NFKBIA*, and *ICAM1* might be potential biomarkers for GDM.

Circ-CBLB is composed of 398 nt and is located on human chromosome 3, and its corresponding parent gene is *CBLB*, which encodes E3 ubiquitin protein ligase; this protein promotes ubiquitin-mediated protein degradation by transferring ubiquitin from E2 ubiquitin-binding enzyme to the matrix. Some studies have shown that CBLB regulates T cell tolerance in peripheral blood and plays a key role in host pathogen defense and antitumor immunity (41). In the process of T cell receptor signal transduction, CBLB can mediate the hydrolysis of the upstream proteins ZAP70 and LCK through the ubiquitin pathway, which is involved in the negative feedback regulation of T cell receptor signaling (42). Interestingly, our microarray results showed that the expression of *CBLB* significantly upregulated in the GDM group and that the expression of the upstream *ZAP70*, *LCK*, and CD3 family molecules (*CD3D*, *CD3E*, *CD3G*, and *CD247*) of the T cell receptor signaling pathway was also significantly upregulated (**Supplemental Figure 6**). The result was also verified by qRT-PCR and strongly suggests that T cell immunity in GDM patients may be in a state of hyperactivation. Hansen also found that the expression of *CBLB* was significantly upregulated in T2D patients (43). Further functional studies showed that the loss of specificity of *CBLB* led to insulin and glucose tolerance test response disorder (44), and blocking CBLB-induced macrophage activation could improve insulin resistance (45). The current study also found that circ-CBLB expression was significantly upregulated in GDM patients, which has not been previously reported in diabetes. Considering the transcriptional regulation of parental genes by circRNAs, circ-CBLB may affect the expression of *CBLB* by regulating the alternative splicing of corresponding mRNA transcripts. However, this possibility still requires further investigation.

Circ-ITPR3 is located on the positive chain of chromosome 6 and consists of 5146 nt. In the current study, it was predicted that miR-24-3p may bind to circ-ITPR3 and then affect the downstream target gene *NFKBIA*. miR-24-3p expression was found to be significantly downregulated in diabetes, and the expression level of miR-24-3p was significantly correlated with serum insulin and HbA1c levels (46). NFKBIA is a member of the NF-κB inhibitor family and can inhibit inflammation. T cell receptor activation and related TNF secretion can induce an increase in *NFKBIA* expression (47). Therefore, it can be inferred that the upregulation of circ-ITPR3 expression may significantly increase *NFKBIA* expression by inhibiting the expression of miR-24-3p, playing a negative feedback role in GDM-related inflammation. In addition, we found that the largest number of circ-ITPR3 binding site was EIF4A3, which is an ATP-dependent RNA helicase and widely involved in RNA splicing and nonsense-mediated mRNA decay. It was also reported that EIF4A3 can regulate cell cycle and apoptosis through TNF-α/NF-κB signaling pathway, which is one of the key signal pathways affecting diabetes (48, 49). Therefore, we speculate that circ-ITPR3 may regulate the expression of *ITPR3* and NF-κB family member *NFKBIA* by recruiting EIF4A3, which subsequently affects the development of GDM.

The parent gene of circ-ITPR3 is *ITPR3*, which encodes inositol 1,4,5-trisphosphate receptors (IP3R), a calcium release channel that responds to the second messenger inositol 1,4,5-trisphosphate (IP3). IP3 can be phosphorylated by inositol 1,4,5-trisphosphate 3-kinase and act as a negative regulator of Ca2+/nuclear factor of activated T cells (NFAT) in the T cell receptor signaling pathway (50). Upregulation of ITPR3 expression may induce autoimmune disorders and promote the occurrence and development of T1D by activating T cells (51). Moreover, genetic studies have shown that the SNPs of *ITPR3* are closely associated with T1D in Caucasian populations (52, 53) and autoimmune diseases (namely, systemic lupus erythematosus, rheumatoid arthritis, and Graves' disease) in Japanese populations (54). Although the present study showed that *ITPR3* expression is significantly upregulated in the GDM group, its potential mechanism still needs to be further studied.

The corresponding parent gene of circ-ICAM1 is *ICAM1*, which encodes intercellular adhesion molecule 1, which is a glycoprotein on the surface of antigen-presenting cells. ICAM1 is an important part of T cell immune synapses and promotes the interaction of T cell receptor signal transduction molecules and the activation of the T cell receptor signaling pathway (55, 56). It has been reported that the plasma ICAM1 concentration of patients with T2D was significantly higher than that of control subjects (57). With the progression of diabetic complications, such as diabetic nephropathy (56), diabetic peripheral neuropathy (58), and diabetic retinopathy (59), the level of ICAM1 further increases. Upregulation of ICAM1 expression was also observed in GDM patients (60), which was consistent with the present result. The potential mechanism suggested that the increase in the ICAM1 levels may cause vascular endothelial dysfunction, leading to vascular inflammation, vascular functional imbalance, and increased oxidative stress, thus promoting the progression of hyperglycemia (61). Although the current study found that circ-ICAM1 expression was significantly upregulated in the GDM group, the target effect of

circ-ICAM1 on diabetes-related diseases has never been reported. The constructed ceRNA network predicted that both miR-874-3p and miR-339-5p significantly interacted with circ-ICAM1. Huo et al. found that the expression of miR-874-3p in diabetic rats was significantly downregulated, which led to increased islet β cell apoptosis and diabetes-induced erectile dysfunction by targeting the inhibition of the nuclear protein 1 (Nupr1)-mediated pathway (62). It was also observed that miR-339-5p and miR-874-3p mediate high glucose-induced endothelial inflammation by targeting interleukin-1 receptor-associated kinase 1 (*IRAK1*) expression (63). The increased *IRAK1* expression was related to the upregulation of *ICAM1* gene expression and the enhancement of monocyte adhesion, which may be due to the weakening of the feedback inhibitory effect of IRAK1 on endothelial inflammation and the decrease in the anti-inflammatory effects of miR-339-5p and miR-874-3p in the diabetic environment (63). Therefore, the significant upregulation of *ICAM1* expression in the current study may be due to the miRNA sponge effect of circ-ICAM1 on miR-874-3p and miR-339-5p in GDM patients.

CONCLUSION

In summary, these findings provide the first demonstration that upregulation of T cell receptor signaling pathway components may be the major pathological mechanism underlying GDM. circ-CBLB, circ-ITPR3, and circ-ICAM1 may serve as GDM-related miRNA sponges and regulate the expression of CBLB, ITPR3, NFKBIA, and ICAM1 in cellular immune pathways, inducing vascular inflammation, immune activation, β cell apoptosis, and other related pathophysiological processes,

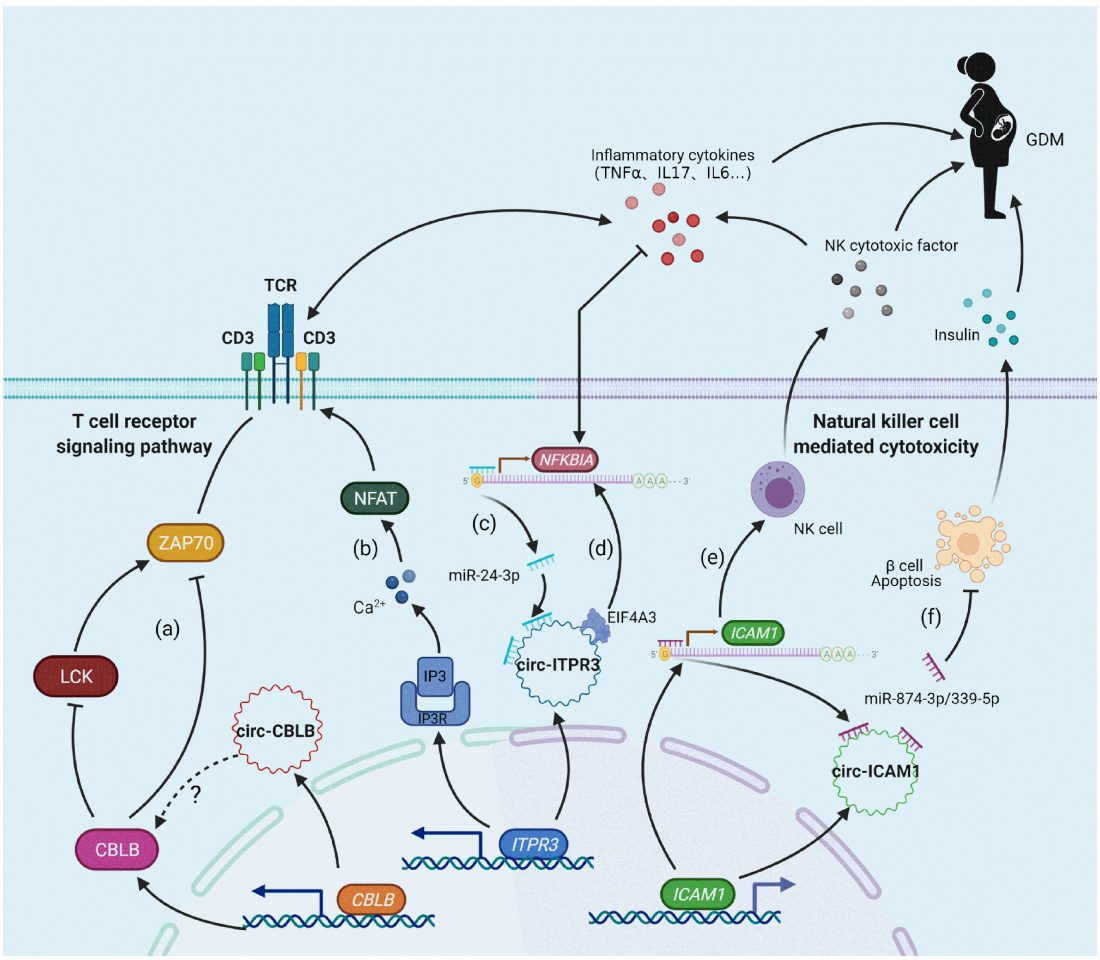

FIGURE 4 | Molecular mechanism of the relationship between candidate circRNAs/mRNAs and GDM. **(A)** CBLB can inhibit LCK and ZAP70 expression levels by ubiquitination, thus exerting negative feedback regulation on T-cell receptor activation; **(B)** ITPR3 can encode IP3R in response to IP3-mediated $Ca2^{+}$ release and promote the activation of the T cell receptor signaling pathway; **(C, E)** circ-ITPR3 and circ-ICAM1 act as miRNA sponges for miR-24-3p and miR-874-3p/miR-339-5p, respectively, which induces upregulation of the downstream target genes NFKBIA and ICAM1; **(D)** circ-ITPR3 regulates the expression of NFKBIA by recruiting EIF4A3. **(F)** The adsorption of circ-ICAM1 to miR-874-3p weakens the anti-apoptotic effect of miR-874-3p, which increases the apoptosis of β cells and decreases the release of insulin.

finally promoting the occurrence and development of GDM (**Figure 4**). T cell receptor immune repertoire analysis is being carried out in GDM patients, which may provide more details. Moreover, functional analyses of the relevant circRNAs, miRNAs, mRNAs, and proteins are necessary, and these analyses may provide a potential approach for the prevention and treatment of GDM.

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 Medical Ethics Committee of Ningbo University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

L-DJ and JX raised the idea for the study. Y-MC, QZ, JC, Z-JZ, and B-BY contributed to the study design, sample collection, and conducted the experiment. Y-MC, L-MZ, L-DJ, and JX analyzed the data and write the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.774608/full#supplementary-material

Supplementary Figure 1 | The expression profiles of mRNAs in the GDM and control groups. **(A)** cluster analysis of mRNA microarray. The red column at the bottom represents the GDM group, and the blue column represents the control group; red indicates relatively high expression, green indicates relatively low expression; **(B)** the number of differentially expressed mRNAs; **(C)** the scatter plot of mRNA signal value; **(D)** the volcano plot analysis of the differentially expressed mRNAs, in which red and green dots represent up- and downregulated mRNAs, respectively.

Supplementary Figure 2 | The expression profiles of circRNAs in the GDM and control groups. **(A)** cluster analysis of circRNA microarray. The red column at the bottom represents the GDM group, and the blue column represents the control group; red indicates relatively high expression, green indicates relatively low expression; **(B)** the number of differentially expressed circRNAs; **(C)** the scatter plot of circRNA signal value; **(D)** the volcano plot analysis of the differentially expressed circRNAs, in which red and green dots represent up- and downregulated circRNAs, respectively.

Supplementary Figure 3 | KEGG pathway analysis of the upregulated and downregulated mRNAs. **(A)** upregulated mRNAs; **(B)** downregulated mRNAs.

Supplementary Figure 4 | circRNA-miRNA-mRNA coexpression network. The circular node represents the candidate circRNA, the square represents the candidate mRNA, and the triangle represents the predicted miRNA. With the number of nodes interacting with each other, that is, the number of degrees, the node changes from small to large. With the differential expression ratio of candidate RNAs between the two groups from low to high, the node color gradually changed from light red to deep red. The predicted miRNA has no expression value and is expressed as a green node.

Supplementary Figure 5 | Prediction of candidate circRNA-binding protein sites. **(A–C)** represent the distribution of RNA-binding proteins and their binding sites of circ-ITPR3, circ-CBLB and circ-ICAM1, respectively.

Supplementary Figure 6 | Microarray data of *ZAP70*, *LCK* and CD3 family molecules. The results are expressed as the mean ± SD The FC value of the control group was set as 1. *represents statistical significance ($P<0.05$) compared with the control group.

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qPCR Analysis Reveals Association of Differential Expression of *SRR*, *NFKB1*, and *PDE4B* Genes With Type 2 Diabetes Mellitus

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Background: Type 2 diabetes mellitus (T2DM) is a heterogeneous, metabolic, and chronic condition affecting vast numbers of the world's population. The related variables and T2DM associations have not been fully understood due to their diverse nature. However, functional genomics can facilitate understanding of the disease. This information will be useful in drug design, advanced diagnostic, and prognostic markers.

Aim: To understand the genetic causes of T2DM, this study was designed to identify the differentially expressed genes (DEGs) of the disease.

Methods: We investigated 20 publicly available disease-specific cDNA datasets from Gene Expression Omnibus (GEO) containing several attributes including gene symbols and clone identifiers, GenBank accession numbers, and phenotypic feature coordinates. We analyzed an integrated system-level framework involving Gene Ontology (GO), protein motifs and co-expression analysis, pathway enrichment, and transcriptional factors to reveal the biological information of genes. A co-expression network was studied to highlight the genes that showed a coordinated expression pattern across a group of samples. The DEGs were validated by quantitative PCR (qPCR) to analyze the expression levels of case and control samples (50 each) using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the reference gene.

Results: From the list of 50 DEGs, we ranked three T2DM-related genes ($p < 0.05$): *SRR*, *NFKB1*, and *PDE4B*. The enriched terms revealed a significant functional role in amino acid metabolism, signal transduction, transmembrane and intracellular transport, and other vital biological functions. *DMBX1*, *TAL1*, *ZFP161*, *NFIC* (66.7%), and *NR1H4* (33.3%) are transcriptional factors associated with the regulatory mechanism. We found substantial enrichment of insulin signaling and other T2DM-related pathways, such as

valine, leucine and isoleucine biosynthesis, serine and threonine metabolism, adipocytokine signaling pathway, P13K/Akt pathway, and Hedgehog signaling pathway. The expression profiles of these DEGs verified by qPCR showed a substantial level of twofold change (FC) expression ($2^{-\Delta\Delta C_T}$) in the genes *SRR* (FC ≤ 0.12), *NFKB1* (FC ≤ 1.09), and *PDE4B* (FC ≤ 0.9) compared to controls (FC ≥ 1.6). The downregulated expression of these genes is associated with pathophysiological development and metabolic disorders.

Conclusion: This study would help to modulate the therapeutic strategies for T2DM and could speed up drug discovery outcomes.

Keywords: cDNA datasets, T2DM, differential expressed genes, enrichment analysis, qPCR, expression profiling

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic and complicated condition characterized by insulin resistance and insulin deficiency due to hyperglycemia (1). Currently, about 285 million individuals are estimated to have type 1 and 2 diabetes, which makes up around 90% of the total (2). Tissues including the pancreas, liver, skeletal muscle, adipose, and intestinal tissues have a role in its progress (3), and despite this, several critical variables such as lifestyle, food, obesity, and genetic factors were identified in insulin and T2DM development (4–6). The fundamental mechanisms are still unclear, and due to its high morbidity and increased clinical impact, this disease has become an extremely severe global issue (2). Due to the multifaceted mechanisms and diverse contributory factors of T2DM, there is an urgent need to identify vital biological molecules as potential therapeutic targets and improve the treatment strategies. Different oral and injectable treatments for T2DM are available; however, the principal difficulties associated with the use of these medications are due to a lack of effectiveness, hypoglycemia, weight gain, and increased susceptibility to infections. It is thus a responsibility of the scientist to study the causes and the mechanisms of the disease to properly manage the risks.

Recent developments have shown that diabetes is diverse, with complicated genetic causes. Many studies have demonstrated the significance of various genes in the etiology of disease. Most genes participating in T2DM have been recognized as prospective therapeutic targets; nevertheless, the limited knowledge of the complexity and interaction of these systems has been a significant problem in the development of effective T2DM treatments. Most cases of diabetes include several genes, each of which contributes to an increased risk of type 2 diabetes (2), and similar genes related to T2DM poorly show recognized pathways of insulin signaling (5). The present techniques for finding statistically significant functional classes of genes associated with T2DM have detected regulatory cell cycles (7, 8). However, in T2DM and in the molecular biology of insulin resistance, the functional categories and the potential therapeutic role of the expressed genes have not been fully understood (8). There are, therefore, still substantial gaps in the clinical outcomes, encouraging scientists to seek further improvements in each of these issues. The differentially expressed genes (DEGs) associated with T2DM, such as *SRR*, *NFKB1*, and *PDE4B*, were examined in cases and controls to identify the genetic causes of the disease.

Based on quantitative PCR (qPCR)-validated genome- and transcriptome-level studies, *CHL1*, *LRFN2*, *RASGRP1*, *NFKB1*, and PPM1K have been substantially linked with insulin secretion and T2DM. In non-diabetic individuals, the influence of genetic diversity on continuous hyperglycemia events has largely shown that insulin secretion has been disturbed (9). Systems biology based on genome-wide association studies (GWAS) reveals the genetic reasons for T2DM pathogenesis and insulin resistance (9). Likewise, aberrant glucagon secretion resulted in inflammation of the islets in T2DM, and interleukin-6 has been found to stimulate secretion (10). Quantitative real-time PCR (qRT-PCR) is a method of choice in gene expression profiling and follow-up validation based on its high accuracy rate with an increased dynamic range and sensitivity (11, 12). An important technique to observe relative gene expression changes in qPCR studies is $2^{-\Delta\Delta C_T}$. This approach is useful in assessing the relative levels of gene expression among various samples by using the qPCR technology directly to compute the threshold cycle (CT) (13).

Genome expression in insulin signaling and integrated pathology can alter any of these genes that might develop clinically important insulin resistance and diabetes (2). The systems biology method may combine these biological networks and assist in uncovering important etiological factors. As genes are crucial to a better understanding of the network of systems biology, complementary DNA (cDNA) microarray technology is an effective tool to simultaneously analyze the expression levels of thousands of genes. The significant number of expression datasets in the public field provides a valuable source of genome-wide information on T2DM and gives an opportunity to investigate the expression of a large number of samples. This work aimed to discover the insulin resistance and T2DM genetic variations by qPCR experimental validation. System-level analysis screened out the *SRR*, *NFKB1*, and *PDE4B* genes from cDNA datasets. qPCR analysis examined the dysregulation of these genes and their pathological role in

T2DM. These findings would help in understanding the genetic basis of the disease and will update therapeutic strategies against T2DM.

MATERIALS AND METHODS

Ethical Approval and Collection of Blood Samples

We collected a total of 100 individual blood samples with an equal ratio of control/cases (n = 50) from the local hospital in Multan, Punjab Province. For qPCR analysis, blood samples were collected from type 2 diabetic patients and healthy individuals based on inclusion and exclusion criteria. The approval of the study and informed consent were obtained from the Research Ethics Committee of the Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan (ref. no. IMBB/2019/002).

Inclusion and Exclusion Criteria

We used 1) pathologically confirmed cases of T2DM; 2) new patients diagnosed by the Nishtar Hospital, Multan, for the first time; 3) patients aged ≥18 years; 4) patients who agreed to provide blood samples for scientific research and consent for the publication of research data; and 5) healthy individuals with no history of diabetes, cancer, and cardiovascular diseases, who were included as controls. The exclusion criteria were: 1) pathologically confirmed local vascular invasion; 2) cases with multiple and complex diseases; 3) cases with cancer or immune disorders; and 4) cases with a history of surgery in the past 3 years.

Normalization and Differential Expression Analysis

Twenty T2DM-related cDNA expression datasets from the NCBI Gene Expression Omnibus (GEO) database were downloaded (**Supplementary Table S1**). DEGs were identified using the Affymetrix U133 Plus 2.0 Array platform and a hgu133plus2 annotation probe. The R platform with Affy, AffyQCReport, AnnotationDbi, Annotate, Biobase, Lima, and hgu133a2cdf Bioconductor packages were used to analyze the computable outcomes. AffyRNAdeg, summary AffyRNAdeg, and plotAffyRNAdeg were used for RNA degradation analysis and for checking the quality of RNA samples. Perfect match (PM) and mismatch (MM) were measured using the Robust Multi-Array Average (RMA) normalization approach, and normalization of the microarray datasets was utilized for comparison. These datasets were grouped into recognizable pheno-data files that include the accession numbers, sample types, number of samples, disease, and clinical conditions (14). RMA was applied for noise reduction from local signals (15, 16). RMA has been a frequently used method to generate an expression matrix from Affymetrix data. The raw expression values of genomic data were background corrected, log2 transformed, and then quantitatively standardized, followed by the linear model being applied to acquire an expression measure for each probe set on each cDNA array. Normalization was carried out to observe the perfect matches through the median level. For normalization and background correction, PM and MM, the following equation was applied:

$$\mathrm{PM}_{ijk} = \mathrm{BG}_{ijk} + \mathrm{S}_{ijk}$$

where PM indicates a perfect match, BG is "background," S is nonspecific binding, and ijk represents the signal for probe j for the probe set k on microarray i.

$$\mathrm{BG}(\mathrm{PM}_{ijk}) = E[S_{ijk} \ \ |\mathrm{PM}_{ijk}] > 0$$

$$S_{ijk} \sim \mathrm{Exp}(\lambda_{ijk})\ \mathrm{BG}_{ijk} \sim N(\beta_i, \sigma^2)$$

In differential analysis, the PM highlights the communal signals of background (BG) and expression (E). The "ArrayQualityMetrics" Bioconductor package was used to measure the quality of the samples, indicating the median expression level. $N(\beta_i, \sigma^2)$ is the normal distribution involving BG_{ijk}. In this case, β_i is the mean regression parameter that assesses the independent variable gene expression i. σ^2 is the least squares regression representing the dependent variables based on the linear model. λ_{ijk} is a distribution rate parameter that reads the transcription of lane k to gene j from sample i. The gene square matrix for each dataset was calculated across the microarrays and missing values were ignored. For a probe set, we utilized the RMA method to compute a summary of the values among samples.

$$X\mathrm{norm} \ = F_2^{-1}(F_1(x))$$

where F_1 and F_2 show the distribution functions of the case and the reference DNA chips, respectively.

In this study, we detected the T2DM-related DEGs from each cDNA dataset by pairwise comparison (17). DEGs and duplicated spots with quality signals were shortlisted. The statistical parameters were recorded and the genes were ranked. False discovery rate (FDR) <0.05, p-value ≤0.05, average expression level (AEL) ≥40%, and an absolute log-fold change (LFC) >1 were used as significant cutoff values (18, 19).

K-Fold Cross-Validation

We applied k-fold cross-validation to assess accuracy in the differential comparison using the Bioconductor "boot" package, and this method had the edges eventually using all samples for the training and test datasets (18). The k-fold approach is usually less biased in comparison with other methods because it ensures that all observations from the original dataset are shown in the training and test sets. In molecular studies, bootstrapping has been successfully used to correct mismatches and background noise (20). The generalized Gaussian linear model was used to check the k-fold cross-validation with the cv.glm technique. It calculates the true error as the average error.

$$E = 1/k\sum_{i=1}^{k} Ei$$

The Gaussian rule is applied based on leave-one-out-cross-validation (LOOCV). In this case, the LOOCV procedure is considered the test set while the remaining data are used as a training set. LOOCV is a modified k-fold, where $k = N$ and $k(i) = i$. For training and other testing, we applied N number of subsets. Increasing the number of samples would decrease errors and improve the validity (18). For the validation and assessment of errors, the following formula was used:

$$E = 1/N\sum_{i=1}^{N} Ei$$

Gene Ontology and Pathway Enrichment Analysis

Gene Ontology (GO) is a common categorization method used to define the key signaling pathways for the biological and molecular activities of DEGs and cellular components. To recognize the function and biological pathways of the T2DM-related DEGs, GO analysis was carried out using a GOnet online web server that shows the gene product and its biological functions (21). The pathway enriched terms ($p < 0.05$) were analyzed using the FunRich tool, version 3.1.3 (22).

Identifying Transcription and Regulatory Motifs

To develop a connected transcription network consisting of transcription factors (TFs) and other signaling molecules, we anticipated the possible regulators of T2DM-related DEGs using the FunRich online tool. These gene regulators have different biological and pathological roles (23). The motifs are signatures of protein families that define the link between the secondary structural components of proteins, and, in every instance, the spatial sequences of the amino acid residues encoded by genes may be comparable in any order. We used the Motif Search online tool to analyze the relationship between the primary sequence and the tertiary structure of proteins to predict protein functions (24).

Mutation Analysis

Mutations resulting from inherited and non-inherited diseases can be understood to decode genetic variations by the association of genotype with phenotype. There are hundreds of single nucleotide variations (SNVs) in the human genome, and many are known to develop diseases. Nearly 21% of the amino acid variations cause single nucleotide missense mutations at different protein sites (known as posttranslational modifications) that are responsible for disease development. Therefore, chemical alterations of amino acids mostly affect the functions of proteins. The online ActiveDriverDB tool was used to analyze the mutations of T2DM-related DEGs (25). In this analysis, the needle plot shows a visual summary of the location, frequency, and functionality of all alterations found in our DEGs. Posttranslational modification (PTM) sites with all changes and the predicted disordered regions of the protein sequences were analyzed. The position of the pins in the plot correlates with the gene and protein sequences, while the associated mutation effect and PTMs may be observed in the figure legend.

Protein Co-Expression Network Analysis

Co-expression network analysis enables the identification of protein modules whose biological activities are characterized by expression patterns. These networks are the most versatile for probing diseases. It correlates at the transcript level, although it may also be used to analyze correlations at all biological levels. The STRING database version 11.0 (26, 27) was used to study the protein–protein interactions (PPIs) of the top-ranked T2DM-related DEGs, and a protein co-expression network was constructed to highlight the important gene signatures directly or indirectly interacting with the DEGs. The protein network based on neighborhood scoring was constructed with high confidence (score > 0.99) (26). In this co-expression network, the RNA levels and protein regulation were analyzed and annotated keywords (FDR < 0.05) were studied.

RNA Extraction and Quantification

Each 300 µl blood sample was transferred to a 700-µl triazole-containing Eppendorf tube (1.5 ml). These tubes were homogenized and incubated gently at 25°C for 5 min. Then, 400 µl of chloroform was added and kept for 3 min, followed by centrifugation at 12,000 rpm for 10 min at 4°C for phase separation. Sequentially, the aqueous top layer was taken into a new tube while keeping it on ice with equal proportions of isopropyl alcohol. These tubes were retained on ice at −20°C for 10 min in a horizontal position to precipitate RNA, followed by centrifugation at 12,000 rpm (4°C) for 10 min while the supernatant was discarded. The pellet was washed twice and air dried for 5 min using 1 ml of 70% ethanol at 7,500 rpm. Forty microliters of RNase-free water was added and then RNA was stored at −80°C (28) using an RNA stabilizer. Finally, RNA was quantified at 260, 280, and 320 nm by Nanodrop (Skanit RE 4.1, Thermo Scientific, Waltham, MA, USA) (29).

cDNA Synthesis

The isolated RNA was converted into cDNA using a cDNA synthesis kit (Vivantis cDSK01-050). As per the manufacturer's protocol, each RNA primer of the DEGs was co-mixed with 10 µl of the cDNA synthesis mix. After centrifugation at 10,000 rpm, the samples were incubated at 40°C for 60 min. The tubes were then incubated at 85°C for 5 min to terminate the reaction. Finally, the tubes were chilled on ice and centrifuged at the same conditions. The synthesized cDNA was directly used for further analysis (29, 30).

Quantitative Real-Time PCR Analysis

The 260:280 ratios between 1.5 and 2.7 indicated high-quality RNA with 800–1250 ng/µl quantity. RNA quantification presented a substantial level for further cDNA synthesis. The relative expressions of the T2DM-related genes *SRR*, *PDE4B*, and *NFKB1* were studied and estimated based on the relative $2^{-\Delta\Delta C_T}$ method. CT values were obtained by absolute quantification presenting quality and significant expression at the real level. We used PrimerBank, an online server, to design the primers of the T2DM-related DEGs (31) (**Table 1**). For optimization, gradient PCRs were carried out using a Galaxy-XP thermal cycler (Bioer, Hangzhou, China) at standard conditions. To validate the DEGs of T2DM, qRT-PCR was performed using MIC-PCR (Bio

TABLE 1 | Primer sequences and amplicon size of the differentially expressed genes *SRR*, *NFKB1*, and *PDE4B* used in the qPCR reaction.

Gene symbol	Probe ID	Protein name	Forward primer	Reverse primer	Amplicon size (bp)
SRR	219204_s_at	Serine racemase	ATGTGTGCTCAGTATTGCATCTC	AAGATTGCGCCCTGTTAGTTG	126
NFKB1	201502_s_at	Nuclear factor NF-kappa-B p105	AACAGAGAGGATTTCGTTTCCG	TTTGACCTGAGGGTAAGACTTCT	104
PDE4B	203708_at	cAMP-3′,5′-cyclic phosphodiesterase 4B	AACGCTGGAGGAATTAGACTGG	GCTCCCGGTTCAGCATTCT	110
GAPDH (reference gene)	–	Glyceraldehyde-3-phosphate dehydrogenase	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	197

Molecular Systems, Upper Coomera, Australia) at optimized conditions (32). A final volume of 10 µl of the reaction mixture was prepared using 2.6 µl of cDNA (1:10), and 5 µl of the SYBR Green Master Mix and 0.4 µl of each gene-specific reverse and forward primer were added. The final composition of the reaction mixture was kept for DEGs and the reference genes. *GAPDH* was used as the internal reference, and a two-step qPCR procedure was applied to measure the expressions of the test and reference genes. The relative expression level of each differential gene was calculated with the *GAPDH* expression level as a "1" standard value. The qPCR cycling conditions were as follows: denaturation at 95°C for 12 min, 40 cycles of 95°C for 15 s, 57°C for 20 s, and 72°C for 20 s. A final extension step was carried out at 72°C for 10 min (33). In amplification, during each cycle, the amplified products were doubled in an exponential form. The PCR products were analyzed by melting the curve graph to assess the dissociation characteristics of double-stranded DNA during heating and to observe the absorbance intensity (34). C_T is a logarithmic value converted to a relative quantity (32), and the average C_T values were measured for both DEGs and the reference gene. In the next step, ΔC_T (delta threshold) was calculated for the target and the reference gene, and the relative expressions of the T2DM-related DEGs were studied and estimated based on the relative $2^{-\Delta\Delta C_T}$ formula.

$$\Delta C_T = C_T \text{value of target gene} - C_T \text{value of the internal reference gene } (GAPDH)$$

The $\Delta\Delta C_T$ (delta–delta threshold) specifies the differences between the expression levels of the T2DM-related DEGs and the reference gene (35). Finally, $2^{-\Delta\Delta C_T}$ was calculated, indicating the fold difference of the expressions of DEGs from that of the control (36). We assessed the absolute correlation of these DEGs to show the complete expression levels. Hierarchical clustering analysis of genes to illustrate the expression pattern was observed (37) using the online one-matrix CIMminer tool (38).

RESULTS

Normalization of Gene Expression Data and Cross-Validation

To study the T2DM-related DEGs, we retrieved 20 publicly available Affymetrix cDNA datasets. Each dataset contains a number of distinct samples and genes obtained by messenger RNA (mRNA) expression profiling utilizing several Affymetrix T2DM platforms. The data were normalized missing values were corrected, and the normalized distance between the DNA chip array and the different arrays of each dataset reveals the quality of the arrays for a medium expression level. The genegene covariance matrix in all arrays of every dataset was calculated by removing the missing values to determine whether the arrays were on the same level. A quality histogram depicting the normalized intensity arrays of the entire DNA chip was constructed during the quantile standardization. Array intensity distributions show boxplots representing summaries of the signal intensity distributions of the arrays and normalization with similar positions and widths. The patterns revealed the distribution of the arrays having similar shapes and ranges (**Figure 1**). From the list of 50 DEGs (**Figure 2**), we identified and selected three DEGs of T2DM—*SRR*, *NFKB1*, and *PDE4B*—based on the FDR (<0.05), *p*-value (≤0.05), and logFC (>1) parameters.

An automated method was employed for comparing biologically similar groups in pairs. We excluded any subgroup without recurrence comparison, accuracy, and differential analysis verification and assessed errors of the cross-validation using the generalized linear model "cv.glm." This generalized linear model normalizes the linear regression, enabling the linear model to be associated with the dependent variables through a correlation and allowing a function of the anticipated value of the variance of each measurement. The Gaussian dispersion criterion was 0.00459, indicating the degree of confidence (**Table 2**). LOOCV is a type of cross-validation technique where each observation is treated as a validation set and the remaining observations ($N - 1$) as a training set. The model was fitted in LOOCV and a validation set was used to estimate it. With *k*-fold valuation, we found the same delta value of 0.0050 we used in the LOOCV approach. The significant codes (0.1, 0.01, 0.001, and 0.05) with residuals of low variance showed that the differential analysis was consistent. We evaluated the confidence of the dataset in the original samples in order to identify the variations at the transcription level. The normalization procedure was utilized to standardize the sampling methods and to evaluate the optimum RNA quality by utilizing refined measures to analyze statistics and algorithms.

Gene Ontology and Pathway Enrichment Analysis

The GO of the T2DM-related DEGs showed significantly enriched terms. *SRR*, *PDE4B*, and *NFKB1* were directly and indirectly linked to cell morphogenesis, anatomical structure development, amino acid metabolism, biosynthetic and catabolic processes, mRNA processing, signal transduction, protein transport,

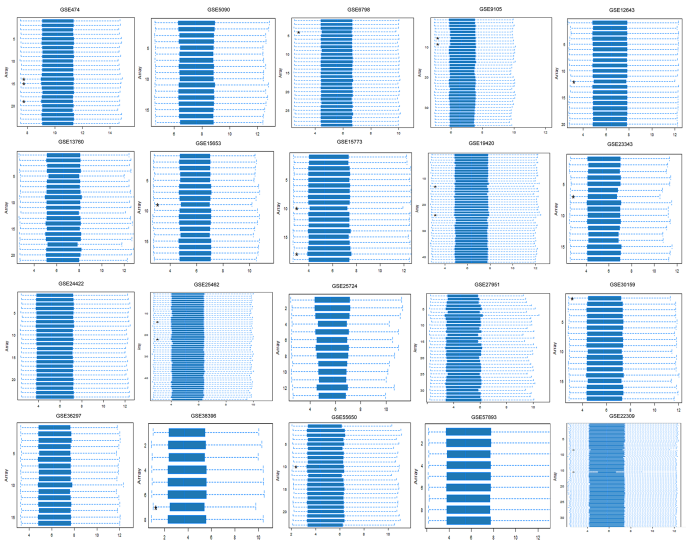


FIGURE 1 | Array intensity distributions showing boxplots representing summaries of the signal intensity distributions of the arrays and normalization with similar positions and widths. Each *box* corresponds to one array. Outlier detection was performed by computing the Kolmogorov–Smirnov K_a statistic between each distribution of the array and the distribution of the pooled data.

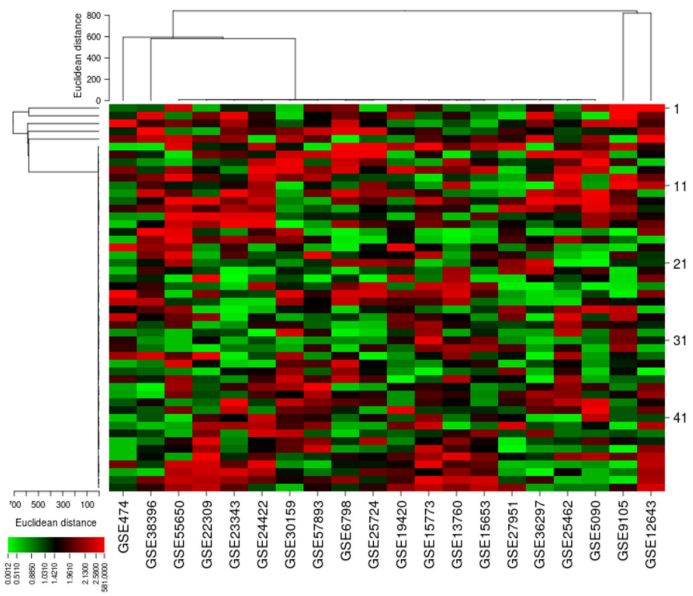


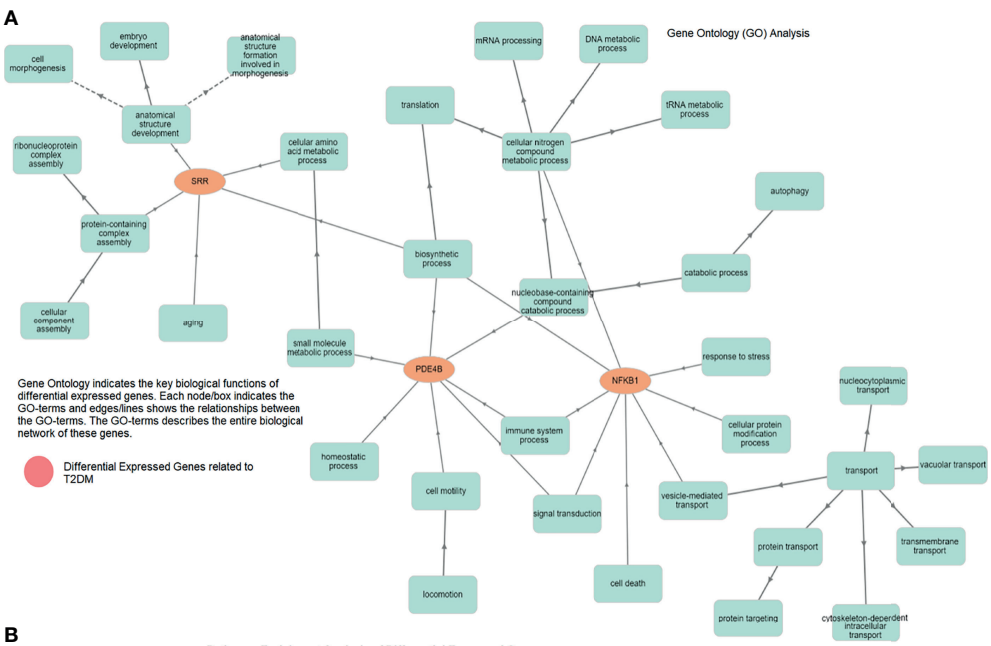
FIGURE 2 | Differential expressions of 50 genes obtained from microarray cDNA datasets. The heat map indicates the differential expressions of these genes in each type 2 diabetes mellitus (T2DM)-related dataset.

transmembrane and intracellular transport, and other vital biological functions ($p < 0.05$). The molecular annotation of *SRR* showed associations with ATP, magnesium, phosphate, calcium, and serine–ammonia lyase binding, and threonine racemase activity. Similarly, at the molecular level, *NFKB1* and *PDE4B* were involved in actinin chromatin binding, transcription regulation, and cAMP-binding phosphodiesterase activity. The role of the gene, its regulation, subtypes, and the cellular processes are essential in the comprehension of biological functions, and abnormalities in these processes can cause T2DM and other metabolic disturbances (**Figure 3A**). The pathway enrichment showed the role of valine, leucine, and isoleucine biosynthesis; glycine, serine, and threonine metabolism; the adipocytokine signaling pathway; the Hedgehog signaling pathway; and insulin

TABLE 2 | *K*-fold cross-validation using the Bioconductor "boot" package based on Gaussian dispersion parameters.

	Estimate	SE	*t* value	Pr(>|*t*|)
(Intercept)	0.000107	0.000211	3.99	$<1.00E^{-13}$***
x_1	0.030034	0.001602	20.018	$<1.00E^{-11}$***
x_2	−0.01041	0.001105	−4.017	$<1.96E^{-10}$***
x_3	0.110112	0.003101	22.015	$<1.00E^{-13}$***
x_4	0.110410	0.001212	20.200	$<1.00E^{-11}$***
x_5	0.016013	0.002130	28.003	$<1.00E^{-10}$***
x_6	0.131220	0.003361	21.012	$<1.00E^{-10}$***
x_7	−0.01201	0.001461	−21.112	$<1.00E^{-9}$***
x_8	0.001212	0.002411	19.115	$<1.00E^{-12}$***
x_9	0.102111	0.003802	62.0716	$<1.00E^{-13}$***
x_{10}	0.010020	0.000500	4.001	$<1.00E^{-11}$***
x_{11}	0.010521	0.001003	22.003	$<1.00E^{-11}$***
x_{12}	−0.01102	0.002014	−2.014	0.0068*
x_{13}	−0.12421	0.002809	−50.023	$<1.00E^{-7}$***
x_{14}	0.010021	0.001230	1.312	$5.28E^{-8}$***
x_{15}	−0.015581	0.001200	−17.102	$<1.00E^{-11}$***

Number of Fisher scoring iterations: 2; $K: [1] 10; $delta: [1] 0.00455 = 0.00459. Null deviance: 100,502.1 with 50,101 degrees of freedom. Residual deviance: 2,504.1 with 40,119 degrees of freedom.
*Signif. codes: 0.001 '**'.*



pathway	description	count in network	strength	false discovery rate
hsa00290	Valine, leucine and isoleucine biosynthesis	2 of 4	2.95	0.00028
hsa00260	Glycine, serine and threonine metabolism	9 of 38	2.62	2.92e-20
hsa00630	Glyoxylate and dicarboxylate metabolism	3 of 30	2.25	6.48e-05
hsa00670	One carbon pool by folate	2 of 20	2.25	0.0037
hsa01230	Biosynthesis of amino acids	6 of 73	2.16	3.67e-10
hsa01523	Antifolate resistance	4 of 31	2.36	4.71e-08
hsa04623	Cytosolic DNA-sensing pathway	5 of 62	2.16	6.86e-09
hsa05220	Chronic myeloid leukemia	6 of 75	2.15	8.57e-10
hsa05120	Epithelial cell signaling in Helicobacter pylori infection	5 of 67	2.12	8.60e-09
hsa04920	Adipocytokine signaling pathway	5 of 69	2.11	9.24e-09
hsa04962	Vasopressin-regulated water reabsorption	3 of 44	2.08	0.00011
hsa04340	Hedgehog signaling pathway	3 of 47	2.06	0.00012
hsa05110	Vibrio cholerae infection	3 of 48	2.05	0.00012
hsa05031	Amphetamine addiction	4 of 66	2.03	7.34e-06

FIGURE 3 | **(A)** Gene Ontology network of the differentially expressed genes indicating important biological functions. **(B)** Pathway enrichment analysis showing the enriched terms associated with type 2 diabetes mellitus (T2DM).

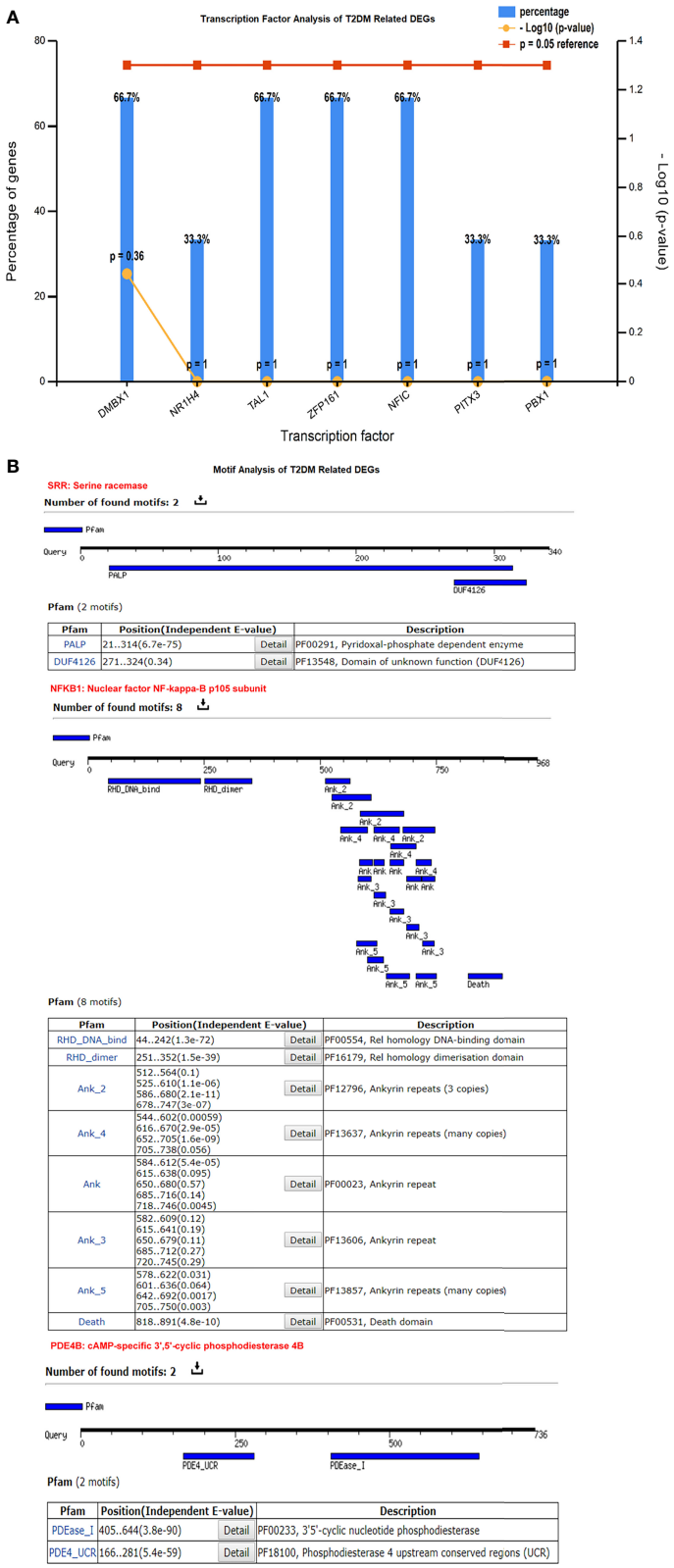


FIGURE 4 | **(A)** Transcriptional factors of the differentially expressed genes (DEGs) showing *DMBX1*, *TAL1*, *ZFP161*, *NFIC*, and other regulatory factors. **(B)** Motif analysis highlights a significant number of functional motifs associated with important biological functions.

and antifolate resistance in T2DM (**Figure 3B**). These DEGs associated with signaling pathways are critically linked to pathophysiological mechanisms.

Transcription and Motif Analysis

We identified the transcriptional factors of the T2DM-related DEGs, such as *DMBX1*, *TAL1*, *ZFP161*, *NFIC* (66.7%), and *NR1H4* (33.3%), and others with a substantial *p*-value (<0.05) (**Figure 4A**). These motifs play a crucial role in the protein interactions among network components. These motifs help to discover TF binding sites and expression of gene regulation. The outcomes showed that the remodeling of a variety of motifs established the promiscuous protein characteristic resulting in different biological functions. We found eight motifs of the nuclear factor NF-kappa-B subunit (NFKB1) protein containing Pfam-annotated DNA binding, cell death, dimerization domain, and ankyrin repeats (proteins that facilitate the attachment of fundamental membrane proteins). Pfam annotation was predicted based on multiple sequence alignments and a hidden Markov model. Under diabetic conditions, the aberrant expression of *NFKB1* ankyrin repeats dysregulate glucose homeostasis and protein kinase (AMP) activity. For the other DEGs, *SRR* contained PALP and DUF4126, while *PDE4B* had the PDEase_1 and PDE4_UCR motifs. These motifs are biologically associated with cyclic nucleotide phosphodiesterase and pyridoxal phosphate-dependent enzyme activity. The dysregulation of these transcriptional regulators changes the phosphodiesterase activity, resulting in insulin resistance and T2DM. The motifs were scanned with a significant cutoff value of $e < 0.0002$ at the default parameters (**Figure 4B**).

Mutation Analysis

NFKB1 had 39 PTM sites with 119 repeated mutations at the positive strand of chromosome 4 encoding 968 protein residues, indicating 29.96% of the predicted disordered region. The mutation visualization plot displayed *NFKB1* isoforms distal, proximal, direct, and network-rewiring mutation impacts between 450 and 900, with reference amino acid (AA) residues Q (Gln), D (Asp), N (Asn), M (Met), H (His), and A (Ala) and mutated amino acid residues E (Glu), K (Lys), R (Arg), D (Asp), T (Thr), and Y (Tyr), and others in the protein. The mutated site of NFKB1-Q900E at position 897 contained S amino acid residues enriched with a phosphorylation network-rewiring mutation impact. *NFKB1* D442Y contained K residues with acetylation and ubiquitination proximal mutation impacts at the 440 position of a protein. On the other hand, *PDE4B* showed 38% disordered regions in a sequence. The *PDE4B* R196S isoform revealed the mutation at position 197 presenting amino acid residue R to the mutated amino acid residue S. At this position, the S amino acid residue sites enriched with phosphorylation indicated network-rewiring PTM impact. PDE4B-A200V contained the S residue with a phosphorylation proximal mutation impact at the 201 position of a protein. Similarly, the differentially expressed *SRR* gene showed a 4.12% disordered region with 44 mutations predicted at chromosome 17 on the positive strand involving 340 amino acid residues and seven PTM sites. The *SRR* R58G isoform showed the mutation at position 58 presenting amino acid residue R to the mutated amino acid residue G with ubiquitination, indicating proximal PTM impact (**Figure 5**). Similarly, at position 203 of *SRR* proteins, the AA residue S was altered by N, indicating proximal PTM impact.

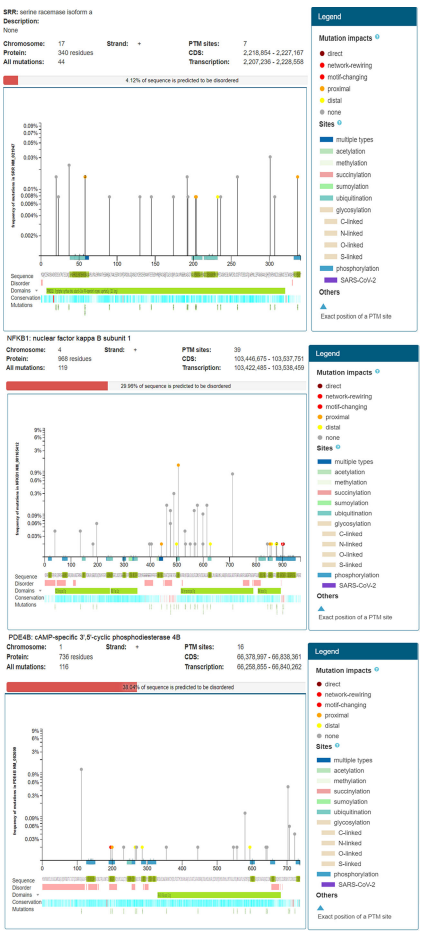


FIGURE 5 | Mutation analysis of type 2 diabetes mellitus (T2DM)-related differentially expressed genes (DEGs) indicating posttranslational modifications with significant cutoff parameters. It highlights the significant disordered regions of the proteins contributing a pathophysiological role and to disease development.

Protein Product Co-Expression Network Analysis

The *SRR*, *PDE4B*, and *NFKB1* genes were studied for possible interactions with each other using the STRING database. It was predicted that these DEGs would have significant interactions. The PPI network contained 33 numbers of nodes (each node indicates proteins), and the edges present the interactions. The *PDE4B* network showed the enriched co-expressed genes (PPI enrichment, $p < 0.05$) functionally associated with adenine phosphoribosyl transferase (*APRT*), deoxycytidine kinase (*DCK*), adenyl succinate lyase (*ADSL*), AMP phosphotransferase (*AK3*), protein phosphatase-1 regulatory subunit 1B (*PPP1R1B*), adenosine kinase (*ADK*), and cAMP-dependent protein kinase catalytic subunit alpha (*PRKACA*). *PDE4B* is directly connected to

the *SRR* source gene *via* DISC1 (schizophrenia 1 protein). *SRR* is linked to D-amino-acid oxidase (*DAO*), l-serine ammonia-lyase (*SDS*), cystathionine beta-synthase-like protein (*CBSL*), phosphoserine phosphatase (*PSPH*), and other gene signatures. Based on the protein product co-expression data, we evaluated the expressions of the genes *SRR*, *PDE4B*, and *NFKB1A* and observed that these were downregulated. Similarly, it has been observed that *NFKB1A* interacts with important target proteins such as transcription factor A (RELA), TNFAIP3-interacting protein 2 (*TNIP2*), an inhibitor of nuclear factor kappa-B kinase subunit alpha (*CHUK*), an inhibitor of nuclear factor kappa B kinase subunit beta (*IKBKB*), and other gene signatures. The RNA co-expression pattern and protein co-regulation showed the significant level of association of the co-expressed genes with annotated keywords: hyperlipidemia, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoproteins (HDL), lipid metabolism, and disease mutation (**Figure 6**).

Validation qRT-PCR Assay and Expression Profiling

The RT-PCR was consistent enough to prevent the reaction from displaying an increased fluorescence signal. The relative expression showed that *SRR*, *NFKB1A*, and *PDE4B* were downregulated with significant fold change values ($2^{-\Delta\Delta C_T}$) compared to controls. These DEGs were aberrantly expressed and related to disease development and growth (**Figure 7A**). Based on the $2^{-\Delta\Delta C_T}$ method, we observed a substantial level (R^2= 0.80, $p < 0.05$) of the relationship between the expression levels of the T2DM-related DEGs assessed by array analysis and the expression levels evaluated by individual qRT-PCR (**Figure 7B**).

In our analysis, the cluster study specified the gene expression profiles of the cases and controls (two groups) with significant expression level differences. **Figure 8** shows that most of the samples have differential expression profiles compared to the left-hand dendrogram, where some genes have similar expression patterns (**Figure 8**). The genes *NFKB1A*, *SRR*, and *PDE4B* were analyzed in 50 cases and controls, and the expression levels of the cases and controls (log fold values) were studied individually and indicated on a heat map. In this figure, the columns indicate the samples and the rows represent the DEGs. These genes may have a parallel biological function or contribute to the same physiological role. Genes with increased differential expression were termed as a gene cluster, and we found important gene clusters that were expressed differently in a number of samples (fold change ≥2 and $p < 0.05$).

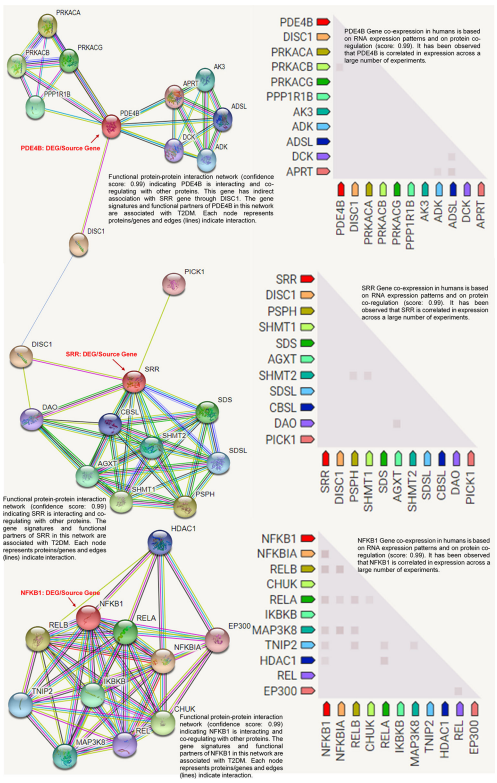


FIGURE 6 | Protein product co-expression network analysis using the STRING database, version 11.0. The protein network was calculated based on the neighborhood score with higher confidence (confidence score > 0.99). *Nodes* represent proteins and *edges* indicate interactions. The co-expression scores based on RNA expression patterns and protein co-regulation were studied and annotated keywords (FDR < 0.05) were observed.

DISCUSSION

In developing countries, T2DM risk factors are frequently recorded, and the prevalence of diabetes is increasing, yet its control worldwide is insufficient (39, 40). Human gene expression has been shown to be of significance for the identification of phenotypic genetic determinants and for discovering complex genetic features. Many genes interact with the most prevalent conditions in individuals and thus need an integrative biological approach to resolve the complications and causes behind these issues (41). Progress in microarray analysis enabled scientists to simultaneously examine a large number of genes and to uncover genetic evidence for different diseases (42). In this study, publicly accessible cDNA datasets were analyzed to identify the DEGs for T2DM. It was revealed that *SRR*, *PDE4B*, and *NFKB1* variants were involved in interactions with known T2DM-associated genes, including *AK3*, *DISC1*, *ADK*, *ASPH*, *SDS*, *RELB*, *HDAC1*, *REL*, and *EP300*. The dysregulation and functional abnormality of these differential genes have been investigated in T2DM development (43–45).

The ontology of these genes and pathway analysis have demonstrated insulin resistance, insulin and adipokine signaling, amino acid metabolism, and T2DM. The amino acid metabolism, biosynthetic and catabolic processes, signal transduction, and other vital molecular functions were directly and indirectly associated with *SRR*, *PDE4B*, and *NFKB1*. We observed that PIk3, S1P1, endothelin, CALM1, TGFB, IL, STAT6, and the adipocytokine signaling pathways linked to these DEGs (40, 44, 46) were regulated by transcriptional factors such as *DMBX1*, *TAL1*, *ZFP161*, *NFIC*, and *NR1H4*. The co-expression network showed the direct and indirect

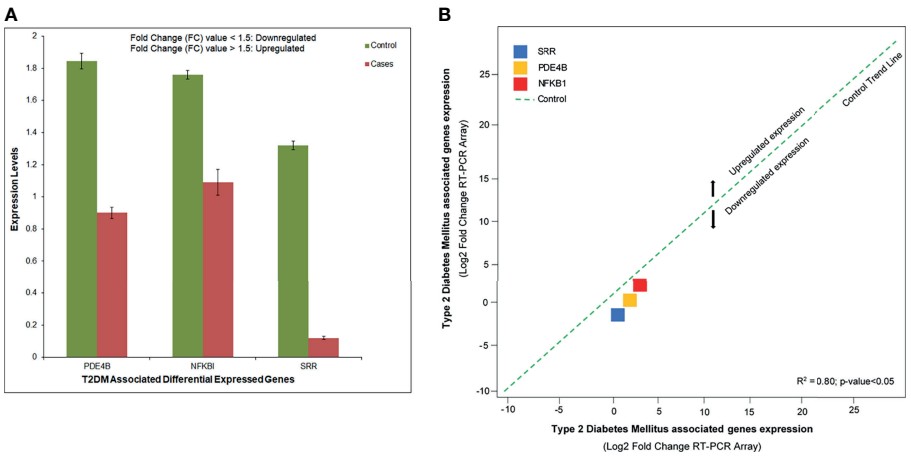


FIGURE 7 | **(A)** Abnormal expression levels of the differentially expressed genes (DEGs) in type 2 diabetes mellitus (T2DM) cases and controls based on the fold change of gene expression. **(B)** Quantitative real-time PCR (qRT-PCR) array validation of the DEGs. Plot graph shows the correlation between the expression levels of type 2 diabetes mellitus (T2DM)-related DEGs measured by array analysis and the expression levels measured by individual qRT-PCR. The $2^{-\Delta\Delta C_T}$ method was applied for this analysis.

functional interactions of the gene signatures with the source genes.

The diverse role of the family of *NFKB1A* genes has been studied in type 2 diabetes (44) associated with impairment of glucose metabolism. *NFKB1A* is the gene expression regulatory factor for many pro-inflammatory proteins. Animal studies have shown that the dysregulated and increased activity of this gene causes the pathogenesis of insulin resistance and muscle atrophy (47). It has been observed that amplified *NFKB1A* signaling may be involved in the pathogenesis of insulin resistance. The modified activity of this TF is connected to muscle loss and weakness (48), common characteristics observed in diabetic individuals.

Increased expressions of interleukin (IL)-1β and *NFKB1* and enhanced infiltration of macrophages have been observed in pancreatic islets of patients with T2DM. Methylation of the *NFKB1* gene was negatively correlated with the levels of IL-1Ra in individuals with T2DM (49).

cDNA differential expression analysis and integrative enrichment studies indicated the substantial association of IL-6, *NFKB1*, and *PIK3CG* with T2DM (50). *NFKB1*, *USF2*, *HINFP*, *MEF2A*, and *SRF* are important genes that are differentially expressed in T2DM (51). In another study, it has been observed that *NFKB1* is engaged in the generation of mild inflammation and oxidative stress, which cause diabetic issues (52).

Similarly, we observed the association of the *PDE4B* gene with cyclic AMP and the *SRC*, *INSRR*, *GRIN*, *DISC1*, *PRKACA*, *PRKACB*, *AK3*, *ADK*, and *APRT* gene signatures, their dysregulation causing insulin resistance and type 2 diabetes (53–55). PDEs are a large family of phosphodiesterases that catalyze cAMP and cGMP to 5′-AMP and 5′-GMP, respectively. It plays a vital role in intracellular signaling pathways and is linked to the regulation of glucagon-like peptide-1 release (56). The dysregulation of this enzyme leads to T2DM (57), making it a potential therapeutic target. PDE4 controls the level of cAMP and forms cAMP signaling, which delicately directs signals from various environmental stimuli to specific microenvironments. PDE4-cAMP signaling dysregulation signifies an important pathophysiological path in a metabolic disorder, as shown by its important role in processes such as inflammation, lipid and glucose metabolism, hepatic steatosis, abnormal lipolysis, and

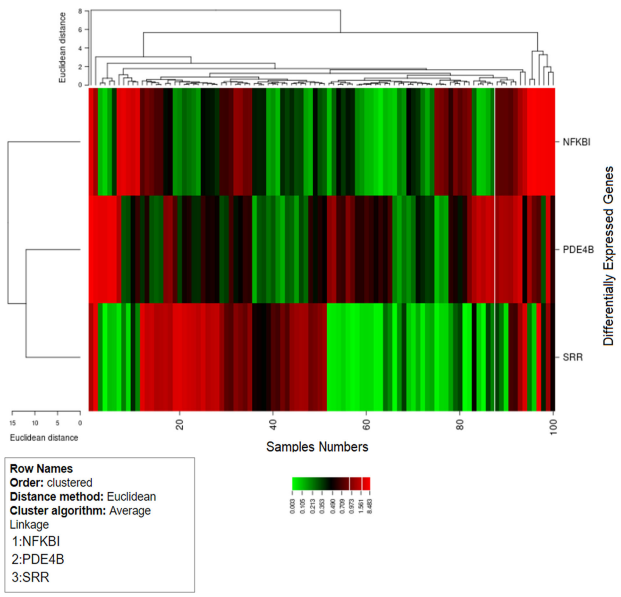


FIGURE 8 | Hierarchical cluster analysis heat map indicates the expression profiles of differentially expressed genes (*SRR*, *NFKB1*, and *PDE4B*). The *columns* in the figure represent the sample numbers and the rows represent the differentially expressed genes.

disturbed neuroendocrine functions (58). It has been observed that PDE3A, PDE3B, PDE4B, PDE4D, and PDE8B in rat islets and in INS-1E cells activate multiple signal pathways critical for pancreatic beta cell function, and their abnormalities are associated with insulin resistance (53). It has been reported that *PDE4B* is a potential therapeutic target to treat obesity-related metabolic diseases. These findings from rodent studies showed that genetic and pharmacological variations in *PDE4B* lead to insulin resistance, insulin and adipocytokine secretion, and T2DM (59).

In another study, it has been observed that the serine racemase (*SRR*) gene is involved in type 2 diabetes (60). GWAS have highlighted the polymorphic association of the *SRR* gene with T2DM (61). This analysis has identified >100 loci independently contributing to T2DM risk, signifying the evidence for a role in insulin secretion of T2DM susceptibility genes, including *PRC1*, *GRIN*, *SRR*, *ZFAND3*, and *ZFAND6* (62). Collectively, we found that the genes *SRR*, *PDE4B*, and *NFKB1A* were directly and indirectly associated with insulin secretion, insulin resistance, and T2DM (49, 58, 63). The pathway analysis diagram (**Figure 9**) showed that source DEGs were primarily enriched in cAMP signaling, amino acid metabolism, protein biosynthesis, the MAPK signaling pathway, regulation of lipolysis in adipocytes, the PI3K/Akt signaling pathway, insulin resistance, and T2DM (40, 57, 62, 64, 65). qPCR analysis was used to validate the outcomes of the differential analysis indicating the significant relationship of *SRR*, *PDE4B*, and *NFKB1* with T2DM. A significant fold variation was observed in the target samples using the $2^{-\Delta\Delta C_T}$ method (66), and real-time expressions of the T2DM-related DEGs demonstrated substantial level expression compared to control.

CONCLUSIONS

In this study, we found a significant correlation of the DEGs *SRR*, *PDE4B*, and *NFKB1* with T2DM in cases compared to controls. Systems biology analysis of the cDNA data allowed us to discover potential therapeutic targets for T2DM, and our comprehensive and integrated steps were helpful in revealing the genome to phenome association with diabetes. The qPCR-based validation and expression profiling of these genes specified their abnormal expression and relates their pathological role in the disease process. These findings clearly demonstrate that the dysregulated expressions of selected genes are correlated with the pathophysiology of T2DM. Therefore, these genes would be considered as possible drug targets that would help update the therapeutic strategies for insulin resistance, T2DM, and other metabolic disorders.

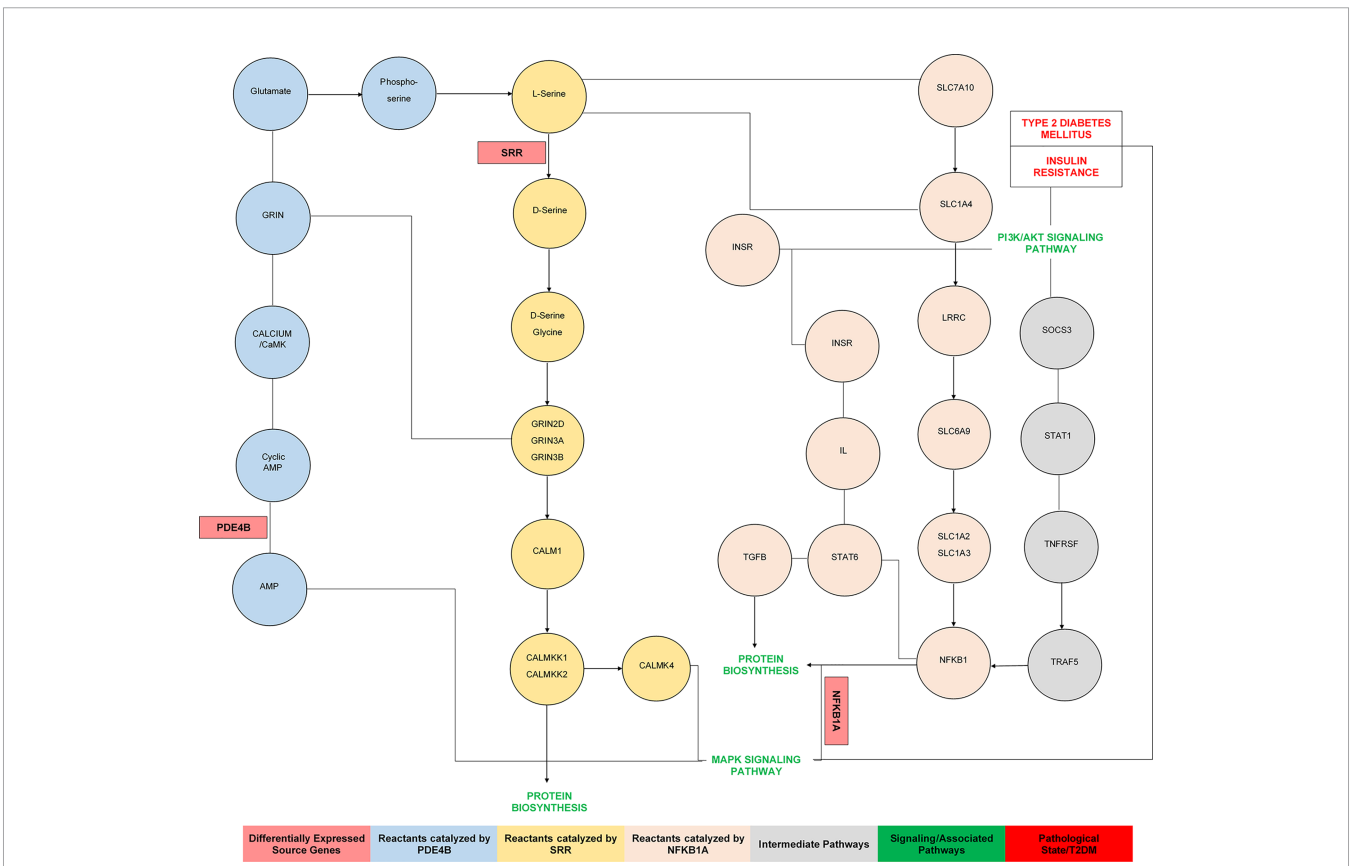


FIGURE 9 | The pathways analysis indicates the mechanism and functional association of source genes involved in the pathophysiology of insulin resistance and type 2 diabetes mellitus.

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 approval of the study and informed consent were obtained from the Research Ethical Committee of Institute of Molecular Biology and Biotechnology, BZ University, Multan (ref. no. IMBB/2019/002). The patients/participants provided written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WR and JG collected the materials and performed the work. MQ and SM designed the study, interpreted the data, and wrote the manuscript. MQ and SM supervised the study. BB interpreted the data. 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.2021.774696/full#supplementary-material

Supplementary Table 1 | List of cDNA datasets analyzed in this study.

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REVIEW
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Novel Nanotechnological Approaches for Targeting Dorsal Root Ganglion (DRG) in Mitigating Diabetic Neuropathic Pain (DNP)

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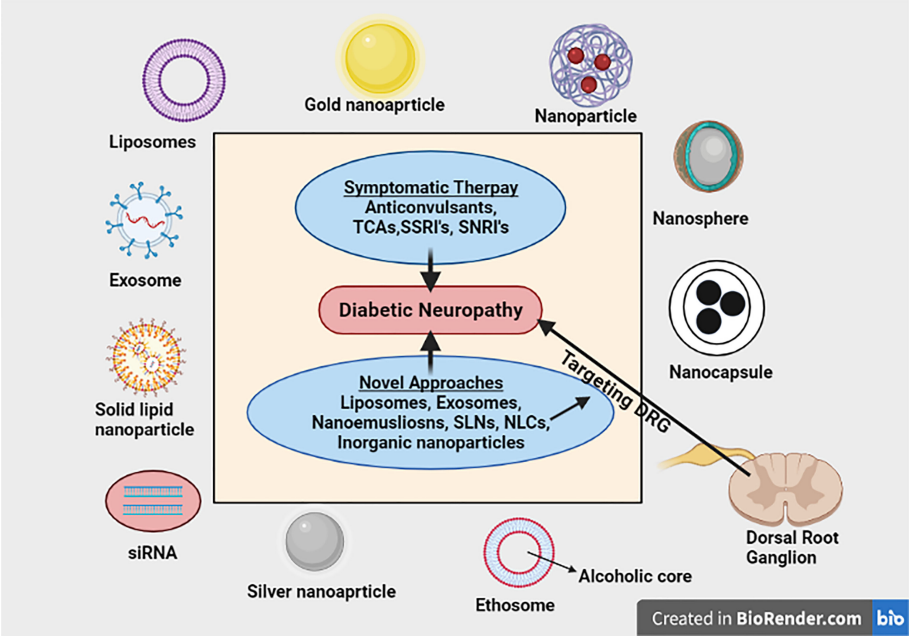
Diabetic neuropathy is the most entrenched complication of diabetes. Usually, it affects the distal foot and toes, which then gradually approaches the lower part of the legs. Diabetic foot ulcer (DFU) could be one of the worst complications of diabetes mellitus. Long-term diabetes leads to hyperglycemia, which is the utmost contributor to neuropathic pain. Hyperglycemia causing an upregulation of voltage-gated sodium channels in the dorsal root ganglion (DRG) was often observed in models of neuropathic pain. DRG opening frequency increases intracellular sodium ion levels, which further causes increased calcium channel opening and stimulates other pathways leading to diabetic peripheral neuropathy (DPN). Currently, pain due to diabetic neuropathy is managed *via* antidepressants, opioids, gamma-aminobutyric acid (GABA) analogs, and topical agents such as capsaicin. Despite the availability of various treatment strategies, the percentage of patients achieving adequate pain relief remains low. Many factors contribute to this condition, such as lack of specificity and adverse effects such as light-headedness, languidness, and multiple daily doses. Therefore, nanotechnology outperforms in every aspect, providing several benefits compared to traditional therapy such as site-specific and targeted drug delivery. Nanotechnology is the branch of science that deals with the development of nanoscale materials and products, even smaller than 100 nm. Carriers can improve their efficacy with reduced side effects by incorporating drugs into the novel delivery systems. Thus, the utilization of nanotechnological approaches such as nanoparticles, polymeric nanoparticles, inorganic nanoparticles, lipid nanoparticles, gene therapy (siRNA and miRNA), and extracellular vesicles can extensively contribute to relieving neuropathic pain.

Keywords: diabetic neuropathic pain (DNP), nanotechnology, dorsal root ganglion (DRG), siRNA, extracellular vesicles, ligand-based targeting, nanoparticles



GRAPHICAL ABSTRACT | Nanotechnology based strategies has been extensively studied for their potential application in improving the delivery of drugs mitigating neuropathic pain to the targeted area with enhanced action. This review has comprehensively summarized and critically discussed the application of various novel nanotechnological approaches for mitigating diabetic neuropathic pain specifically targeting DRG.

HIGHLIGHTS

- Diabetes mellitus is a common metabolic disorder characterized by diabetic neuropathy, influencing around 90% of patients.
- Symptoms of diabetic neuropathic pain become unpleasant and disturbing at night and usually involves burning sensation, acute cricking, plunging, and body aches, especially in the lower part.
- Studies suggest that the dorsal root ganglion (DRG) is an active participant in peripheral processes, including platelet activation factor (PAF) damage, inflammation, and the production of neuropathic pain.
- Nanotechnology plays a significant role in effectively delivering drugs (analgesics) to specific sites, thus mitigating chronic pain.

1 INTRODUCTION

Diabetes mellitus (DM) is a common metabolic disorder characterized by diabetic neuropathy, influencing around 90% of patients (1). Neuropathy develops gradually, usually after 25 years of disease (2). The pervasiveness of painful diabetic neuropathy (PDN) ranges from 10% to 20% in diabetics (3). In 2020, approximately 34.2 million people had diabetes, and out of that, around 26.9 million people, including adults, were undiagnosed (4). Distal symmetrical peripheral neuropathy (DSPN) is the most dominant form of diabetic neuropathy, affecting 20% of type-I diabetic patients worldwide. Normally, it affects the distal foot and toes, which gradually approaches the lower part of the legs (5). The clinical manifestations of DSPN include foot ulceration and serious neuropathic pain (5). Symptoms of diabetic neuropathic pain (DNP) become unpleasant and disturbing at night and usually involves burning sensation, acute cricking, plunging, and body aches, especially in the lower part. Sometimes, diabetic neuropathy leads to neuropathic cachexia, accompanied by depression and loss of weight (1). Many apparent mechanisms have been put forward to elucidate the pain related to diabetic neuropathy, including auto-oxidative stress, hyperglycemia, agitated polyol pathway, enhanced levels of advanced glycation end products (AGEs), and rise in protein kinase C (PKC) (mainly β-isoform). As compared to nerves, dorsal root ganglion (DRG) is more assailable to oxidative stress (6). Recent studies have shown that DRG neurons offer a plausible target and are linked with various problems of diabetic neuropathy (6). DRG possesses many applications, particularly for DNP (7). Nowadays, the stimulation of DRG has been considered a new neuromodulation paradigm. Various techniques are being employed or utilized for DRG stimulation, but implantable devices are gaining recognition to a greater extent (8). DRG neurons emerge from the dorsal root of spinal nerves, conveying sensory signals to the central nervous system (CNS) for a response to various receptors (9). Studies suggest that DRG is an active participant in peripheral processes, including platelet activation factor (PAF) damage, inflammation, and the production of neuropathic pain (9). Peripheral damage to the

nerves in neuropathic pain leads to overexpression of the P2X3 receptor in the DRG (10). Apart from the P2X3 receptor, studies suggested that the P2X4 receptor also plays a key role in neuropathic pain. DRG consists of satellite glial cells (SGCs), which are mainly involved in the expression of the P2X4 receptor. Whenever there is nerve impairment, it is accompanied by the liberation of ATP, which further stimulates P2X4 receptors on SGCs, thereby generating neuropathic pain (11). Transient receptor potential vanilloid (TRPV) is also concerned with DNP, as it plays a vital role in nociceptive transference under clinical forms of pain (11, 12). The primary key in controlling DNP is symptomatic treatment (13). Various drugs have been recommended to minimize neuropathic pain either alone or in combination. The USA has given regulatory allowance to three drugs in treating DNP: pregabalin, duloxetine, and tapentadol (5). Although there are numerous therapeutic agents utilized in the treatment of DNP, half of the population is not able to achieve adequate pain relief. This failure is not due to the lack of efficacy of the drug but due to inadequate drug delivery at the site of action (14). Therefore, we need to incorporate innovative drug delivery systems to overcome the limitations offered by conventional ones. Nanotechnology plays a major role in effectively delivering drugs (analgesics) to specific sites, thus mitigating chronic pain. The main drawback offered by analgesics was their toxicity; thus, incorporating them into nanocarriers greatly enhanced their efficacy and reduced their toxicity. Some of the common analgesics, namely, baclofen, bupivacaine, and morphine, were formulated with liposomes, polyesters, poly (lactic-co-glycolic acid) (PLGA), nanoemulsions, etc., to improve their efficacy (11). It is reported that P2X3 receptor activation leads to allodynia in rat models of diabetes (15). DM rats, when treated with NONRATT021972 [long non-protein-coding RNAs (lncRNAs) siRNA], have shown that the expression of the DRG P2X3 receptor is significantly decreased as compared to type 2 diabetes mellitus (T2DM) rats in which no treatment is given. Unlike aqueous drugs, baclofen-loaded PLGA nanoparticles enhanced the retention duration of drug in the brain in order to mitigate neuropathic pain and turned out to be a suitable carrier for baclofen (16). Similarly, another emerging technology involves ribonucleic acid interference (RNAi) that mainly blocks gene assertion after transcription. Due to this inhibition, there is stimulation of RNA-induced silencing complex (RISC), which further hampers the protein synthesis. Potential benefits of bupivacaine were analyzed after its local delivery in people suffering from constant DRG compression (17). In the following review, novel approaches for targeting the DRG with the illustration of physiology of DRG and pathophysiology of DNP are discussed.

1.1 Epidemiology

One of the most recognized complications of DM is DNP. In various studies across India, PN prevalence ranges from about 10.5% to 32.2% in diabetic patients (18). Compared to the West, it has a higher prevalence of DM in India (4). Nowadays, practically in every country, diabetes impacts the population and increases medical load. Diabetes has become an epidemic globally; nearly 463 million adults in the age group of 20–79 years had diabetes in 2019, and this number is projected to grow to 700 million by 2045 (19). In Indian epidemiological studies from different areas, the average prevalence of PN in various community studies ranged from 5 to 2,400 per 10,000 population (20). Pain is one of the most pronounced symptoms of diabetic polyneuropathy. The incidence of diabetic peripheral neuropathy (DPN) was 46% in the African population in a survey conducted in 2020. Apart from this, the highest prevalence was reported in West Africa, accounting for about 49.4% (21). In autonomic neuropathy, the extent to which symptoms occur is relatively low (0%–10%), except impotence, whose chances of occurrence are about 5%–50% (22). As per reports from Europe and the USA in the year 2007, it has been revealed that the prevalence of DPN ranges from 6% to 51% with successive years of follow-up (13–14 years) (23). The pervasiveness of DPN in adults increased to 30% from 6% in type 1 diabetic patients as per the study conducted by Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) (24). A survey from the Consensus Development Conference on Diabetic Foot Wound Care suggested that around 26% of youth with type 2 diabetes developed DPN, thereby concluding that type 2 diabetics are more prone to develop neuropathic pain (25). Foot ulceration is one of the common manifestations of diabetic neuropathy. In some patients (14%–24%), foot ulceration is so severe that, sooner or later, it requires amputation (26). Patients with a previous history of foot ulcers, foot malformation, poor sugar control, smoking, etc., are at higher risk of amputation (27). Older adults are more prone to diabetic neuropathy who have had chronic diabetes for a long time (28). Some studies demonstrated that diabetic neuropathy is less observed in the Asian population, although there was no evidence or finding supporting this particular statement (29). More recently, DPN's prevalence has been reevaluated in young people with shorter durations of diabetes.

1.2 Physiology of Dorsal Root Ganglion

DRG is one of the most condemning structure in sensory signaling and modulation, along with pain transmission (30). A very thin boundary of cerebrospinal fluid (CSF) surrounds the sural sheath in which DRG is located (31). DRG is a mere extension of the dorsal root that usually accommodates cell bodies of primary sensory neurons (PSNs). The diameters of cell bodies can be classified as large-light neurons (which are generally known as A-neurons, and these usually transmit non-noxious information) or small-dark neurons (traditionally known as C-neurons, which transmit painful signals) (32). The axon soon gets bifurcated into a T-like fashion into a peripheral branch, which is connected with somatic and visceral receptors, and finally enters into a central component that ends up into a cord (33). The DRG's root sheath covers the dorsal root cord and traverses the subarachnoid space toward it. The proximal part usually consists of numerous tiny rootlets entering the dorsolateral cord in a defined manner (33). The DRG central projections typically end up in the corresponding segment. DRG

is in close association with the sympathetic chain *via rami communicantes* nerves. Sometimes, these nerves can act as channels for discogenic afferents that can deliver spinal pain signals to the DRG (34) (**Figure 1**).

1.2.1 Changes in Spinal Cord

The increased spinal neuronal activity due to neuropathy can be linked to the enhanced activity of the dorsal horns (35). In animal models having nerve injury, animal models have the stimulation of various protein kinases protein kinase A (PKA), p38, Src, extracellular signal-regulated kinase (ERK), calcium/calmodulin-dependent protein kinase (CaMK)II, and mitogen-activated protein kinase (MAPK). In addition, many CNS changes are associated with the production of inflammatory mediators. For example, the dorsal horn neurons possess increased expression of chemokines such as SDF-1α/CXCL12 and CXCL13 in rat models (36).

1.2.2 Initial Pathological Changes in the Dorsal Root Ganglion (Primary Triggers for Neuropathic Pain)

Major changes in primary sensory neurons altered gene/protein expression. Due to the destruction of peripheral sensory fibers, hyperalgesia occurs due to upregulation in the face of Cav α2 δ-$_1$ channel subunit, the Nav 1.3 sodium channel, and bradykinin (BK) B1 and capsaicin TRPV1 receptors (37). In addition, there is an immense increase in the expression of neurotrophic factors such as nerve growth factor (NGF) and neurotrophin-3 (NT-3). These neurotrophins are present in satellite glial cells (SGCs), which usually surround neuron cell bodies in DRG (38).

1.3 Pathophysiology of Diabetic Neuropathic Pain

The pathophysiology of DNP is quite complex, and there is no complete evidence to understand it entirely. Arteries endowing peripheral nerves undergo numerous changes and have been considered one reason behind aches and pains related to diabetic neuropathy. Recently, variations in sodium and calcium channel expression and central pain mechanisms have been linked to pain (39). Moreover, DNP is manifested by various risk factors, including old age, smoking, alcohol intake, and long-term diabetes (40). Due to a reduction in heat- and cold-specific C fibers and Aδ fibers, respectively, neuropathy leads to cold and heat allodynia (41). Mitochondria malfunctioning causes many problems in the body such as induction of neuropathic pain and changes in the peripheral nervous system (42). Various mitochondrial mechanisms, including calcium regulation (43), production of reactive oxygen species (ROS) (44), and apoptotic signaling pathways (45), are significantly involved in the development of neuropathic pain. Therefore, it is not only one single pathway that causes pain, but so many interconnected pathways operate together to start the cascade leading to neuropathic pain.

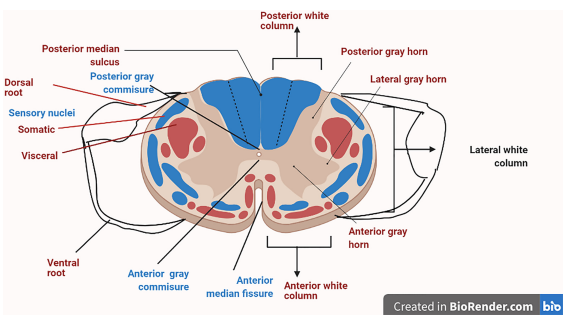


FIGURE 1 | Sectional organization of the spinal cord showing the dorsal root ganglion.

Changes in sodium channel expression appear to be triggered by hyperglycemia. In pain models of neuropathy, upregulated sodium channels (voltage-gated) were commonly seen in the DRG (46). Impairment of $Na^{(+)}$-$K^{(+)}$ pump occurs basically due to hyperglycemia, and it affects $Na^{(+)}$ currents to a great extent (47). Along with their transmission, these channels impact action potential processing and can be regarded as tetrodotoxin sensitive (TTX-S) (48). Tetrodotoxin-sensitive Nav1.3 channels are usually upregulated in diabetic animal models (49) and Nav1.7 in the DRG (50) (51, 52). Na^+ channels are repeatedly opened due to sensory neurons of DRGs, and their opening duration has also been seen to be prolonged to elevate the levels of intracellular sodium ions. Due to polarization of the neuron, there is increased opening of calcium channels that further leads to hyperpolarization (47). Rats in which nerves of the spinal area are injured show the oversensitivity of nociceptive responses to harmless mechanical stimulation due to overexpression of α2δ-1 subunit of the calcium channel (53). Due to this overexpression, more calcium enters the cell, leading to various signaling cascades (53). Also, the release of glutamate in the presynaptic zone leads to stimulation of N-methyl-D-aspartate (NMDA) receptors. This activation of the NMDA receptor will enhance the influx of calcium into the cell, thus rising calcium levels intracellularly (54). In response to the hyperpolarization of cells, mitochondria start releasing more calcium in the cytoplasm from its intercellular stores. As calcium concentration elevates inside the cell, it leads to activation of various signaling cascades mainly involving phosphorylation of PKC (55), causing an upregulation of TRPV (56), which directly causes variations in the sensory neurons, which result in a hyperresponsive state. There is the generation of nitrogen and oxygen-free radicals due to the upregulation of TRPV, leading to neuronal cytotoxicity (57).

TRPV1 coresides with transient receptor potential ankyrin 1 (TRPA1) in particular neurons of DRG, and it is proven to have a role in the generation of the pain signals and in inflammation that may occur due to various irritants such as chemical agents, ROS, or nitrogen radicals (58). Increased permeability of mitochondrial permeability transition pores (mPTPs) due to hyperpolarization inside the neuronal body may cause the release of cytochrome C that further begins apoptotic cascades. During the apoptotic pathways, caspases get activated, which can cause the destruction of neuronal bodies and can cause cellular toxicity (59). Consequently, the number of cold-specific Aδ fibers

and heat-specific C fibers starts reducing from the epidermis, known as loss of intraepidermal nerve fibers, and loss of nociceptors has also been observed that will result in the hyperresponsive state of the remaining nociceptors (60). Various inflammatory mediators involving tumor necrosis factor (TNF)-α, interleukin-1 (IL-1), and IL-6 are also seen to be involved in this signaling cascade. Cytokines, after binding to their receptors, lead to activation of PKC and MAPK that further corresponds to the development of neuropathic pain (61). These inflammatory cytokines usually enhance the expression of various ion channels involving sodium channels that causes neuronal excitotoxicity and significantly contributes to neuropathic pain pathogenesis (62). The pathogenesis of DNP interconnecting different pathways is represented in **Figure 2**.

1.3.1 Oxidative and Nitrosative Stress

The main trigger for the generation of oxidative stress in DNP could be activating the polyol pathway. However, some other factors can also contribute to the initiation of oxidative stress

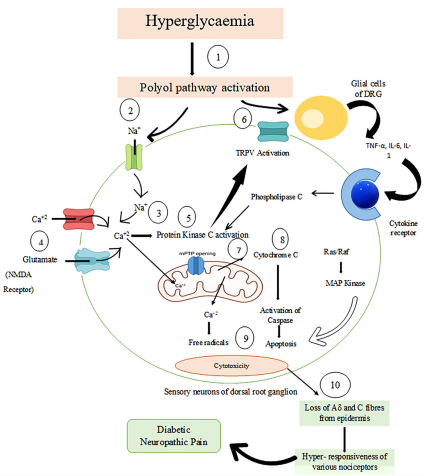


FIGURE 2 | Pathophysiology of diabetic neuropathic pain. 1) Hyperglycemia stimulates the polyol pathway, which leads to the destruction of sodium currents. 2) Na^+ channels were repeatedly opened due to sensory neurons of DRG, thus leading to increased sodium ions intracellularly. 3) As a result of polarization, there is the further opening of calcium channels. 4) In the presynaptic zone, glutamate causes activation of NMDA receptors and enhances the entry of calcium. Due to increased calcium levels, this triggers more calcium release from mitochondrial stores. 5) Activation of protein kinase C takes place due to increased calcium levels. 6) Transient receptor potential vanilloid (TRPV) phosphorylation and activation occur *via* protein kinase C due to which sensory neurons become hyperresponsive and also there is ROS and nitrogen radical generation, which causes cellular toxicity. 7) After the opening of mPTPs, there is the release of cytochrome C, 8) which initiates apoptotic avalanche with activation of caspases leading to sensory neuronal destruction 9) and finally leads to apoptosis. 10) Epidermis may lose some Aδ and C fibers, which causes hyperresponsiveness of various nociceptors. Inflammatory mediators such as IL-1, IL-6, and TNF-α are also involved in this process and play an essential role in developing neuropathic pain. DRG, Dorsal Root Ganglion; NMDA, N-methyl-D-aspartate; TRPV, Transient Receptor Potential Vanilloid; ROS, Reactive Oxygen Species; mPTP, mitochondrial permeability transition pores; IL-6 & IL-1, Interleukin 1 & 6; TNF-alpha, Tumour Necrosis Factor-alpha.

such as glucose auto-oxidation, rise in AGE levels intracellularly, enhanced expression of AGE receptors, and at last hyperactivity hexosamine pathway. Some evidence highly supports the fact that there is generation of oxidative stress due to glucose metabolism itself. Apart from oxidative stress, another key player that plays a crucial role in the development of diabetic complications is reactive nitrogen species, mainly peroxynitrite. In the animal models of diabetes, it has been observed that there are insignificant tissue concentrations of carbonyl compounds. The catalase and glutathione levels are precisely the same in DNP patients compared with non-diabetic neuropathic ones. This oxidative stress plays a significant role in the generation of chronic pain mechanisms and DNP (5).

1.3.2 Pro-Inflammatory Signaling in Diabetic Neuropathic Pain

The progression of diabetic neuropathy is associated with the acquirement of the pro-inflammatory process endured by nerve tissues. There is enhanced nerve conduction velocity (NCV) delay due to cytokine release hindrance and macrophage migration inhibition. The innate immune system is triggered by low-grade inflammation and plays a vital role in the pathogenesis of DNP. Inflammation is arbitrated by protein high-mobility group box 1 (HMGB1) released by immune cells. HMGB1 signaling was considered as one of the most poorly regulated pathways. This observation was made while comparing the differentially expressed genes between diabetic and non-diabetic patients. HMGB1 signaling is induced *via* Receptor for advanced glycation endproducts (RAGE) and Toll-like receptors (TLRs), both of which are involved in DPN. Therefore, these dysregulations of pathways linked with transcription implicate a lot in the pathophysiology of DPN (63).

1.3.3 Pharmacogenetic Analysis of Diabetic Neuropathic Pain

A genome-wide association study (GWAS) was conducted to determine the genetic contributors involved in DNP. The study involved monitoring patients having neuropathic pain consuming at least one of the five drugs [duloxetine, gabapentin, pregabalin, capsaicin cream (or patch) and lidocaine patch] indicated. However, diabetic individuals with no history of consuming these drugs were taken as control. Individuals who had a prescription history of amitriptyline, carbamazepine, or nortriptyline were not included as controls because these drugs are often used for the treatment of other medical conditions, as well as neuropathic pain. After the successful analysis, it was observed that sex-specific narrow sense heritability was higher in males (30.0%) as compared to females (14.7%). This specific GWAS analysis provides ample evidence about the involvement of sex-specific Chr1p35.1 (ZSCAN 20-TLR 12P) and Chr8p23.1 (HMGB1P46) in DNP. Here, abbreviations has been explained of ZSCAN 20-TLR. Zinc finger and SCAN domain containing 20 (ZSCAN20), TLRs, HMGB1 (64).

Another study evaluated the impact of *CYP2D6* genotype on amitriptyline efficacy for the treatment of DPN. Randomly, 31 participants were selected and given low-dose amitriptyline, and

after some time, their *CYP2D6* gene was sequenced. As a result, fewer side effects were observed in patients possessing ultrarapid metabolizer phenotypes. Therefore, this study can guide drug therapy for DNP shortly (65). There are numerous drugs in the market for the treatment of neuropathic pain. Furthermore, we describe competitive market landscape, market potential, and limitations of current therapy.

1.3.4 Protein–Protein Interaction

Having a deep insight of molecular mechanisms associated with a particular disease is the foremost goal of modern medical research. In order to understand this, a study was done that generated a comprehensive network of 1,002 contextualized protein–protein interactions (PPIs) that are particularly related to pain. The PPIs possess an extremely coherent and interlinked structure. In this specific study, the purpose and reliability of pain-related PPIs using network have been explored *via* gene bias assessment methods. Out of the most enriched proteins in the network, majority of them play an important role in the pathology of pain for e.g., OPRM1, TPRV1, and NGF. As per the results, around 144 interactions are associated with neuropathic pain in the given dataset. Out of these 144 interactions, around 122 contribute to the pathology of pain. Neuropathic pain network contains 127 proteins out of which 8 enriched proteins are mainly involved such as GRIN2B, NOS1, MAPK14, IL-6, DLG2, CX3R1, P2RX4, and VGF. This method of utilizing disease-specific interactions presents an appreciable advancement in specificity and relevance (66).

1.4 Competitive Market Landscape

TABLE 1 | Marketed drugs for alleviating diabetic neuropathic pain (67).

S. No.	Medication	Indication	Brand name	Company	Drug class
1	Pregabalin (systemic)	DPN	Lyrica	Pfizer	Gamma-aminobutyric acid analogs
2	Topiramate (systemic)	DPN	Topamax	Mylan	Carbonic anhydrase inhibitor (anticonvulsants)
3	Duloxetine (systemic)	DPN	Cymbalta	Eli Lilly	SNRIs
4	Capsaicin cream (topical)	DPN	Zostrix, Capzasin	–	Miscellaneous topical agents
5	Carbamazepine	Neuropathic pain	Tegretol	Novartis	Dibenzapine anticonvulsants
6	Gabapentin	Neuropathic pain	Neurontin	Pfizer	Gamma-aminobutyric acid analogs
7	Nortriptyline hydrochloride or desipramine hydrochloride)	Chronic pain	Pamelor	Mallinckrodt Pharmaceuticals	Tricyclic antidepressants
8	Venlafaxine ER	DPN	Effexor	Pfizer	SNRIs

DPN, diabetic peripheral neuropathy; SNRI, serotonin–norepinephrine reuptake inhibitor.

1.5 Limitations of Current Therapy

The main drawback offered by drugs used in the treatment of diabetic neuropathy was their toxicity. Hence, incorporating them into nanocarriers greatly enhanced their efficacy and reduced their toxicity. In addition, many side effects were accounted for with traditional treatment such as lack of specificity and adverse effects such as light-headedness, languidness, and multiple daily doses (68). The latest treatments do not provide adequate pain relief for about half of the patients and offer many undesired side effects such as somnolence and dizziness and the requirement of a complex dose regimen to reduce patient compliance. Standard agents for topical administration are there for the treatment of DNP, such as capsaicin cream, which is without any side effects. Still, they have low efficiency, and complex multiple administration is required, which can cause discomfort, and also the chances of contamination of sensitive body areas are also there, both of which can lead to poor patient compliance (69) (**Table 1**). The basis of this study is to incorporate novel nanotechnological approaches in mitigating DNP by targeting the DRG. Previously, opioid analgesics were widely used to treat DPN (13). Unfortunately, severe side effects were seen in patients exposed to this drug therapy. It mainly arises due to its action on the receptors present in the CNS, leading to respiratory depression, sedation, dizziness, etc. Here, nanotechnology outperforms in every aspect by delivering sensitive and targeted treatment. Another point to be taken into account is the uncontrolled drug delivery and frequent administration of drugs offered by traditional delivery systems. This probably leads to changes in plasma drug levels, thus increasing the demand for novel approaches (70).

With recent progress in identifying pain-generating processes and adopting evidence-based treatments, patients suffering from DPN are still difficult to cure. The latest treatments do not provide adequate pain relief for about half of the patients and offer many undesirable side effects such as somnolence and dizziness and the requirement of a complex dose regimen that reduces patient compliance. In addition, due to the lack of specificity of drugs, there is inadequate relief of pain. Ultimately, more understanding of the basic pathophysiological processes that lead to this complication should make it possible to devise optimal therapies for individual patients suffering from neuropathic pain (69).

1.6 Market Potential

Nowadays, diabetes is one of the most widespread and long-term diseases affecting most people globally (71). As per recent estimates, in the course of a year (2020–2021), the global diabetic neuropathy market is appraised to expand at a compound annual growth rate (CAGR) of 5.9%. In 2011, around 366 million people had diabetes, and the count is estimated to significantly rise to 522 million by 2030, as per approximation given by the International Diabetes Federation (72). Therefore, we can say that shortly the DNP market has stupendous opportunities to flourish. However, most of the formulations are sold by their generic names due to which there is an excellent hindrance in introducing all new and innovative therapeutic agents.

On the other hand, there has been a significant emergence and rise in the diabetic drug market after approval by the Food and Drug Administration (FDA) on using novel drugs for

treating DNP. Various medications were approved, out of which two were widely used, namely, Nucynta ER and Lyrica, in 2015. The rise in the market is commonly observed in five areas, namely, Asia Pacific, South America, North America, Europe, and Africa. Among all these, North America holds the biggest market for diabetic neuropathy, where around 7.9% of adults have a chance of developing diabetes. Moreover, type 2 diabetes is directly related to obesity, hence in the US, with rising cases of obesity, there are great chances of developing DNP, thus depicting enormous market scope (73).

Hence, with comprehensive understanding about the disease, we move forward to understanding about the novel nanotechnological as well as other approaches for targeting the DRG for the treatment of neuropathic pain.

2 NOVEL APPROACHES FOR TARGETING THE DORSAL ROOT GANGLION IN MITIGATING DIABETIC NEUROPATHIC PAIN

2.1 Nanoparticles

Nanoparticles represent a massive variety of particles, mainly particulate materials less than 100 nm (74). Nanoparticles exhibit remarkable and distinctive mechanized, chemical, and optical characteristics, making them a consummate agent for treating DNP. A study indicated that the CeO_2 (cerium oxide) nanoparticles play a significant role in combating oxidative impairment and showed protective actions on diabetic neuropathy. Compared to the control group, diabetic rats showed a higher nociceptive threshold. After treatment with CeO_2 nanoparticles, the pain threshold was reinstituted to the standard level. This study proved to be significantly successful in revealing the CeO_2 nanoparticle as an excellent agent that suppresses nerve damage due to diabetes (75). Another study demonstrated the potential benefits of curcumin incorporated into nanoparticles in mitigating DNP arbitrated by P2Y12 receptor on SGCs in DRG. In diabetic rats, thermal hyperalgesia occurs due to modulation of IL-1 and Cx43. When curcumin nanoparticles were administered in the DRG of rats, the expression of IL-1 and Cx43 reduced significantly. Therefore, it can be said that curcumin nanoparticles are an effective therapeutic agent for treating DNP (76). One study examined the effects of emodin nanoparticles on DNP initiated by P2X3 receptors in DRG. After administration of emodin in DRG of rats, there is a significant reduction in the modulation of P2X3 receptors, thus alleviating DNP and suppressing all the channeling related to P2X3 receptors in DRG neurons (77).

2.1.1 Polymeric Nanoparticles

Polymeric nanoparticles comprise nanospheres and nanocapsules, colloidal systems ranging from 10–1,000 nm in size (78). A preclinical study in rats evaluated the efficacy of baclofen-loaded PLGA nanoparticles in managing neuropathic pain. Results revealed that baclofen polymeric nanoparticles significantly reduced toxicity and increased cell feasibility on a Neuro 2a cell line. Also, in contrast to aqueous drugs, the retention time of these PLGA nanoparticles was enhanced in the brain, thus depicting it as a suitable agent in mitigating neuropathic pain (16). Bupivacaine is a local anesthetic that is commonly used to treat pain. Another study looked into the influence of bupivacaine on pain management in animals with chronic compression of the DRG. For this purpose, bupivacaine was incorporated into PLGA nanoparticles and then administered parenterally into L3 and L4 DRGs of mice. The size of nanoparticles prepared ranges from 150 ± 10 nm in diameter. Results showed that DRG administered with drug (bupivacaine) alone developed allodynia and hyperalgesia in the hind paw of mice. Whereas bupivacaine nanoparticles significantly suppressed both complications and brought the mechanical sensitivity within the range of typical values as obtained for healthy animals (17).

2.1.2 Inorganic Nanoparticles

Metallic nanoparticles are composed of metals such as silver (Ag), gold (Au), and copper (Cu) along with certain metallic oxides, namely, TiO_2 and ZnO, which impart rigid and flexible structure (79). Out of all the metals involved, silver is one of the most widely employed due to its excellent characteristics such as the large surface area-to-volume ratio (70). ROS are significant contributors to neuropathic pain. Silver nanoparticles can easily combat ROS production by binding to membrane proteins (80). Previously, many techniques were adopted to synthesize silver nanoparticles, but those methods were rejected due to toxicity of utilized chemicals. This led to the idea of employing medicinal plants in the development of silver nanoparticles (81). One study involved *Nigella sativa* extracted in the green synthesis of silver nanoparticles and determined its beneficial effects in diabetic neuropathy. An experiment was carried out in which a healthy control group of rats was compared to the diabetic neuropathy-induced group to estimate the potential actions of nanoparticles administered. Results revealed that neuropathy-induced group showed significant demodulation in brain tropomyosin receptor kinase A (trKA) levels and increased inflammatory mediators. However, the group treated with silver nanoparticles experienced less pain and enhanced retention time. Thus, due to its antidiabetic, anti-inflammatory, and antioxidant effects, silver nanoparticles combined with *N. sativa* could be an innovative treatment option against diabetic neuropathy (82).

2.1.3 Gene-Based Nanoparticles (siRNA)

siRNA is a double-stranded RNA molecule that causes obtrusion in the genetic expression of complementary base pairs of mRNA and leads to knockdown of expression (83). Microglia homing peptide molecules are sound delivery systems for siRNA due to their potent knockdown efficacy. One of the most frequently employed homing peptides for the siRNA–interferon regulatory factor-1 complex is MG1. Compared to standard siRNA and other peptide molecules, the siRNA delivery system was eminent in reducing hyperalgesia-associated nerve damage. Such shreds of evidence suggest siRNA delivery candidates as a plausible therapeutic in alleviating neuropathic pain. Calcitonin gene-related peptide located in the DRG primarily impacts nociception in afferent transmission input. This activation of afferent neurons leads to the release of the calcitonin gene-related

peptide in the spinal cord. Due to glutamate release, NMDA receptors activate, which further enhances calcium influx in the cell, triggering the release of more calcium from stores. As a result, enhanced calcium ion levels actuate various protein kinases involved in the pathophysiology of neuropathic pain (55). siRNA delivery device mitigates neuropathic pain by suppressing the $P2X_3$ receptor in the DRG and leads to inhibition of expressed Calcitonin gene-related peptide in the spinal cord (84). There is the release of numerous cytokines, which stimulates kinase activated process. siRNA delivery device halts this activation thereby, alleviating neuropathic pain (61).

Lentivirus-containing siRNA was introduced into the spinal cord *via* the intrathecal route in a rat model. The results revealed a diminution in nociception due to the sequential inhibition of mRNA and expressed protein GluN2B. Furthermore, the lentiviral delivery device successfully introduced GluN2B to the dorsal horn, thus reducing neuropathic pain (85). **Figure 3** corresponds to the mechanism of siRNA-based nanocarriers in alleviating neuropathic pain.

2.1.4 Solid Lipid Nanoparticles and Nanostructured Lipid Carriers

Solid lipid nanoparticles (SLNs) promise drug delivery systems that consist of solid lipid particles such as fatty acids and waxes to which surface-active agents have been added to form a stable matrix system. SLNs can easily inculcate both hydrophilic and hydrophobic drugs in their matrix, thus preventing the medicine from any deterioration (79). However, SLNs possessed some drawbacks due to which concept of nanostructured lipid carriers (NLCs) came into existence. NLCs outperform SLNs in terms of good drug loading capability (78). Capsaicin is widely used in treating DNP due to its ability to bind to TRPV1 present on Aδ and C-nerve fibers (86). Capsaicin (0.25%)-loaded lipid nanoparticles were developed from capsicum extract in the study. There was strategic incorporation of capsaicin into SLNs and NLCs without any toxic solvent involvement. The particle size of prepared nanoparticles was less than 200 nm. Compared to SLNs, NLCs offer enhanced encapsulation capability and better capsaicin liberation, thereby augmenting its release into deeper skin layers. Hence, through the above study, capsaicin-loaded lipid nanoparticles could be an excellent therapeutic agent for pain management (87).

2.2 Nanoemulsion

One of the highly recommended drug delivery systems, nanoemulsion, consists of oil, surfactant, and water in relevant ratios. Nanoemulsion bears an average atom size of 1–100 nm (68). These are widely used (88). Various experiments have been conducted to determine the potential of nanoemulsion incorporating *Bauhinia variegata* to treat diabetic peripheral neuropathic pain *via* the acupuncture technique. Due to its polyphenol and flavonoid content, *Bauhinia* exhibits free radical-scavenging properties. Experimental rats were administered streptozocin, employing intraperitoneal injection to induce diabetes. Administration of *Bauhinia variegata* nanoemulsion normalized blood glucose levels compared to the control group. Long-term treatment with nanoemulsion effectively reduced hind paw abolition latency and alleviated allodynia. Therefore, through the above experiment, one can presume that *Bauhinia* nanoemulsion could effectively relieve peripheral neuropathic pain (89). Furthermore, α-eloestearic acid, one of the main

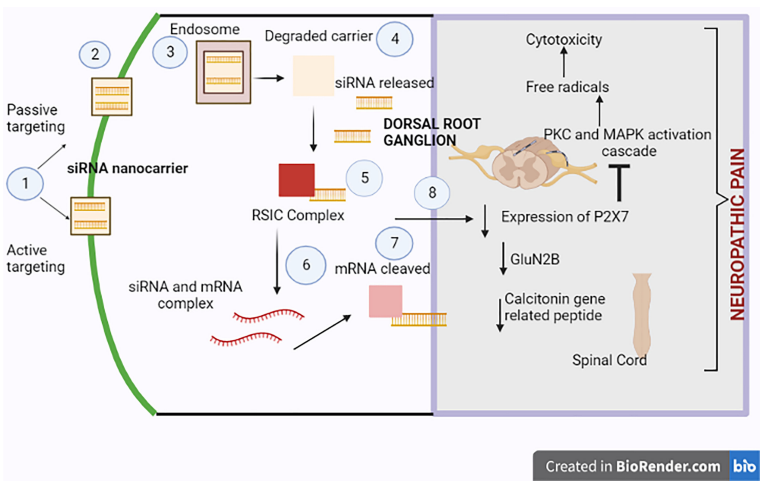


FIGURE 3 | Neuropathic pain-relieving molecular mechanism of siRNA-based nanocarriers. 1) Either passive or active targeting allows the siRNA delivery device to penetrate the cell. The attachment of antibodies or aptamers, which improve the device's specificity, aids in active targeting. 2) The siRNA nanocarrier then enters the cell. 3) There is the engulfment of the delivery device by the endosome. 4) As a result, the outer carrier breaks down, releasing free siRNA therapeutics. 5) An RNA-induced silencing complex (RISC) is formed due to the siRNA formation. 6) To progress the knockdown of the chosen mRNA, the mRNA and siRNA interact with one another. 7) The RISC cleaves the mRNA to silence proteins implicated in neuropathic pain disease. 8) P2X7 receptor expression in the dorsal root ganglion, GluN2B peptide, and the calcitonin gene-related peptide in the spinal cord are suppressed. The siRNA delivery device reduces neuropathic pain by inhibiting excitation transmission through the P2X3 receptor in the DRG and inhibiting expressed calcitonin gene-related peptide in the spinal cord, which changes the calcium-augmented pathways in neuropathic pain.

constituents of the bitter gourd when administered orally in the form of nanoemulsion to diabetic rats, showed promising effects by reducing neuropathic pain (90).

2.3 Liposomes

Liposomes are the most widely used nano delivery system, as they can significantly increase drug efficacy while minimizing their side effects. Liposomes consist of an aqueous core encircled by phospholipid bilayers (91). Ropivacaine is a widely used anesthetic for mitigating neuropathic pain (92). To alleviate long-term neuropathic pain, it was seen that liposomal preparation of ropivacaine (Rop-DPRL) could lead to the cytotoxicity of cancerous cells *via* nutrient destitution. Another study demonstrated the effects of zoledronic acid (ZOL) in mitigating neuropathic pain. The most pronounced drawback of ZOL is its pharmacokinetic outline. Hence, an animal model developed and assessed ZOL incorporating PEGylated liposomes (LipoZOL) for its action in attenuating neuropathic pain. There is partial or complete disorganization of the blood–brain barrier (BBB) in chronic neuropathic pain, which permits the safe entry of nanocarriers such as LipoZOL. Changes in BBB due to sciatic nerve destruction encourage the invasion of LipoZOL in the dorsal horn of the spinal cord, thereby administering adequate concentrations of ZOL in the CNS. This further regulates the phenotypical shift of glial cells, thus alleviating neuropathic pain (93).

2.4 Exosomes/Extracellular Vesicles

Exosomes are small vesicles that are seen in body fluids. Exosomes are acknowledged for their excellent capacity for loading nucleic acids and are less toxic than other novel carriers such as carbon nanotubes and fullerenes (94). They are primarily apprehended for their enhanced action, as they serve as carriers for numerous molecules, including proteins, nucleic acids, and lipids. As we know, RNAse leads to the destruction of miRNA, so it was loaded into extracellular vesicles to prevent its degradation. These exosome-loaded miRNAs impact physiological responses in beneficiary cells by controlling gene expression (95). Superoxide dismutase (SOD)-loaded polymersomes are highly beneficial in treating neuropathic pain, as they possess antioxidant action. These SOD-loaded polymersomes have several advantages such as appropriate interaction of the enzyme with ROS due to porous membrane and enzymes maintained their original shape. Treatment with SOD-loaded polymersomes is much effective for treating neuropathic pain as compared to SOD alone after painful DRG compression (96).

3 LIGAND-BASED TARGETING TO DORSAL ROOT GANGLION

Recently, the concept of small peptide aptamer is gaining a lot of undivided attention in treating neuropathic pain as these target protein–protein connections in pain pathways. Also, these aptamers have been considered as a useful clinical tool in alleviating chronic pain (97). Compared to gene delivery strategies such as RNA interference, this peptide aptamer hindrance can effectively slab various interactions selectively, causing functional knockdown. Ca^{2+} channel-binding domain 3 (CBD3), in association with the TAT motif (TAT-CBD3), is a famous example of a peptide inhibitor that can prevent the pain caused by different conditions. However, after proving its excellent efficacy results, CBD3 in conjunction with TAT has been utilized widely as an alternative to mitigate chronic pain (98). A study suggested that Adeno-associated viruses (AAV) injection can lead to uninterrupted CBD3 expression in DRG neurons, relieving pain with reduced or no toxic effects. Here, voltage-gated Ca^{2+} channels (VGCCs) were selected as molecular targets, as they play a significant role in synchronizing neuron excitability and transmission *via* synapses (99). Ultimately, small interfering peptides can be utilized as an effective alternative and strategy for treating neuropathic pain (97). One study suggested that chemokine receptor CXCR3 is involved in generating chronic pain. It is present in spinal cord, and the pain is generated due to mast cell destruction due to which there is release of histamine. In this situation, histamine antagonists H1 and H4 can be used as plausible ligands to stop the release by blocking CXCR3 receptors (100).

4 OTHER APPROACHES

4.1 Neuromodulation

Neuromodulation is a rapidly emerging area of pain medicine that influences hundreds of thousands of patients dealing with several disorders globally (101). It involves the utilization of noninvasive and surgical electrical therapies. In the case of PDN, neuromodulation seems to be a very effective treatment option for those patients who are generally insensitive to conventional pharmacotherapy (102). Therefore, the exemplary treatment method, namely, tonic spinal cord stimulation (t-SCS), has been incorporated. It mainly involves the entry of regular electrical pulses into the dorsal column *via* epidural electrodes. The electrical pulses are delivered at a frequency of around 50 Hz (103). DRG stimulation or neuromodulation can effectively cause a reduction in chronic pain associated with PDN (104). DRG and DNP-DRG may be particularly susceptible to this disease for several reasons such as the following:

a) DRG consists of sensory neurons, which are not protected from the BBB, and the ambient oxygen tensions in DRG are pretty low. These physiological conditions may suggest that these may be vulnerable to microangiopathy, which is a complication related to diabetes (105).

b) Also, the involvement of sensory neurons in early diabetic polyneuropathy may put forward the fact that diabetes specifically targets DRG. Certain features associated with DRG might imply that it would be exposed to changes known to occur in diabetes such as excessive polyol flux, microangiopathy, and protein glycosylation (106).

c) Several studies, including streptozocin-induced DPN, also highlighted the fact that DRG is closely related to painful DPN acquiring several metabolic and immunological processes (107).

d) Certain receptors such as TRPV1 present in DRG are closely associated with DNP (108).

4.2 Precision Medicine

Several techniques are available to mitigate DNP, but neither glucose control nor the symptomatic treatment is very successful in doing so. Therefore, to overcome this issue, a concept has been taken into account that hypothesizes the study of patient characteristics. The concept could possibly be helpful to stratify individuals, thus providing them specific and targeted therapy to get better pain relief. This whole concept of studying patient characteristics [clinical features, quantification sensory testing (QST), genetics, and cerebral imaging] and then developing targeted therapy is termed "precision medicine" (109).

5 CONCLUSION

Diabetic neuropathy is the most entrenched complication of diabetes. Typically, it affects the distal foot and toes, gradually approaching the lower part of the legs. Diabetic foot ulcer (DFU) could be one of the worst complications of DM. Long-term diabetes leads to hyperglycemia, which is considered to be the utmost contributor to neuropathic pain. Therefore, using antidepressants, GABA analogs, opioids, and topical agents to treat pain in PDN is recommended. Currently available systemic medications provide adequate pain relief for approximately half of affected patients and are limited by unwanted adverse reactions and multiple-dose regimens. So, other treatment options need to be explored to treat this widespread complication of diabetes, mainly involving novel nanotechnological approaches. Nanotechnology plays a significant role in effectively delivering drugs (analgesics) to a specific site, thus mitigating chronic pain. Some of the standard painkillers, namely, baclofen, bupivacaine, and morphine, were formulated with liposomes, polyesters, PLGA, and nanoemulsions, etc., to improve their efficacy. siRNA can also be used as potential therapeutics to treat DPN but are limited by its unstable nature under normal physiology in the blood, wherein it undergoes digestion by nuclease enzymes. Therefore, innovative nanotechnological approaches such as liposomes, niosomes, nanoemulsions, SLNs, and NLCs have been utilized to overcome conventional therapy's drawbacks.

6 FUTURE PERSPECTIVES

Despite having so many alternative therapeutic options for treating DNP, still, pharmacological treatment remains a big never-ending issue for physicians. Therefore, there is a need to find out various important target areas that can be utilized directly to mitigate DPN. We can also expect multiple novel nanotechnology-based products in the market to treat diabetic neuropathy, which can adequately manage the condition with enhanced effects. Moreover, natural plant-based products are also being studied to a large extent to provide more safe and cheap treatment to the patients. Furthermore, many advancements have been made about gene therapy, including new therapeutic approaches that may become combination therapies with various siRNAs targeting various survival pathways or a combination of specific siRNAs that may sensitize the treatment of DNP with other pain-relieving drugs. Therefore, in association with novel technological approaches, conventional medicines can significantly enhance their action toward diabetic neuropathy, and we can expect plenty of nano-based products in the market for the mitigation of diabetic neuropathy.

AUTHOR CONTRIBUTIONS

RB: conceptualization, methodology, writing—review, editing and visualization, literature search. AS: literature search, data collection, and writing. AK: final supervision. All authors contributed to the article and approved the submitted version.

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The Association Between FokI Vitamin D Receptor Polymorphisms With Metabolic Syndrome Among Pregnant Arab Women

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Metabolic syndrome (MetS) is a serious health condition that is becoming extremely threatening in Saudi Arabia. The link between vitamin D receptor (VDR) gene polymorphisms and maternal MetS has been observed in several ethnic groups, but is yet to be clarified in the Arabian population. This study aims to investigate the relationship between the *FokI* VDR genotype and the risk of MetS and its components in pregnant Saudi women. A cross-sectional study was conducted using 368 pregnant Saudi women on first trimester screened for MetS (44 with MetS and 324 without MetS). Measurements included anthropometrics, glycemic and lipid profile and 25(OH)D. TaqMan genotyping assay was used to determine *FokI* VDR genotype of participants. Vitamin D deficiency (25 (OH)D <50nmol/l) was seen in 85% of the participants. An estimated 12% of participants had MetS. In the MetS group, the *FokI* VDR genotyping frequencies for *FF, Ff*, and *ff* genotypes were 50%, 36.4% and 13.6%, respectively. In controls, the frequencies were 62.7%, 31.4% and 5.9%, respectively. No significant association between the individual MetS components and *FokI* VDR genotypes were observed. Nevertheless, carriers of the *ff* allele had a significant risk for full maternal MetS [Odds Ratio 4.2 (95% Confidence Interval 1.4-12.2; adjusted p=0.009). The study suggests that the *ff FokI* VDR genotype is a genetic marker of maternal MetS in pregnant Arabian women. Prospective studies that include neonatal outcomes may confirm present findings.

Keywords: metabolic syndrome, vitamin D polymorphisms, pregnancy, gestational diabetes mellitus, genetic marker

INTRODUCTION

Metabolic syndrome (MetS) is a cluster of conditions that is present when three or more of the following five characteristics are met: central obesity, high cholesterol, elevated blood pressure, dyslipidemia, and elevated fasting blood glucose (1). MetS is a well-established independent risk factor for type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (2). The prevalence of MetS is increasing globally, rising from a rate of 20% among adult populations to 40% (3). Almost 25% of the adult population in the United States suffers from MetS (4), but still lower as compared to Saudi Arabia, where 35% of the adult population has full MetS (5). Among at risk populations, women with MetS are at a higher risk of developing maternal complications like preeclampsia and coma, in addition to experiencing adverse perinatal effects like preterm birth, jaundice, neonatal macrosomia, and malformations (1, 4). The presence of MetS during pregnancy alone increases maternal CVD by as much as seven-fold (6), with offspring of high-risk pregnancies also having a higher risk of developing CVD, MetS, and T2DM (1, 7). The Riyadh Mother and Baby Study (RAHMA), a large cohort study in Saudi Arabia, revealed that more than 68% of the 14,568 pregnant women studied were either overweight or obese, while 24% had developed gestational diabetes mellitus (GDM), considered among the highest in the world (8). Aside from MetS and its components as GDM risk factors (9), an emerging GDM risk factor considered unique in the Arabian population is vitamin D deficiency (10).

On the physiological level, 1.25-dihydroxyvitamin D is the active form of vitamin D, and its activity is mediated *via* the cellular vitamin D nuclear receptor (VDR) protein (11). VDR is abundantly expressed in most body tissues, including uteroplacental parts, and is important in bone metabolism, calcium homeostasis and other non-calcemic functions (12, 13). In normal pregnancy, VDRs can regulate implantation as well as hormonal and immune modulations in the placenta (14). Similar to the nature of most nuclear receptors, the full function of VDR can be altered by several single nucleotide polymorphisms (SNPs), including the *FokI* VDR gene polymorphism (15).

The *FokI* VDR gene polymorphism is of particular interest, given that it occurs within the transcription start codon (11). Furthermore, its polymorphic form (*f*) shifts the start codon position, meaning that the expression of the VDR protein is three amino acids longer than the wild-type (*F*) allele (11). The shorter-wildtype version of the VDR protein is more active than the longer VDR protein. In some epidemiologic studies, the shorter *FokI* VDR allelic variation has been associated with a host of adverse disease outcomes (16, 17), including increased risk to MetS in adults (2, 3, 15, 16, 18, 19). Furthermore, increased susceptibility to GDM from expectant mothers having the *FokI* variant were observed among expectant mothers coming from Turkey (20) and Iran (21), but not Chinese (22) and Saudi Arabians (23), which means that *FokI* may confer susceptibility only to select populations (24). To date, there is scarcity of evidence linking MetS and *FokI* VDR SNPs in pregnant women, more so in understudied ethnic groups such as Saudi Arabians. Since vitamin D deficiency is endemic in the Saudi population, this group is susceptible to the adverse effects of vitamin D-related disorders, given the presence of the relatively ineffective longer VDR protein found in women with the *ff* VDR genotype. This study is therefore intended to explore the link between VDR *FokI* genotypes and the risk of MetS and its components in Saudi Arabian pregnant women.

MATERIALS AND METHODS

Study Design and Sample Population

This study comprises part of a larger prospective cohort study, "*Vitamin D and Pregnancy in Saudi Women*." This cross-sectional study focuses on a subset of study participants—324 healthy pregnant women and 44 pregnant women with MetS—for a total of 368 participants. These women visited one of three Saudi hospitals in their second trimester of pregnancy (24–28 weeks) between December 2013 and January 2016: King Khaled University Hospital (KKUH), King Salman bin Abdulaziz Hospital, or King Fahad Medical City (KFMC), all in the capital city of Saudi Arabia, Riyadh. All of the necessary approvals were collected, meaning that this study has full ethical approval to collect samples and patient data. Approval was secured from the Ethics Committee of the College of Medicine (Approval No. E-13-1013, February 11, 2014) King Saud University, Riyadh. Each patient also provided his or her express written consent.

Inclusion and Exclusion Criteria

The study focused on healthy Saudi women who were pregnant and aged between 18 and 40 years. These participants were enrolled in the study before 16 weeks of gestation and exhibited no previous history of DM. Participants were excluded if they were pregnant non-Saudis over 16 weeks age of gestation, those on vitamin D supplements, oral glucocorticoids, calcium, cardiac medications or any drug known to interfere with vitamin D, calcium absorption, or parathyroid disorders, those with malabsorption syndrome, hypertension and other pre-existing conditions including previous history of GDM.

Anthropometric Measurements

Anthropometric measurements included height (cm) and weight (kg) in order to calculate BMI (kg/m^2) as well as pre-pregnancy weight (kg) and pre-pregnancy BMI (kg/m^2). Blood pressure information (mmHg) was also noted. Body weight measurements were taken without shoes, and the participants used lightweight clothing. This measurement was recorded to the nearest 0.1 kg (Digital Person Scale, ADAM Equipment Inc., USA). The pre-pregnancy weight was self-reported during the prenatal visit, and this was used to classify the patients according to the World Health Organization's (WHO) BMI definitions: underweight: < 18.5 kg/m^2; normal weight: 18.5–24.9 kg/m^2; overweight: 25.0–29.9 kg/m^2; or obese: ≥ 30.0 kg/m^2 (25). The height of the participants was measured to the nearest 0.5 cm using the Digital Pearson Scale at the first pregnancy visit. During the measurement, patients were standing upright and

without shoes. A mercurial sphygmomanometer was used to measure blood pressure (mmHg).

Biochemical Assessment and MetS Screening

Participants were asked to fast for > 10 hours before fasting blood samples of 10ml were taken. These samples were collected with a sterile vacutainer blood collection apparatus. Samples were aliquoted and moved to a -80°C freezer. The Chair for Biomarkers of Chronic Diseases (CBCD) in King Saud University, Riyadh, Saudi Arabia was responsible for storing and analyzing the blood samples. Electro-chemiluminescence binding assay 2012 (ECLIA) (Roche Diagnostics GmbA, Mannheim, Germany) and commercially available IDS kits (IDS Ltd., Boldon Colliery, Tyne & Wear, UK) were used to measure serum 25(OH)D. The inter- and intra-assay coefficients of variation (CV) for 25(OH)D ELISA are 5.3% and 4.6%, respectively, with 100% cross-reactivity to 25(OH)D3 and 75% cross-reactivity to 25(OH)D2. According to national and regional guidelines, the cutoff values for vitamin D deficiency are less than 50 nmol/L for serum 25(OH)D and, for sufficiency serum 25(OH)D, more than 50nmol/L (26, 27).

A chemical analyzer (Konelab, Vantaa, Finland) was used to measure triglycerides (TG), fasting serum glucose (FBG) and lipid profile [including total cholesterol (TC)], high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) (28). Point-of-care (POC) devices (Accu-Check Active, Roche Diagnostics, Mannheim, Germany) were used to measure the HbA_{1C} from the whole blood.

Under the definition of MetS proposed for the obstetric population, a pregnant woman can be defined as having MetS if she has three or more of these risk factors: a pre-pregnancy body mass index (BMI) > 30 kg/m^2; level of serum triglycerides (TG) ≥ 1.7mmol/L; serum high-density lipoprotein (HDL)-cholesterol level < 1.3mmol/L; fasting serum glucose level ≥ 5.6mmol/L, and blood pressure level ≥ 130/85 mmHg (10). The participants who fell outside of this definition were grouped as controls. **Figure 1** shows the flowchart of participants.

DNA Extraction and Quantification

For extracting Genomic DNC from the whole blood, innuPREP mini blood kits (Analytik Jena, Germany) were used according to the instructions of the manufacturer. In brief, a 1.5 ml reaction tube containing lysis buffer and Proteinase K hosted 200 µl of a whole blood sample. This was mixed using vortex and incubated for 10 minutes at 60°C. An appropriate quantity of binding solution was also added before the tube itself was transferred to a spin filter column and centrifuged for a full minute at 12,000 rpm. The washing of the DNA was performed using washing solution C—included with the kit—followed by washing solution BS. For the final step, the spin filter was inserted into a 1.5 m elution tube while 200 µl of elution buffer was pre-warmed and added. Once centrifugation was completed, the DNA was saved for further analysis at a temperature of -20°C. A Nano-Drop spectrophotometer was used to determine the concentration and purity (260/280) of the DNA (29).

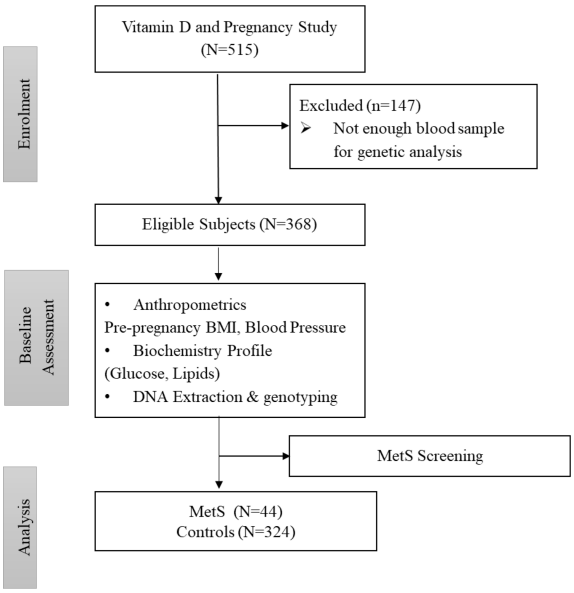

FIGURE 1 | Flowchart of participants.

FOK-I SNP Genotyping

The *Fok-I* SNP (rs 2228570) was assessed using a pre-designed TaqMan genotyping assay from Applied Biosystems, Foster City, CA, USA (assay ID: C_12060045_20). Amplification reactions were performed in a volume of 10 µL containing 1X TaqMan genotyping Master Mix (Applied Biosystems), 1X mix of unlabeled PCR primers and TaqMan MGB probes, and 30 ng of template DNA. All amplification and detection was conducted in 96-well PCR plates using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Milan, Italy). Thermal cycling was initiated with a denaturation step of 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 90 s at 60°C. After PCR was completed, allelic discrimination was analyzed using the Bio-Rad CFX Manager Software (Version 1.6, Bio-Rad). Genotype assignment was determined by plotting the end point relative fluorescent units (RFU) for one fluorophore (allele 1 on the x-axis) against the RFU for the other fluorophore (allele 2 on the y-axis) on the allelic discrimination. All PCR reactions were set up in a dedicated PCR area with dedicated PCR pipettes and reagents.

Sample Size and Statistical Analysis

Based on the association between HDL-cholesterol and FoKI polymorphism reported in previous literature with the effect size of 0.162 (17), the required sample size for this study at 95% and 80% power is N=366. Data were analyzed using SPSS (version 21.0, IBM). Continuous data are presented as mean ± standard deviation (SD) for Gaussian variables, and non-Gaussian variables are presented as median (25^{th} and 75^{th}) percentiles. Categorical data are presented as frequencies and percentages (%). All continuous variables were checked for normality using a Kolmogorov-Smirnov test. Non-Gaussian variables were log-transformed prior to parametric analysis. Independent T-tests, analysis of variance

(ANOVA), and Mann-Whitney and Kruskal-Wallis H tests were used to compare mean differences in Gaussian and non-Gaussian variables. Odds-ratio and X^2 tests were done using binary logistics and multinomial logistic regression. A *P*-value < 0.05 was considered statistically significant.

RESULTS

General Characteristics and Components of MetS

Participant characteristics are presented in **Table 1**. The prevalence of MetS in our study was 12%. The MetS group in our study was significantly older and had a higher pre-pregnancy BMI than the controls ($p < 0.02$ and < 0.01, respectively). There was no significant difference in circulating 25(OH)D levels. Compared with the controls, TG, insulin, HbA_{1C}, fasting serum glucose, HOMA-IR, and diastolic blood pressure were significantly higher in the MetS group ($p < 0.01$ for all variables), while HDL cholesterol was found to be significantly lower (1.6 ± 0.4 vs. 1.3 ± 0.5 mg/dl, $p < 0.01$).

FokI VDR Gene Polymorphisms and MetS Risk

The disparity between the two groups in the presence of genotypes in *FokI* was discovered to be statistically significant even after adjustment for age (**Table 2**). The frequencies of genotypes *FF*, *Ff*, and *ff* in the control group were 62.7%, 31.4%, and 5.9%, respectively, while for women with MetS, those numbers were 50%, 36.4%, and 13.6%, respectively. The genotype *ff* is a risk factor for MetS (OR = 4.17; 95% CI, 1.42-12.2, $p = 0.009$). The *f* allele is also a genetic risk factor for MetS in this population, given that the prevalence of *F* and *f* alleles for the *FokI* VDR polymorphisms in the two groups was statistically significant (allele *F* vs. *f*; $p = 0.017$).

Clinical Characteristics of the FokI VDR Gene Polymorphisms in MetS and Control Groups

Table 3 shows the clinical variables that were observed in MetS participants and pregnant controls according to the *FokI* VDR genotype. Those without MetS with the *FF* genotype had a higher pre-pregnancy and current BMI than those with either the F*f* or *ff* genotypes (*p-values* 0.02, 0.03, respectively). There was no association between individual components of MetS and the VDR genotype in participants.

VDR FokI Polymorphism Versus Components of MetS

Table 4 shows the multinomial logistic regression between the different genotypes and alleles of *FokI* VDR polymorphisms and obesity, hypertriglyceridemia, low HDL, hypertension, dyslipidemia, hypercholesterolemia, and GDM. The odds ratio for the risk of having any component of MetS was not significant among varying *FokI* VDR genotypes. Participants who had homozygous *ff* genotype were found to have elevated blood pressure (OR = 1.85, 95% CI: 0.79, 4.34), low HDL (OR = 1.14, 95% CI: 0.70, 1.87), dyslipidemia (OR = 1.60, 95% CI: 0.63, 4.10), and GDM (OR = 1.08, 95% CI: 0.66, 1.76). Maternal obesity was present in 27% of participants. Moreover, 12%, 29%, 63%, 55%, 34%, and 47% of participants had elevated blood pressure, GDM, hypercholesterolemia, hypertriglyceridemia, low HDL, and dyslipidemia, respectively (**Figure 2**).

MetS and Its Components Versus Vitamin D Status

Differences in MetS and control groups in terms of anthropometric and biochemical characteristics were presented in **Supplementary Table 1**. Vitamin D deficiency was present in 70.7% of the participants. Those participants also had higher fasting insulin,

TABLE 1 | Demographic and biochemical characteristics of MetS vs. control groups.

Parameters	All	MetS	Control	*P*-value	*P*-value*
N	368	44 (12.0)	324 (88.0)		
Age (years)	29.1 ± 5.6	31.3 ± 6.9	28.7 ± 5.3	0.02	
Pre-pregnancy BMI (kg/m^2)	26.9 ± 5.9	33.3 ± 4.1	25.9 ± 5.5	<0.001	
Current BMI (kg/m^2)	28.2 ± 6.2	34.7 ± 4.5	27.5 ± 5.9	<0.001	0.96
Parity	2 (1–4)	2 (1–5)	2 (1–4)	0.21	0.54
Systolic Blood Pressure (mmHg)	113.3 ± 12.9	119.1 ± 15.7	112.3 ± 12.1	0.02	0.27
Diastolic Blood Pressure (mmHg)	66.9 ± 9.2	71.7 ± 11.4	66.1 ± 8.5	0.010	0.03
Hba1c (%)	4.8 ± 0.5	5.1 ± 0.7	4.8 ± 0.8	<0.001	0.005
Fasting Glucose (mmol/L)	4.6 ± 1.0	5.6 ± 1.5	4.5 ± 0.8	<0.001	<0.001
HOMA-IR	1.5 (0.9-2.5)	3.1 (1.7-5.2)	1.3 (0.9-2.3)	<0.001	<0.001
Insulin (uU/ml)	7.5 (4.5-13.1)	14.2 (7.9-22.8)	6.9 (4.1-11.9)	<0.001	0.003
HDL-Cholesterol (mmol/L)	1.5 ± 0.4	1.3 ± 0.5	1.6 ± 0.4	<0.001	<0.001
LDL-Cholesterol (mmol/L)	3.9 ± 1.3	3.9 ± 1.4	3.9 ± 1.3	0.70	0.27
Total Cholesterol (mmol/L)	6.2 ± 1.2	6.4 ± 1.3	6.2 ± 1.5	0.27	0.08
Triglycerides (mmol/L)	1.8 (1.4-2.3)	2.6 (2.1-3.4)	1.7 (1.3-2.2)	<0.001	<0.001
25(OH)D (nmol/L)	33.4 (21.3-53.7)	29.9 (17.9-44.6)	33.7 (21.7-54.4)	0.23	0.19
Vitamin D deficiency (<50nmol/L)	314 (85.3)	38 (86.4)	276 (85.2)	0.29	0.20

*Pre-pregnancy BMI, Pre-pregnancy body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; HbA1c, hemoglobin A1c or glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Data presented as mean ± SD and median (25th–75th) percentiles for Gaussian and non-Gaussian variables. *P-value adjusted for age and pre-pregnancy BMI; significant at 0.05 and 0.01.*

TABLE 2 | Genotype distribution of VDR *FokI* polymorphisms in MetS and control groups.

FokI Genotype	All	MetS	Control	β	OR (95% CI)	*P-Value	Adjusted OR	**P-Value
FF	225 (61.1)	22 (50.0)	203 (62.7)		1		1	
F*f*	118 (32.1)	16 (36.4)	102 (31.4)	0.37	1.45 (0.73-2.88)	0.29	1.42 (0.71-2.9)	0.31
ff	25 (6.8)	6 (13.6)	19 (5.9)	1.07	2.91 (1.05-8.1)	0.04	4.17 (1.42-12.2)	0.009
F*f*+*ff*	143 (38.9)	22 (50.0)	121 (37.3)	0.52	1.68 (0.89-3.16)	0.11	1.76 (0.92-3.36)	0.09
F	568 (77.2)	60 (68.2)	508 (78.4)		1	0.03	1	0.02
f	168 (22.8)	28 (31.8)	140 (21.6)	0.53	1.69 (1.04-2.75)		1.84 (1.05-1.14)	

*OR, odds ratio (95% CI); *P-value significant at < 0.05, **P-value adjusted for age and significant at < 0.05, 0.01.*

TABLE 3 | Clinical characteristics of *FokI* VDR gene polymorphisms in MetS and control groups.

Parameters	FF	F*f*	*ff*	*P*-value
		MetS		
N	22	16	6	
Age (years)	29.1 ± 5.4	28.5 ± 5.3	26.8 ± 3.6	0.19
Pre-pregnancy BMI (kg/m^2)	34.2 ± 4.8	33.3 ± 2.6	30.3 ± 4.3	0.13
Current BMI (kg/m^2)	35.3 ± 4.8	34.8 ± 3.1	32.6 ± 6.3	0.43
Parity	2.0 (1.0-5.0)	3.0 (1.0-7.0)	4.0 (2.0-6.0)	0.64
Systolic BP (mmHg)	111.9 ± 12.3	112.9 ± 11.4	112.3 ± 14.2	0.87
Diastolic BP (mmHg)	65.6 ± 8.3	67.2 ± 9.1	65.3 ± 7.9	0.47
Hba1c (%)	4.7 ± 0.4	4.8 ± 0.6	4.8 ± 0.4	0.64
Fasting Glucose (mmol/L)	4.5 ± 0.9	4.4 ± 0.7	4.5 ± 0.3	0.59
HOMA-IR	3.7 (1.9-5.3)	3.7 (1.7-5.9)	2.1 (1.1-2.7)	0.13
Insulin (uU/ml)	7.4 (4.1-12.3)	6.4 (4.2-10.9)	6.4 (3.6-17.9)	0.91
HDL-Cholesterol (mmol/L)	1.6 ± 0.4	1.5 ± 0.4	1.6 ± 0.4	0.95
LDL-Cholesterol (mmol/L)	3.8 ± 1.3	3.9 ± 1.2	3.7 ± 1.0	0.75
Total Cholesterol (mmol/L)	6.2 ± 0.6	6.2 ± 1.4	6.2 ± 1.2	0.99
Triglycerides (mmol/L)	2.7 (2.1-3.2)	2.5 (2.2-3.4)	3.3 (2.2-3.4)	0.76
25(OH)D (nmol/L)	29.9 (18–47)	37.3 (20–61)	22.2 (16–28)	0.23
		Control		
N	203	102	19	
Age (years)	32.4 ± 7.1	31.8 ± 6.6	26.3 ± 5.5	0.14
Pre-pregnancy BMI (kg/m^2)	26.6 ± 5.9	24.7 ± 4.3	25.9 ± 5.7	0.02
Current BMI (kg/m^2)	27.9 ± 6.3	25.9 ± 4.7	27.0 ± 5.9	0.03
Parity	2.0 (1.0-4.0)	2.0 (1.0-3.0)	1.0 (1.0-2.0)	0.44
Systolic BP (mmHg)	120.1 ± 15.3	119.3 ± 15.5	112.0 ± 22.5	0.72
Diastolic BP (mmHg)	70.8 ± 11.6	73.4 ± 10.3	69.7 ± 18.5	0.80
Hba1c (%)	5.1 ± 0.6	5.0 ± 0.8	5.2 ± 0.9	0.84
Fasting Glucose (mmol/L)	5.4 ± 1.5	6.2 ± 1.6	5.0 ± 0.9	0.16
HOMA-IR	1.4 (0.8-2.3)	1.2 (0.8-2.1)	1.3 (0.8-3.6)	0.62
Insulin (uU/ml)	16.2 (9.3-22.7)	15.5 (6.7-24.7)	7.9 (6.0-11.7)	0.08
HDL-Cholesterol (mmol/L)	1.3 ± 0.5	1.4 ± 0.5	1.2 ± 0.2	0.80
LDL-Cholesterol (mmol/L)	4.1 ± 1.6	3.7 ± 1.2	4.1 ± 0.9	0.74
Total Cholesterol (mmol/L)	6.4 ± 1.3	6.4 ± 1.4	6.6 ± 0.9	0.92
Triglycerides (mmol/L)	1.7 (1.3-2.2)	1.7 (1.2-2.2)	1.6 (1.4-2.3)	0.73
25(OH)D (nmol/L)	34.3 (23-54)	31.4 (19-52)	40.4 (23-85)	0.09

FF denotes normal homozygous, Ff denotes heterozygous and ff denotes homozygous genotypes. Data represent mean ± SD and median (25th and 75th) percentile for Gaussian and non-Gaussian variables. P-value significance at 0.05.

HOMA-IR, and systolic blood pressure (SBP) (p = 0.007, 0.02, 0.03, respectively). For participants who were not deficient in vitamin D, HDL-C and TC were significantly higher (p = 0.001 and 0.049, respectively). Furthermore, those with the *ff* genotype and MetS have a significant and inverse association between serum 25(OH)D and fasting serum glucose (r = -0.92) (**Figure 3A**). A more significant inverse association between serum 25(OH)D and diastolic blood pressure was found in controls, regardless of the genotype (r = -0.16; p<0.05) (**Figure 3B**). Those who carried the *FF* genotype demonstrated a strong association between 25(OH)D, HDL-cholesterol, LDL-cholesterol, and total cholesterol (r = 0.22, 0.19, 0.25, respectively) (**Figures 3C–E**).

DISCUSSION

The present study is the first evaluation of the links between *FokI* VDR gene polymorphisms and MetS among Saudi Arabian pregnant women. *FokI* VDR polymorphisms may exert a strong impact on MetS susceptibility. It appears that the *f* allele and the *ff* genotype could be risk factors for MetS, whereas the *F* allele and the *FF* genotype could play a protective role. These findings correspond with those of Aslani et al. on postpartum Iranian women with MetS and found that the frequency of the *ff* genotype was two-fold when compared to healthy participants in a postpartum follow-up exercise (OR = 2.1; 95% CI: 0.90–4.89;

TABLE 4 | Risk of the *FokI* VDR gene polymorphisms with MetS and its components.

Parameters	Yes	β	OR (95% CI)	P-Value
BMI ≥ 30 kg/m^2	101			
FF	66 (65.3)		1	
Ff	28 (27.4)	0.03	1.03 (0.40-2.63)	0.95
ff	7 (7.4)	-0.36	0.70 (0.41-1.18)	0.18
Ff+*ff*	35 (34.8)	-0.29	0.75 (0.46-1.22)	0.24
F	160 (28.3)		1	
F	42 (25.1)	-0.17	0.84 (0.56-1.27)	0.84
Blood Pressure > 130/85	44			
FF	19 (46.2)		1	
Ff	19 (46.2)	0.46	1.59 (0.32-7.89)	0.57
ff	6 (7.7)	0.62	1.85 (0.79-4.34)	0.16
Ff+*ff*	25 (53.9)	0.59	1.81 (0.79-4.11)	0.16
F	57 (10.3)		1	
F	31 (14.7)	0.41	1.51 (0.80-2.83)	0.20
HDL-C < 1.3 mmo/L	126			
FF	76 (60.0)		1	
Ff	42 (33.0)	0.07	1.07 (0.44-2.66)	0.88
ff	8 (7.0)	0.13	1.14 (0.70-1.87)	0.59
Ff+*ff*	50 (40.0)	0.12	1.13 (0.71-1.79)	0.60
F	194 (33.8)		1	
F	58 (35.8)	0.09	1.09 (0.75-1.59)	0.65
Triglycerides > 1.7 mmol/l	202			
FF	127 (62.8)		1	
Ff	60 (29.6)	0.13	1.14 (0.49-2.65)	0.76
ff	15 (7.5)	-0.24	0.79 (0.50-1.23)	0.30
Ff+*ff*	75 (37.1)	-0.18	0.84 (0.55-1.28)	0.42
F	314 (55.6)		1	
F	90 (53.6)	-0.08	0.92 (0.65-1.31)	0.66
Total Cholesterol > 5.7 mmol/l	233			
FF	137 (58.5)		1	
Ff	78 (33.6)	0.50	1.65 (0.66-4.12)	0.28
ff	18 (7.9)	0.24	1.27 (0.79-2.03)	0.32
Ff+*ff*	96 (41.5)	0.28	1.33 (0.85-2.07)	0.21
F	352 (62.1)		1	
F	114 (68.1)	0.26	1.31 (0.90-1.89)	0.16
Dyslipidemia	171			
FF	88 (51.5)		1	
Ff	69 (40.5)	-0.004	1.00 (0.57-1.75)	0.32
ff	14 (8.0)	0.47	1.60 (0.63-4.10)	0.99
Ff+*ff*	83 (48.5)	0.09	1.09 (0.65-1.84)	0.74
F	245 (71.6)		1	
F	97 (29.4)	0.10	1.11 (0.72-1.71)	0.65
GDM	108			
FF	65 (60.2)		1	
Ff	36 (33.3)	-0.04	0.96 (0.38-2.40)	0.93
ff	7 (6.5)	0.06	1.08 (0.66-1.76)	0.76
Ff+*ff*	43 (39.8)	0.06	1.06 (0.66-1.68)	0.81
F	166 (76.9)		1	
F	50 (23.1)	0.03	1.03 (0.70-1.50)	0.70

FF denotes normal homozygous, Ff denotes heterozygous and ff denotes homozygous genotypes. Data represent unstandardized β, odds ratio, and (95% CI). The P-value is significant at < 0.05.

$p = 0.09$) (21). The same population did not discover significant links between the *FokI* VDR polymorphism and MetS in adult males or females (30). This finding proves to be consistent with additional studies conducted in China (3), the United Arab Emirates (UAE) (18) and Brazil (2). The differences between the studies could be attributed to a range of environmental differences experienced by the populations (31). Likewise, the difference in findings in the Iranian studies demonstrates that there is a substantial difference between ethnic groups in terms of the frequency of the VDR *FokI* genotypes and alleles—even with identical population samples (18).

The association between *FokI* VDR polymorphisms and obesity has been uncovered in a previous study. Mackawy and Badawi (19) found that, in Egypt, the *ff* genotype had a link with higher waist circumference in diabetic patients with MetS when compared to the FF genotype in the same participants. Zaki et al. (16) discovered that obese Egyptian women with a vitamin D deficiency exhibited higher frequencies of the mutant allele *f* when compared to healthy obese women who registered a healthy level of vitamin D. Haris and Baig noted the potential of a link between the *ff* genotype and obesity in their preliminary study on obese Pakistanis (32). Zhao et al. (3) supported this

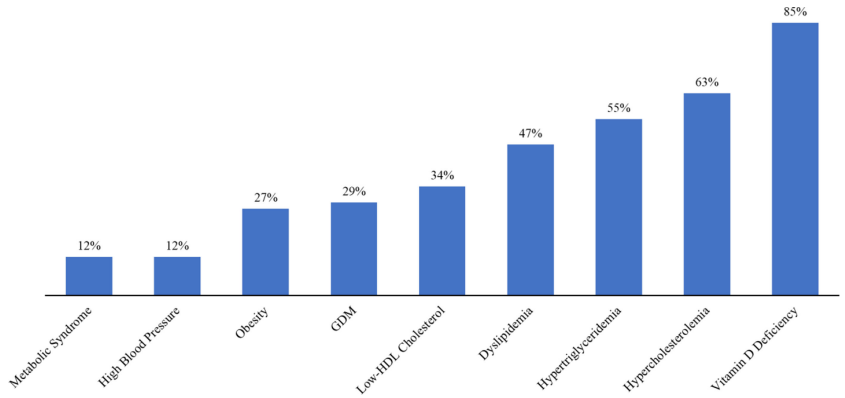

FIGURE 2 | Presence of metabolic disorders among pregnant Saudi women in their first trimester.

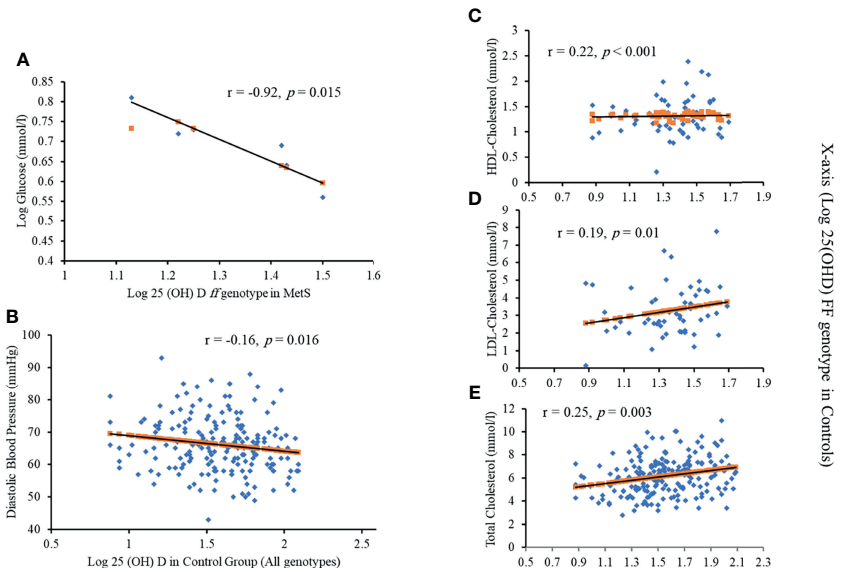

FIGURE 3 | Significant correlations between Log 25(OH)D and **(A)** Log glucose in *ff* genotype in MetS; **(B)** diastolic blood pressure in all genotypes; **(C)** HDL cholesterol in FF genotype in controls; **(D)** LDL cholesterol in FF genotype in controls and **(E)** Total cholesterol in FF genotype in controls

finding, discovering that the *FF* genotype bears a link with lower BMI in Chinese adults with MetS than other genotypes. A significant association between the *f* allele and pre-pregnancy obesity was observed in pregnant Iranian women by Aslani et al. (21). In this study, the control-group participants who carried the *FF* genotype exhibited a higher pre-pregnancy BMI than those who carried Ff and *ff* genotypes. While not deemed significant, this finding may indicate that the *ff* genotype could be protective against obesity.

The study also discovered a significant inverse correlation between serum 25(OH)D and diastolic blood pressure across all genotypes in non-MetS participants. Those who carried the mutant genotype *ff* also had a higher propensity for hypertension than homozygous and heterozygous genotypes. These results though not significant reflect Zaki et al.'s (16) study that Egyptian women with genotypes *Ff* and *ff* demonstrate higher blood pressure than those who had the more common homozygous genotype *FF*. Two additional studies uncovered a strong link between the *FF* genotype and a risk of high blood pressure. The first studied Emirati women and linked the *FF* genotype to higher systolic blood pressure (18), and the second study uncovered a link between the *FF* genotype and hypertension among Indian adults (15). Both studies attributed elevated blood pressure or hypertension to enhanced production of renin and angiotensin II due to the effect of *FF* homozygotes. Cottone et al. (33) explored hypertensive Italian participants and did not detect a significant link between the *FokI* VDR polymorphism and blood pressure or between blood pressure

and serum 25(OH)D levels. Future research may demonstrate a strong link between *FokI* VDR polymorphisms and maternal hypertension. Until then, it is important to understand that when compared to healthy pregnant women, those with hypertension have an increased risk of premature birth and other complications, such as a defect in endothelial-dependent vascular function (4).

Many studies have uncovered a link between the VDR gene polymorphism and dyslipidemia, supporting this relationship (18, 19, 33, 34). Schuch et al. (2) found, for example, that participants who did not suffer from MetS and carried the mutant homozygous genotype (*ff*) exhibited significantly higher TG levels and lower HDL levels when compared to study participants who possessed the heterozygous (F*f*) or normal homozygous (*FF*) genotypes. Reciprocal outcomes have been reached by a host of other researchers. Mackawy and Badawi (19) observed, for example, that when compared to carriers of the *FF* genotype, diabetic, non-MetS carriers of the *ff* genotype exhibited higher plasma TC, TG, and LDL-C with lower HDL-C levels. Hasan et al. (18) studied Emirati carriers of the *ff* genotype and found that when compared to other genotypes of the *FokI* VDR polymorphism, they exhibited higher serum TC levels. These results are consistent with those of this study, which found that when compared to participants carrying the normal homozygous and heterozygous genotypes, those with the mutant, homozygous genotype exhibited a higher risk of developing dyslipidemia. These results are not, however, statistically significant. Moreover, all participants with MetS—regardless of genotype—exhibited a significant association between HDL and serum 25(OH)D levels. In all non-MetS participants carrying *FF* genotypes, serum 25(OH)D levels were found to be significantly associated with HDL, LDL, and TC. Surprisingly, it was discovered that compared to participants with vitamin D deficiency, non-deficient women exhibited a higher lipid profile, similar to the study of Al-Ajlan et al. (10), which discovered that serum vitamin D levels were significantly and positively correlated with serum TG and TC levels in a subgroup of pregnant Saudi women with a vitamin D deficiency. Researchers have speculated that the cause for this outcome could be a link between the high metabolic demands of pregnancy and vitamin D deficiency (10). An increase in lipid levels is a normal physiological consequence of pregnancy, but it is important to understand that by disrupting normal placentation and damaging the vessel wall *via* increased oxidative stress, maternal hyperlipidemia can lead to a host of serious health issues in the fetus (6). Previous research has discovered a strong link between preterm birth and hyperlipidemia, a leading cause of prenatal morbidity and mortality (35, 36).

Many researchers were inspired to explore the link between VDR gene polymorphisms and glucose homeostasis due to the presence of VDR in human tissue and pancreatic β-cells (18, 21). In Brazil, for example, Schuch et al. (2) recorded a significantly higher level of β-cell secretion (HOMA-β) in individuals carrying the *f* allele when compared to participants carrying the F allele. In Brazilian and Egyptian populations, respectively, Schuch et al. (2) and Zaki et al. (18) discovered an important link between the *ff* genotype and a higher HOMA-IR when compared to the *FF* and *Ff* genotypes of the *FokI* VDR polymorphism. Mackawy et al. (21) also discovered that Egyptian patients who carried the *ff* genotype, were diabetic, and suffered from MetS exhibited higher insulin levels and HOMA-IR than those with the *FF* and *Ff* genotypes. These results support our findings of a statistically significant and inverse correlation between serum 25 (OH)D levels and fasting serum glucose in pregnant women carrying the *ff* genotype in the MetS group.

Very few studies have explored the effect of the *FokI* VDR polymorphism on GDM. Contradictory results were achieved in a pair of studies exploring Iranian (21) and Saudi populations (24), which explored the relationship between the *FokI* VDR polymorphism and GDM risk. In Iran, Aslani et al. (21) discovered that the frequency of the *ff* genotype in GDM patients was higher than in normal pregnant women (10.6% vs. 6.2%). Healthy participants were also discovered to exhibit a higher frequency of the *F* allele, leading to a suggestion that the *F* allele could play a role as a protective factor against GDM (30). El-Beshbishy et al. (24) conducted their research in Saudi Arabia and found that when compared to their GDM group, there was a higher frequency of the F allele (56.4% vs. 35.7%) and a lower frequency of the *f* allele (43.6% vs. 64.3%) in their control group. However, these results were not statistically significant ($P = 0.100$). The authors of this research concluded that no significant association existed between the *FokI* VDR polymorphism and GDM in the Saudi population (24). The results from this study support this assertion, given that we did not uncover a statistically significant link between GDM and the *FokI* VDR polymorphism. In logistic regression analysis, participants with the *ff* genotype are at higher risk of developing GDM compared to carriers of other genotypes of the *FokI* VDR polymorphism (odds ratio = 1.27; 95% CI: 0.76–2.14; $P = 0.360$, after adjustments for age and BMI). It is widely accepted that while both conditions cause defects in insulin secretory response, *GDM* mimics T2DM in its pathology. We posit that a larger cohort study that had the necessary statistical power would affirm the potential of VDR genetic variation in predicting GDM.

This study had its limitations. A limited frequency of the mutant *ff* genotype meant that we were unable to record a link between *FokI* and components of MetS. For prospective studies, we believe it is important that sufficient statistical power is present, particularly when it comes to the study of the effect of the mutant *ff FokI* genotype on MetS and its components. The study also analyzed the VDR gene at only one SNP, though this SNP was one that translates structurally diverse VDR proteins, which vary in their potential to elicit vitamin D-mediated gene expression. Unfortunately, the study did not accommodate a range of factors that may impact the risk of MetS, such as dietary habits, physical activity, or other lifestyle factors. Despite these limitations, this study is the first to explore the link between the *FokI* VDR polymorphism and MetS and its components in pregnant women from Saudi Arabia. To our knowledge, no previous study has explored these associations in pregnancy among any population. The accuracy of the vitamin D measurements can be assured, given that we explored the role

of vitamin D status and correlated it with different variables related to the components of MetS in all *FokI* genotypes. In this study, unlike previous epidemiological studies, precise measurement was used for each MetS component. Blood pressure and the fasting serum samples—including the lipid, glucose, and insulin concentrations—were precisely measured and not self-reported.

CONCLUSION

Our study uncovered a statistically significant association between the *FokI* VDR polymorphism and an increased risk of MetS, particularly in participants who carried the *ff* genotype. This link could be used as a prognostic tool to predict maternal MetS risk in Saudi women. This study proposes that the *FokI* VDR polymorphism could have an impact upon key components of MetS, including obesity, dyslipidemia, and GDM. While the link is not statistically significant, our findings should be investigated further in larger cohorts, and additional associative studies should be conducted on other VDR gene polymorphisms.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the College of Science, King Saud University, Riyadh, Saudi Arabia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MA, NA-D, and RW contributed in study conception and design. SA, MF, AA-A, and DB dealt in recruitment of participants and procurement of samples. SS and AM analyzed samples and data and MA wrote the manuscript. SS reviewed the manuscript. MA and NA-D supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Kynurenic Acid Acts as a Signaling Molecule Regulating Energy Expenditure and Is Closely Associated With Metabolic Diseases

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Kynurenic acid (KYNA) is an important bio-active product of tryptophan metabolism. In addition to its well-known neuroprotective effects on mental health disorders, it has been proposed as a bio-marker for such metabolic diseases as atherosclerosis and diabetes. Emerging evidence suggests that KYNA acts as a signaling molecule controlling the networks involved in the balance of energy store and expenditure through GPR35 and AMPK signaling pathway. KYNA plays an important role in the pathogenesis and development of several endocrine and metabolic diseases. Exercise training promotes KYNA production in skeletal muscles and increases thermogenesis in the long term and limits weight gain, insulin resistance and inflammation. Additionally, KYNA is also present in breast milk and may act as an anti-obesity agent in infants. Although we are far from fully understanding the role of KYNA in our body, administration of KYNA, enzyme inhibitors or metabolites may serve as a potential therapeutic strategy for treating metabolic diseases. The present review provides a perspective on the current knowledge regarding the biological effects of KYNA in metabolic diseases and perinatal nutrition.

Keywords: kynurenic acid, inflammation, physical exercise, perinatal nutrition, metabolic diseases

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INTRODUCTION

Kynurenic acid (KYNA) is one of the metabolites of tryptophan catabolism formed *via* the kynurenine pathway. It is first known for its neuro-protective effect as it is the only known broad-spectrum endogenous antagonist for ionotropic glutamate receptors. As such, a large number of studies have been carried out to investigate the role of KYNA in the physio-pathology of central nervous system (CNS) such as depression, Alzheimer's diseases and schizophrenia in the past two decades. In addition to such neuronal contributions, KYNA can also be found with higher concentration in urine, pancreatic mucus, serum and breast milk than in cerebrospinal fluid (1). It has been subsequently found to be involved in immune (2) and digestive system (1) in the periphery. Increasing reports have concentrated on the role of KYNA outside the CNS.

More recently, evidence suggests that physical exercise may also influence this pathway and KYNA has emerged as a signaling molecule for energy homeostasis in peripheral tissues. Some metabolomic and

epidemiological studies have proposed that KYNA can serve as an early bio-marker for diabetes and some other metabolic diseases (3, 4). Furthermore, in some animal studies, KYNA has been considered as a significant protector against such metabolic diseases as obesity and nonalcoholic fatty liver disease (NAFLD) (5, 6). This essay summarizes recent advances of how KYNA is involved in the regulation of energy homeostasis of peripheral tissue and its potential role in the onset and progression of such metabolic diseases as obesity and diabetes.

THE ENDOGENOUS PRODUCTION OF KYNA IN PERIPHERAL TISSUES

The endogenous production of KYNA in human brain has been well studied over the past few decades because of its neuroprotective effects. However, increasing evidence showed that KYNA can also be found, even in higher concentration, in many body fluids in humans such serum (7), saliva (8), bile (9) and breast milk (10). In humans and rodents, the production of KYNA has been described in a number of peripheral tissues such as muscle (11), liver (12), kidney (12), pancreas (9, 13), endothelial cells (14) and immune cells (15) under physiological conditions. KYNA is produced mainly through the side branch of tryptophan/kynurenine pathway. Approximately 95% of tryptophan is metabolized through kynurenine pathway, and about 0.3% is converted to KYNA (10 μmol/day) (16).

In this catabolic pathway, the first step is catalyzed by the enzymes indol-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) to generate N-formyl-L-kynurenine, an unstable compound which is rapidly converted to L-kynurenine (L-KYN) by ubiquitous aryl formamidase (AFMID). TDO is mainly expressed in the liver and has a K_m value of 190 μM for TRP, which ensures that TDO is active to convert TRP to L-KYN at higher than physiological concentrations (about 80 μM) (17, 18). Another limiting enzyme, IDO1, is expressed under the induction of proinflammatory cytokines (19). IDO1 has a K_m value of 20 μM for tryptophan, which is much lower than TDO (17). IDO2, a newly discovered enzyme, has some homology to IDO1, but its K_m value for TRP is much higher than that of IDO1 and TDO, and has little effect on the production of TRP downstream metabolites (20). After this step, L-KYN can be either converted to nicotinamide by a series of reactions or enter into a side branch to produce KYNA. KYNA is supposed to be a final catabolic product of this side branch of kynurenine pathway because no further metabolite is reported in mammals (but can be further catabolized by intestinal flora).

The KYNA branch of the kynurenine pathway is mainly regulated by the activity of kynurenine aminotransferase (KYAT, EC 2.6.1.7), whose abbreviation is recently updated from KAT to KYAT. Four different isozymes of KYAT (KYAT1-4) are identified in mammalian cells. They are all members of the pyridoxal-5'-phosphate-dependent enzyme family and require an α-ketoacid as the amino group acceptors.

KYAT1 catalyzes the transamination of L-KYN to form KYNA and its K_m for L-KYN is around 4.7 mM (21). It should be noted that KYAT1 possesses broad amino acid specificity and also catalyzes the transamination such as glutamine to α-ketoglutaramate, thus it is also known as glutamine transaminase K (GTK, EC 2.6.1.15). Leucine, glutamate, methionine also seem to be the preferred substrates of human KYAT1 (21). But, to date, its most biologically significant product is KYNA. KYAT1 also possesses cysteine S-conjugate beta-lyases (CCBL1) activity and catalyzes the beta-elimination of cysteine S-conjugates to generate pyruvate and thioacylating fragments. This activity plays an important role in the bio-activation of cysteine S-conjugates found in garlic and onion (22) and in the toxification of some toxins like 5-S-l-cysteinyldopamine. Competitive inhibition test suggests KYAT1 has two active sites, one for KYAT and the other for GTK. The active sites of KYAT and CCBL1 may be the same (23). KYAT1 is both cytosolic and mitochondrial because it exists two different mRNA variants coding for proteins with and without mitochondria targeting sequence. In rats, KYAT1 mRNA can be detected in most of tissues such as small intestine, pancreas, lung, liver, heart, kidney, brain, muscle, testis, ovary (23, 24). In brain, the activity of KYAT1 is critical to the formation of KYNA, its activity is associated with schizophrenia (25). A missense mutation in KYAT1 was identified in spontaneously hypertensive rats. This mutation led to abnormally low KYNA levels in the area of central nervous system that controls blood pressure (26). Although the role of KYAT1 in brain has mostly been discussed, its expression is much higher in livers and kidneys than in brains (27). Its role in peripheral tissues did not receive much attention until the discovery of the immunomodulator and metabolic effect of KYNA.

KYAT2, the second isoform of the KYAT family, also possesses a broad-spectrum transamination activity with no S-conjugate beta-lyases activity. It is also known as alpha-aminoadipate aminotransferase (AADAT). Similar to KYAT1, its K_m for L-KYN is around 4.7 mM (28). KYAT2 can be detected in most tissues, but it is not detectable in murine skeletal muscle (11). KYAT2 appears to play a more important role in rat's brain because KYAT2 is highly expressed in astrocytes than other KYATs and KYNA is reported to be predominantly generated by it in the brain (29, 30).

Among the four KYATs, KYAT3 shares similar sequence and expression pattern to KYAT1 and is also known as glutamine transaminase L (GTL) and CCBL2 (EC 4.4.1.13). Likewise, KYAT3 is a multifunctional aminotransferase and catalyzes glutamine, methionine, phenylalanine, tyrosine and cysteine as transamination subtract, although it displayed no activity toward leucine (31). Its expression is much higher in kidney, liver and neuroendocrine tissues than in brain (27, 32).

KYAT4 is the last discovered KYAT. In fact, it is better known as mitochondrial glutamic-oxaloacetictransaminase 2 (EC 2.6.1.1), an essential player in the malate-aspartate shuttle in mitochondria and in the synthesis of glutamate (33). It is highly expressed in most tissues and organs because malate-aspartate shuttle is a general feature of cells with functional mitochondria, except for white adipose tissue (34).

Kynurenine 3-monooxygenase (KMO; EC 1.14.13.9) is an important regulator of KYNA synthesis as it is a kynurenine-consuming enzyme competing with KYAT for substrate. KMO is an NADPH-dependent flavin monooxygenase located in the outer membrane of mitochondria. It catalyzes the conversion of

kynurenine to 3-hydroxykynurenine, a cytotoxic metabolite involved in the generation of ROS and activation of inflammatory response. KMO is widely expressed in our body, for it has been discovered in liver, kidney, pancreas, brain, macrophages, and monocytes (3, 13, 35, 36), with the highest KMO levels found in liver and kidney. Moreover, the activity of KMO in liver and kidney decreases significantly with aging (37).

KMO possesses higher affinity to kynurenine (7-14 μM for human KMO (38, 39), 15-16 μM for rodent (40) than all four types of KYATs. KMO inhibition by pharmacological inhibitor significantly increases KYNA levels (41). Also, in KMO knockout mice, the level of KYN and KYNA was significantly increased in the periphery (42). The characteristics of enzymes related to KYNA metabolism are summarized in **Table 1**.

Under some physiological or pathological conditions, KYNA can be produced from indole-3-Pyruvic Acid or from kynurenine by scavenging free radicals. These pathways represent alternative routes of KYNA production. Although the contributions of these alternative routes remain unclear, these could be very important in such metabolic diseases as obesity and diabetes, because these diseases share common factors such as oxidative stress and inflammation. This information has been reviewed by Ramos-Chávez et al. (47).

THE TRANSPORT OF KYNA

KYNA is able to cross the plasma membrane through organic anion transporters 1 and 3 (OAT1/SLC22A6 and OAT3/SLC22A8) (48). The proximal tubule of the kidney, where OATs are found, is not simply for renal elimination of KYNA and it also senses tryptophan metabolites levels and responds to changes in their intracellular abundance (49). It remains to be further investigated whether there exists an exocytosis gated KYNA secretion.

While KYNA does not easily cross the blood-brain barrier, KYNA synthesized by brain cannot be directly exported to the periphery and *vice versa* (50). However, since its precursor L-KYN can cross the blood-brain barrier (51), KYNA can be synthesized by KYAT *in situ* using transported L-KYN in central nervous system under certain conditions (51). Moreover, a recent study showed that in *Caenorhabditis elegans*, an ortholog of the human LAT1 transporter, AAT-1, imports L-KYN into sites of KYNA production (52). Another study showed that five amino acids, including leucine, isoleucine, methionine, phenylalanine and tyrosine, act as LAT substrates and inhibit brain KYNA synthesis by blocking L-KYN transport (53). Similar to this transport mechanism in brain, the uptake of KYNA in T cells can be mediated by the uptake of L-KYN *via* L-amino acid transporter SLC7A5 (54).

TABLE 1 | The characteristics of enzyme related to KYNA metabolism.

Enzyme	K_m	Substrate	References
TPH1	8 μM	Trp	(43)
TPH2	41.3 μM	Trp	(44)
IDO1	20 μM	Trp	(45)
TDO2	190 μM	Trp	(17)
KMO	7-16 μM	KYN	(38–40)
Kase	493 μM	KYN	(46)
KYAT1	4700 μM	KYN	(21)
KYAT2	4700 μM	KYN	(28)
KYAT3	1500 μM	KYN	(31)

LINKS BETWEEN KYNA AND COMMON METABOLIC DISEASES

Inflammation, the First Link Between KYNA and Metabolic Diseases

The production of KYNA is directly correlated to inflammation as KYNA acts as an important immune regulated during inflammation response. KYNA inhibits TNF-α at transcriptional level and suppresses the secretion of TNF-α in mononuclear cells and in $CD14^+$ monocytes (55). Oral administration of KYNA decreases the activity of the peripheral blood leukocytes in mice (56).

The immune response signaling pathway and metabolic regulation signaling pathway, especially insulin signaling pathway, are highly integrated, because organism would need to redistribute its energy resources during the activation of immune response (57). Chronic inflammation is activated in overweight individuals as a consequence of adipose expansion. Recent insights suggest that it may play an indispensable role in the over-nutrition induced insulin resistance (58). During the past two decades, it became clear that nutrient excess and activation of the innate immune system are highly associated in most organs such as adipose tissues, liver, gut, muscle, and islets (58). Low-grade chronic inflammation or metabolically triggered inflammation is considered as a fundamental characteristic of metabolic diseases particularly in the context of obesity and type 2 diabetes. Targeting inflammation has been suggested as an important strategy to prevent and control these diseases (58).

Numbers of studies suggest that KYNA is produced during inflammation and it has been shown to mediate various immunomodulatory effects under inflammatory conditions (2). Since inflammation is one of the main factors in many metabolic diseases, it can be foreseen that KYNA may also play an important regulatory role in the metabolic diseases.

Increased tryptophan/kynurenine metabolite levels are frequently observed in overweight individuals (3). An increased serum KYNA level can be found in Zucker fatty rats (59), and in HFD fed LDL receptor knockout mice (60). Clinically, serum KYNA has been found positively correlated with BMI in overweight individuals (3, 61). The elevation of serum KYNA concentrations is closely associated with the activation of immune cells as increased IDO1 activity in macrophages and increased serum KYNA levels have been reported in obese animal models. The increased KYNA levels may result from an up-regulated biosynthesis in the omental adipose tissue (but not in subcutaneous adipose tissue), as the expression levels of IDO1, KYAT1 and KYAT3 were significantly higher in overweight individuals than in lean individuals (3). Furthermore, the activation of KYNA production was not restrained in resident immune cells of adipose tissue as the increased expression of IDO1, KYAT2 and KYAT3 can also be found in adipocytes.

This up-regulation may be due to the increased production of pro-inflammatory cytokines from resident immune cells since adipocyte does not express KMO (3). However, in another study, Pyun et al. found a negative correlation between serum KYNA levels and BMI (6). These controversial observations may be due to the different criteria. Another possible explanation to the controversy is that the KYNA determination method used in these studies are different: the last one used an ELISA kit to determinate serum KYNA levels, while the others used the HPLC-MS/MS method. It needs to be further verified whether these divergent results are due to some immeasurable confounding factors.

Liver seems to be another important source of serum KYNA in overweight individuals as TDO and KYATs are highly expressed in this organ (17, 18). Moreover, overweight is frequently associated with a low-grade chronic inflammation with an induction of IDO1 in liver (62). Furthermore, both of the liver and adipose tissue have a closed crosstalk between resident immune cells (Kupffer cells or macrophages) and metabolic cells (adipocytes or hepatocytes). However, to the best of our knowledge, neither the production of KYNA nor the regulation of IDO, TDO and KYATs in hepatocytes under metabolic challenge have been reported.

Physical Exercise, the Second Link Between KYNA and Metabolic Diseases

Physical exercise has been described as a promising non-pharmacological treatment for overweight and some other metabolic diseases (63). In addition to its role in energy expenditure regulation, skeletal muscle is increasingly considered as one of the largest endocrine organs in our body. It secretes a variety of myokines and bioactive metabolites, which exerts important effects on the regulation of metabolism and inflammation. KYNA can also be synthesized by skeletal muscle and its production is closely correlated to the physical exercise in both human and mouse (11, 64).

All the four KYATs are expressed in skeletal muscle, but KYAT isoforms display fiber-type specific expression. KYAT1, KYAT3 and KYAT4 are more abundant in oxidative type I than glycolytic type II fiber (65). Accordingly, an increased serum KYNA level has been found in individuals after endurance exercise (64). Within the first hour after aerobic exercise, there is an increase in plasmatic KYNA and this effect lasts for 2 hours after exercise. In contrast, high-intensity eccentric exercise did not lead to increased plasmatic KYNA concentration (64). Regarding the effect of long-term exercise on KYNA, a recent study of 4-week physical exercises on human found that physical exercises promote an increase in the amount of KYNA in sweat on day 14. The KYNA level returned to baseline on day 28 (66). Additionally, inhibition of KYATs reduces myotube oxidative capacity and exercise performance in mice (67).

Physical exercise induces peroxisome-proliferator activated receptor γ coactivator 1α (PGC-1α) expression in skeletal muscle (11). PGC1-1α coordinates the expression of several genes involved in the adaptive energy metabolism and fatigue-resistance such as mitochondrial biogenesis and fatty acid oxidation. Recently, the canonical and longest transcript variant of PGC-1α, PGC-1α1, is reported to up-regulate KYAT2 and KYAT4 expression (11). Such mechanism in skeletal muscle during physical exercise may be primarily aimed at enhancing the malate-aspartate shuttle as both KYATs are important enzymes in the malate-aspartate shuttle (67). Consequently, this mechanism shifts the kynurenine metabolism to KYNA production.

This exercise-induced KYNA production is originally described as the crosstalk between skeletal muscle and the brain to elucidate the effectiveness of exercise in reducing depressive symptoms. A recent study by Agudelo et al. demonstrated that KYNA increases energy utilization by activating G-protein-coupled receptor 35 (GPR35), which stimulates lipid metabolism, thermogenic, and anti-inflammatory gene expression in adipose tissue (68). Also, GPR35 agonists was reported to suppress high fat diet-induced fatty liver development (5). These data uncovered that skeletal muscle derived from KYNA may be a potential regulator of energy homeostasis and a coordinator of exercise-induced adaptations in other organs including liver, adipose tissue and brain.

However, it should be noted that physical exercise induces strong and transit increases in KYNA levels while inflammation leads to mild and sustained increases in KYNA levels.

Perinatal Nutrition, The Third Link Between KYNA and Metabolic Diseases

Epidemiological and experimental data have suggested that perinatal nutrition has a significant role in the development of lifelong metabolic disorders (69). KYNA may also act as a link between perinatal offspring and mother. KYNA can pass through the placenta into the fetus (70). However, placental and fetal KYNA were not affected by placental infusion of L-KYN in mice (70). Also, under physiological conditions, KYNA was higher in the liver and brain of mouse's fetuses than in the placenta, and KYNA in the fetus was not affected during oral maternal administration of KYNA. It can be hypothesized that maternal KYNA cannot affect fetus through placenta (70, 71).

Although maternal KYNA cannot directly affect the fetus, some studies found the KYNA content in breast milk gradually increases in different lactation periods (10). Epidemiological studies showed a slower body weight gain in naturally fed newborns compared to artificially fed ones (72). Although the formula milk powder for infants in different periods are different, studies have found that KYNA content in formula milk powder is much lower than that in breast milk (10). Rats postnatally exposed to KYNA supplementation were observed to have a significant reduction of body weight gain, but no changes in total body surface and bone mineral density. The rat offspring supplemented with KYNA presents a lower mass gain during the first 21 days of life, which indicates that KYNA may act as an anti-obese agent (10).

Another potential mechanism is that perinatal KYNA may be protective against overweight by modulating the gut microbiota. Formula feeding appears to promote the microbiota associated with overweight (73), while KYNA stimulates the growth of certain probiotics (74). It still needs to be further explored whether the presence of KYNA in breast milk acts as a modulator of gut microbiota.

POTENTIAL MECHANISM OF KYNA INVOLVED IN COMMON METABOLIC DISEASES

Glutamate Receptors

KYNA is well-known for its role as an endogenous N-methyl-D-aspartate receptor (NMDAR) antagonist in the brain. In the periphery, the expression of functional NMDA receptor is reported in the pancreatic β-cell. Activation of NMDA receptor reduces the glucose-stimulated insulin secretion. Likewise, NMDA receptor knockout in mouse islets increases glucose-stimulated insulin secretion. NMDA activation in β-cells also promotes cell death under stress. In microphages, activation of NMDA receptor induces ABCA1 degradation which promotes cholesterol accumulation and foam cell formation (75). In liver, NMDA receptor is present on the surface of Kupffer cells, and its activation has been reported to limit inflammasome activation (76).

KYNA acts as a low affinity competitive antagonist of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors. It directly acts on the glutamate binding domain. Meanwhile, low concentrations (0.03–30μM) of KYNA potentiate AMPA receptor responses (77). Therefore, KYNA has a dual action on AMPA receptor responses.

KYNA also directly interacts with the glutamate-binding domain of kainate receptors. Its antagonistic effect on this type of receptor is the least potent (IC50 500μM) among the 3 types of glutamate receptors (78).

However, serum KYNA level hardly reaches to micromolar levels. It is unclear whether KYNA in serum or in the periphery is sufficient to antagonize these glutamate receptors.

Other High Affinity Receptors

The G-protein-coupled receptor 35 (GPR35) is an orphan receptor that was identified in 1998 (79). It was originally described as a receptor for zaprinast, a phosphodiesterase (PDE) inhibitor. Recently, the KYNA was identified as an endogenous ligand for GPR35 with an EC_{50} of 39μM in human and 7.9μM in rat (80). Although the plasmatic concentration of KYNA is often in the nanomolar range in humans, it can become micromolar under inflammatory conditions.

GPR35 is associated with Gi/G0 and G13 proteins (81). Thus, activation of GPR35 reduces the activity of adenylate cyclase (Gi/G0) and/or increases that of the RhoA pathway (G13). GPR35 is expressed in central nervous system and in many peripheral tissues. In humans, significant expression of GPR35 has been detected in the colon, pancreas, small intestine, spleen and immune cells (monocytes, neutrophils, T cells and dendritic cells). The level of its expression is lower in the stomach, skeletal muscle, adipose tissue, pancreatic islets, kidney, liver, and thymus (82). Activation of GPR35 by KYNA has anti-inflammatory effect (83) by inducing autophagy-dependent degradation of NLRP3 in macrophage (84). It also plays anti-nociceptive (85) and anti-asthmatic (86) roles. Furthermore, KYNA enhances Pgc-1α1 and UCP1 expression GPR35 signaling in adipocytes, which suggests KYNA is a signaling molecule which directly controls energy homeostasis (68).

More recently, KYNA has been discovered to significantly increase AMP-activated protein kinase (AMPK) phosphorylation and to ameliorate palmitate-induced inflammation and insulin resistance. It potentially alleviates inflammation and insulin resistance in skeletal muscle and adipose tissues through GPR35/AMPK and SIRT6-mediated pathways (87). It may also ameliorate hepatic steatosis *via* the AMPK/autophagy- and AMPK/ORP150-mediated suppression of endoplasmic reticulum stress (6).

KYNA is also identified as an endogenous ligand for Aryl hydrocarbon receptor (AhR). AhR was originally described as a xenobiotic receptor, also known as the dioxin receptor. It is activated by exogenous ligands, such as flavonoids, natural plant polyphenols, indoles and dioxins. AhR plays multiple roles in xenobiotic metabolism, the regulation of inflammation, development, and the homeostasis of several organs (88). DiNatale and colleagues (89) showed that KYNA is a potent endogenous agonist of AHR with an EC_{25} around 100nM. Activation of AHR by KYNA may lead to IL6 expression in tumor cells.

Moreover, studies have shown that KYNA can also act at nicotinic receptors as a potent noncompetitive antagonist, particularly at the α7 subunit of the nicotinic receptor (90). KYNA inhibits CHRNA7 in a non-competitive manner at physiological concentrations. CHRNA7 was found to be expressed in glutamatergic axon terminals. Activation of CHRNA7 enhances glutamate release. Thus, KYNA may also be involved in the repression of glutamate release at the presynaptic level. This represents another mechanism by which KYNA exerts its anti-glutamatergic effect (90). The characteristics of KYNA receptors are summarized in **Table 2**.

Scavenger of Free Radicals

In addition to its receptor-dependent effects, KYNA at high concentrations (100-300 μM) also acts as a potent endogenous antioxidant, as it is a scavenger of free radicals such as hydroxyl radicals (OH■), superoxide anion (O_2^-) and peroxynitrite ($ONOO^-$) (92). Since oxidative stress is also critical for the pathogenesis of metabolic diseases (93), the antioxidative properties of KYNA represent an important mechanism in preventing the onset of metabolic diseases.

Interestingly, another study showed that KYNA is not a guaranteed protector against oxidative stress. It exhibits a strong pro-oxidative effect combined with δ-aminolaevulinic acid (ALA), an endogenous precursor of heme and source of hydroxyl radical, and elevates deoxyribose deterioration by 9 times compared to ALA alone (94).

Mitochondrial Homeostasis

The mitochondrial localization of KYAT suggests a direct release of KYNA into the mitochondria. KYNA plays a key role in the

TABLE 2 | The characteristic of KYNA receptor.

Receptor	KYNA	Affinity	References
NMDA	antagonist	IC_{50} 7.9-20μM	(70, 91)
Kainate	antagonist	IC_{50} 500μM	(78)
CHRNA7	antagonist	IC_{50} 7μM	(90)
GPR35	agonist	EC_{50} 8-40μM	(80)
AHR	agonist	EC_{25} 100nM	(89)

TABLE 3 | The roles of KYNA in metabolic diseases.

Organ/cell	KYNA production	Effects	Associated metabolic disease
Liver	High expression levels of TDO/IDO/KYATs were detected in liver (12)	Activation of GPR35 inhibits the development of NAFLD (5)	NAFLD
		Inhibition of Kuffer cells NMDA receptor by KYNA limits inflammasome activation (76)	Metabolic inflammation
		Activation of AMPK/autophagy- and AMPK/ORP150 pathway by KYNA ameliorate endoplasmic reticulum stress and hepatic steatosis (6)	Hepatic steatosis
Adipose tissue/ adipocyte	Expression of IDO1/KYAT1/KYAT3 were detected in adipocytes (3)	Activation of GPR35 by KYNA promotes the expression of PGC1-α and UCP1 (67)	Insulin resistance
		Activation of GPR35/AMPK and SIRT6 pathways by KYNA reduces inflammation and insulin resistance in adipocytes (87)	Metabolic inflammation
Muscle	Endurance essences enhance KYATs expression and promote KYNA production (64, 65, 67)	Activation of GPR35/AMPK and SIRT6 pathways by KYNA reduces inflammation and insulin resistance in skeletal muscle (87)	Insulin resistance
			Metabolic inflammation
Immune cell	KYAT1/KYAT2 expressions were detected in in both unstimulated and stimulated macrophage (107)	Antagonize NMDA receptor by KYNA inhibits ABCA1 degradation (75)	Cholesterol accumulation
		Activation of GPR35 by KYNA induces autophagy-dependent degradation of NLRP3 in macrophage (84)	Metabolic inflammation
Pancreas	KYNA were detected in pancreas fluid, expression of TDO/KYATs were detected in pancreatic islets (9, 13)	High concentration of KYNA enhances glucose stimulated insulin secretion (13)	Type 2 diabetes
Mammary gland	KYNA content in breast milk gradually increases in different lactation periods (10)	KYNA may act as an anti-obese agent for children (10)	Obesity

redox balance in the mitochondria. The expression and function of KYATs has been shown to be diminished in rat model with mitochondrial dysfunction (95, 96).

Firstly, the formation of KYNA diverts the pathway from *de novo* synthesis of NAD+/NADH, which regulates the mitochondrial TCA cycle, oxidative state and mitochondrial dynamics, suggesting the involvement of KYNA in the mitochondria energy metabolism regulation. MPTP and 3-nitropropionic acid (3-NA) have inhibitory effects on mitochondrial respiratory chain complexes and on KYAT1 and KYAT2, thus compromise the ATP and KYNA production in the mitochondria (96). Experimentally, FK506, a neuroimmunophilin drug, not only enhanced the formation of KYNA, but abolished the inhibition of KYNA synthesis caused by MPTP and 3-NA. This result suggested that the restoration of respiratory chain function may activate the KYNA synthesis pathway (97). In the case of monogenic form of Leigh Syndrome, the loss-of-function mutation in LRPPRC causes mitochondrial RNA metabolism disorder. The metabolic signature demonstrated a decrease in kynurenine, the precursor of KYNA (98). In patients with Schizophrenia, the prescription of N-acetylcysteine has shown inhibitory effect on KYAT, decreasing the deleterious effect of elevated KYNA on glutamate and dopamine signaling (99). Furthermore, the plasmatic KYNA is positively correlated with fatty acid oxidation and mitochondrial proliferation in the liver of rat (100).

Secondly, KYNA has shown scavenging property of OH■, O_2^-, $ONOO^-$ (92, 101). In a preparation of oocytes, KYNA significantly reduced ROS and lipid peroxidation induced by $FeSO_4$. For 3-Methylglutaric acid (3MGA) which accumulates in the brains of children coursing with metabolic acidurias, experiments showed that 3MGA induced an increase in ROS production and lipid peroxidation and a decrease in mitochondrial function. Addition of KYNA showed antagonist effects (102).

Thirdly, as has been discussed in 4.1, KYNA has potent antagonist effects over NMDAR (103), which may reduce de notorious effect of excitotoxicity on mitochondria, *via* decreasing excessive intracellular Ca^{2+} as example (104).

Finally, KYNA was shown to directly impair respiratory parameters of heart mitochondria. Moreover, the effect is selective for complex I (105, 106). However, this respiratory chain-modulating property was only observed in heart mitochondria, and is absent for brain and liver mitochondria, suggesting profound differences between tissular mitochondria content and helping to explain the tissue-specific effect of KYNA. It should be pointed out that these *in vivo* experiments were carried out with high concentration of KYNA (125-1000 μM) which is rarely achieved under physiological conditions (92).

CONCLUSION

Increasing evidence indicates that KYNA can act as a signaling molecule to regulate energy expenditure in a network integrating nutrition, physical exercise, inflammation and metabolic diseases besides its neuro-protector role in the central nervous system (**Table 3**). Targeting KYNA signaling network or its metabolic pathway harbors high potentials to expand the range of strategy to prevent and treat metabolic diseases.

AUTHOR CONTRIBUTIONS

ZS designed and reviewed the article. DZ wrote the draft. XDZ revised the content. JL collected references. All authors contributed to the article and approved the submitted version.

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miRNAs as Predictive Factors in Early Diagnosis of Gestational Diabetes Mellitus

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Introduction: Circulating miRNAs are important mediators in epigenetic changes. These non-coding molecules regulate post-transcriptional gene expression by binding to mRNA. As a result, they influence the development of many diseases, such as gestational diabetes mellitus (GDM). Therefore, this study investigates the changes in the miRNA profile in GDM patients before hyperglycemia appears.

Materials and Methods: The study group consisted of 24 patients with GDM, and the control group was 24 normoglycemic pregnant women who were matched for body mass index (BMI), age, and gestational age. GDM was diagnosed with an oral glucose tolerance test between the 24th and 26th weeks of pregnancy. The study had a prospective design, and serum for analysis was obtained in the first trimester of pregnancy. Circulating miRNAs were measured using the NanoString quantitative assay platform. Validation with real time-polymerase chain reaction (RT-PCR) was performed on the same group of patients. Mann-Whitney U-test and Spearman correlation were done to assess the significance of the results.

Results: Among the 800 miRNAs, 221 miRNAs were not detected, and 439 were close to background noise. The remaining miRNAs were carefully investigated for their average counts, fold changes, p-values, and false discovery rate (FDR) scores. We selected four miRNAs for further validation: miR-16-5p, miR-142-3p, miR-144-3p, and miR-320e, which showed the most prominent changes between the studied groups. The validation showed up-regulation of miR-16-5p ($p<0.0001$), miR-142-3p ($p=0.001$), and miR-144-3p ($p=0.003$).

Conclusion: We present changes in miRNA profile in the serum of GDM women, which may indicate significance in the pathophysiology of GDM. These findings emphasize the role of miRNAs as a predictive factor that could potentially be useful in early diagnosis.

Keywords: gestational diabetes, miR-16-5p, miR-142-3p, miR-144-3p, epigenetics, serum profiling, biomarkers, miRNA

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INTRODUCTION

Gestational Diabetes Mellitus (GDM) is one of the leading diseases during pregnancy. According to the newest edition of the International Diabetes Federation (IDF) Diabetes Atlas, GDM affected nearly 17 million live births in the last year (1). Extensive hormonal changes during pregnancy are one of the reasons for increased insulin resistance. For instant, the hyperestrogenemic state observed during pregnancy contributes to alterations in insulin sensitivity. Estrogen may bind directly to insulin or its receptors, making them unavailable for insulin (2). Furthermore, human placental lactogen (hPL) decreases maternal insulin sensitivity in order to provide the fetus with sufficient nutrition (3). When the insulin release is insufficient and a glucose-lowering response is not achieved, the risk of GDM development is high (4). Meta-analysis showed that the most relevant risk factors for GDM are high BMI and thyroid disease (5). Another risk factors are increased fasting glycemia in the first trimester of pregnancy, abdominal obesity, family history of diabetes mellitus, genetic factors, environmental factors including lifestyle and diet, comorbidities like polycystic ovary syndrome (PCOS) (6, 7). Combinations of several risk factors more confidently indicate women at high risk of developing GDM (8). Considering, that utility of risk factors, such as i.e first-trimester fasting blood glucose concertation is limited (9), it is essential to search for the most ideal non-invasive biomarker for early GDM detection or even a predisposition to develop GDM.

MiRNAs are a group of non-encoding RNA molecules of 19-22 nucleotides that play a key role in the regulation of post-transcriptional gene expression (10, 11). Notably, one miRNA has the ability to bind with many genes by recognizing the not-necessarily complementary sequence at the end of the 3'-untranslated region (3'UTR) of the target mRNA (12). In this way, endogenous miRNAs control the expression of many genes and influence the processes that take place in cells, such as cell metabolism, proliferation, DNA repair, and apoptosis. Furthermore, data suggest that extracellular miRNAs act as modulators during physiological and pathological processes by transferring information between cells (13). Depending on which gene that the miRNA impacts, it can be either a stimulator or a suppressor of a pathological state (14).

MiRNA is detectable in various biological fluids, such as blood, urine, tears, saliva, and cerebrospinal, amniotic, or synovial fluid (15). In contrast to other RNA molecules, an important feature of miRNA is their stability and resistance to external factors, such as RNAse (16). This is due to the form in which they occur in biofluids. MiRNA forms complexes with lipoproteins or proteins (17). Moreover, the protective effect may be a result of their encasement inside membrane structures like exosomes, microparticles, or apoptotic bodies (17, 18). It has also been shown that repeated cycles of freezing and thawing do not cause significant changes in miRNA content in the serum (19). These mechanisms and non-invasive collection mean that circulating miRNAs have good potential as a biomarker.

In recent years, there have been a number of reports on changes in miRNA expression in various diseases, including metabolic disorders. One of the ultimate purposes of most of the studies is finding miRNAs that could help with identifying pathological processes, estimate the success of a patient's response to therapy (20), or support the identification of high-risk groups (21). Zhao et al. were some of the first to describe changes in the sera of pregnant women with GDM (22). Since that time, many scientists have focused on changes in miRNA expression in GDM, but the available data are not consistent. Thus, the purpose of this study was to compare the miRNA expression profile in a group of patients in the first trimester of pregnancy and GDM diagnosed in the second trimester of pregnancy with that of a healthy control group. Then, based on these results, we sought to identify potential biomarkers of early GDM diagnosis.

MATERIALS AND METHODS

Study Population

Project included four meetings, in the first trimester (9-12 week), in the second (24-26 week), in the third trimester (34-37 week) and three months after delivery. During the first trimester of pregnancy, fasting venous blood samples were collected into S-Monovette Gel Clotting Activator tubes (Sarstedt, Numbrecht, Germany). After complete clotting and centrifugation, the serum to be used for miRNA analysis was separated, transferred into DNase- and RNase-free tubes (Eppendorf, Hamburg, Germany), and stored at -80°C until they were assayed. To diagnose GDM all patients underwent a 75g oral glucose tolerance test (OGTT) in the second trimester, between 24^{th} and 27^{th} weeks of pregnancy. GDM was diagnosed according to the World Health Organization (WHO) criteria (23). In the experiment the serum from the first trimester was examined while both groups revealed normoglycemia. The study group (GDM) (n=24) and control group with normal glucose tolerance (NGT) (n=24) were carefully matched for pre-pregnancy body mass index (BMI), age, and gestational age. Women with the history of GDM, stillbirth, childbirth with congenital anomalies, pregnancy-induced hypertension, preeclampsia, cholestasis, premature delivery, acute or chronic inflammation, multiple pregnancy, pre-existing glucose intolerance, and active smokers were excluded from the study. Written informed consent was obtained from each patient, and the study was approved by the local ethics committee (Medical University of Bialystok).

Biochemical Methods

Plasma glucose concentrations were measured using an enzymatic method with hexokinase (Cobas C11, Roche Diagnostics Ltd, Switzerland), and the serum insulin level was evaluated by an immunoradiometric method (DiaSource Europe SA, Belgium) using a Wallac Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Life Science, Turku, Finland). Glycated hemoglobin (HbA1c) was assayed by high-performance liquid chromatography (Bio-Rad D-10, Bio-Rad Laboratories, Hercules, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) and homeostatic model assessment of β-cell function were calculated for all women in each trimester

of pregnancy. Moreover, in the second trimester, insulin sensitivity was measured using the OGTT insulin sensitivity index of Matsuda and DeFronzo (ISI_{OGTT}).

miRNA Isolation

MiRNA was isolated using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany) by following the manufacturer's protocol. The isolation method is based on the innovative spin-column separation method with a silica membrane. The use of this kit allows us to obtain miRNA of high quality and purity, which is necessary for the subsequent stages of the experiment. The content of miRNA in extracted samples was checked with a fluorometer (Qubit 3.0, Thermo Fisher Scientific, Waltham, USA).

Nanostring Analysis

For miRNA profiling, we used NanoString technology with a digital color-coded barcode for direct and multiplex marking of target sequences of 800 miRNAs. The method uses about 50 nucleotide probes per 1 miRNA. At the 5' end, a set of 6 fluorescently labeled "barcodes" is placed, and at the 3' end, a "capture probe" with biotin is placed. One set allows for simultaneous determination of 800 miRNAs in 12 samples.

Due to the procedure used, the cDNA synthesis and amplification stages were omitted, which allows us to reduce the probability of laboratory error. The results were read out on a NanoString nCounter scanner. The first stage of the analysis was the hybridization of individual miRNAs with specific probes, and the next was the purification and placing of hybridized samples on a specially standardized plate. The last stage was reading of the obtained results. The method allowed for the exact number of miRNA copies to be specified in each sample.

RT-PCR Validation

Validation of the results was carried out on the same group of patients (24 women in the NGT control group and 24 in GDM study group). To validate the results, the real-time PCR method was used. In the first step, reverse transcription was performed to transcribe miRNA to cDNA using the miRCURY LNA RT Kit (Qiagen, Germany) in accordance with the manufacturer's procedure on a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, USA). Subsequently, we performed RT PCR reaction using the miRCURY LNA SYBR Green PCR Kit (Qiagen, Germany) and specific primers for each of the analyzed miRNAs (Qiagen, Germany) on a LightCycler 480 thermal cycler (Roche Diagnostics Ltd, Switzerland). Expression of circulating miRNAs was evaluated using miR-103a-3p as an endogenous control gene. All samples were assayed in duplicate, and the comparative Ct method was used to calculate the relative changes in gene expression.

Data Analysis

Analysis of raw miRNA data obtained using NanoString technology was performed in nSolver software version 4.0. Data were normalized by the average geometric mean of the top 100 probes detected. The miRNAs' expression values in RT-PCT validation were calculated based on the ΔΔCT method. The differences in miRNA expressions between groups were calculated by the Mann-Whitney U test using Statistica 13 for Microsoft Software (StatSoft Inc., Tulsa, USA). The relationships between variables were tested using the Spearman rank correlation coefficient. Results were considered statistically significant with p-value less than 0.05.

RESULTS

Characteristics of the Groups Studied

The clinical characteristics of the studied groups are presented as medians and interquartile ranges (**Tables 1**, **2**). In the 1st trimester of pregnancy, there were no significant differences between groups. Women in both groups were normoglycemic. Most patients had normal pre-pregnancy BMI (n=10 in GDM group and n=11 in NGT group had BMI >25 kg/m^2 indicating overweight). In the 2nd trimester, groups revealed significant differences in fasted and post-loaded glucose measurements (glucose at 0, 30, 60, and 120 minutes: p=0.0001, p=0.0000, p=0.0000, and p=0.001, respectively). The GDM group had a higher insulin level at 60 minutes (p=0.02), insulin level at 120 minutes (p=0.004), and HOMA-IR (p=0.02). Fasting insulin and

TABLE 1 | Clinical characteristics of groups studied in the 1st trimester.

	NGT	GDM	p-value
n	24	24	
Age (years)	28 (26-31.5)	26 (24-30.5)	0.36
Pre-pregnancy BMI (kg/m^2)	21.8 (20.0-28.2)	23.5 (21.6-26.8)	0.73
Gestational age (week)	11 (10-12)	10 (9.5-11)	0.24
Fasting glucose (mg/dl)	86 (84-88)	87.5 (85-90)	0.29
Fasting insulin (μU/ml)	10.7 (9.1-12.9)	11.3 (10.2-13.3)	0.25
HOMA-IR	2.3 (1.9-2.8)	2.5 (2.1-2.9)	0.18
HOMA-β	168.0 (145.5-187.5)	166.5 (146.9-201.0)	0.97
HbA1c (%)	5.0 (4.9-5.4)	5.0 (4.9-5.4)	0.98
Total cholesterol (mmol/l)	170 (149.5-191.5)	169.5 (156.5-186)	0.81
HDL-cholesterol (mmol/l)	81 (69.5-90.5)	73.5 (59.5-84.5)	0.19
LDL-cholesterol (mmol/l)	78.2 (64.3-91.6)	81 (64.9-95.3)	0.78
Triglycerides (mmol/l)	72 (60.5-109.5)	95.5 (68.5-118)	0.14

Data are shown as medians (interquartile range); The difference between NGT vs GDM group was compared with the Mann-Whitney U-test.

TABLE 2 | Clinical characteristics of groups studied in the 2nd trimester.

	NGT	GDM	P value
n	24	24	
Gestational age (week)	25 (25-26)	25 (25-26)	0.81
Fasting glucose (mg/dl)	82.5 (79-85)	92 (84-94)	0.0001
Glucose 30' (mg/dl)	127.5 (121-140)	158 (148-165)	< 0.0001
Glucose 60' (mg/dl)	122 (101.5-141.5)	169 (136.5-184)	< 0.0001
Glucose 120' (mg/dl)	105.5 (86-119)	125.5 (111.5-166)	0.001
Fasting insulin (μU/ml)	11.2 (8.6-13.3)	13.4 (10.1-18.2)	0.08
Insulin 30' (μU/ml)	74.2 (60.0-110.9)	80.1 (61.0-137.3)	0.62
Insulin 60' (μU/ml)	80.1 (54.3-107.2)	106.5 (74.2-174.0)	0.02
Insulin 120' (μU/ml)	56.0 (42.0-72.4)	108.8 (60.5-131.0)	0.004
HOMA-IR	2.3 (1.7-2.7)	3.0 (2.1-4.4)	0.02
HOMA-β	188.3 (168.8-282.0)	191.4 (149.4-240.3)	0.21
ISI OGTT	4.4 (3.4-5.4)	2.8 (2.1-3.9)	0.002
HbA1c (%)	4.8 (4.7-5.1)	4.9 (4.6-5.1)	0.74
Total cholesterol (mmol/l)	267.5 (203-287.5)	238 (188-257)	0.046
HDL-cholesterol (mmol/l)	96.5 (85.5-108.5)	85 (69.5-104.5)	0.13
LDL-cholesterol (mmol/l)	131 (101.4-171.2)	119.2 (85.0-140.4)	0.12
Triglycerides (mmol/l)	138 (120.5-170.5)	159 (135.5-204.5)	0.13

Data are shown as medians (interquartile range); The difference between NGT versus GDM group was compared with the Mann-Whitney U-test.

insulin after 30 minutes post-loading were also higher in the study group than in the NGT group, but the differences were insignificant. Moreover, the GDM group demonstrated lower ISI_{OGTT} (p=0.002) and lower total cholesterol (p=0.046) than the NGT group.

Nanostring Profiling

We identified 28 miRNAs with expression that was significantly altered in the GDM group compared to the NGT group (p-value p<0.05). A careful analysis was done while considering not only the p-value, but also the false discovery rate, count ranges, fold change, and standard deviation. The results pointed out miR-16-5p (p=0.07), miR-142-3p (p=0.02), miR-144-3p (p=0.003), and miR-320e (p=0.02) for further validation. Changes in expression of miR-16-5p were not significant, whereas the mean value ranges of the counts were high (GDM=1056.03 versus NGT=756.86) with a wide standard deviation. Considering the method of simultaneous determination of many miRNAs and high count number, we decided to evaluate these molecules in further analysis.

Validation of the Results

NanoString results were validated by RT-PCR. The fold change of gene expression was calculated using the ΔΔCt method, and then log transformation was used to avoid a non-normal distribution of the results. We obtain confirmation of three miRNAs: miR-16-5p (p<0.0001), miR-142-3p (p=0.001), and miR-144-3p (p=0.003), which were significantly upregulated in the GDM group. No significant difference was observed for miR-320e (p=0.16) (**Figure 1**).

ROC curve analysis was performed for significant miRNAs in the 1st trimester of pregnancy as parameters to discriminate those who are at high risk group of developing GDM in the 2nd trimester of pregnancy (**Figure 2**). The AUC for miR-16-5p was 0.868 (95% confidence interval: 0.757–0.98; p<0.0001). AUC was 0.778 (95% confidence interval: 0.644-0.913; p<0.0001) for miR-142-3p, and for miR-144-3p, AUC was 0.756 (95% confidence interval: 0.613-0.898; p=0.0004).

The relationships between prominent molecules' expressions and other variables were checked. Across the study population, 1st-trimester miR-16-5p expression correlated positively with fasting plasma glucose concentration in the 2nd trimester (R=0.56, p<0.05), plasma glucose concentration at 30 minutes post-loading (R=0.43, p<0.05), and HOMA-IR (R=0.36, p<0.05). Its expression negatively correlated with ISI_{OGTT} (R=-0.34, p<0.05). MiRNA-142-3p positively correlated with plasma glucose levels post-loading with indexes as follows: 30 minutes (R=0.35, p<0.05), 60 minutes (R=0.37, p<0.05), and 120 minutes (R=0.36, p<0.05). Furthermore, there were correlations between miR-144-3p and plasma glucose concentration at 30 minutes post-loading (R= 0.41, p<0.05) and the plasma glucose level at 60 minutes post-loading (R=0.42, p<0.05), as well as a negative correlation with ISI_{OGTT} (R=-0.33, p<0.05). Multiple regression analysis confirmed the dependences described except for the association of miR-16-5p and ISI_{OGTT}.

DISCUSSION

Researchers for many years have been trying to find the most ideal GDM biomarker. Among many significant features of the perfect indicator the most relevant is prediction value (24). In case of GDM, the diagnosis nowadays is based on OGTT performed in the second trimester of pregnancy. Considering complications during pregnancy and delivery and a high risk of long-term complications for the child and the mother the GDM a biomarker revealed before changes in the glycemia occur seems to be crucial. Yoffe et al. (25) studied women between 9th and 11th weeks of pregnancy and showed an up-regulation of the miR-223 and miR-23a in plasma of GDM women. Interesting point of view was presented by Wander et al. connection of the miR-21-3p and miR-210-3p with GDM diagnosed in overweight and

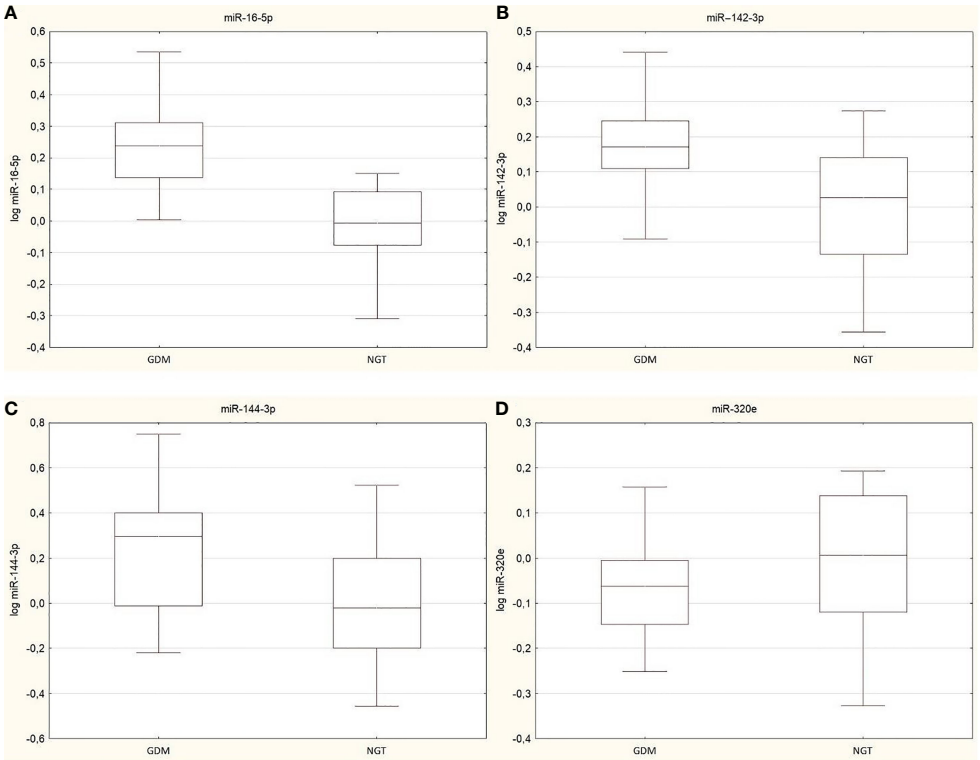

FIGURE 1 | Box plots presented changes in expression of validated miRNAs between GDM group and NGT, **(A)** miR-16-5p ($p<0.0001$), **(B)/** miR-142-3p ($p=0.001$), **(C)** miR-144-3p ($p=0.003$) and **(D)** miR-320e ($p=0.16$). Data are presented by median indicated by line in each box and interquartile range. Maximum and minimum values are represented by whiskers.

obese women (26). Lamadrid-Romero et al. (27) showed that miR-183-5p was increased in every trimester in serum collected from women diagnosed with GDM. Simultaneously, the higher expression of miR-125b-3p, miR-200b-3p and miR-1290 were observed in the first trimester of pregnancy.

Our study shows that circulating miR-16-5p is upregulated in women before the onset of GDM, which is consistent with the results obtained by other studies. Zhu et al. (28) conducted studies on women at 16-19 weeks of pregnancy and described five molecules that were upregulated in the GDM group (e.g., miR-16-5p). Other studies reported increased expression of miR-16-5p in serum at 24 -28 weeks of pregnancy (29). Our results show this difference earlier between the 9th and 12th weeks of pregnancy. Moreover, we observed a positive correlation with HOMA-IR, which was also described by Cao et al. (29). Apart from miR-16-5p they described an up-regulation of the miR-17-5p and miR-20a-5p which was not observed in our experiment.

Attempts were made to determine miR-16-5p in leukocytes of women with GDM, but no significant differences were observed (30, 31). It turns out that high miR-16-5p expression also persists after pregnancy and correlates with high cardiovascular risk (32). This indicates that epigenetic changes during GDM are permanent, and women with a history of GDM are predisposed to the development type 2 diabetes (T2D) or cardiovascular disease in the following years (33). Another study revealed increased expression of miR16-5p in overweight women before the 20th week of pregnancy. In contrast to previously cited reports, that study was conducted on European women (34). On the other hand, Martinez-Ibarra et al. demonstrated no significant changes in miR16-5p expression in serum collected in the 2nd trimester from GDM patients compared to NGT (35). A similar result was obtained by scientists from South Africa (36).

Considering that miRNAs could be related to genetic and environmental factors, Sørensen et al. proposed ethnicity as a potential explanation of differences in obtained results (34). Furthermore, they also suggested age, which is a known risk factor for GDM. The idea was supported by the correlation obtained between age and miR-16-5p expression. However, this dependence was not observed in our study.

Available data show that miR-16-5p is one of the most potent regulating molecules in the insulin-signaling pathway. Target genes for miR-16-5p encode insulin receptor substrate (IRS) proteins 1 and 2 and the insulin receptor itself (INSR) (37, 38). These proteins are crucial factors in a proper insulin signaling pathway, and their downregulation results in insulin resistance and metabolic disorders like diabetes. Additionally, miR-16-5p-targeted genes are involved in pancreatic β-cell proliferation and apoptosis (39). Target genes for miR-16-5p that are downregulated in type 2 diabetes are located in not only β-

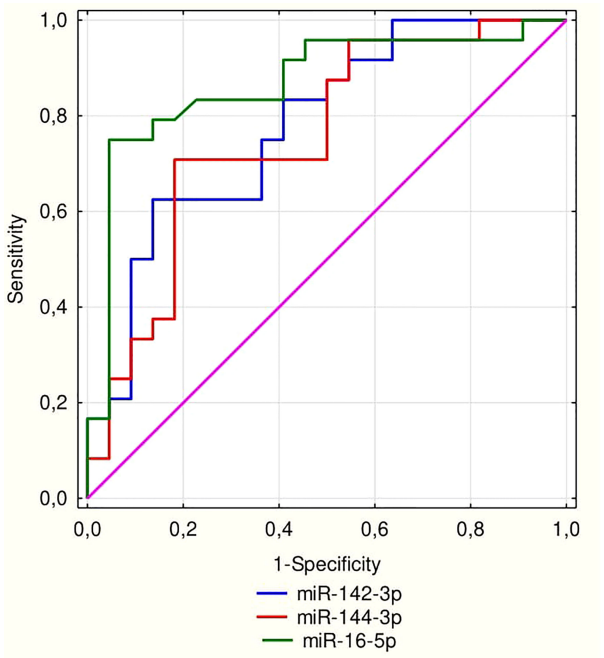

FIGURE 2 | ROC curve for miRNA 16-5p (AUC = 0.868; p < 0.0001), miR-142-3p (AUC = 0.778; p < 0.0001) and for miR-144-3p (AUC = 0.756; p = 0.0004).

cells on pancreatic islets, but also peripheral blood mononuclear cells (PBMCs), the liver, and skeletal muscle (40).

An experimental study on *Cmah*-null mice showed that diabetic mice have upregulated miR-16-5p (among others) and downregulated IRS1, IRS2, AKT1, and mTOR mRNA (41). As a result of these changes, the crucial pathway in insulin-signaling PI3K-Akt-mTOR is dysregulated (42). Interestingly, Lee et al. (43) demonstrated a decrease in miR-16-5p expression in insulin-resistant skeletal muscle. Moreover, their *in-vitro* study revealed that miR-16-5p is involved in autophagy through controlling Bcl-2 protein synthesis. Also, an overexpression of miR-16-5p was accompanied by decreased mTOR content. Based on these findings, the inhibition of miR-16-5p expression might be important in treatment (44).

There are two reports on miR-142-3p in GDM. However, neither of these studies considers circulating human miR-142-3p. Collares et al. (45) described nine miRNAs (e.g., miR-142-3p) that are upregulated in PBMC obtained from type 1 diabetes (T1D), T2D, and gestational diabetes mellitus. The study did not associate the molecule with a specific gene, but its involvement in diabetes in general was noticeable. A study conducted on GDM-induced mice reported an overexpression of miR-142-3p in the circulating blood and embryonic tissue of GDM mice. Data demonstrated that *in-vitro* up-regulation of miR-143-3p has a positive effect on β-cells by promoting their proliferation, as well as inhibiting apoptosis by blocking the expression of p27, Bax, and caspase-3. In addition, bioinformatic analysis indicated forkhead box protein O1 (FOXO1) as a target gene for miR-142-3p (46). FOXO1 is known as a multifunctional protein, and besides controlling glycogenolysis and gluconeogenesis, it regulates the differentiation of β-cells and promotes their apoptosis (47). This could be a self-protective effect of miR-142-3p.

Escalated expression of miR-142-3p has been described in obese adults as a parameter that is strongly associated with insulin, HOMA-IR, BMI, adiponectin, and leptin levels (48). Similar results were obtained in the case of childhood obesity, which revealed an increased concentration of miR-142-3p and a positive correlation with BMI, fat mass, adipose tissue distribution, and HOMA-IR. Interestingly, during a 3-year follow-up, upregulation in the expression of this molecule was observed solely in the serum of patients whose BMI remained stable or decreased (49). The data showed that the expression of the miR-142-3p may be sex-related.

Overexpression of miR-142-3p in the group of patients with pre-diabetes and diabetes was found only among women (50). The studies mentioned the possibility of age affecting these results because the male group was significantly younger. However, there may also be an influence from the distribution of adipose tissue according to the studies cited. In contrast to our study, Liang et al. showed a decreased expression of miR-142-3p in the serum of T2D patients, and a negative correlation with HOMA-IR was observed (51). In the present study, a positive correlation was revealed between miR142-3p and plasma glucose post-loading.

Another study has shown that miR-144-3p is upregulated in the liver, pancreas, skeletal muscle, adipose tissue, and blood of a diabetic rat model. The result was confirmed in circulating blood obtained from human T2D patients. In addition, a study of pancreatic cells cultured from rats revealed an increased level of miR-144-3p in a high glucose environment, and similar to miR-16-5p, it caused a downregulation of the expression of IRS1 (52). Moreover, the upregulation of miR-144-3p was observed in PBMCs collected from patients with T1D, T2D, and GDM (45). However, Akerman et al. (53) investigated patients with T1D and did not observe an elevation of serum miR-144-3p levels, but there was a positive correlation with islet antigen 2 antibodies (IA2A), indicating a possible relationship with the assessment of those at risk for T1D development.

Upregulated expression and a positive correlation with HOMA-IR of circulating miR-144-3p were observed in a Chinese cohort with impaired fasting glucose (IFG). Furthermore, high miR-144-3p was a predictor of T2D development (51). Interestingly, Wang et al. (54) showed an increased expression of miR-144-3p in T2D patients but solely in a Swedish population, not in patients from Iraq. Thus, this report confirms the contribution of environmental factors to epigenetic changes mentioned above. In a meta-analysis, Zhu and Leung (55) selected eight molecules as potential biomarkers of T2D, including miR-142-3p and miR-144-3p.

In summary, we found significantly upregulated expression of miR-16-5p, miR-142-3p, and miR-144-3p in the serum of patients in their 1st trimester of pregnancy who suffered from GDM diagnosed in the 2nd trimester. NanoString technology allowed us to study a wide panel of miRNA profiles. Considering

the research on miRNAs, a strong point of our experiment was the large number of patients in the studied groups. Although our findings are limited by the validation using the same group of women, our observations strongly suggest that changes taking place in the miRNA profile occur earlier than changes in glucose levels, and research on the more sensitive and specific biomarkers of GDM should be continued.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

Conceptualization: IJ and MK. Methodology: MN, IJ, IS and AB. Formal analysis: MZK and AK. Writing—original draft preparation: IJ and MK. Writing—review and editing: AJK and JS. Supervision: JS and AJK. All authors contributed to the article and approved the submitted version.

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The *CDKAL1* rs7747752-Bile Acids Interaction Increased Risk of Gestational Diabetes Mellitus: A Nested Case-Control Study

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Aims: The study aimed to explore additive interactions of *CDKAL1* rs7747752 and GUDCA/DCA for GDM risk and whether the interactive effects on the risk of GDM was mediated *via* increasing lysophosphatidylcholines (LPC) 18:0 and/or saturated fatty acid (SFA) 16:0.

Methods: A 1:1 age-matched study nested in a prospective cohort of pregnant women (207 pairs) was organized in Tianjin, China. Additive interactions were used to test interaction effects while mediation analyses and Sobel tests were used to test mediation effects of LPC18:0 and SFA16:0 between copresence of rs7747752 and low GUDCA/DCA, and GDM risk.

Results: The *CDKAL1* rs7747752 was associated with GDM (P<0.05). The rs7747752 C polymorphism markedly enhanced ORs of low GUDCA from 4.04 (0.72-22.8) to 9.02 (1.63-49.7) and low DCA from 1.67 (0.68-4.11) to 4.24 (1.84-9.76), both with significant additive interactions. Further adjustment for LPC18:0 attenuated the interactive effects of rs7747752 and low DCA, with a significant mediation effect (P=0.003). High SFA16:0 did not mediate the interactive effects of rs7747752 and low DCA/GUDCA on GDM risk.

Conclusions: The *CDKAL1* rs7747752 C carrier status and low GUDCA/DCA had significant additive interactions on the risk of GDM with the effect from interaction with DCA being partially mediated *via* increasing LPC18:0.

Keywords: *CDKAL1*, rs7747752, glycoursodeoxycholic acid, deoxycholic acid, gestational diabetes mellitus

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INTRODUCTION

The prevalence of gestational diabetes mellitus (GDM) has been rapidly increasing worldwide (1). GDM is not only associated with perinatal adverse outcomes but also predispose women to increased risk of diabetes and cardiovascular disease in later life, and their offspring to increased risk of childhood obesity (2, 3). On the other hand, intensive management of GDM does not have a detectable effect on the risk of postpartum diabetes in the mothers (4) and childhood obesity in their offspring (5). How to prevent GDM has become increasingly important to reduce the burden of GDM. However, our meta-analysis of 29 randomized controlled trials shows that lifestyle interventions within 15 weeks of pregnancy can only achieve as low as 20% reduction in risk of GDM while later lifestyle interventions are ineffective in reducing the risk (6). Indeed, a better understanding of the pathophysiology of GDM and identification of its novel biomarkers are critically important for the prediction and prevention of GDM in early pregnancy.

GDM is a complex disease determined by a constellation of factors, including genetic, metabolic, and other environmental factors (7). Among genetic factors, single nucleotide polymorphisms (SNP) within the cyclin-dependent kinase 5 regulatory subunit-associated protein1-like 1 (*CDKAL1*) locus was found to be strongly associated with GDM by genome-wide association analysis (GWAS) and dozens of replication studies in different populations (8–10). *CDKAL1* gene is located on the short arm of human chromosome 6 and encodes a protein of 579 amino acids, which is indeed a member of the methylthiotransferase that specifically modifies transfer ribonucleic acid (tRNA) Lys in mammals (11). The *CDKAL1* gene plays a role in the cell cycle control of beta cells by inhibiting the activity of cyclin-dependent kinase 5 (CDK5) and acting as a tRNA-modifying enzyme (12). The association between *CDKAL1* rs7747752 polymorphism and GDM was also reported in Chinese pregnant women (8).

In addition to genetic origin and environmental factors, various metabolic factors, such as bile acids (BAs) and saturated fatty acids (SFAs), can act as risk factors (13, 14). In this connection, our group found that serum glycoursodeoxycholic acid (GUDCA) ≤ 0.07 nmol/mL and deoxycholic acid (DCA) ≤ 0.28 nmol/mL in early pregnancy were independently associated with markedly increased risk of GDM in Chinese pregnant women (15). Interestingly, adjustment for LPC18:0 attenuated the risk associations of both low DCA and low GUDCA with GDM while low DCA and low GUDCA enhanced the risk association between LPC18:0 and GDM (16). The interaction between genetic disposition and metabolic factors plays a critical role in the development of GDM (17). Indeed, the CDKAL1 gene was related to defects in the conversion of proinsulin (18) and insulin response under glucose stimulation (19, 20). It is known that defection of insulin response reduces its ability to inhibit lipolysis in adipose tissue, further resulting in increased flow of SFAs into the circulation (21). In line with these findings from mechanistic investigations, our group reported that high SFA16:0 played a vital role in the risk of GDM, which had a significant interactive effect with *CDKAL1* rs7747752 on the risk of GDM (14). In addition, an animal study showed that defection of *CDKAL1* gene led to impaired insulin secretion and longitudinal fluctuations in insulin sensitivity during high-fat feeding in mice (22). Notably, BAs, as cholesterol-derived metabolites, have a well-established role in the digestion and absorption of dietary fats (23); furthermore, BAs also affect insulin secretion (24, 25). This observation suggests that BAs and *CDKAL1* variants may have a synergistic effect on the risk of GDM, possibly being mediated by LPC18:0 or SFA16:0. Indeed, it is worthwhile to explore whether abnormal BAs have any interactions with *CDKAL1* gene variants towards increased risk of GDM. It is also quite interesting to understand potential interrelationships among *CDKAL1*, BAs, SFAs, and LPCs and their roles in the etiology of GDM.

Using an age-matched case-control study nested in a large population-based cohort of pregnant women in Tianjin, China, this analysis aimed to explore 1) additive interactions between *CDKAL1* rs7747752 polymorphism and low GUDCA/DCA for the risk of GDM; and 2) whether the additive interactive effect if any between rs7747752 polymorphism and low GUDCA/DCA on the risk of GDM was mediated *via* LPC18:0 and/or SFA16:0.

MATERIALS AND METHODS

Research Design and Population

The design and method of this study have been described previously (26). To be brief, we set up a prospective cohort study of 22 302 pregnant women at their first antenatal care with median 10th gestational weeks in Tianjin, China, from October 2010 to August 2012. The ethics of the study protocol was approved by the Ethics Committee of Tianjin Women and Children's Health Center (TWCHC), and written informed consent was obtained before data collection.

A two-step screening procedure was used to identify GDM. First, a 1-h 50-g glucose challenge test (GCT) was performed on pregnant women at 24-28th gestational weeks in the primary hospital. Second, a 2-hour 75-g oral glucose tolerance test (OGTT) was performed on pregnant women with GCT ≥ 7.8 mmol/L at the GDM clinic in TWCHC. The diagnosis of GDM is based on the cutoff points of the International Association of Diabetes and Pregnancy Study Group (27). Of the 22 302 participants, 2 991 pregnant women donated fasting blood samples. We finally included 207 GDM cases and 207 non-GDM controls who were matched by maternal age (± 1 year) (15). Data from the 207 pairs of women with high-quality SNP (28) were used to explore the research questions of this study. The flowchart of the study participants was available elsewhere (14).

Data Collection Procedures

Data were collected during the first antenatal care visit, GCT, OGTT, and the postpartum period, which has been described in detail (26). Demographic, clinical and lifestyle information included age, weight, height, systolic/diastolic blood pressure (SBP/DBP), ethnicity, education level, parity, family history of diabetes, in first degree relatives, smoking and drinking habits before and during pregnancy.

Measurement of Serum BAs, LPCs and SFAs

Liquid chromatography-tandem mass spectrometry (LC–MS/MS) was used to assay the concentrations of serum BAs, LPCs, and SFAs. The detailed measurement methods of serum BAs, LPCs, and SFAs were available elsewhere (14–16).

Genotyping

Genotyping was conducted using the Illumina Infinium® Global Screening Array and genotype data was imputed using minimal 3 with the 1000 Genomes Project phase 3 v 5 as reference panel. Genotyping data from specific candidate SNP (rs7747752) were extracted from the genome-wide genotyping data. The overall genotype call rate was 99.4%.

Statistical Analysis

Power and sample size analysis was performed using PASS 15 (NCSS, LLC. Kaysville, Utah, USA). In the 1:1 age-matched case-control study, we assumed that the probability of exposure among sampled control patients is 10% and the correlation coefficient for exposure between matched case and control patents is 0.2, and type I error is set at 0.05. The sample size required to achieve 85% power for an odds ratio (OR) of 2.0 is 256 (128 GDM and 128 control). Therefore, the current sample size of our study (n=414) had more than 85% power to detect the assumed risk association.

All other statistical analyses were performed using Statistical Analysis System (SAS) release 9.4 (SAS Institute, Cary, NC). Quantitative data were compared between the GDM group and the non-GDM group, using the paired Student's t-test or Wilcoxon signed-rank test. The categorical data were compared using the McNemar test or Fisher's exact test. In addition, Spearman correlation analysis was used to calculate the correlation coefficients of these serum BAs and rs7747752 with neonatal birth weight. In addition, Kruskal-Wallis test was used to test the differences of serum levels of BAs between different rs7747752 genotypes. In this analysis, a P-value < 0.05 was regarded to be statistically significant.

In our previous analysis, we detected that GUDCA ≤ 0.07 nmol/mL, DCA ≤ 0.28 nmol/mL, LPC18:0 ≥ 18.0 nmol/mL and SFA16:0 ≥ 17.1 nmol/mL were independently associated with markedly increased risk of GDM in restricted cubic spline analysis (14–16). In this analysis, we used the same cutoff points of GUDCA, DCA, LPC18:0 and SFA16:0 to define low GUDCA, low DCA, high LPC18:0 and high SFA16:0. Conditional logistic regressions were used to obtain the ORs and their 95% confidence intervals (CIs) of rs7747752 and GUDCA/DCA for the risk of GDM in unadjusted models and adjusted models. The adjusted models considered confounding effects of traditional risk factors of GDM, including pre-pregnancy body mass index (BMI), family history of diabetes in first degree relatives, SBP, current smoker before pregnancy, and weight gain to the time of GCT. Next, the combined effects of rs7747752 genotypes (CC vs. CG vs. GG) and low serum levels of GUDCA/DCA on GDM susceptibility were examined to explore whether the rs7747752 and low GUDCA/DCA have potential interactive effects on the risk of GDM. Then, based on the results of combined effects of rs7747752 genotypes (CC vs. CG vs. GG) and low GUDCA/DCA on GDM, we further tested the additive interactions between rs7747752 C risk allele and low GUDCA/DCA for GDM. Three measures, i.e., relative excess risk due to the interaction (RERI), attributable proportion due to the interaction (AP), and synergy index (SI) were used to judge additive interactions (29). Any of RERI > 0, AP > 0 or SI > 1 indicates a significant additive interaction.

In order to shed light on the potential mechanisms of the additive interactions between CDKAL1 rs7747752 C allele and low GUDCA/DCA on the risk of GDM, we further conducted mediation analyses to examine whether high LPC18:0 and/or SFA16:0 can account for the association between copresence of CDKAL1 rs7747752 C allele carrier status and low GUDCA/DCA, and the increased risk of GDM. First, we estimated the ORs of copresence of rs7747752 and low GUDCA/DCA for high LPC18:0 and high SFA16:0, and the ORs of high LPC18:0 and high SFA16:0 for GDM. Then, we calculated the ORs of copresence of rs7747752 and low GUDCA/DCA for GDM after adjusting for high LPC18:0 and high SFA16:0. At last, Sobel test was used to assess the mediation effects of LPC18:0 and SFA16:0 (30).

RESULTS

Characteristics of the Research Participants

The clinical and biochemical information of the study participants was shown in **Table 1**. The mean age of participants at the first antenatal care visit was 29.24 ± 3.04 standard deviation (SD) years. There were no differences in height, ethnicity, education level, parity, weight gain to GCT, smoking and drinking habits before and during pregnancy among the controls and GDM groups (P > 0.05). Women with GDM had significantly higher weight, BMI, SBP, DBP and GCT glucose levels than the controls (P < 0.05). Women with GDM also had a higher proportion of family history of diabetes in first degree relatives than the non-GDM group. Women with GDM had a lower serum level of GUDCA and DCA while had a high serum level of LPC18:0 and SFA16:0, compared with non-GDM women. The frequencies of the heterozygote (CG) and homozygous (CC) of the *CDKAL1* rs7747752 were found to be significantly higher in women with GDM than in the controls (non-GDM) (P < 0.05). Whereas, we failed to find any significant differences of serum levels of GUDCA and DCA between rs7747752 genotypes (**Table S1**).

In addition, Spearman correlation analysis was used to calculate the correlation coefficients of these serum BAs and rs7747752 with neonatal birth weight. The correlation coefficients of DCA, GUDCA and rs7747752 with birth weight were 0.063 (P = 0.199), -0.022 (P = 0.658) and -0.017 (P = 0.728), respectively, all being not significant.

Associations of *CDKAL1* rs7747752 and Low Serum Levels of GUDCA/DCA With GDM

The GUDCA ≤ 0.07 nmol/mL was associated with increased risk of GDM (OR: 5.84, 95% CI: 2.13-16.0) after adjustment for

TABLE 1 | Clinical and biochemical characteristics of GDM and non-GDM women.

Characteristic	Non-GDM (n = 207)	GDM (n = 207)	*P* value
Variables at registration			
Age, years	29.23 ± 3.34	29.25 ± 2.74	0.480*
Height, cm	162.98 ± 4.54	163.25 ± 5.04	0.509*
Weight, kg	58.58 ± 9.78	63.87 ± 10.54	<0.001*
BMI, kg/m^2	22.04 ± 3.46	23.95 ± 3.66	<0.001*
Systolic blood pressure, mmHg	104.21 ± 10.60	108.21 ± 10.54	<0.001*
Diastolic blood pressure, mmHg	67.91 ± 7.66	70.72 ± 7.93	<0.001*
Han ethnicity	200 (96.62)	202 (97.58)	0.564**
Education > 12 years	113 (54.59)	109 (52.66)	0.683**
Parity ≥ 1	10 (4.83)	13 (6.28)	0.532**
Family history of diabetes in first degree relatives	13 (6.28)	26 (12.56)	0.033**
Current smoker before pregnancy	13 (6.28)	14 (6.76)	0.841**
Alcohol drinker before pregnancy	52 (25.12)	63 (30.43)	0.564**
Variables during pregnancy			
Current smoker during pregnancy	1 (0.48)	2 (0.97)	0.564**
Alcohol drinker during pregnancy	2 (0.97)	2 (0.97)	1.000**
gestational weeks at GCT, week	25.16 ± 2.28	24.95 ± 1.44	0.033*
Weight gain to GCT, kg/week	0.58 ± 0.21	0.56 ± 0.23	0.532*
GCT glucose, mmol/L	6.39 ± 1.35	9.30 ± 1.45	<0.001*
Bile acid species			
GUDCA ≤ 0.07 nmol/mL	167 (80.68)	199 (96.14)	<0.001**
DCA ≤ 0.28 nmol/mL	111 (53.62)	139 (67.15)	0.006**
Lysophosphatidylcholines species			
LPC18:0 ≥ 18.0 nmol/mL	40 (19.32)	175 (84.54)	<0.001**
Saturated fatty acids species			
SFA16:0 ≥ 17.1 nmol/mL	87 (42.03)	125 (60.39)	<0.001**
***CDKAL1* gene**			
rs7747752 (C/G)			0.003**
GG	64 (30.92)	43 (20.77)	
CG	101 (48.79)	103 (49.76)	
CC	42 (20.29)	61 (29.47)	

GDM, gestational diabetes mellitus; BMI, body mass index; GCT, glucose challenge test; GUDCA, glycoursodeoxycholic acid; DCA, deoxycholic acid; LPC, Lysophosphatidylcholines. SFA, Saturated fatty acid; CDKAL1, cyclin-dependent kinase 5 regulatory subunit-associated protein1-like 1.

Data are reported in mean ± SD or number (percentages).

**Derived from paired t-test or Wilcoxon signed-rank test.*

***Derived from McNemar test or Fisher's exact test.*

traditional risk factors, including pre-pregnancy BMI, family history of diabetes in first degree relatives, SBP, current smoker before pregnancy and weight gain to the time of GCT. Similarly, after adjustment for traditional risk factors, DCA ≤ 0.28 nmol/mL was also associated with increased risk of GDM (OR: 2.28, 95% CI: 1.43-3.64). In the cohort, the C allele frequency for rs7747752 was 49.89%. The *CDKAL1* rs7747752 C allele was significantly associated with GDM in the unadjusted model (OR: 1.48, 95% CI: 1.12-1.96) and adjusted model (OR: 1.76, 95% CI: 1.26-2.44). After adjustment for traditional risk factors, heterozygote (CG) and homozygote (CC) genotypes of *CDKAL1* rs7747752 were all associated with increased risk of GDM with ORs being 1.95 (95% CI: 1.15-3.31) and 3.07 (95% CI: 1.59-5.95), respectively. In dominant and recessive models, the ORs of the genotype distribution of rs7747752 in the adjusted model were 2.19 (95% CI: 1.31-3.65) and 1.88 (95% CI: 1.13-3.15), respectively (**Table 2**).

Combined Effects With rs7747752 Genotypes (CC vs. CG vs. GG) and Low Serum Levels of GUDCA/DCA for the Risk of GDM

Using the GG genotype of rs7747752 and serum GUDCA > 0.07 nmol/mL as the reference, GUDCA ≤ 0.07 nmol/mL combined with CG and CC were associated with significantly increased risk of GDM after adjustment for traditional GDM risk factors, and the ORs were 9.58 (95% CI: 1.60-57.5) and 16.5 (95% CI: 2.59-106), respectively. In the same vein, using the GG genotype of rs7747752 and DCA > 0.28 nmol/mL as the reference, the combinations of rs7747752 CG or CC genotypes and DCA ≤ 0.28 nmol/mL were associated with markedly increased risk of GDM and ORs were 3.51 (95% CI: 1.50-8.24) and 8.06 (95% CI: 2.85-22.8), respectively (**Table 3**).

Additive Interactions Between rs7747752 C Allele (C vs. G) and Low Serum Levels of GUDCA/DCA for the Risk of GDM

Using rs7747752 G allele and GUDCA > 0.07 nmol/mL as the reference, the presence of rs7747752 C allele markedly increased the OR of GUDCA ≤ 0.07 nmol/mL for GDM in adjusted model from 4.04 (95% CI: 0.72-22.8) for GUDCA ≤ 0.07 nmol/mL alone to 9.02 (95% CI: 1.63-49.7) for the presence of both. There was a significant additive interaction between *CDKAL1* rs7747752 C allele and GUDCA ≤ 0.07 nmol/L for GDM (AP: 0.50, 95% CI: 0.17-0.83).

Similarly, *CDKAL1* rs7747752 C allele also markedly increased OR of DCA ≤ 0.28 nmol/mL for GDM in adjusted model from 1.67 (95% CI: 0.68-4.11) for DCA ≤ 0.28 nmol/mL

TABLE 2 | Odds ratios of the *CDKAL1* rs7747752 and bile acid metabolisms for increased risk of gestational diabetes mellitus.

	Unadjusted Model		Adjusted Model	
	OR (95% CI)	***P* value**	**OR (95% CI)**	***P* value**
Metabolites				
GUDCA ≤ vs. > 0.07 nmol/mL	7.40 (2.91-18.8)	<0.001	5.84 (2.13-16.0)	<0.001
DCA ≤ vs. > 0.28 nmol/mL	1.74 (1.17-2.59)	0.007	2.28 (1.43-3.64)	<0.001
Genetic variants				
rs7747752				
GG	1.00	–	1.00	–
CG	1.51 (0.95-2.41)	0.080	1.95 (1.15-3.31)	0.014
CC	2.19 (1.24-3.85)	0.007	3.07 (1.59-5.95)	0.001
Additive (CC vs. CG vs. GG)	1.48 (1.12-1.96)	0.007	1.76 (1.26-2.44)	0.001
Dominant (CC/CG vs. GG)	1.68 (1.08-2.62)	0.023	2.19 (1.31-3.65)	0.003
Recessive (CC vs. CG/GG)	1.63 (1.04-2.57)	0.034	1.88 (1.13-3.15)	0.016

OR, odds ratios; CI, confidence intervals; GUDCA, glycoursodeoxycholic acid; DCA, deoxycholic acid.
Adjusted Model, adjusted for traditional risk factors, including pre-pregnancy body mass index, family history of diabetes in first degree relatives, systolic blood pressure, current smoker before pregnancy and weight gain to the time of glucose challenge test.

alone to 4.24 (95% CI: 1.84-9.76) for copresence of both. The additive interaction was also significant (AP: 0.46, 95%CI: 0.09-0.83) (**Table 4**).

Mediation Effects of LPC18:0 and SFA16:0 for Copresence of rs7747752 C Allele (C vs. G) and Low Serum Levels of GUDCA/DCA to the Risk of GDM

In adjusted model analysis, copresence of rs7747752 C allele and DCA ≤ 0.28 nmol/mL was associated with markedly increased risk of high LPC18:0 (OR: 4.51, 95% CI: 1.79-11.3). Adjustment for LPC18:0 greatly attenuated the OR of copresence of rs7747752 C allele and DCA ≤ 0.28 nmol/mL from 4.24 (1.84-9.76) to 2.67 (0.71-9.99). The mediation effect of LPC18:0 on the risk association of copresence of rs7747752 C risk allele and DCA ≤ 0.28 nmol/mL with GDM was statistically significant (P of Sobel test: 0.003) (**Table 5**). On the other hand, the mediation effect of LPC18:0 on the risk association of rs7747752 C risk allele and GUDCA ≤ 0.07 nmol/mL with GDM was non-significant (P of Sobel test: 0.114). Whereas, further adjustment for SFA16:0 markedly enhanced the interactive effects of rs7747752 C risk allele and low GUDCA on the risk of GDM up to 7.20 (95% CI: 0.87-59.4), and slightly enhanced the risk association of rs7747752 C risk allele and low DCA with GDM (OR: 4.36, 95% CI: 1.83-10.4), both without any mediation effects (**Table S2**).

DISCUSSION

Our study found significant additive interactions between *CDKAL1* rs7747752 C allele carrier status and low serum levels of GUDCA and DCA for the markedly increased risk of GDM in Chinese pregnant women. The additive interactive effect of *CDKAL1* rs7747752 C allele carrier status and low serum levels

TABLE 3 | Risk associations of combinations of rs7747752 genotypes (CC vs. CG vs. GG) and low serum levels of GUDCA/DCA with gestational diabetes mellitus.

Genetic variants	Metabolites	Non-GDM (n=207)	GDM (n=207)	Unadjusted Model		Adjusted Model	
				OR (95% CI)	***P* value**	**OR (95% CI)**	***P* value**
Combinations of rs7747752 genotypes and GUDCA							
rs7747752	GUDCA (in nmol/mL)						
GG	>0.07	10 (4.83)	2 (0.96)	1.00	–	1.00	–
GG	≤0.07	54 (26.09)	41 (19.81)	6.32 (1.07-37.2)	0.042	4.88 (0.80-29.8)	0.086
CG	>0.07	22 (10.63)	5 (2.42)	1.81 (0.26-12.4)	0.546	2.42 (0.34-17.3)	0.378
CG	≤0.07	79 (38.16)	98 (47.34)	10.4 (1.79-60.5)	0.009	9.58 (1.60-57.5)	0.013
CC	>0.07	8 (3.86)	1 (0.48)	0.54 (0.04-6.74)	0.634	0.48 (0.03-6.95)	0.589
CC	≤0.07	34 (16.43)	60 (28.99)	15.9 (2.61-97.1)	0.003	16.5 (2.59-106)	0.003
Combinations of rs7747752 genotypes and DCA							
rs7747752	DCA (in nmol/mL)						
GG	>0.28	27 (13.04)	15 (7.25)	1.00	–	1.00	–
GG	≤0.28	37 (17.87)	28 (13.53)	1.40 (0.63-3.09)	0.406	1.70 (0.69-4.22)	0.250
CG	>0.28	44 (21.26)	34 (16.43)	1.38 (0.61-3.09)	0.435	1.57 (0.62-3.95)	0.341
CG	≤0.28	57 (27.54)	69 (33.33)	2.13 (1.03-4.38)	0.040	3.51 (1.50-8.24)	0.004
CC	>0.28	25 (12.08)	19 (9.18)	1.43 (0.58-3.53)	0.436	2.03 (0.71-5.75)	0.185
CC	≤0.28	17 (8.21)	42 (20.29)	4.66 (1.90-11.4)	<0.001	8.06 (2.85-22.8)	<0.001

OR, odds ratios; CI, confidence intervals; GUDCA, glycoursodeoxycholic acid; DCA, deoxycholic acid.
Adjusted Model, adjusted for traditional risk factors, including pre-pregnancy body mass index, family history of diabetes in first degree relatives, systolic blood pressure, current smoker before pregnancy and weight gain to the time of glucose challenge test.

TABLE 4 | Additive interactions between rs7747752 C allele (C vs. G) and low GUDCA/DCA for the risk of gestational diabetes mellitus.

	Unadjusted Model		Adjusted Model	
	OR/Estimate (95% CI)	***P* value**	**OR/Estimate (95% CI)**	***P* value**
Additive interaction between rs7747752 risk allele C (vs. G) and low GUDCA				
rs7747752 G allele & GUDCA > 0.07 nmol/mL	1.00	–	1.00	–
rs7747752 G allele & GUDCA ≤ 0.07 nmol/mL	5.52 (1.02-29.8)	0.047	4.04 (0.72-22.8)	0.113
rs7747752 C allele & GUDCA > 0.07 nmol/mL	1.20 (0.21-6.95)	0.838	1.43 (0.23-8.93)	0.700
rs7747752 C allele & GUDCA ≤ 0.07 nmol/mL	10.1 (1.90-53.5)	0.007	9.02 (1.63-49.7)	0.012
RERI	4.36 (-2.53-11.3)		4.54 (-2.66-11.7)	
AP	0.43 (0.11-0.76)		0.50 (0.17-0.83)	
SI	1.92 (0.90-4.13)		2.31 (0.85-6.24)	
Additive interaction between rs7747752 risk allele C (vs. G) and low DCA				
rs7747752 G allele & DCA > 0.28 nmol/mL	1.00	–	1.00	–
rs7747752 G allele & DCA ≤ 0.28 nmol/mL	1.36 (0.62-2.99)	0.444	1.67 (0.68-4.11)	0.266
rs7747752 C allele & DCA > 0.28 nmol/mL	1.34 (0.63-2.88)	0.448	1.64 (0.68-3.93)	0.269
rs7747752 C allele & DCA ≤ 0.28 nmol/mL	2.55 (1.26-5.17)	0.009	4.24 (1.84-9.76)	<0.001
RERI	0.85 (-0.27-1.96)		1.93 (-0.09-3.96)	
AP	0.33 (-0.12-0.79)		0.46 (0.09-0.83)	
SI	2.20 (0.33-14.8)		2.48 (0.66-9.34)	

OR, odds ratios; CI, confidence intervals; GUDCA, glycoursodeoxycholic acid; DCA, deoxycholic acid; RERI, relative excess risk due to interaction; AP, attributable proportion due to interaction; SI, synergy index.

Adjusted model, adjusted for traditional risk factors, including pre-pregnancy body mass index, family history of diabetes in first degree relatives, systolic blood pressure, current smoker before pregnancy and weight gain to the time of glucose challenge test.

of DCA on GDM was partially mediated *via* high LPC18:0. However, we failed to confirm that the copresence of *CDKAL1* rs7747752 C allele carrier status and low GUDCA on the risk of GDM was also mediated *via* high LPC18:0. In addition, the additive interactions between *CDKAL1* rs7747752 C allele carrier status and low serum levels of GUDCA and DCA on GDM risk were independent of high SFA16:0.

The role of BAs in the regulation of glucose metabolism is a hot topic of recent diabetes research. Several studies in pregnant women have attempted to address associations between BAs and GDM with inconsistent findings. A nested case-control study of 131 women with GDM and 138 controls in early pregnancy did not find significant differences in DCA, ursodeoxycholic acid (UDCA), and GUDCA between GDM and non-GDM women, but did observe that the primary BAs were down-regulated while some other secondary BAs were up-regulated in GDM versus non-GDM in the univariate analysis (31). Two other studies also did not detect a significant difference in DCA and UDCA between GDM women and their control group at 28th gestational weeks (13, 32). Using the restricted cubic spline technique, our group found that GUDCA and DCA had clear threshold effects on the risk of GDM, with the GUDCA ≤ 0.07 nmol/mL and the DCA ≤ 0.28 nmol/mL being associated with markedly increased risk of GDM (15). Our findings further indicated that women in low serum levels of GUDCA and DCA with rs7747752 C risk alleles had markedly increased the risk of GDM. It is worthwhile to investigate whether population differences in proportions of *CDKAL1* rs7747752 C allele carriers contributed to the inconsistent findings regarding the association between BA metabolites and the risk of GDM in different studies.

CDKAL1 gene has a strong association with GDM among the genes identified to date. *CDKAL1* genetic variants also have been shown to predict GDM and related glycemic traits (10), suggesting that it might have a synergistic effect with other risk

TABLE 5 | Mediation effect of LPC18:0 for interaction between rs7747752 C allele (C vs. G) and low DCA to increased risk of gestational diabetes mellitus.

	Beta (SD)	**OR (95% CI)**	***P* value**
Model A (LPC18:0 ≥ 18.0 nmol/mL as the outcome)			
rs7747752 C allele & DCA ≤ 0.28 nmol/mL	1.51 (0.47)	4.51 (1.79-11.3)	0.001
Model B (GDM as the outcome)			
LPC18:0 ≥ vs. < 18.0 nmol/mL	2.89 (0.38)	18.1 (8.51-38.3)	<0.001
Model C (GDM as the outcome)			
rs7747752 C allele & DCA ≤ 0.28 nmol/mL	0.98 (0.67)	2.67 (0.71-9.99)	0.145
Sobel test for mediation effect[†]			0.003

SD, standard definition; OR, odds ratios; CI, confidence intervals; LPC, lysophosphatidylcholines; GDM, gestational diabetes mellitus; DCA, deoxycholic acid.

*Model A was adjusted for the variables listed in the adjusted model in **Table 4**.*

*Model B was adjusted for traditional risk factors in the adjusted model in **Table 4**.*

*Model C was further adjusted for LPC18:0 ≥ 18.0 nmol/mL in addition to the variables listed in the adjusted model in **Table 4**.*

[†]P value of Sobel test < 0.05 indicating significant mediation effect.

factors. The rs7747752 under study was significantly associated with increased GDM risk. Similar to our findings, another study also found that Chinese women with the rs7747752 polymorphism were predisposed to a high risk of GDM (8). Previous studies have reported significant interaction effects of *CDKAL1* with lifestyle interventions, SFAs, and vitamin D on GDM risk (14, 17, 33). In a recent study, our group found that *CDKAL1* genetic variants had a significant additive interaction with serum SFAs, resulting in a markedly increased risk of GDM (14). The interaction between both risk factors may suggest that GDM develops when impaired beta-cell function cannot produce enough insulin in response to increased insulin resistance as manifested by high SFA16:0. In this analysis, we further found that the *CDKAL1* rs7747752 C allele carrier status had significant additive interactions with low GUDCA and low DCA for the risk of GDM, independent of high SFA16:0. Interestingly, LPC18:0 mediated the interactive effect of *CDKAL1* rs7747752 and low DCA on the risk of GDM.

To our knowledge, our study was the first that investigated additive interactions between the *CDKAL1* genetic variants (rs7747752) and low serum levels of GUDCA/DCA for the risk of GDM. We also observed that LPC18:0 but not SFA16:0 mediated the interactive effects of rs7747752 and low DCA on the risk of GDM. The functions of *CDKAL1* and methylthiotransferase in the regulation of metabolism are not fully understood, so do the mechanisms underlying the additive interactions between *CDKAL1* rs7747752 and low GUDCA/DCA. Recent studies have suggested several biological links between BAs and glucose regulation. First, a biological link between decreased DCA/GUDCA and GDM is plausible. Some BAs can activate FXR in the intestine stimulate the synthesis of fibroblast growth factor (FGF) 15/19 (34) and upregulate expression of pancreatic beta cells (35), which exerts pleiotropic effects on hepatic BAs metabolism as well as lipid, protein, and glucose metabolism (34). What's more, BAs-mediated TGR-5 signaling increases the release of GLP-1, which augments glucose-stimulated insulin secretion from pancreatic beta cells (36). Notably, the *CDKAL1* gene is tightly associated with impaired beta-cell function (37) and insulin secretion (38), thus being associated with increased risk of GDM. Hence, it is possible that copresence of low GUDCA/DCA and *CDKAL1* genetic variants markedly increased the risk of GDM *via* impaired beta-cell function. Second, multiple lines of evidence support a pathway from the additive interactions of low DCA/GUDCA and *CDKAL1* to increased LPC18:0 could play a role in the etiology of GDM. A mouse study found that increased intestinal taurine-beta-muocholic acid, an antagonist of FXR, reduced high-fat diet-induced increase in LPCs by inhibiting the activity of FXR (39). In this regard, another study showed that the biological link of gut microbiota-GUDCA-FXR was related to glucose intolerance (40). Furthermore, LPCs, the active metabolites of SFA16:0, promoted insulin resistance and cell death in diabetes by activating endoplasmic reticulum (ER) stress (41, 42). In this regard, our study found that high SFA16:0 enhanced the risk association of copresence of low DCA/GUDCA and *CDKAL1* genetic variants with the risk of GDM. Thus, it is also possible that impaired beta-cell function cannot produce enough insulin to cope with increased insulin resistance as caused by high LPC18:0, thereby triggering a high risk of GDM. Hence, the markedly increased susceptibility to GDM due to exposure to both *CDKAL1* rs7747752 C allele and low GUDCA/DCA is biologically plausible although we are not sure whether impaired insulin secretion or increased insulin resistance plays a dominant role. Indeed, molecular mechanisms underlying the interactions between rs7747752 and low GUDCA/DCA for GDM warrant further investigations.

Our findings have public health implications. GDM begets adverse short- and long-term health risks to the mother, developing fetus, and their offspring. Thus, understanding the pathophysiology of GDM and identifying potentially modifiable risk factors and biomarkers for early diagnosis of GDM is essential. In the contention, our study corroborated the link of the *CDKAL1* rs7747752 with GDM in Chinese pregnant women and confirmed that the additive interactions between rs7747752 and low serum levels of GUDCA/DCA for markedly increased risk of GDM, were independent of serum levels of SFA16:0. Notably, the additive interaction effect between rs7747752 and low DCA on the risk of GDM was partly mediated *via* increasing levels of LPC18:0. These risk genetic and metabolomics markers may be useful in the identification of women at high risk of GDM in early pregnancy.

Our research has several limitations. First, our study was a case-control study nested in a population-based prospective cohort of pregnant women in Tianjin, China. Our findings need to be replicated in other Chinese and non-Chinese cohorts of pregnant women. Second, due to busy clinical settings and a limited budget, lifestyle factors such as dietary habits were not collected. Third, we used a two-step GDM screening procedure to identify GDM and some GDM cases might have been missed. Fourth, universal OGTT in early pregnancy was not conducted due to tight budget and we cannot exclude the possibility that some women with GDM may have had prepregnancy impaired glucose tolerance.

In conclusion, we found that *CDKAL1* rs7747752 and serum GUDCA ≤ 0.07 nmol/mL and DCA ≤ 0.28 nmol/mL had significant interactive effects on the risk of GDM in Chinese pregnant women, independent of serum levels of SFA16:0. The additive interaction effect between *CDKAL1* rs7747752 and low DCA on GDM was partially mediated *via* increasing levels of LPC18:0. The discovery of the interactions between *CDKAL1* rs7747752 C risk allele and low DCA/GUDCA is an important step towards precision prevention of GDM in early pregnancy. The additive interaction has potential values in the prediction of GDM in early pregnancy that may benefit from specific intervention if validation studies can confirm our findings in other cohorts of pregnant women populations. It is also warranted to investigate the molecular roles of *CDKAL1* gene and methylthiotransferase in the regulation of metabolic and signaling pathways of BAs, SFAs, and LPCs for increased risk of GDM for better understanding of the etiology of GDM.

DATA AVAILABILITY STATEMENT

The data and code used during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Tianjin Women and Children's Health Center (TWCHC). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XYang, YW and ZF conceived the idea and designed the study. JLeng and WL collected the clinical data. ZF measured the metabolomics and integrated metabolomics data. YW measured the genetics and integrated the genetic data. HW and JLi analyzed the data and wrote the first draft. All the authors gave critical comments and edited the manuscript. XYang and HW took full responsibility for the work, including the study design, access to the data, and decision to submit. 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.2022.808956/full#supplementary-material

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The Nursing Effect of Individualized Management on Patients With Diabetes Mellitus Type 2 and Hypertension

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It focused on clinical effects of individualized nursing and health education (INHE) on patients with diabetes mellitus type 2 (T2DM) and hypertension. 68 patients were randomly rolled into two groups, 34 cases in the control group (group A) received routine nursing and remaining 34 cases in the experimental group (group B) received INHE. The disease knowledge mastery (DKM) and the effect of rehabilitation nursing of patients were compared. The results suggested that DKM of patients in group B was obviously greater ($P < 0.05$). The total effective rate (TER) in group B was 91.45%, which was observably greater than that (76.35%) in group A ($P < 0.05$). After nursing, the fasting plasma glucose (FPG), 2-hour postprandial glucose (2h PG), systolic blood pressure (SBP), and diastolic blood pressure (DBP) levels of all patients decreased, and those in group B were much lower ($P < 0.05$). Scores of the Self-Rating Anxiety Scale (SAS) and Self-Rating Depression Scale (SDS) of the two groups were 56.34 ± 8.12 points and 56.33 ± 8.01 points in group A, respectively; and those in group B were 42.52 ± 6.77 points and 41.71 ± 7.23 points, respectively; and they were all decreased and those in the group B were obviously smaller ($P < 0.05$). In summary, INHE can effectively improve the psychological cognition of patients with T2DM and hypertension and strengthen the control of blood pressure and blood sugar.

Keywords: individualized nursing and health education, individualized management, diabetes mellitus type 2, hypertension, rehabilitation efficacy

INTRODUCTION

As the rapid growth of the world economy and people's living standards, the dietary structure is changing greatly day by day, and factors such as faster and faster pace of life and lack of exercise have increased the incidence and prevalence of diabetes (1). Diabetes is a public health issue of general concern around the world. It is a lifelong chronic disease that can affect multiple organs; it is the third most common, frequently-occurring, and chronic non-communicable disease after

cerebrovascular disease and tumors; and it poses a serious threat to human health (2). Statistics show that there are about 347 million patients suffering from diabetes mellitus type 2 (T2DM) in the world so far, and its prevalence in low-income countries is more serious (3). Statistics from the Chinese Medical Association (CMA) Diabetes Association in 2007 showed that the prevalence of the disease in men and women over 20 years old in China is 10.6% and 8.8%, respectively, and the total prevalence is 9.7%. Based on this estimate, the total number of diabetic patients in China is currently around 92.4 million, and there are 14.82 million patients in the pre-diabetes stage (fasting plasma glucose (FPG) > 6.1 mmol/L, or 2-hour postprandial glucose (2h PG) > 7.8 mmol/L, but they have not yet reached the diagnosis criteria of diabetes). It accounts for nearly 10% and 15% of the total population, which is 4 times the statistical data in 1994 and twice the statistical data in 2001 (4, 5).

T2DM is an endocrine disease, which is very common clinically. It is characterized by high blood sugar, and it is closely related to factors such as weight (obesity) and genetics. In addition, the clinical manifestations are relatively mild at the initial stage of the illness. After the disease worsens, the symptoms of polydipsia, polyphagia, polyuria, and weight loss will occur, which greatly affect the patient's quality of life (6). Nowadays, evidence-based medicine and clinical studies have shown that prevalence of hypertension in T2DM patients is much greater than that of those without diabetes. In clinical hypertension, it is a very common complication of diabetic patients (7). A report from World Health Organization (WHO) showed that the prevalence of concurrent hypertension in diabetic patients is 20 - 40%, which is about 40 - 50% in China. A surgery from National Health and Nutrition Examination Survey III (NHANES III) found that 71% of 1500 diabetic patients have hypertension at the same time, and the incidence of concurrent hypertension in diabetic patients is 1.5 - 3.0 times that of patients without diabetes. On the contrary, hypertension patients are more likely to develop diabetes than patients with normal blood pressure, and the incidence of diabetes is 2.0 - 2.5 times that of the normal blood pressure group (8, 9). Through epidemiological investigations, it is found that nearly 40% of T2DM patients will have hypertension, and 5% - 10% of primary hypertension may suffer from T2DM (10). These data suggest that T2DM is inseparable from hypertension. The prevention and treatment of diabetes complicated by hypertension has to be solved urgently, so as to improve people's quality of life and health.

Incidence of hypertension in T2DM patients is increasing, and the patient's condition will become worse when comorbidities occur. If the symptomatic treatment is not timely, it will cause anxiety and depression symptoms of the patient, and cause damage to the eyes, nerves, heart, and other parts of the patient. What is more, serious comorbidities such as kidney failure, myocardium, and cerebral infarction will occur (11, 12). At the same time, the symptoms of anxiety and depression will in turn affect the patient's blood sugar and blood pressure control, making the symptoms of anxiety and depression more serious, and then forming a vicious circle. T2DM patients with hypertension have a very obvious curative effect in terms of drug treatment. However, symptoms such as anxiety and depression will seriously affect the occurrence and development of the disease (13).

In the past few years, the general nursing mode such as monitoring the patient's blood sugar and blood pressure has been mostly used. However, because most diabetic patients are accompanied by hypertension and are prone to some cardiovascular and cerebrovascular diseases, this nursing mode is not effective. Therefore, while actively treating patients, it is particularly important to use a scientific nursing model. The INHE, through scientific guidance on patients' lifestyle and medication habits, improves the quality of life of patients. For example, it can correct the bad habits of the patient's diet and ensure a balanced intake of nutrients, so as to effectively control the weight gain, so that the incidence of diabetes is greatly reduced. It can guide patients to carry out reasonable and effective aerobic exercise, help expand the peripheral blood vessels of the body, and reduce peripheral resistance, thereby reducing the occurrence and development of hypertension. There are relatively many clinical studies on the individualized care model or health education for patients with diabetes and hypertension, but relatively few studies on the combination of the two methods (14, 15). Therefore, it explored the clinical effect of the combination of INHE on patients with T2DM and hypertension in this work.

RESEARCH MATERIALS AND METHODS

General Data of Included Patients

68 patients with T2DM complicated with hypertension admitted to the Endocrinology Department of our Hospital from March 2018 to February 2020 were rolled into a group B and a group A using the random number table method. 34 cases in the control group (group A) received routine nursing and remaining 34 cases in the experimental group (group B) received INHE. Patients included had to meet the following criteria. First, patients suffered from the symptoms in line with the diagnostic criteria for diabetes defined by World Health Organization (WHO) and complied with the diagnostic criteria for hypertension defined by the *Guidelines for the Prevention and Treatment of Hypertension* in China. Second, the age of the patient was between 34 and 78 years old, and the course of the disease was 3 to 20 years. Third, the measured FPG was ≥ 7.0 mmol/L, the measured 2h PG was ≥ 11.1 mmol/L, the systolic blood pressure (SBP) was ≥ 140 mmHg, and the diastolic blood pressure (DBP) was ≥ 90 mmHg. If following items can be meet, the patients had to be excluded from this study: patients with severe functional disorders of any organ; patients with cancer or secondary diabetes; patients with hearing and speech impairment and dementia; and patients being unable to cooperate and accept the treatment. The general conditions of patients were shown in **Table 1**.

No statistically obvious difference was found between the two groups of patients in gender, age, course of disease, and other data ($P > 0.05$). The patients and their families in this study had fully understood the situation and signed the informed consent

TABLE 1 | General data of patients.

	Group B	Group A
Number of cases	34 cases	34 cases
Gender ratio	Males: females = 20:14	Males: females = 23:11
Age	46 ~ 78 years old	43 ~ 74 years old
Average age	56.37 ± 3.56 years old	56.71 ± 3.82 years old
Course of disease	5 ~ 13 years	5 ~ 12 years
Average course of disease	8.02 ± 1.83 years	7.87 ± 1.80 years
FPG	9.73 ± 1.78 mmol/L	9.61 ± 1.72 mmol/L
2h PG	15.53 ± 2.65 mmol/L	15.57 ± 2.64 mmol/L

forms. The medical ethics committee of the hospital had known and agreed to implement it.

Methods of Individualized Management

Patients in group A underwent routine nursing, which referred to routine (admission and diet) guidance, disease introduction, regular monitoring of patients' FPG and 2h PG levels, and changes in blood pressure. It should monitor the blood sugar and blood pressure control situation of patients, arrange medication plan accordingly, and guide the patient to use medication rationally. Patients in the group B were given INHE on the basis of treatment ways in the group A. The detailed methods were as follows:

The health education was implemented as follows. After the patient was admitted to the hospital, a specific person would be arranged to carry out admission publicity and education to the patient and his family with a kind attitude to tell the patient and his family about the disease-related knowledge, preventive measures, medication plan, emergency management, drug effects and adverse reactions, and exercise plan during the rehabilitation period. The person should give timely and effective responses to patients' doubts, and regularly distribute materials and play videos to explain relevant knowledge. It should strengthen the awareness of diabetes and confidence in treatment through visits and questionnaire surveys. In this way, it aimed to build a harmonious relationship between doctors and patients and allow patients to actively cooperate with treatment.

The individualized care model was implemented as follows.

First, after the patient was admitted to the hospital, the nurses carefully told the patient and their family about the ward's environmental equipment, the doctor in charge, and the nurse with a kind attitude, give them more care and nursing, eliminate their bad mood, and make the patient actively cooperate with the treatment.

Second, it can plan the patient's personal meal plan. For example, it should confirm the total calories required per day and the distribution method of nutrients required for each meal; instruct them to eat less high-cholesterol and high-fat foods, eat more high-potassium and high-calcium foods and more dietary fiber and vitamin content food, avoid drinking black tea, and control the intake of sodium, fat, and carbohydrates.

Third, it should strictly abide by the doctor's orders, and explain in detail the mechanism of action and adverse reactions of the drug to patients and their families. At the same time, it should inform the patient that metformin drugs should be taken after meals, sulfonylurea hypoglycemic drugs should be taken 30 minutes before meals, and α-glucose anhydrase inhibitors should be taken with the first meal. If symptoms of hypotension (dizziness, nausea, vomiting, etc.) occur, it can slowly move to the supine position with head down and feet up to increase the return to the heart. When paleness, palpitations, and low blood sugar reactions occur, the sugar can be dissolved in warm water and used, and the above reactions can be quickly relieved. If the symptoms are not relieved, they should be sent to a doctor immediately; and the patient and their family members are advised to act mildly to avoid the occurrence of orthostatic hypotension and cerebrovascular disease.

Fourth: it should measure and record blood pressure at 7:00 ~ 8:00 and 19:00 ~ 20:00 every day. When blood pressure was stable, it can be measured 1 ~ 2 times a week, and when there is fluctuation, it can be measured 1 ~ 2 times a day. In addition, it can monitor and record blood glucose levels before and after breakfast, lunch and dinner, and before bedtime. The blood lipids, glycosylated hemoglobin (Hemoglobin, HbA1c), urine glucose (Urine glucose, GLU), and urine ketone levels should be measured and recorded. It should instruct the patient's family to use blood pressure monitor, blood glucose meter correctly.

Fifth, it should explain to patients and their families the importance of exercise for disease recovery, so that patients can better control blood sugar without metabolic disorders. It should instruct patients to choose appropriate exercise (walking, square dancing, swimming, cycling, etc.) and time 1 hour after meals according to their own conditions, and exercise 3 to 5 times a week. At the same time, it should make regular health assessments, and go out to exercise to prepare candy or other sugary substances to prevent hypoglycemia.

Observation Index and Effect Evaluation Standard

The disease knowledge mastery (DKM) of patients was analyzed and compared after the intervention from 5 aspects to understand the mastery status, including diet, rehabilitation, nursing, medication guidance, and precautions. The total score of each aspect was 10 points, and the score was proportional to the degree of DKM.

The clinical efficacy was compared and graded into three levels. Grade I (excellent) meant that the symptoms of diabetes and hypertension were effectively controlled, the nursing effect was maintained for more than 1 year, the blood pressure level was less than 130/85 mmHg, the FPG level was 3.9 ~ 6.1 mmol/L,

and the 2h PG was less than 10.0 mmol/L. Grade II (good) meant that diabetes and hypertension were basically controlled, but the effect was relatively unstable, and a phenomenon greater than the prescribed value occurs within 1 year. Grade III (poor) meant that blood pressure and blood sugar did not meet the specified value standards. The total effective rate (TER) = (level I + level II) / total number of cases × 100%.

Changes in blood sugar and blood pressure of patients were compared before and after nursing. PFG in the morning and 2h PG were detected by enzyme-linked immunosorbent assay (ELISA) using a Pumen automatic chemiluminescence analyzer (eCL8000, Shenzhen Hewlett Packard Medical Technology Co., Ltd.). PG level. The adult desktop sphygmomanometer from Shanghai Mediffin was adopted to monitor the patient's DBP and SBP (the patient was instructed to rest quietly for more than 5 minutes before the test, do not smoke and drink coffee, and empty the bladder half an hour before). The average of the two monitoring results was calculated and recorded. If the difference in DBP and SBP was greater than 5 mmHg, then the average of the three monitoring results was calculated and recorded.

The mental states of patients before and after nursing were analyzed and compared. *Self-rating Depression Scale* (SDS) and *Self-Rating Anxiety Scale* (SAS) were used to evaluate the anxiety and depression of the two groups of patients before and after nursing. There were 20 items for each scale, each with 0 ~ 4 points. The critical value of the SDS scale was 53 points, 53 ~ 62 indicated the depression was mild, 63 ~ 72 indicated the depression was moderate, and > 72 indicated the depression was severe. The critical value of the SAS scale was 50 points, 50 - 59 indicated mild anxiety, 60 - 69 indicated moderate anxiety, and greater than 70 indicated severe anxiety.

Statistical Analysis

The statistical data was processed by SPSS22.0. The count data was expressed in the form of N/%, using the χ^2 test, and the measurement data was expressed as $\bar{x} \pm s$, using the t test. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

DKM

The DKM of two groups of patients was analyzed, as shown in **Figure 1**.

Figure 1 illustrated that DKM of the patients in the group B was remarkably greater than that of the group A, showing statistically great difference ($P < 0.05$).

Clinical Efficacy

Comparison on the clinical efficacy of the two groups were shown in **Figure 2**.

As shown in **Figure 2**, there were 10 cases of grade I and 16 cases of grade II in group A; and 19 cases of grade I and 12 cases of grade II in group B. From the equation of TER = (grade I + grade II)/total number of cases × 100%, it can calculate the TER of the two groups of patients. The TER of patients in group B was 91.18%, which was significantly greater than that of group A (76.47%), and the difference was statistically significant ($P < 0.05$).

Blood Sugar, Blood Pressure, and Rehabilitation Effect Before and After Nursing

The levels of FPG, 2h PG, SBP, DBP, and rehabilitation effect of the two groups of patients before and after nursing were shown in **Figures 3**, **4**.

As illustrated in **Figures 3**, **4**, after nursing, the levels of FPG, 2h PG, SBP, and DBP in the two groups of patients were significantly decreased, and those in group B were much lower than group A ($P < 0.05$). Similarly, the rehabilitation effect of patients in group B was significantly better than that in group A, and the difference was statistically significant ($P < 0.05$).

Mental State Before and After Nursing

The scores of the Self-rating Depression Scale (SDS) and the Self-rating Anxiety Scale (SAS) of the patients in the two groups before and after nursing were shown in **Figures 5**, **6**.

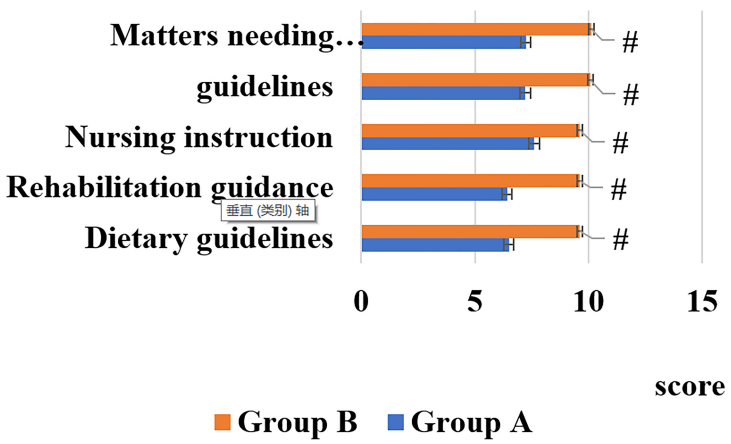

FIGURE 1 | DKM of patients. [# indicated that compared with group A, the difference was statistically significant ($P < 0.05$)].

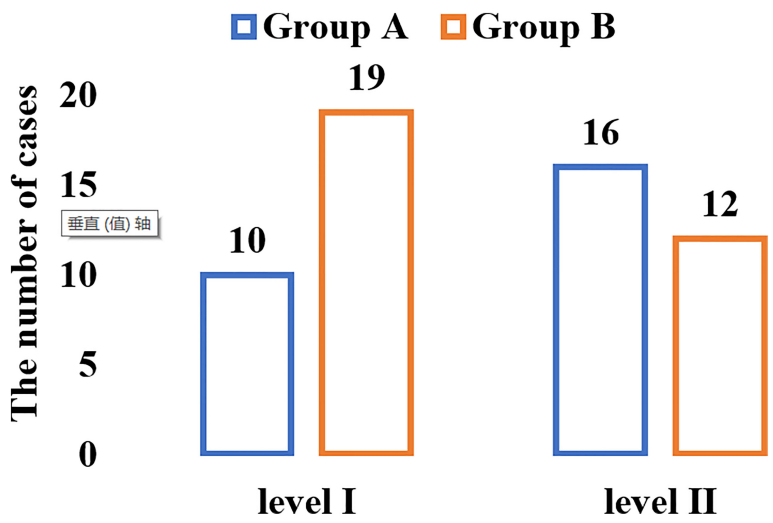

FIGURE 2 | The results of the comparison of the clinical efficacy of the two groups of patients.

After nursing, the scores of the SDS and SAS of all patients in groups A and B decreased, and those in the group B were significantly smaller than the scores in the group A, showing statistically visible differences ($P < 0.05$).

DISCUSSION

T2DM is a chronic disease caused by insufficient insulin secretion by pancreatic β-cells or caused by insensitivity of target cells to insulin. Its course is longer, the condition is more complicated, and there are many complications, the prognosis is poor, and it is easy to relapse (16). One of the very common complications of T2DM is hypertension, which is prone to cause cardiovascular and cerebrovascular diseases. The mortality and disability rate are relatively high. It is one of the important causes of death in diabetic patients and seriously threatens the patient's life safety (17). Some scholars believe that the cause of hypertension associated with T2DM is that the increase in blood sugar inhibits the relaxation of vascular endothelium, stimulates the transcription of vascular smooth factor genes, and leads to the emergence of hypertension. In addition, the main risk factors for increased mortality from cardiovascular and cerebrovascular diseases are hypertension, hyperglycemia, and lipid metabolism disorders (18, 19). Therefore, increasing the regulation of blood sugar and blood pressure in patients with T2DM accompanied by hypertension is of high significance for the prevention of cardiovascular and cerebrovascular diseases.

At present, methods such as reducing blood sugar and blood lipids, adjusting body weight, and changing lifestyles are commonly used clinical methods to treat T2DM with hypertension. However, most patients have symptoms such as anxiety and depression due to long treatment time and repeated illnesses, resulting in poor control of blood sugar and blood pressure levels, resulting in a low treatment rate (20). Therefore, while actively treating patients, it is particularly important to carry out a scientific and reasonable nursing model. The implementation of individualized health education combined with a nursing model can enhance the patient's enthusiasm for cooperating with treatment, eliminate bad emotions such as anxiety and depression, and effectively improve the patient's quality of life.

After INHE, the levels of FPG, 2h PG, SBP, and DBP, and the scores of SDS and SAS in the two groups were decreased, and those in group B were significantly lower than those in group A ($P < 0.05$). The DKM and rehabilitation effect of patients in group B were significantly higher than those in group A ($P < 0.05$); and the TER of patients in group B was 91.18%, which was higher than 76.47% in group A ($P < 0.05$). It can be found that INHE can effectively improve the clinical treatment effect of patients, and has very good professionalism, pertinence, and effectiveness. In this work, the psychological and physiological characteristics of patients are combined for health education for them, which strengthened the patients' awareness of the disease and better cooperates with clinical treatment. Combined with the individualized care model, effective psychological counseling is provided to patients, to eliminate negative emotions, and to promote patients' mastery of the use of various commonly used drugs. Thereby, it can effectively prevent abnormal blood pressure and blood sugar of patients and improve the quality of life of patients.

CONCLUSION

INHE was implemented for patients with T2DM complicated with hypertension to observe and compare DKM and

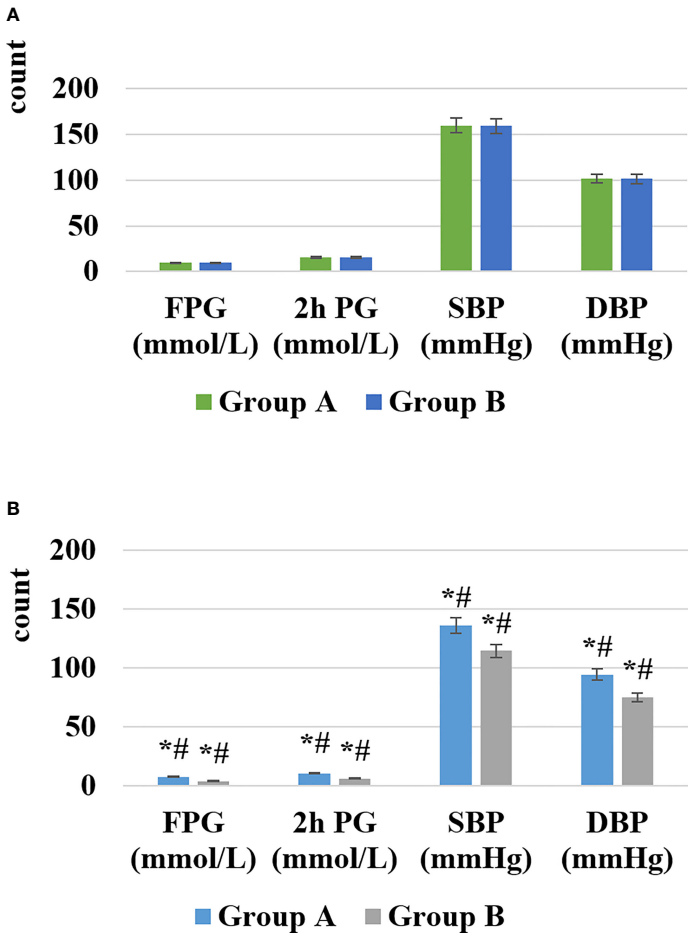

FIGURE 3 | Comparison of blood glucose and blood pressure before and after nursing [**(A)** showed the change before nursing, **(B)** showed the change after nursing; *# represented the comparison with the case in the same group before nursing, the difference was statistically significant ($P < 0.05$)].

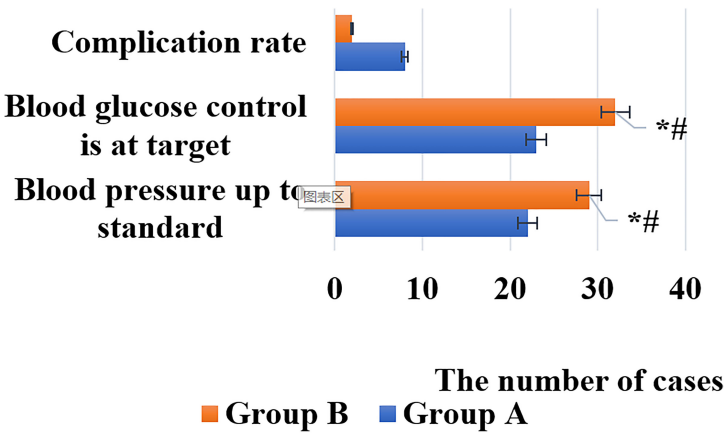

FIGURE 4 | Comparison of rehabilitation effects between the two groups of patients [*# indicated that compared with group A, the difference was statistically significant ($P < 0.05$)].

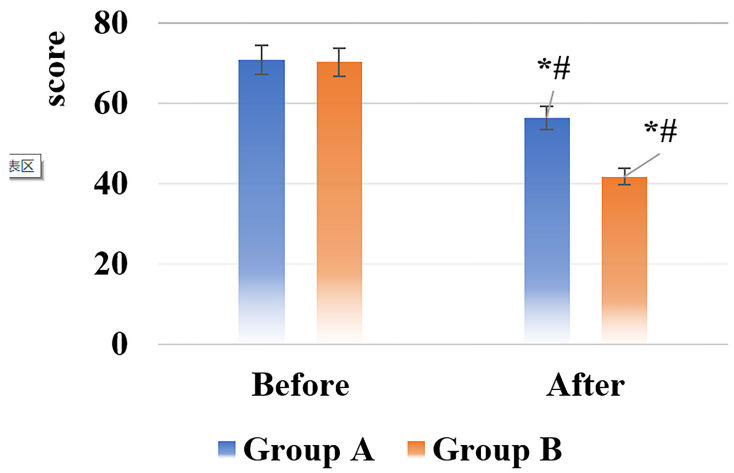

FIGURE 5 | SDS score results of the two groups of patients before and after nursing [*# indicated that compared with the same group before nursing, the difference was statistically significant ($P < 0.05$)].

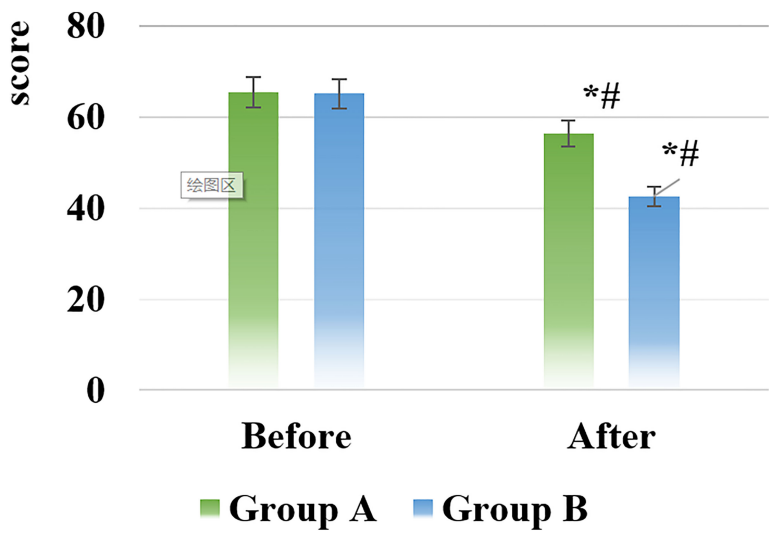

FIGURE 6 | SAS score results of the two groups of patients before and after nursing [*# indicated that compared with the same group before nursing, the difference was statistically significant ($P < 0.05$)].

rehabilitation effect of patients in the group B and the group A of patients. It was found that INHE can effectively improve the psychological cognition of patients with T2DM by hypertension, strengthen the control of blood pressure and blood sugar, and improve their quality of life. The innovation of this work lied in the combination of individualized nursing intervention and health education, which was more conducive to the rehabilitation of patients with diabetes and hypertension. However, the sample size was small, and it needed to further expand the research in the future, aiming to use more clinical experiments to verify the conclusion.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RL: conceptualization, data collection and analysis, and writing–original draft. WX: data collection and wrting–original draft. PY: visualization and data collection. LT: software and visualization. ZL: writing–editing and reviewing. XG: writing–editing and reviewing, and project administration. All authors contributed to the article and approved the submitted version.

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Characterizing and Evaluating Diurnal Salivary Uric Acid Across Pregnancy Among Healthy Women

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Uric acid levels during pregnancy have been examined as a potential indicator of risk for gestational diabetes mellites, hypertension, and related adverse birth outcomes. However, evidence supporting the utility of serum uric acid levels in predicting poor maternal and fetal health has been mixed. The lack of consistent findings may be due to limitations inherent in serum-based biomeasure evaluations, such as minimal repeated assessments and variability in the timing of these assessments. To address these gaps, we examined repeated measurements of diurnal salivary uric acid (sUA) levels in a sample of 44 healthy women across early-mid and late pregnancy. We assessed potential covariates and confounds of sUA levels and diurnal trajectories, as well as associations between maternal weight gain and blood pressure during pregnancy and sUA concentrations. Using multilevel linear models, we found sUA increased across pregnancy and displayed a robust diurnal pattern with the highest concentrations at waking, a steep decline in the early morning, and decreasing levels across the day. Maternal pre-pregnancy BMI, age, prior-night sleep duration, and fetal sex were associated with sUA levels and/or diurnal slopes. Maternal blood pressure and gestational weight gain also showed significant associations with sUA levels across pregnancy. Our results expand upon those found with serum UA measurements. Further, they demonstrate the feasibility of using at-home, minimally-invasive saliva sampling procedures to track UA levels across pregnancy with potential applications for the long-term monitoring of maternal cardiometabolic risk.

Keywords: uric acid (UA), saliva, pregnancy, blood pressure (BP), body mass index - BMI, diurnal pattern, APrON study

1 INTRODUCTION

Gestational diabetes mellitus (GDM) and hypertensive disorders are two of the most common complications experienced during pregnancy. Hypertensive disorders are the second leading obstetric cause of maternal death in the world, and both GDM and high blood pressure (BP) during pregnancy are related to poor health outcomes for mothers and their children, including increased risk of type 2 diabetes mellitus (T2DM) and cardiovascular conditions after pregnancy, problems with fetal growth, and birth complications (e.g., pre-term birth, cesarean delivery) (1–4). Thus, identifying and intervening with women at high risk of GDM and hypertensive disorders during pregnancy is important for supporting maternal and child health globally (5–7). A key research priority in this effort is the development and validation of novel biomarkers of risks during pregnancy that can be assessed on a large-scale, at low cost, and in ecology-valid settings (5, 7). To begin to address this need, we conducted a rigorous examination of uric acid (UA) levels measured in saliva, and the potential covariates and confounds of UA concentrations, using at-home, minimally-invasive biospecimen collection protocols.

A large body of research has examined UA levels in blood during pregnancy as a potential indicator of increased risk of gestational diabetes, hypertension, preeclampsia, and other related adverse health outcomes. Serum UA levels, particularly in early pregnancy, have been associated with increased risk of developing GDM (8–11). High concentrations of serum UA during pregnancy have also been associated with high BP and preeclampsia, as well as related poor birth outcomes such as lower birth weight and earlier gestational age at birth (12–19). Further, among women with GDM or hypertensive disorders in pregnancy, UA levels may help identify a subset of women at particularly high risk of poor maternal or fetal outcomes, such as preterm birth, small for gestational age, and preeclampsia (16, 20–23). The literature connecting serum UA with GDM and hypertensive disorders in pregnancy, however, is mixed with marked heterogeneity across study designs, samples, and testing frequency protocols (16). For example, a recent meta-analysis found no evidence that serum UA predicted preeclampsia, eclampsia, fetal/neonatal death, low birthweight, or preterm birth among pregnant women with high BP (16). While another meta-analysis published in the same year comparing serum UA in pregnant women with and without preeclampsia found serum UA levels, particularly in the third trimester, predicted hypertensive disorders during pregnancy including preeclampsia and eclampsia (15). Notably, both studies highlighted the need for more rigorous and large-scale investigations and rated the quality of the evidence reviewed as low to moderate (15, 16). Despite this, the International Society for the Study of Hypertension in Pregnancy recommends monitoring UA levels of women with chronic hypertension in pregnancy to track risk of poor maternal and fetal outcomes (24). Thus, the utility of UA as an indicator of risk among pregnant women and the role of UA during pregnancy remains unclear (16, 25–29).

While serum UA concentrations in the general population correlate with, and in some cases predict, cardiometabolic conditions such as T2DM, hypertension, cardiovascular disease, and obesity (30–34), UA levels undergo natural changes during pregnancy that could alter these associations. Early in gestation, serum UA levels decrease as a result of increased blood volume, increased estrogen, and changes in UA reabsorption in the kidneys (27, 35, 36). UA levels rise across pregnancy, reaching pre-pregnancy levels toward the end of the third trimester (14, 36, 37). While UA may represent a byproduct of metabolic and hypertensive risk among pregnant women, indicating pre-existing obesity, metabolic conditions, or renal problems (14, 27), UA may also play an active role in the development of these disorders through increased inflammation, oxidative stress, and endothelial dysfunction (26, 27, 29, 38). High levels of UA in early pregnancy, and changes from early to late pregnancy, may be important predictors of GDM and hypertensive disorders (9–11, 13, 14, 39). However, few studies have examined UA longitudinally across pregnancy (see (13, 14) for exceptions), and those that have measured UA across gestation vary in the timing and frequency of assessments. For example, Powers and colleagues examined serum UA multiple times across gestation to evaluate patterns of UA across pregnancy among healthy women and those with preeclampsia (14). However, their findings were limited by differences in the number UA assessments across study groups and a lack of standardization in the timing of biospecimen collections (14). A recent prospective examination of repeated measurements of salivary UA (sUA) across pregnancy found sUA levels predicted preeclampsia and pregnancy-induced hypertension (13). However, this study did not consider potential confounding variables, such as obesity and race/ethnicity, and only examined sUA levels in the early morning (13). Diurnal variation in UA levels, and how variability in UA levels across the day may be related to health, represents another significant gap in our understanding of UA's role during pregnancy. UA has been shown to display a marked diurnal pattern in adults (40–43). Insights from patients with gout and those with hypertension highlight the importance of diurnal variation in symptom severity and suggest potential involvement of UA levels in these associations (44, 45). Two small studies conducted over 30 years ago suggest that the diurnal pattern of UA is generally conserved during pregnancy, especially among women with hypertension (46, 47). However, a thorough examination of diurnal UA across pregnancy has not been conducted.

Recent advancements allowing for the measurement of UA in saliva (48) provide opportunities to study the dynamic pattens of UA across the day and across pregnancy. Salivary UA levels are correlated with blood levels (correlation coefficients= .47-95) (41, 48–50). The few studies that have examined sUA during pregnancy have found significant relations between higher sUA levels and high BP, preeclampsia, pre-term birth, and lower birth weight, but no differences between sUA levels in women with and without GDM (13, 50, 51). These studies, however, include only one assessment of UA per day or only one assessment across pregnancy, and there is variability in the timing of these samples across the day. Furthering our understanding of sUA levels in pregnancy has important implications for advancing minimally-

invasive and inexpensive screening and monitoring practices for pregnant women at risk of GDM and hypertensive disorders. With self-collection and at-home saliva sampling protocols well-established, extended sUA monitoring during pregnancy could occur alongside current recommended procedures for blood sugar and BP monitoring among high-risk pregnant women (4, 24).

In the present study, we used data from a longitudinal investigation of women across pregnancy to address gaps in our understanding of the measurement and utility of sUA during pregnancy and how it relates to maternal sociodemographic and health characteristics. Using multiple assessments of sUA collected during early-mid and late pregnancy from a sample of low-risk, mid/high-socioeconomic status (SES), generally healthy women, we addressed three aims: 1) characterize the diurnal pattern of sUA among pregnant women and assess changes in this pattern across pregnancy; 2) examine potential covariates and confounds of sUA levels across the day and across pregnancy; and 3) assess relations between maternal weight gain and blood pressure during pregnancy and maternal sUA levels and diurnal patterns.

We expected that sUA diurnal trajectories would follow the same pattern as previously reported for serum UA among non-pregnant women and men with higher levels in the morning and declining levels across the day (40). We anticipated lower sUA levels in earlier pregnancy than in later pregnancy (37), and that maternal body mass index (BMI) and age would be positively associated with sUA levels while the duration of sleep the prior night would show inverse associations with sUA (20, 52). The importance of other maternal and saliva sample characteristics, such as maternal education, socioeconomic status, race/ethnicity, prior pregnancies, medication use, oral health, and salivary flow rate, in predicting sUA concentrations and/or confounding the relations of interest in the study were explored based on prior work (14, 20, 53–55). Finally, we expected excessive weight gain and hypertension during pregnancy would be positively associated with sUA levels.

2 MATERIALS AND METHODS

2.1 Study Sample

This study used data from a subsample of participants enrolled in the Fetal Programming study. Detailed descriptions of the Fetal Programming study participants and procedures have been previously reported (56). The Fetal Programming study is a prospective cohort study of 272 women recruited from prenatal clinics and the community in 2011-2012 who were part of a larger study called the Alberta Pregnancy Outcomes and Nutrition (APrON) study (57, 58). Inclusion criteria required that participants be at least 16 years old, pregnant with a singleton fetus, and able to read and write in English. Exclusion criteria were: taking a steroid medication, smoking, consuming alcohol or drugs, or known fetal or pregnancy complications (e.g., fetal genetic anomalies, gestational diabetes or hypertension). A subsample of 44 Fetal Programming participants was selected for this study based on the volume of saliva remaining in archived biospecimens collected during the initial study procedures and without consideration of maternal nor fetal/infant characteristics (see **Supplemental Materials** for additional information about the subsample).

2.2 Procedures

Data for this study were primarily collected at two prenatal time points: time 1 (T1) in early-mid pregnancy [5-21 weeks gestation, MN(SD)=14.01 weeks (3.41)], and time 2 (T2) in late pregnancy [30-34 weeks gestation, MN(SD)= 32.35 weeks (0.70)]. At each time point, mothers self-collected salivary biospecimens in their homes over two consecutive days (excluding weekends). On each day, participants provided four whole unstimulated saliva samples using Salivabio oral swabs placed under the tongue (total of 16 sampling occasions per participant). A personal digital assistant (PDA) was provided to each participant to assist with data collection. The PDA signaled saliva sampling instructions upon waking (allowing for individualized wake times), 30 minutes after waking, and at 1130h and 2100h. Participants were instructed to refrain from consuming food, caffeine, citric drinks, and dairy, and to avoid vigorous exercise and brushing their teeth in the 30 minutes prior to saliva collection. Participants reported adherence to these guidelines in the PDA. They were also instructed to reschedule saliva collections if they had recent dental work or acute illness. For each saliva sample, except those collected upon waking, the PDA recorded the sample collection start and end times. To reduce participant burden, women were not asked to record collection duration times nor adherence to the eating, teeth brushing, and exercise instructions for the waking saliva samples. After collection, participants stored their samples in their home freezers. Saliva samples were transferred frozen to the Institute for Interdisciplinary Salivary Bioscience Research at the University of California, Irvine and archived. All participants provided written informed consent, and the study procedures were approved by the University of Calgary Conjoint Health Research Ethics Board.

2.3 Measures

2.3.1 Uric Acid

Participants' archived saliva samples were assayed for UA in duplicate using a commercially available kit (Catalog #1-3802, Salimetrics, Carlsbad, CA). The test volume was 10 μL, and the range of the assay was 0.07 to 20 mg/dL. The inter- and intra-assay CVs were 4.57% and 4.45%, respectively.

2.3.2 Saliva Sample Characteristics and Confounds

The diurnal timing of each saliva sample was indexed as time since waking (minutes) using participant-reported waking and sample collection times. Data regarding common confounders of salivary analyte levels, including flow rate, and recent (within the 30 minutes prior to collection) eating, teeth brushing, and exercising (yes/no), were available for all non-waking saliva samples. Flow rate (mL/minute) was calculated using PDA-recorded sample collection durations (minutes) and saliva volume (mL) estimated by sample weight.

2.3.3 Maternal Sociodemographic, Health, and Pregnancy Characteristics

Upon enrollment, mothers reported their age (years), race/ethnicity, education, family income, number of prior pregnancies, pre-pregnancy height and weight (used to calculate pre-pregnancy BMI), and the sex of their fetus. Due to the distribution of the data in our sample, education and family income data were dichotomized as Completed University (yes/no) and Family Income <$100,000 (yes/no). The majority of participants were white (n_{white}=35; n_{Asian}=6; n_{Arab}=1; $n_{Latin\ American}$=2), so the relations between race/ethnicity and sUA levels were only examined in sensitivity analyses.

Self-reported medication use and overall oral health were assessed twice (once at T1 and once at T2) during an interview with study staff. Participants rated their oral health on a scale from 1 (poor) to 3 (good), and data were dichotomized (good/adequate) based on the distribution of responses. Medication use was rare (any use n=9 and 12 at T1 and T2, respectively), and a wide range of medications were reported. Therefore, relations between medication use and sUA levels were only examined in sensitivity analyses.

Prior night sleep duration was assessed on all four days of data collection using self-reported sleep and wake times. Within- and between-individual effects of prior night sleep duration were modeled separately. Within-individual effects were indexed by the difference between a participant's sleep duration (hours) on a given day and her average duration of sleep across the study period. Between-individual effects were indexed by the average duration of prior night sleep across the four study days.

2.3.4 Maternal Weight Gain and Blood Pressure During Pregnancy

Each participant was assigned a gestational weight gain (GWG) Group (i.e., below, within, or above the recommended gains) using self-reported pre-pregnancy BMI, measurements of maternal weight assessed by trained study staff in the 1st, 2nd, and 3rd trimesters, and self-reported data about women's highest weight during pregnancy (assessed at a 3-month postpartum study visit) (59, 60). Total GWG and pre-pregnancy BMI were used to classify women as either below, within, or above the total GWG recommendations of the Institute of Medicine (59, 60).

Systolic and diastolic BP data were extracted from participants' medical records. Thirty participants had BP data available at both prenatal time points (T1 and T2). These assessments were conducted by medical staff during clinical visits. The number of BP assessments per participant ranged from 1 to 13 (MN(SD)=8.38(2.95)) with 0 to 5 conducted during gestational weeks 5-21 (MN(SD)=2.00(1.36)) and 1 to 3 conducted during weeks 30-34 (MN(SD)=2.31(0.66)). Each BP assessment was assigned a BP category (normal, elevated, hypertension-stage 1, hypertension-stage 2, or hypertensive crisis) based on the American Heart Association guidelines (61). BP categories were coded from 0-4 (normal-hypertensive crisis). Two indictors were computed and examined: 1) an Overall Average BP Category Score across pregnancy to index between-person BP effects; and 2) a Within-individual BP Change index was created for each participant by computing the difference between the average BP category score for a given timepoint (T1 or T2) and the average BP category score across both timepoints.

2.3.5 Pregnancy Stage

To examine changes in sUA levels and diurnal patterns across pregnancy, and whether maternal characteristics were differentially associated with sUA levels in early-mid *vs*. late pregnancy, a Pregnancy Stage variable was created to code data collected in early-mid (T1; 5-21 weeks gestation) *vs*. late pregnancy (T2; 30-34 weeks gestation). Gestational age at the time of data collection was calculated using maternal-report of the first day of her last menstrual period.

2.4 Statistical Approach

2.4.1 Analytic Sample and Preliminary Analyses

The distribution and range of all data were examined, and sUA data were assessed for normality and kurtosis. Raw sUA data were plotted across the day and across pregnancy (**Figure 1**; **Supplementary Figure 1** and **Supplementary Table 1**). Tests of association (e.g., Spearman's and Pearson's correlations, t-tests and Kruskal-Wallis rank tests) explored relations between sUA concentrations at each sampling occasion and the potential covariates. A Wilcoxon signed-rank test assessed changes in average BP category scores from T1 to T2. Spearman's correlations evaluated the relations between pre-pregnancy BMI and Overall Average BP Category Scores across pregnancy and within-individual changes in BP category scores from T1 to T2.

2.4.2 Characterization of Diurnal Salivary UA Across Pregnancy

We used multilevel linear mixed models to examine change in sUA across the day and across pregnancy. Three-level models predicted sUA levels using data from all 16 saliva samples per participant. The models included random intercepts for day and participant to account for the nesting of sampling occasions (level 1) within day (level 2) and participant (level 3). The slope of change in sUA levels across the day (indexed using Time Since Waking (minutes); level 1) and Pregnancy Stage (early-mid *vs*. late; level 2) were the only independent variables included in these models. Given the non-linear change in sUA across the day (**Figure 1**), we used a piecewise approach to model the diurnal slopes of sUA. Rather than modeling Time Since Waking continuously, it was modeled using two variables- one indexing the slope of change in sUA levels from 0 to 34 minutes post-waking (i.e., Morning Slope) and one indexing the slope of change in sUA levels from 34 minutes post-waking to the time of the last saliva sample of the day (i.e., Afternoon/Evening Slope). The break at 34 minutes post-waking was determined using the median reported time of the 30-minutes post-waking saliva sample. Parameter estimates for the two slope terms (level 1) and Pregnancy Stage (level 2) were examined to assess the changes in sUA levels across the day and differences in sUA levels across pregnancy stage. Interactions between Pregnancy Stage and each slope term were examined to test whether the Morning and Afternoon/Evening Slopes of sUA were different in early-mid *vs*. late pregnancy.

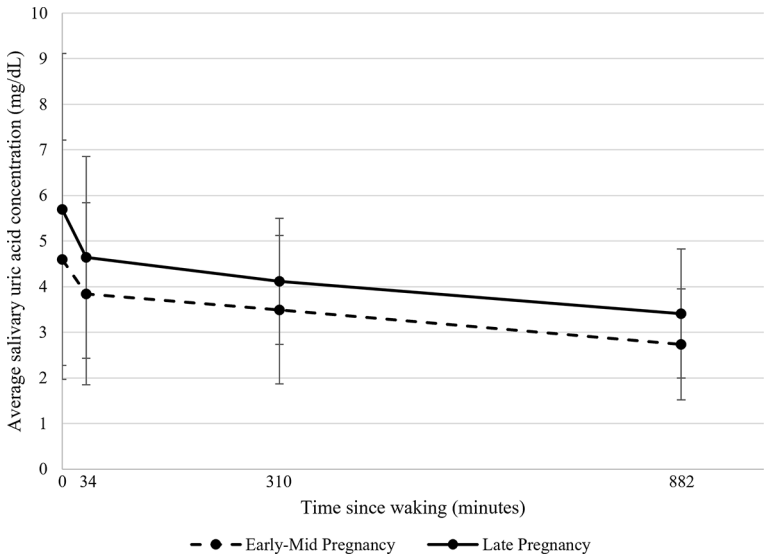

FIGURE 1 | Average salivary uric acid concentrations (mg/dL) across the day during early-mid and late pregnancy among healthy women (*N*=43). Raw salivary uric acid (sUA) concentrations are presented with data averaged across all participants for two days in early-mid (5-21 weeks gestation; shown in dotted lines) and two days in late pregnancy (30-34 weeks gestation; shown in solid lines). On each day of data collection, participants were asked to self-collect saliva samples at home upon waking, 30 minutes after waking, at 1130h, and at 2100h. For presentation purposes, the median collection times across all participants and days were used to plot average sUA concentrations as a function of time since waking. Data at 0 minutes represent average sUA for the waking samples; data at 34 minutes represent average sUA for the 30-minutes post-waking samples; data at 310 minutes represent average sUA for the 1130h samples; and data at 882 minutes represent average sUA for the 2100h samples. Error bars represent the standard deviation of the sUA data.

2.4.3 Associations Between Maternal Sociodemographic, Health, and Pregnancy Characteristics and Salivary UA During Pregnancy

The relations between maternal sociodemographic, health, and pregnancy characteristics and sUA levels and diurnal slopes were assessed by adding each maternal/pregnancy characteristic variable to the unadjusted model for sUA described above as either a level 3 (Maternal Age, Completed University, Family Income <$100,000, Number of Prior Pregnancies, Pre-pregnancy BMI, Fetal Sex, and Average Prior Night Sleep Duration) or level 2 (Self-reported Oral Health, Within-individual Change in Prior Night Sleep Duration) independent variable. Each variable was examined in a separate model. We assessed the effect of each variable on overall sUA levels as well as the significance of interaction terms between the variables and the Pregnancy Stage and Morning and Afternoon/Evening Slope parameters. These interaction terms tested whether the relations between the variables and sUA levels were different in early-mid *vs*. late pregnancy (covariate × Pregnancy Stage) and if there were differential effects of the variables on Morning or Afternoon/Evening Slopes of sUA (covariate × diurnal slope parameters). Variables with significant main effects and/or interaction terms were retained and included in a fully-adjusted model.

2.4.4 Relations Between Maternal Weight Gain and Blood Pressure During Pregnancy and Salivary UA During Pregnancy

GWG Group (level 3) and BP indices [Overall Average BP Category Score across pregnancy (level 3) and the Within-individual BP Change index (level 2)] were added separately as independent variables to fully adjusted models for sUA. We evaluated the effect of each variable on overall sUA levels and examined their interactions with Pregnancy Stage and the Morning and Afternoon/Evening Slope parameters to assess whether GWG or the BP indices were differentially associated with sUA during early-mid *vs*. late pregnancy and/or differentially related to changes in sUA levels across the morning or across the afternoon/evening. The model assessing relations between BP indices and sUA only included women with BP data available at both T1 and T2 (*n*=30).

2.4.5 Model Fit and Sensitivity Analyses

The range, distribution, and heteroskedasticity of the residuals were examined for each final model using Q-Q and scatter plots. Residuals were plotted against independent variables and interaction terms to identify potentially influential data points. Participants identified as potentially influential and those with residuals >|3| SD from the mean were excluded in sensitivity analyses.

Sensitivity analyses also assessed the effect of medication use on sUA levels and the impact of excluding women reporting any medication use or complications during pregnancy (e.g., stressful pregnancies, at-risk of diabetes) on the findings. We also tested whether adjusting for the number of BP assessments across pregnancy affected the associations between BP indices and sUA, and we added race/ethnicity (white/non-white) to each final model to examine the impact on model findings. All analyses were conducted using Stata/SE 15.1.

3 RESULTS

3.1 Analytic Sample and Preliminary Analyses

sUA data were moderately normally distributed (skew=-0.02-2.58; kurtosis=1.93-13.55; **Supplementary Figure 1**). Therefore, analyses were conducted using the raw data, and model assumptions and the distribution of residuals were assessed after model estimation. One saliva sample with a sUA concentration below the assay's lowest level of measurement was replaced with half the lower measurement threshold (62). One participant had sUA levels >4 SD from the mean at four sampling occasions (range of sUA at these occasions: 11.18-29.58 mg/dL). Including this participant in the sample significantly influenced the model results, so this individual was removed from the analytic sample. Characteristics of the analytic sample are shown in **Table 1**.

Across pregnancy, the majority of participants (54%; *n*=21) had an average BP category score that placed them in a higher than Normal to Elevated Risk category (according to the AHA guidelines; range of Overall Average BP Category Scores across pregnancy for these participants= 0.09 to 0.83). Approximately 28% of participants (*n*=11) were in the Normal BP category at all assessments conducted across pregnancy, and 15% (*n*=6) had an average BP category score across pregnancy that placed them in a higher than Elevated Risk to Hypertension-stage 1 category (according to the AHA guidelines; range of Overall Average BP Category Scores across pregnancy for these participants= 1.2 to 2.00). Only one participant had an Overall Average BP Category Score across pregnancy that exceed 2, placing her in a category higher than Hypertension-stage 1 (Overall Average BP Category Score across pregnancy for this participant= 2.67). The within-individual change in average BP category scores from T1 to T2 was not statistically significant (change from T1 to T2: MN(SD)= 0.11(0.69); range= -2.00 to 1.67). Pre-pregnancy BMI was positively associated with the Overall Average BP Category Score across pregnancy ($\rho(28)=0.55$, $p<0.01$). However, pre-pregnancy BMI was not significantly related to the within-individual change in BP category scores from T1 to T2.

3.1.1 Saliva Sample Characteristics and Confounds

Among the saliva samples for which there were available data, the effects of Flow Rate, and Recent Eating, Exercising, and Teeth Brushing on sUA levels were minimal. There were no statistically significant correlations between Flow Rate and sUA levels. No differences in sUA levels between participants reporting eating or brushing their teeth in the 30 minutes prior to sample collection *vs.* those reporting not eating or brushing their teeth were statistically significant after adjusting for multiple testing with a Bonferroni correction (see **Supplemental Materials**). Too few women reported exercising in the 30 minutes before the sample collection (*n*=0-2 participants across all sampling occasions) to statistically assess the effect of exercise on sUA levels. Given these findings, and the selected missingness of these variables, Flow Rate, and Recent Eating, Exercising, and Teeth Brushing were not included as covariates in the models assessing sUA across the day and across pregnancy. The potential effects of recent eating, teeth brushing, and exercise were further examined in sensitivity analyses (see **Supplemental Materials**).

TABLE 1 | Sample characteristics (N=43 healthy pregnant women).

	MN	SD
Age (years)	31.12	3.67
White [*n* (%)]	34	79%
Annual Family Income >$100,000 [*n* (%)]	20	47%
Completed University [*n* (%)]	30	71%
Number of Prior Pregnancies	1.81	1.10
Carrying a Female Fetus [*n* (%)]	27	63%
Average Hours of Sleep the Night Before Assessments in Early-Mid Pregnancy[a]	7.31	1.15
Average Hours of Sleep the Night Before Assessments in Late Pregnancy[a]	7.24	1.26
Pre-pregnancy BMI	25.08	5.18
Self-reported Oral Health in Early-Mid Pregnancy		
Good	30	70%
Adequate	13	30%
Self-reported Oral Health in Late Pregnancy		
Good	31	74%
Adequate	11	26%
Gestational Weight Gain Group [*n* (%)]		
Below	8	19%
Met	16	37%
Above	19	44%
Average Blood Pressure in Early-Mid Pregnancy[b]		
Systolic	108.21	10.83
Diastolic	64.41	8.30
Blood Pressure Category Score	0.38	0.63
Average Blood Pressure in Late Pregnancy[b]		
Systolic	110.82	11.33
Diastolic	67.01	8.17
Blood Pressure Category Score	0.46	0.75
Overall Average Blood Pressure Across Pregnancy[b]		
Systolic	110.91	10.18
Diastolic	66.81	7.31
Blood Pressure Category Score	0.52	0.66

Early-Mid Pregnancy= 5-21 weeks gestation; Late Pregnancy= 30-34 weeks gestation. All data are complete (N=43), except: maternal education n=42 (2% missing data); hours of sleep in Early-Mid Pregnancy n=41 (5% missing data); hours of sleep in Late Pregnancy n=42 (2% missing data); oral health in Late Pregnancy n=42 (2% missing data); blood pressure in Early-Mid Pregnancy n=30 (30% missing data); blood pressure in Late Pregnancy n=39 (9% missing data); blood pressure across all of pregnancy n=39 (9% missing data). Gestational weight gain group is based on Institute of Medicine guidelines. MN, sample mean; SD, sample standard deviation.

[a]*Data were collected on two days in Early-Mid and two days in Late pregnancy and averaged across days to generate Early-Mid and Late means.*

[b]*Blood pressure was assessed multiple times across pregnancy. Blood pressure category scores were assigned according to the American Heart Association guidelines and were as follows: 0, normal; 1, elevated; 2, hypertension-stage 1; 3, hypertension-stage 2; and 4, hypertensive crisis. See section 2.3 Measures for details.*

3.2 Characterization of Diurnal Salivary UA Across Pregnancy

On average, sUA levels were highest upon waking and exhibited a steep decline from the waking to the 30-minutes post-waking sample (**Table 2**). After the 30-minutes post-waking assessment, sUA levels continued to decline until nighttime, although at a slower rate than in the early morning (**Table 2**). The change in sUA slope at 34-mintues post-waking was statistically significant ($\chi^2(1)=17.30$, $p<0.001$). Variability in sUA concentrations was highest at waking and tended to decline across the day (**Table 2**, **Figure 1** and **Supplementary Figure 1**; **Supplementary Table 1**). Overall, sUA

TABLE 2 | Results from unadjusted and adjusted models predicting salivary uric acid concentrations across four days of pregnancy among healthy women (N = 43).

		b	SE	95% CI	*p*-value
Unadjusted Model					
Independent Variables	Morning Slope	-0.032	0.007	-0.046, -0.018	<0.001
	Afternoon/Evening Slope	-0.001	0.000	-0.002, -0.001	<0.001
	Pregnancy Stage	0.699	0.104	0.495, 0.902	<0.001
	Intercept	4.899	0.278	4.355, 5.444	<0.001
Random Intercepts	*Participant*	1.066	0.256	0.666, 1.707	
	Day	0.080	0.067	0.015, 0.412	
Residuals by Saliva Sample Type	*Wake*	6.915	0.778	5.547, 8.621	
	30-minutes post-waking	2.878	0.343	2.279, 3.634	
	1130h	1.196	0.158	0.922, 1.551	
	2100h	0.736	0.112	0.546, 0.993	
Fully Adjusted Model					
Independent Variables	Morning Slope	-0.015	0.011	-0.037, 0.007	0.171
	Afternoon/Evening Slope	-0.001	0.000	-0.002, -0.001	<0.001
	Pregnancy Stage	2.843	0.822	1.231, 4.454	0.001
	Fetal Sex	1.278	0.524	0.251, 2.304	0.015
	Fetal Sex × Morning Slope	-0.024	0.014	-0.051, 0.003	0.077
	Maternal Age	0.073	0.039	-0.002, 0.149	0.058
	Maternal Age × Pregnancy Stage	-0.063	0.027	-0.116, -0.010	0.019
	Maternal Pre-pregnancy BMI	0.079	0.026	0.028, 0.130	0.002
	Overall Average Prior-night Sleep Duration	-0.005	0.003	-0.010, 0.000	0.072
	Overall Average Prior-night Sleep Duration × Pregnancy Stage	-0.005	0.002	-0.009, -0.001	0.009
	Within-individual Change in Prior-night Sleep Duration	-0.0004	0.001	-0.002, 0.002	0.671
	Intercept	6.076	1.226	3.674, 8.478	<0.001
Random Intercepts	*Participant*	0.614	0.156	0.373, 1.011	
	Day	0.042	0.062	0.002, 0.762	
Residuals by Saliva Sample Type	*Wake*	6.787	0.767	5.438, 8.470	
	30-minutes post-waking	2.935	0.347	2.327, 3.700	
	1130h	1.182	0.154	0.915, 1.528	
	2100h	0.719	0.107	0.537, 0.961	

Morning Slope, change in uric acid from waking until 34-minutes post-waking; Afternoon/Evening Slope, change in uric acid from 34-minutes post-waking until the last saliva sample collected in the evening. Reference groups are: early-mid pregnancy and male fetal sex. All continuous variables are centered. b, coefficient; SE, standard error; CI, confidence interval; BMI, body mass index.

levels were higher in late compared to early-mid pregnancy (**Table 2**), and there were no significant differences in the Morning nor Afternoon/Evening Slopes of sUA in early-mid *vs*. late pregnancy (**Figure 1**).

3.3 Associations Between Maternal Sociodemographic, Health, and Pregnancy Characteristics and Salivary UA During Pregnancy

Maternal Pre-pregnancy BMI, Age, Prior-night Sleep Duration, and Fetal Sex were significantly associated with sUA levels and/or diurnal slopes (**Table 2** and **Figure 2**). In fully adjusted models, women with higher pre-pregnancy BMIs had, on average, higher levels of sUA during pregnancy (**Table 2**). Maternal Age significantly interacted with Pregnancy Stage such that the difference in sUA levels from early-mid to late pregnancy decreased as a function of increasing age (**Table 2**). For example, the predicted increase in sUA from early-mid to late pregnancy for a 25-year-old woman in the sample was more than twice that predicted for a 34-year-old woman in the sample (1.10 mg/dL predicted increase *vs*. 0.53 mg/dL predicted increase).

The effect of prior-night sleep duration on sUA levels also varied by Pregnancy Stage with individuals who had, on average, longer sleep durations across the study period exhibiting lower sUA levels, particularly in late pregnancy (**Table 2**; effects of Average Prior-night Sleep Duration in early-mid pregnancy: b=-0.005, SE=0.003, p=0.07, 95% CI [-0.01, 0.0004], and in late pregnancy: b=-0.01, SE=0.003, p<0.001, 95% CI [-0.01, -0.004]). The difference in sUA levels from early-mid to late pregnancy therefore decreased with increasing average sleep duration. For example, the predicted increase in sUA from early-mid to late pregnancy for a woman reporting sleeping, on average, 6 hours per night was more than twice that predicted for a woman reporting sleeping an average of 8 hours per night (1.09 mg/dL predicted increase *vs*. 0.50 mg/dL predicted increase).

Finally, mothers carrying female fetuses had, on average, higher levels of sUA during pregnancy (average marginal effect of Fetal Sex: b=0.64, SE=0.29, p<0.05, 95% CI [0.07, 1.21]) and exhibited marginally steeper morning declines in sUA compared to women carrying male fetuses (**Table 2** and **Figure 2**; average Morning Slope of sUA in mothers carrying female fetuses: b=-0.04, SE= 0.01, p<0.001, 95% CI [-0.06, -0.02] *vs*. mothers carrying male fetuses: b=-0.02, SE=0.01, p=0.17, 95% CI [-0.04, 0.01]).

In our sample of mid/high-SES, low sociodemographic risk women, sUA levels across the day and across pregnancy did not vary significantly by maternal education, family income, self-reported oral health, the number of prior pregnancies, nor Within-individual Changes in Prior-night Sleep Duration.

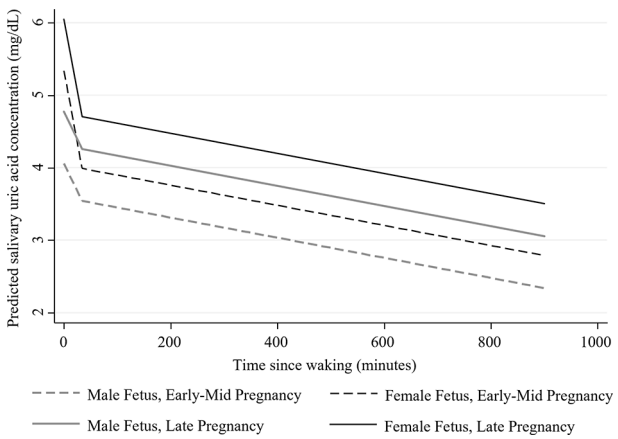

FIGURE 2 | Estimated adjusted diurnal concentrations of salivary uric acid (mg/dL) during early-mid and late pregnancy among women carrying male and female fetuses (*N*=43). Data were collected on two days in early-mid (5-21 weeks gestation; shown in dotted lines) and two days in late pregnancy (30-34 weeks gestation; shown solid lines). Estimates were generated from the fully adjusted model for salivary uric acid. See **Table 2** for the covariates included in this model.

3.4 Relations Between Maternal Weight Gain and Blood Pressure During Pregnancy and Salivary UA During Pregnancy

Women who gained less than the recommended amount of weight during pregnancy showed the highest sUA concentrations overall, and the difference in sUA levels was statistically significant when comparing women who were below the recommended weight gain level to those who were within the weight gain recommendations (within *vs*. below the recommendations: b=0.72, SE=0.37, p<0.05, 95% CI [0.008, 1.44]). All other relations between maternal/pregnancy characteristics and sUA levels and slopes in this model were similar to those reported in **Table 2**, including the effect of Pre-pregnancy BMI on sUA levels (b=0.07, SE=0.03, p<0.01, 95% CI [0.02, 0.12]). There were no significant interactions between GWG Group and Pregnancy Stage nor the Morning nor Afternoon/Evening Slope parameters.

In the model assessing relations between maternal BP indices and sUA, the variance associated with the random effect for day (level 2) was too small to be estimated. Therefore, for this model, we removed the random intercept for day. The results showed that higher Overall Average BP Category scores across pregnancy were significantly associated with higher sUA levels during late, but not early-mid, pregnancy (Overall Average BP Category Score across pregnancy by Pregnancy Stage interaction: b=0.40, SE=0.17, p<0.05, 95% CI [0.07, 0.72]; effect of Overall Average BP Category score on sUA levels in late pregnancy: b=0.65, SE=0.30, p<0.05, 95% CI [0.06, 1.23]). In addition, within-individual increases in BP category scores across Pregnancy Stage were associated with higher overall sUA levels (b=0.34, SE=0.16, p<0.05, 95% CI [0.02, 0.65]). In this model, all other relations between maternal/pregnancy characteristics and sUA levels remained similar to those reported in **Table 2**, except the interaction between Fetal Sex and the Morning Slope of sUA was not statistically significant nor trending and the interaction between Maternal Age and Pregnancy Stage was only marginally significant (b=-0.05, SE=0.03, p=0.07, 95% CI [-0.10, 0.004]). Neither the between- nor within-individual effects of BP category score showed significant interactions with the sUA diurnal slope parameters.

3.5 Model Fit and Sensitivity Analyses

Sensitivity analyses excluding participants with high residuals (n=4 for all models) or potentially influential cases (n=2 for the unadjusted, fully adjusted, and GWG Group models; n=4 for the BP model) revealed an overall robustness of the sUA patterns of change across pregnancy and across the day. However, the effects of Fetal Sex and GWG Group, and the interaction between Fetal Sex and the Morning Slope of sUA, were sensitive to the exclusion of some cases. Further analyses revealed that the relation between GWG Group and sUA levels was driven by two participants who were below the GWG recommendations and had relatively high sUA concentrations. The relations between the BP indices and sUA were also sensitive to the exclusion of potentially influential cases (n=4) as excluding these participants reduced both the within-individual effect of changes in BP and the interaction between Overall Average BP category score and Pregnancy Stage to non-significant. Examination of the excluded cases in these analyses revealed that the observed BP effects were driven by the participants with especially high overall BP (n=2) and very large within-person changes in BP (n=1). One of these participants also reported having undiagnosed preeclampsia. Excluding this participant from the BP model reduced the significance of the Overall Average BP Category score by Pregnancy Stage interaction to non-significant (with no significant effects of Overall BP Category score within Pregnancy Stage) and reduced the within-individual effect of change in BP category score to marginally significant. All other sensitivity analyses provided results similar to those presented above. See the **Supplemental Materials** for detailed descriptions of these results.

4 DISCUSSION

Our findings provide novel information about the dynamics and correlates of sUA during pregnancy. While preliminary, they demonstrate the feasibility and advantages of measuring UA in saliva. Our results highlight the importance of repeated assessments of UA across the day and across pregnancy while also demonstrating the relative robustness of sUA levels and diurnal patterns to potential confounds. Overall, our findings support the prospective utility of sUA as an easily-measurable and inexpensive biomeasure for monitoring and tracking risk across pregnancy. Further, they provide important, new information about the assessment of sUA that can be used by future studies to advance GDM and cardiometabolic health research among pregnant women.

Consistent with studies of UA in blood, we found sUA increased from early-mid to late pregnancy and exhibited a clear diurnal pattern with the highest concentrations at waking and declining levels across the day (37, 40). The factors significantly related to sUA levels in our sample, including pre-pregnancy BMI, age, and prior-night sleep duration, are also well aligned with findings from prior studies examining other populations and assessing UA in blood (20, 52). Our results build on these findings to further suggest that older women and those with longer average night sleep durations may show smaller changes in UA concentrations across pregnancy. These findings are important as prior studies have examined change in UA across pregnancy as a possible indicator of risk for hypertensive disorders (13, 14). Our results highlight potential confounds that may be important for clarifying relations between UA and cardiometabolic risk in pregnancy.

In contrast, our findings suggest the diurnal pattern for sUA is relatively robust with no significant differences in the diurnal slopes of sUA in early-mid *vs*. late pregnancy and no differences in the slopes associated with the maternal characteristics examined. While morning declines in sUA tended to vary by fetal sex, these findings were only marginally significant and did not hold up to robustness and sensitivity checks of our models. To our knowledge, no studies have examined associations between the UA diurnal patterns and health and disease risk across pregnancy. For other biomeasures, such as cortisol, the diurnal pattern has been shown to be important for understanding physiologic function and predicting adverse health outcomes [e.g (63–66)]. Our own work with this study sample has shown that the diurnal patterns of salivary cortisol and alpha-amylase during pregnancy are associated with depression, anxiety, and stress, as well as history of previous miscarriage (67, 68). While additional research is needed to assess whether the diurnal pattern of sUA is clinically meaningful, our findings lay the foundation for this work and support the measurement of diurnal trajectories of UA using minimally-invasive and easily-implemented at-home saliva collection protocols.

We found preliminary support for the positive association between BP and sUA levels during pregnancy. Women with higher average BP scores exhibited higher sUA levels during late pregnancy, and within-individual increases in BP across pregnancy stage were associated with higher overall levels of sUA. These findings are consistent with studies assessing UA in pregnancy as a risk factor for hypertensive disorders (12–19) and may reflect UA's role in regulating BP *via* activation of the renin-angiotensin system and by increasing oxidative stress, inflammation, endothelin, and endothelial dysfunction (31). If confirmed and extended, our finding that increases in BP across pregnancy was positively associated with sUA levels during pregnancy could have important implications for identifying women at high risk of BP complications during pregnancy. In our sample of healthy and low risk women, however, these associations were small and driven by the highest risk women. Additional research is needed with larger, higher-risk samples to confirm these findings. Further studies are also needed to understand the relations observed between GWG and sUA in our sample as these associations were in the opposite direction as expected and also driven by select cases in our sample. Despite their limitations, these findings suggest the potential added value of monitoring UA across pregnancy *via* minimally-invasive, at-home methods as these associations were observed after adjusting for maternal pre-pregnancy BMI and, therefore, could represent unique mechanisms conveying maternal health risks.

The role of UA in the development of poor maternal, as well as fetal, health outcomes is not fully understood. High UA levels during pregnancy may be due to the increased breakdown of maternal, placental, or fetal tissues and/or decreased clearance of UA by the kidneys (27, 35). Elevated pre-pregnancy UA may potentiate increases in UA during pregnancy, making it difficult to dissociate UA's role as a reflection of or contributor to poor health outcomes (27). For example, high UA is strongly associated with obesity among adults, and elevated levels of UA are linked with both increased fat deposition in the liver and with diets high in fat and fructose (31). This suggests that women with high UA before pregnancy may be at increased risk for poor health and pregnancy outcomes due to pre-existing risk factors. However, there are several mechanisms by which UA may also be directly involved in the development of maternal and fetal health problems. For example, UA may disrupt placental development and function resulting in impaired blood flow and fetal growth restrictions (27, 35). Increased inflammation and oxidative stress, stimulated by high UA levels, may also have negative effects on maternal and fetal health, including mitochondrial damage in the mother's liver, an increase in maternal oxidized fats, and a decrease in maternal adiponectin levels (27, 29, 35). Furthermore, UA can lead to endothelial dysfunction in the mother and affect fetal growth hormone levels (27, 35). While few studies have examined diurnal variation in UA during pregnancy (46, 47), change in UA levels across the day may be related to circadian patterns of renal function, urine production, and purine metabolism (43, 69). How these processes are reflected in sUA diurnal slopes, and the clinical significance of changes in these trajectories, has not yet been evaluated. Future studies tracking UA levels and diurnal patterns during pregnancy along with maternal and fetal health are essential next steps in this research. Such studies would provide novel information that would complement recent findings that pregnant women with hypertensive and glucose metabolic disorders show dysregulated circadian patterns of melatonin secretion (70). Our findings suggest that these inquiries can be addressed using multiple, repeated measurements of UA in saliva, rather than serum. They also provide new information about key covariates of sUA levels (e.g., maternal age) that should be considered when designing new studies of sUA and pregnancy-related health outcomes. Thus, these findings support the advancement of GDM and cardiometabolic health research among pregnant women and may help open up new opportunities for evaluating, identifying, and preventing key health problems during pregnancy and their long-term consequences for maternal and child health.

4.1 Limitations

There are several limitations to our study that warrant discussion. First, our small sample size (*N*=30-43) restricted our power to find significant associations in our models and highlights the need to replicate the findings as statistically significant effects in our small sample may not be replicated by larger studies. The homogeneity and low-risk nature of our sample further limits the generalizability of our findings and likely hindered our ability to find robust associations between UA levels and maternal BP and GWG indices. Also, we did not assess biomarkers of diabetes nor gestational diabetes (e.g., blood glucose levels) which limits the implications of our findings for GDM research. It is also important to note that our findings may not be generalizable to women with pregnancy complications, such as gestational diabetes or hypertension, as women with known pregnancy complications were excluded, and our sample was comprised of mostly low-risk and generally healthy pregnant women. Given our sample and the relative novelty of sUA research, we cannot make assumptions about the clinical nor biological significance of our findings. There are no known thresholds for sUA levels that convey risk for maternal and/or fetal health. The homogeneity of the sample also likely limited our ability to identify significant covariates of UA levels and trajectories, such as age, income, and race/ethnicity [e.g (43)]. Many of the variables examined also relied on self-report data, and BP measurements were not standardized across participants nor available for all women in our sample. Further, we were not able to assess the effect of diet on sUA levels which is strongly associated with UA and may be an unmeasured confound affecting our findings (31). Despite these limitations, many of our findings are consistent with those reported by other studies of UA measured in either serum or saliva [e.g (12, 13, 50)]. To confirm and extend these findings, additional research with larger, more diverse samples that include at-risk women and standardized, high-quality measures is needed. Our paper represents the first step in a wider conversation related to the clinical utility of sUA during pregnancy, and clinical interpretations of sUA depend on additional studies using sUA for this purpose.

Future research should also include additional assessments of sUA across pregnancy to allow for a more granular examination of the change in sUA concentrations from early to late pregnancy. Our study design only allowed us to compare sUA levels from early-mid *vs.* late pregnancy using data from two prenatal time points, and there was a wide range during which women were assessed in early-mid pregnancy (between 5- and 21-weeks gestation). This raises important questions regarding the timing and magnitude of changes in sUA across pregnancy. Additional studies addressing these questions will be important for future research and clinical work aiming to assess changes in sUA across pregnancy as a potential indicator of maternal or fetal health.

While the measurement of UA in saliva presents exciting opportunities to conduct in-depth studies of UA and maternal and fetal health on a large-scale, it also introduces new concerns regarding measurement validity and reliability. There are several factors that may affect the integrity of analyte levels measured in saliva to serve as proxies for serum levels. These include oral health conditions that affect the composition of saliva and can increase blood levels; recent food/drink intake which can alter saliva quality and pH and affect bioassay procedures; and biospecimen collection and cold chain procedures which are more difficult to control when samples are collected outside the laboratory or clinical setting. While not directly assessed in this study, prior reports suggest that UA has a strong serum-saliva correlation, that levels in saliva are not significantly associated with markers of oral inflammation, and that collection technique (e.g., swab *vs.* passive drool) does not significantly affect the concentration of UA measured in a sample (41, 48–50, 71, 72). We evaluated the effects of flow rate, oral health, and recent food intake and teeth brushing in our sample and found minimal effects on sUA levels. However, these measures were largely based on self-report, data were not available for all biospecimens, and, in general, few participants reported eating or brushing their teeth in the 30 minutes prior to sample collection. These limitations likely hindered our ability to find differences in sUA levels related to these factors. Future research is needed to fully examine the sensitivity of sUA concentrations to these methodologic and oral-specific confounds. The investigation of oral health and its associations with sUA, GDM, and hypertensive risk is especially important as sUA levels may vary by periodontal disease status and these effects may be different for hypertensive or preeclamptic women (54, 73, 74).

4.2 Conclusions

Salivary assessment of UA levels offers the opportunity to conduct long-term, repeated, minimally-invasive, at-home monitoring of women at risk of metabolic or hypertensive disorders during pregnancy. Our preliminary findings demonstrate the feasibility of such monitoring and suggest that the data generated may be useful in tracking maternal health risks. Our results also suggest that some of the inconsistencies in prior studies assessing UA as an indicator of health risks during pregnancy may be related to limitations inherent in serum-based biomeasure evaluations, such as minimal repeated assessments of UA and variability in the timing of these assessments. Future research evaluating sUA during pregnancy among larger, more diverse, and at-risk samples, and with standardized assessments of maternal and fetal health across pregnancy, will be essential to expanding our understanding of the role of UA in pregnancy and fetal development and the potential utility of sUA as a clinical marker of maternal or fetal health.

DATA AVAILABILITY STATEMENT

The data analyzed in this study are subject to the following licenses/restrictions: The datasets presented in this article are not readily available. The data are available upon request to GG and the corresponding author and with the permission of the other lead researchers of this project. Requests to access these datasets should be directed to GG (ggiesbre@ucalgary.ca).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Calgary Conjoint Health Research Ethics Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GG, TC, and NL designed and conducted the original study and data collection procedures. DG collaborated with GG on the original study design. JR and DG conceived of the current study. JR and SC performed the statistical analyses in consultation with DG and GG. JR drafted the manuscript. All authors revised the report. 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.2022.813564/full#supplementary-material

Supplementary Figure 1 | Salivary uric acid concentrations (mg/dL) across the day on four days of data collection during early-mid and late pregnancy (*N*= 40-43 healthy pregnant women). Raw salivary uric acid (sUA) concentrations are presented for two days in early-mid (5-21 weeks gestation; shown in dotted boxes) and two days in late pregnancy (30-34 weeks gestation; shown in solid boxes). On each day of data collection, participants were asked to self-collect saliva samples at home upon waking, 30 minutes after waking, at 1130h, and at 2100h. W, data from the waking sample; W+30, data from the sample collected 30-minutes post-waking; 1130h, data from the sample collected at 1130h; 2100h, data from the sample collected at 2100h. Participants with outside values were not extreme relative to the rest of the study sample on any of the potential covariates and confounds examined in this study (see *Measures*).

Supplementary Table 1 | Descriptive statistics for salivary uric acid concentrations (mg/dL) across the day on four days of data collection during early-mid and late pregnancy.[a]Data were collected on two days in early-mid (5-21 weeks gestation) and two days in late pregnancy (30-34 weeks gestation). SD, sample standard deviation.

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Hepatic IGF2/H19 Epigenetic Alteration Induced Glucose Intolerance in Gestational Diabetes Mellitus Offspring *via* FoxO1 Mediation

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Objective: The offspring of women with gestational diabetes mellitus (GDM) have a high predisposition to developing type 2 diabetes during childhood and adulthood. The aim of the study was to evaluate how GDM exposure in the second half of pregnancy contributes to hepatic glucose intolerance through a mouse model.

Methods: By creating a GDM mouse model, we tested glucose and insulin tolerance of offspring by intraperitoneal glucose tolerance test (IPGTT), insulin tolerance test (ITT), and pyruvate tolerance test (PTT). In addition, we checked the expression of genes IGF2/H19, FoxO1, and DNMTs in the mouse liver by RT-qPCR. Pyrosequencing was used to detect the methylation status on IGF2/H19 differentially methylated regions (DMRs). *In vitro* insulin stimulation experiments were performed to evaluate the effect of different insulin concentrations on HepG2 cells. Moreover, we detect the interaction between FoxO1 and DNMT3A by chromatin immunoprecipitation–quantitative PCR (Chip-qPCR) and knock-down experiments on HepG2 cells.

Results: We found that the first generation of GDM offspring (GDM-F1) exhibited impaired glucose tolerance (IGT) and insulin resistance, with males being disproportionately affected. In addition, the expression of imprinted genes IGF2 and H19 was downregulated in the livers of male mice *via* hypermethylation of IGF2-DMR0 and IGF2-DMR1. Furthermore, increased expression of transcriptional factor FoxO1 was confirmed to regulate DNMT3A expression, which contributed to abnormal methylation of IGF2/H19 DMRs. Notably, different insulin treatments on HepG2 demonstrated those genetic alterations, suggesting that they might be induced by intrauterine hyperinsulinemia.

Conclusion: Our results demonstrated that the intrauterine hyperinsulinemia environment has increased hepatic FoxO1 levels and subsequently increased expression of DNMT3A and epigenetic alterations on IGF2/H19 DMRs. These findings provide potential molecular mechanisms responsible for glucose intolerance and insulin resistance in the first male generation of GDM mice.

Keywords: gestational diabetes mellitus (GDM), epigenetic regulation, DNA methylation, fetal-origin diseases, insulin resistance

INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as glucose intolerance first detected during pregnancy and is one of the most common complications of pregnancy (1). The estimated prevalence of GDM is about 15% among pregnant women (2). Increasing evidence indicates that maternal GDM influences their offspring's health, notably resulting in a higher risk of cardiometabolic diseases such as type 2 diabetes and obesity (3, 4). Currently, there are no definitive treatments for GDM except lifestyle change and limited insulin therapy (5). Therefore, we aimed to explore the potential mechanisms related to the impact of GDM on the long-term health of the offspring.

Recently, an increasing field of data articulates an interest in the potential mechanism between epigenetic regulation and the susceptibility to chronic cardiometabolic diseases in GDM offspring (6, 7). DNA methylation is the most extensively studied epigenetic modification. Hjort et al. (8) performed a genome-wide DNA methylation study on peripheral blood in adolescent offspring (9–16 years old) of GDM women, and the results indicated that intrauterine exposure to hyperglycemia was related to DNA abnormal methylation in 76 differentially methylated CpG sites (8). Cote et al. (9) put forward that DNA hypermethylation of peroxisome proliferator-activator receptor-gamma, co-activator 1, alpha (PPARGC1α) in the placenta was involved in the association between maternal hyperglycemia and elevated cord blood leptin levels, which is considered as a marker for adiposity in offspring (9). Ding et al. (10) found that the expression of imprinted gene IGF2/H19 was downregulated in mouse pancreatic islets of GDM offspring due to hypermethylation status in the differentially methylated region (DMR) (10).

Normal pregnancy is typically a state of insulin resistance because of a surge of placental anti-insulin hormones (11). Moreover, hyperglycemia in GDM patients is induced by insulin secretion that is inadequate to compensate for the concurrent insulin resistance. Therefore, chronic hyperinsulinemia is a crucial element of the pathophysiology of GDM (12). However, the concrete mechanism between intrauterine exposure to hyperinsulinemia and diabetes offspring is limited, since most studies have focused on hyperglycemia in the intrauterine environment (13, 14).

Under normal physiological feeding conditions, insulin canonically modulates hepatic gluconeogenesis to lower glucose concentration by combining its receptor (IR) and insulin receptor substrates (Irs) and then initiating the phosphorylation of Akt protein kinases (15, 16). Forkhead box O1 (FoxO1) inhibits the canonical Akt-induced insulin pathway as a critical liver transcription factor in reducing genes coding gluconeogenic enzymes (17, 18). Accordingly, FoxO1 is active during fasting by collaborating with co-activators PPARGC1α to coordinatively upregulate the expression of G6pc (glucose-6-phosphatase, catalytic subunit) and Pck1 (cytosolic phosphoenolpyruvate carboxykinase 1) (19, 20) and is suppressed by Akt-mediated phosphorylation after feeding (21). FoxO1 knockout in the mouse livers could afford an improvement in glucose homeostasis and subsequently reduce insulin resistance (16).

Consequently, in our study, we hypothesized that intrauterine hyperinsulinemia in GDM induced abnormal DNA methylation through insulin pathway transcription factor FoxO1, which subsequently causes abnormal expression of imprinted gene IGF2/H19, ultimately resulting in glucose intolerance and insulin resistance in adult GDM offspring.

MATERIALS AND METHODS

Animal Care and Model

The Zhejiang University Animal Care and Use Committee (IACUC) approved all the animal care and treatment protocols (ZJU2015-323-01). All the experiments were conducted by the Institute of Cancer Research (ICR) mice, which were purchased from Shanghai SLAC Laboratory Animal Company (Shanghai, China). Virgin and healthy ICR females (age 3–4 weeks) were fed with a high-fat diet (HFD) containing 60 kcal% fat (D12492, Research Diets, New Brunswick, NJ, USA) or chow diet. The mice were then mated with normal ICR males. A copulation plug present overnight was considered a pregnancy (day 0.5). The pregnant ICR females were divided into two groups, control (Ctrl) and GDM, and fed with an HFD during the whole course of pregnancy. On day 6 and day 12 of pregnancy, GDM dams were fasted 8 h and received a streptozotocin (STZ) injection (100 mg/kg i.p.) (Sigma-Aldrich, St. Louis, MO, USA) (22, 23) (**Figure 1A**). Control dams received an equal volume of citrate buffer. We tested the blood glucose level *via* the tail vein 48–72 h after the second STZ injection, and diabetes was defined as a blood glucose level over 14 mmol/L (252 mg/dl) (24), which is confirmed by continuous monitoring in the following days. Both the control and GDM pups were fostered by normoglycemic females until they were 3 weeks old. Mice were weighed at ages 3, 8, and 16 weeks.

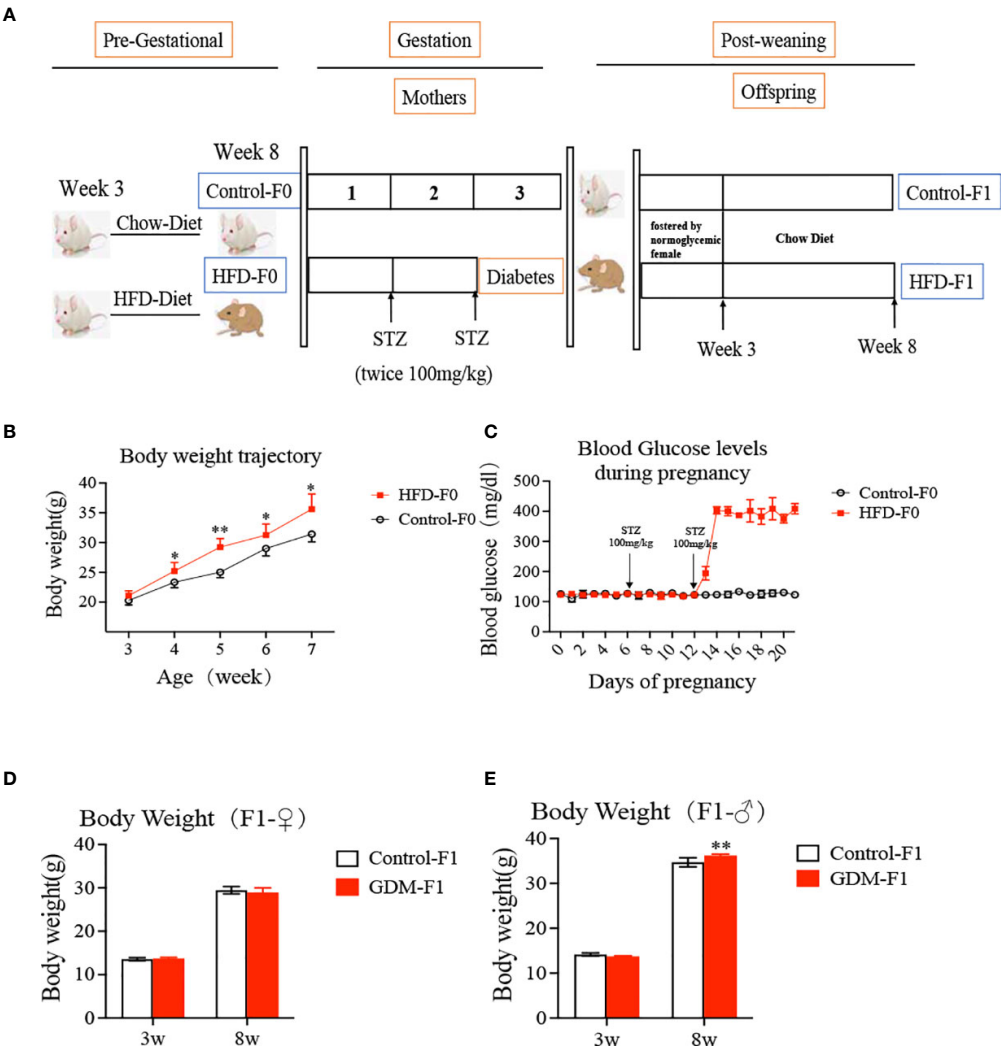

FIGURE 1 | Experimental design, offspring growth curves. **(A)** Experimental design; the ICR female mice (age 3-4 weeks) divided into two group, fed with a high-fat diet or chow diet for 4 weeks. Then the mice were mated with nonnal ICR males. The pregnancy ICR females were injected STZ or citrate buffer on day 6 and day 12 of pregnancy. Both the control and GDM pups were fostered by normoglycemic female until they were three weeks old, and then fed by chow diet. **(B)** body weight of mice both fed by high fat diet or chow diet at different developmental weeks; **(C)** Blood glucose levels during pregnancy for mice both fed by high fat diet or chow diet; **(D)** Body weight of female F1 offspring; **(E)** Body weight of male F1 offspring. In all panels, data are presented as mean ± SD, $^{*}P < 0.05$. $^{**}P < 0.01$, n = 6 mice per group. Significance was determined by Student *t* test.

Glucose, Insulin, and Pyruvate Tolerance Tests

Glucose tolerance tests (GTTs) were performed in overnight (16 h) fasted animals as previously described (25). Each mouse was administered an intraperitoneal (i.p.) injection of 2 g/kg body weight (BW) glucose (DeltaSelect, Munich, Germany) in sterile saline. Blood glucose concentrations were measured collection from the tail vein at 30, 60, and 120 min after the injection.

Insulin tolerance tests (ITTs) were performed in *ad libitum* fed mice as the previous study reported (26919426). Each animal received an i.p. injection of insulin at 0.8 U/kg (Humulin R; Eli Lilly, Indianapolis, IN, USA). Blood glucose levels were then measured at 30, 60, and 120 min after insulin injection.

Pyruvate tolerance test (PTT) was performed after overnight fasting of 12 h. Animals received an i.p. injection of 1.5 g/kg BW sodium pyruvate (Cat#P5280, Sigma-Aldrich, St. Louis, MO, USA). Blood glucose levels were then measured at 30, 60, and 120 min after the injection. The area under the curve (AUC) of glucose against time was calculated in order to analyze glucose tolerance as previous studies described (10, 26).

Serum Analysis

Serum was collected from 16-week-old mice by fasting for 12 h. Serum insulin level was evaluated at an overnight state (Crystal

Chem, Downers Grove, IL, USA). HOMA-insulin resistance (IR) was calculated as our previous study reported (22): fasting serum insulin concentration (μU/ml) multiplied by fasting blood glucose level (mg/dl) divided by 405.

RNA Extraction and Quantitative PCR Analysis

Total RNA was extracted from the mouse liver tissue samples (16 weeks old) and fetal liver tissue (day 18.5) with the TRIzol Reagents (Invitrogen Life Technologies, Carlsbad, CA, USA). Complementary DNA was synthesized using oligo-deoxythymidylic acid and random primers (RR037A, Takara, Maebashi, Japan). Quantitative PCR was performed using ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA) with SYBR green detection (RR420A, Takara, Japan). Glyceraldehyde-3-phosphate dehydrogenase was the internal control. The primer sequences are provided in **Supplementary Table 1**.

DNA Isolation and Bisulfite Conversion

Total genomic DNA was isolated from the mouse liver tissue by using the Genomic DNA Purification Kit (Cat. K0512, Invitrogen Life Technologies). Bisulfite conversion was performed by the EpiTect bisulfite kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions; the details are shown in a previous study (27).

DNA Methylation Analysis by Pyrosequencing

The methylation status was analyzed by pyrosequencing (27). In brief, pyrosequencing primers were designed by Qiagen PyroMark Assay Design 2.0 software (Qiagen) (**Supplementary Table 2**). PCR product was checked by agarose gel analysis. Pyrosequencing was carried out on a PyroMark Q96 instrument (Qiagen) according to the manufacturer's instruction. The Pyro Q CpG software (Qiagen) was used to calculate the percentage of methylation.

Cell Culture, Insulin Treatment, and siRNA Transfection

The established human HepG2 hepatoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Beijing, China), supplemented with 10% fetal bovine serum (FBS) (Gibco, Beijing, China) and 1% penicillin and streptomycin (PS) at 37°C in 5% humidified CO_2 tissue culture incubator. Generally, the cells were seeded in 24-well plates for 12 h and then incubated in an FBS-free DMEM for 24 h, followed by different insulin concentrations (0, 50, 100 nmol/L) treatment for 24h.

For siRNA transfection, the sequences of siRNA targeting FoxO1 (siFoxO1) are as follows (5′–3′): sense: GCAG CAGACACCAUGCUAUTT; anti-sense: AUAGCAUG GUGUCUGCUGCTT. The sequences of negative control (siCon) are as follows (5′–3′): UUCUCCGAACGUGUCA CGUdTdT; anti-sense, ACGUGACACGUUCGGAGAAdTdT. siFoxO1 or siCon was mixed with 25 μl of OPTI-MEM (Cat. 31985-070, Gibco) by gentle pipetting. Meanwhile, 1 μl of Lipofectamine 3000 reagent (Cat. L3000008, Thermo Fisher Scientific, Waltham, MA, USA) was mixed with 25 μl of OPTI-MEM. Then the Lipofectamine or siRNA from two tubes was mixed together and incubated for 5 min at room temperature (RT). Then cell pellet ($5 * 10^4$ cells) was resuspended in a total 50 μl solution and incubated at RT for 10 min, 450 μl of DMEM containing FBS was added, and the cell suspension was transfected into one well of a 24-well plate.

Western Blotting

The protein was extracted from HepG2 cells with lysis buffer, which was separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed using polyvinylidene fluoride membrane and the antibodies for FoxO1 (Cell Signaling Technology, Danvers, MA, USA; 2880, used at a dilution 1:1,000), DNMT3A (Cell Signaling, 32578, used at a dilution of 1:1,000), and Actin (Abcam, Cambridge, UK; ab8227, used at a dilution 1:1,000). The enhanced chemiluminescence system (Pierce, Rockford, IL, USA) was used to visualize protein bands.

Chromatin Immunoprecipitation–Quantitative PCR

The detailed protocol is described in a previous study (28). Briefly, all experiments were performed on a 10-cm plate scale by Chromatin Prep Module Kit (Cat. 26158, Thermo Fisher Scientific) according to the manufacturer's instructions. Agarose beads were used to pre-bind overnight with antibodies against FoxO1 (Abcam, ab39670, used at a dilution of 1:500). HepG2 cells were cross-linked with 1% formaldehyde at RT for 10 min and then stopped by 1* glycine. Chips were performed at 4°C. Primers for the specific promoter regions of DNMT3A are provided in **Supplementary Table 3**. The relative enrichment of the indicated DNA regions was measured according to the manufacturer's instruction and was normalized to % input.

Statistical Analysis

All data were presented as mean ± SD. Statistical analysis was performed by two-tailed Student's *t*-test and one-way ANOVA as described in the table and figure legends, using SPSS 18.0 software. $p < 0.05$ and $p < 0.01$ were considered statistically significant.

RESULTS

Gestational Diabetes Mellitus Environment Induced Increased Birth Weight in Male Adult Offspring

We established a mouse model of hyperglycemia during the midlate stage of pregnancy mimicking the clinical GDM. The bodyweight of HFD-fed mice was significantly higher than that of chow diet-fed mice (**Figure 1B**), while plasma glucose concentrations at day 0 were similar in two groups (**Figure 1C**). After two lose-dose STZ injections, the plasma glucose levels of GDM dams averagely reached 403 mg/dl

(**Figure 1C**). Notably, increased BW was seen in GDM-F1 male adult offspring at 8 weeks of age (**Figure 1E**). However, these associations were not observed in GDM-F1 female adult offspring (**Figure 1D**).

Gestational Diabetes Mellitus Exposure Induced Glucose Intolerance, Insulin Resistance, and Enhanced Gluconeogenesis in GDM-F1 Offspring

Fasting blood glucose levels of 8-week-old GDM-F1 offspring did not differ from those in the corresponding controls, and there was no gender difference (**Figure 2A**). We further performed a GTT by i.p. injection of glucose (2 g/kg body wt). There was no difference in the glucose levels and GTT AUC in 8-week-old female offspring between GDM-F1 and Control-F1 (**Figure 2B**). However, impaired glucose tolerance (IGT) was found in both 8-week-old mice of the GDM-F1 group, whose blood glucose level significantly increased at 30 min after injection (**Figure 2B**); along with this, we found increased fasting insulin concentration in GDM-F1 male mice (**Figure 2A**). In addition, decreased insulin sensitivity was observed in 8-week-old male mice of the GDM-F1 group in the ITT. Interestingly, the difference between GDM-F1 and Control-F1 female mice was not obvious (**Figure 2C**), consistent with the results of HOMA-IR

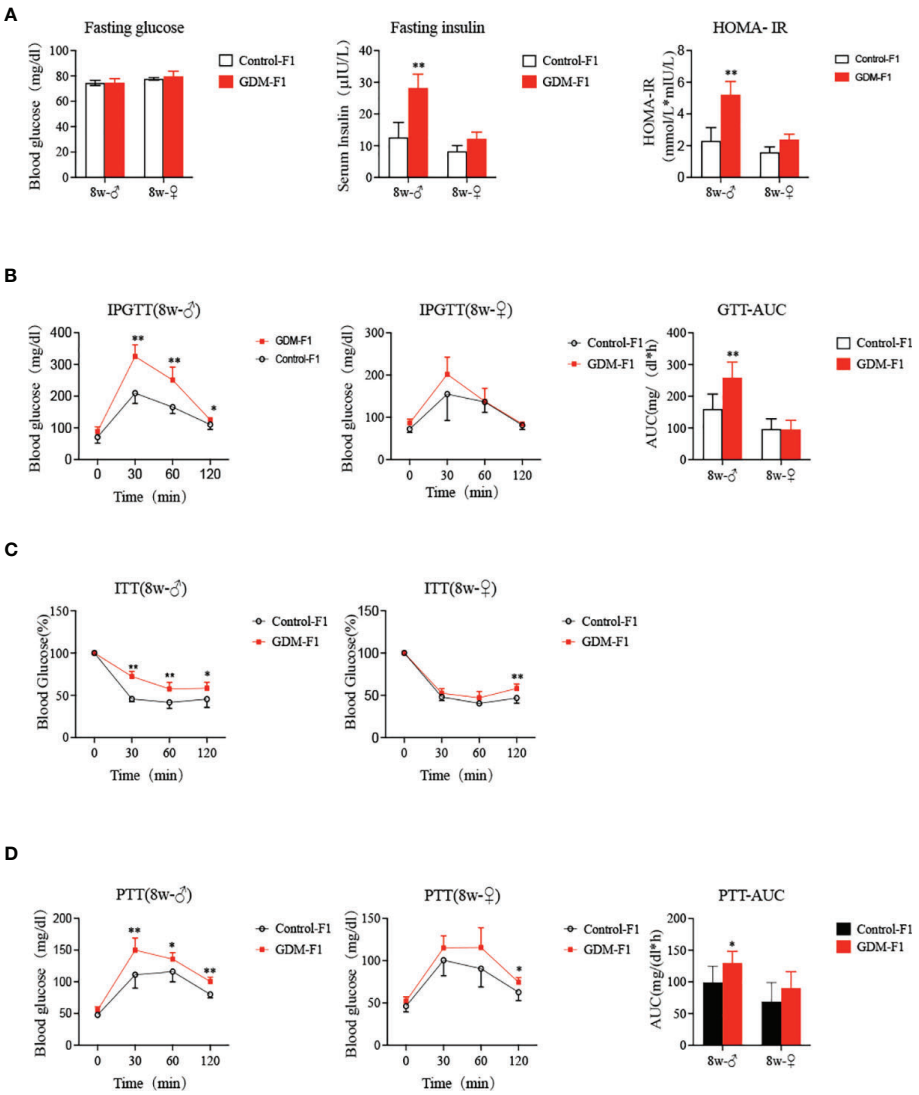

FIGURE 2 | Glucose Homeostasis on GDM-F1 and control-F1 mice. **(A)** fasting serum glucose concentration, fasting insulin concentration, HOMA-IR of 8-week-old F1 offspring; **(B)** Glucose tolerance test (GTT, 2 g/kg glucose ip) and Area under the curve (AUC) of 8-week-old F1 offspring over the course of 120 min; **(C)** insulin tolerance test (ITT, 0.75 U/kg insulin ip) of 8-week-old F1 offspring over the course of 120 min; **(D)** Pyruvate tolerance test (PTT, 2g/kg pyruvate ip) and AUC of 8-week-old F1 offspring over the course of 120 min; data are presented as mean ± SD, $^{*}P < 0.05$. $^{**}P < 0.01$, n = 6 mice per group. Significance was determined by Student *t* test.

(**Figure 2A**). Regarding hepatic gluconeogenic activity, the PTT displayed that the GDM-F1 male mice showed enhanced blood glucose levels compared with the Control-F1 group (**Figure 2D**). In contrast, excessive gluconeogenesis was not observed in 8-week-old female GDM-F1 mice (**Figure 2D**).

Intrauterine Hyperinsulinemia Downregulated the Expression of IGF2 and H19 in Male Mouse Liver

We collected the liver from 8-week-old male mice and found that the relative mRNA levels of IGF2 and H19 were both significantly lower in GDM-F1 male mice (**Figures 3A, B**), which was consistent with the islet results previously published in *Diabetes* (10). In order to verify the direct effect of hyperinsulinemia on liver development, HepG2 cells were cultured in a medium containing different insulin concentrations for 24 h. We found that the IGF2 and H19 mRNA levels were significantly lower in HepG2 cells exposed to a high insulin concentration (100 mmol/L) than those in the control or a low concentration of insulin (50 nmol/L) (**Figures 3C, D**).

Intrauterine Hyperinsulinemia Induced Hypermethylation of IGF2/H19 Differentially Methylated Region in Male Mouse Liver

As imprinted genes, IGF2 and H19 allelic expressions in mice are regulated by allele-specific methylation at four DMRs (10, 29) (**Figure 4A**). We collected liver from a 16-week-old male of the control and GDM-F1 groups. We analyzed the methylation levels of 8 cytosine phosphate guanine (CpGs) of the IGF2-DMR0, 2 CpGs of the IGF2-DMR1, 6 CpGs of the IGF2-DMR2, and 9 CpGs of the H19-DMR by pyrosequencing. In the IGF2-DMR0 and IGF2 DMR1, the methylation status was significantly higher in GDM-F1 groups compared with that in control except for site 2 CpG in IGF2-DMR0 (**Figures 4B, C**). Furthermore, only site 4 CpG exhibited significant hypermethylation in IGF2-DMR2 of GDM-F1 liver (**Figure 4D**). Though the H19 DMR upstream of the H19 gene (located 90 kb 3′ of IGF2) acted as a methylation-sensitive boundary component, there was no significant difference in these two groups (**Figure 4E**).

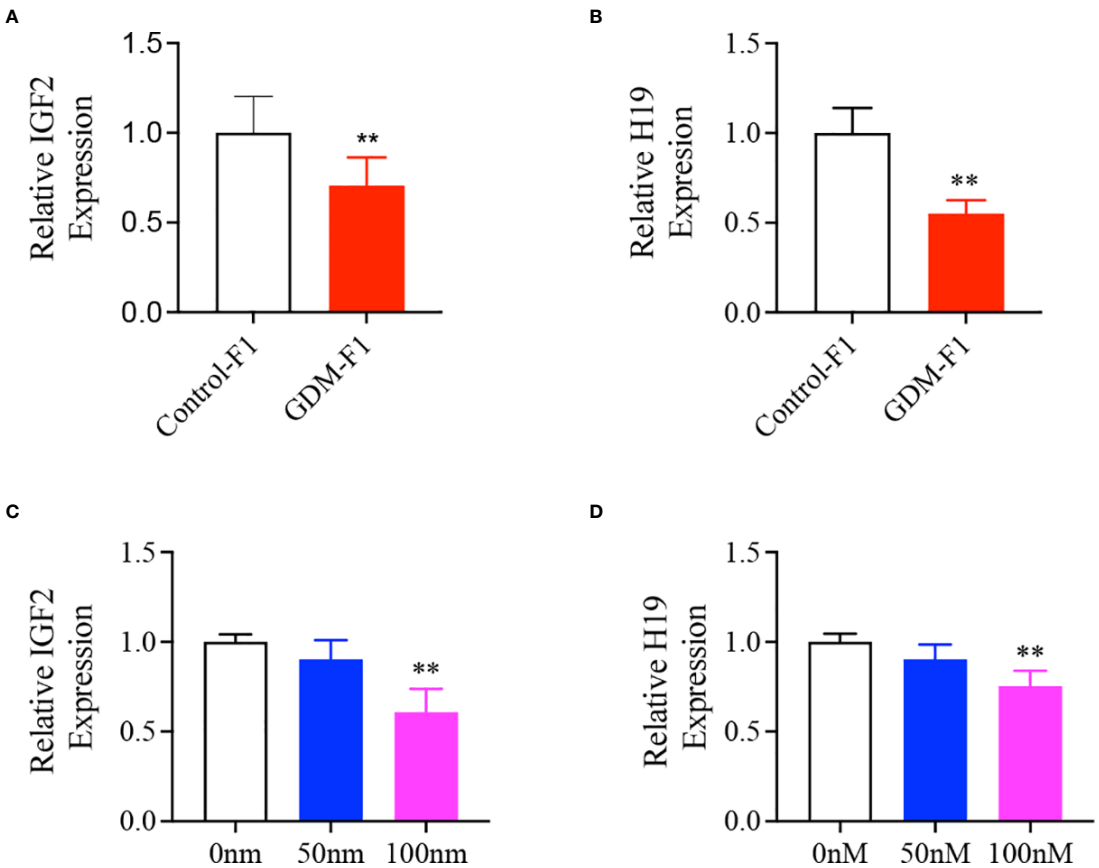

FIGURE 3 | Representative mRNA levels of Igf2, H19 in the liver of 8-week-old F1 male mice and HepG2 cells treated with different insulin concentration. **(A, B)** IGF2/H19 mRNA expression in the liver of 8-week-old mice (every group contains 10 mice); **(C, D)** IGF2, H19 gene expression in HepG2 cells treated with 0, 50, 100 mnol/L insulin concentration (n = 4 replicates/group in at least three independent isolations). Data were analyzed with the Eq. $2^{-\Delta\Delta CT}$ where ΔΔCT = ΔCT (treatment group) -ΔCT (control group), and ΔCT = ΔCT (sample) - ΔCT (intemal control). The values were normalized to ACTIN mRNA levels, ACTIN was an intemal control. Data are presented as mean ± SD, $^*P < 0.01$. Significance was determined by Student *t* test.

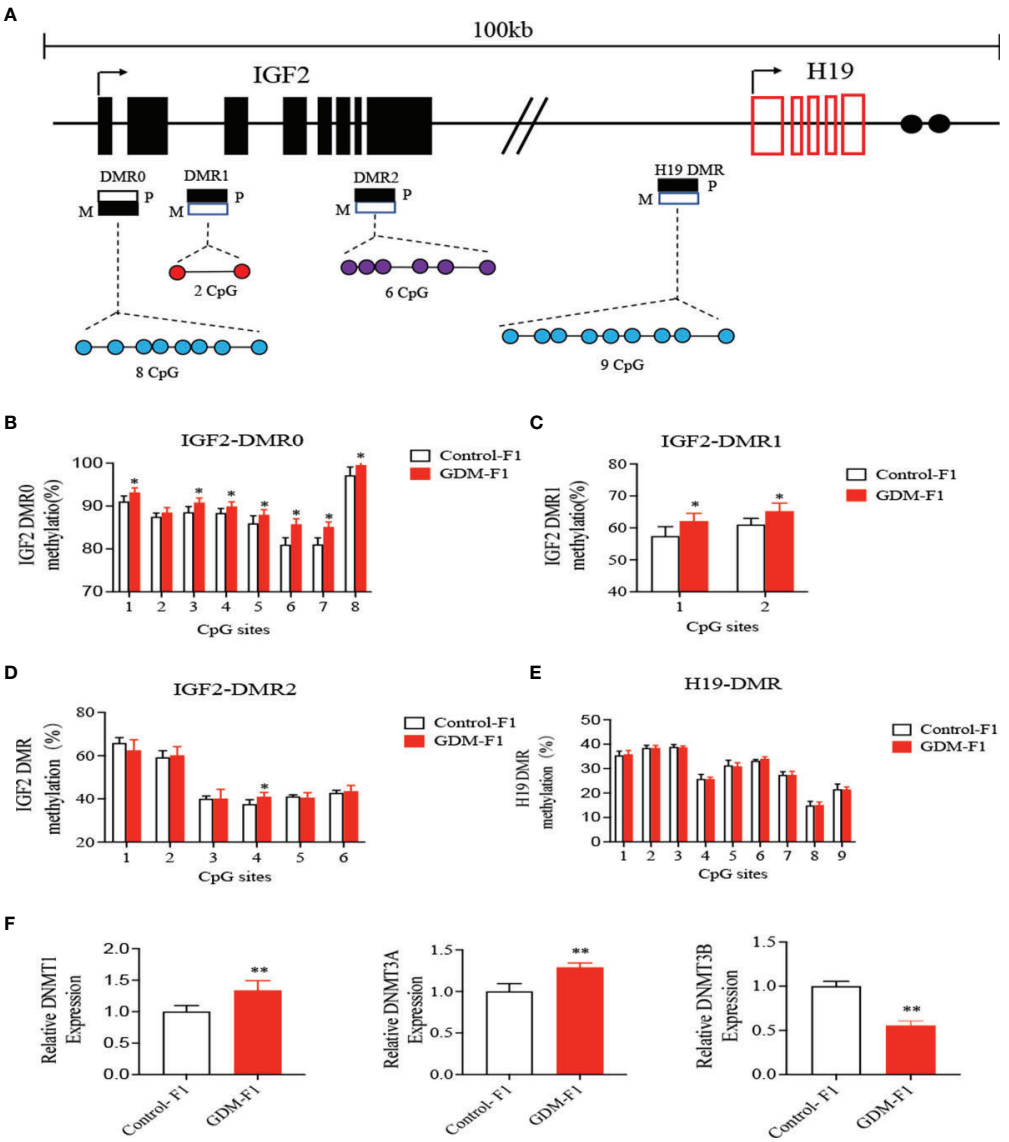

FIGURE 4 | Methylation analysis of IGF2/H19 DMRs by pyrosequencing. **(A)** Schematic representation of mouse imprinted locus, showing the relative positions of the IGF2 and H19 genes and indicating the location of the four DMR knows to contribute to IGF2 imprinting. Enhancers **(E)** are indicated as black circles. The locations of the four DMRs within the IGF2/H19 imprinted locus represented by boxes are shaded to indicate preferential methylation of the maternal (M) or paternal (P) allel in each region. **(B–E)** Percentage of methylation for each CpG cytosine is calculated. **(B)** Methylation status of individual DNA strands of IGF2-DMR0 containing 8 CpG sites; **(C)** IGF2-DMRI containing 2 CpG sites; **(D)** IGF2-DMR2 containing 6 CpG sites; **(E)** H19-DMR containing 9 CpG and the average methylation ratio in each CpG site. **(F)** The relative gene expression of DNMTI, DNMT3A, DNMT3B in the liver of 8-week-old mice. Data were analyzed with the Eq. $2^{-\Delta\Delta CT}$, where ΔΔCT = ΔCT (treatment group) -ΔCT (control group), and ΔCT = ΔCT (sample) -ΔCT (internal control). The values were normalized to ACTIN mRNA levels. For pyrosequencing and RT-qPCR, every group contains 10 mice. In all panels, data are presented as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, Significance was determined by Student *t* test.

DNA methylation is processed by a family of enzymes called the DNA methyltransferases (DNMTs), which include DNMT1, DNMT3A, and DNMT3B (30). DNMT1 is conservatively expressed and responsible for the maintenance of methylation, while DNMT3A and DNMT3B are required for *de novo* methylation (31). We performed qPCR to detect the expression of DNMTs, and the results indicated that there was a significant elevation of DNMT1 and DNMT3A, while there was a significant reduction of DNMT3B in GDM-F1 mouse livers compared with Control-F1 mouse livers (**Figure 4F**).

Intrauterine Hyperinsulinemia Induced Elevated Expression of DNMT3A *via* Activating FoxO1 Expression

FoxO1 plays a major role in the regulation of insulin sensitivity, and the liver is one of the critical sites of action. In our study, we

found that the relative expression of FoxO1 significantly increased in the GDM-F1 male mice through RT-qPCR compared to Control-F1 male mice (**Figure 5A**). Chromatin immunoprecipitation coupled with qPCR (Chip-qPCR) demonstrated the association between FoxO1 with DNMT promotor region. The binding of FoxO1 onto the promotor of DNMT3A was increased in the 8-week-old liver of GDM-F1 male mice compared to that in Control-F1 male mice (**Figure 5E**). In order to evaluate whether intrauterine hyperinsulinemia induced the abnormal expression of FoxO1 and DNMT3A, we cultured HepG2 in different insulin concentrations, and the results indicated that the expression of FoxO1 and DNMT3A gradually augmented as the insulin concentration increased, at both the mRNA and protein levels (**Figures 5B–D**). In addition, we also measured the effect of FoxO1 deletion on DNMT3A in the HepG2 cells. The DNMT3A

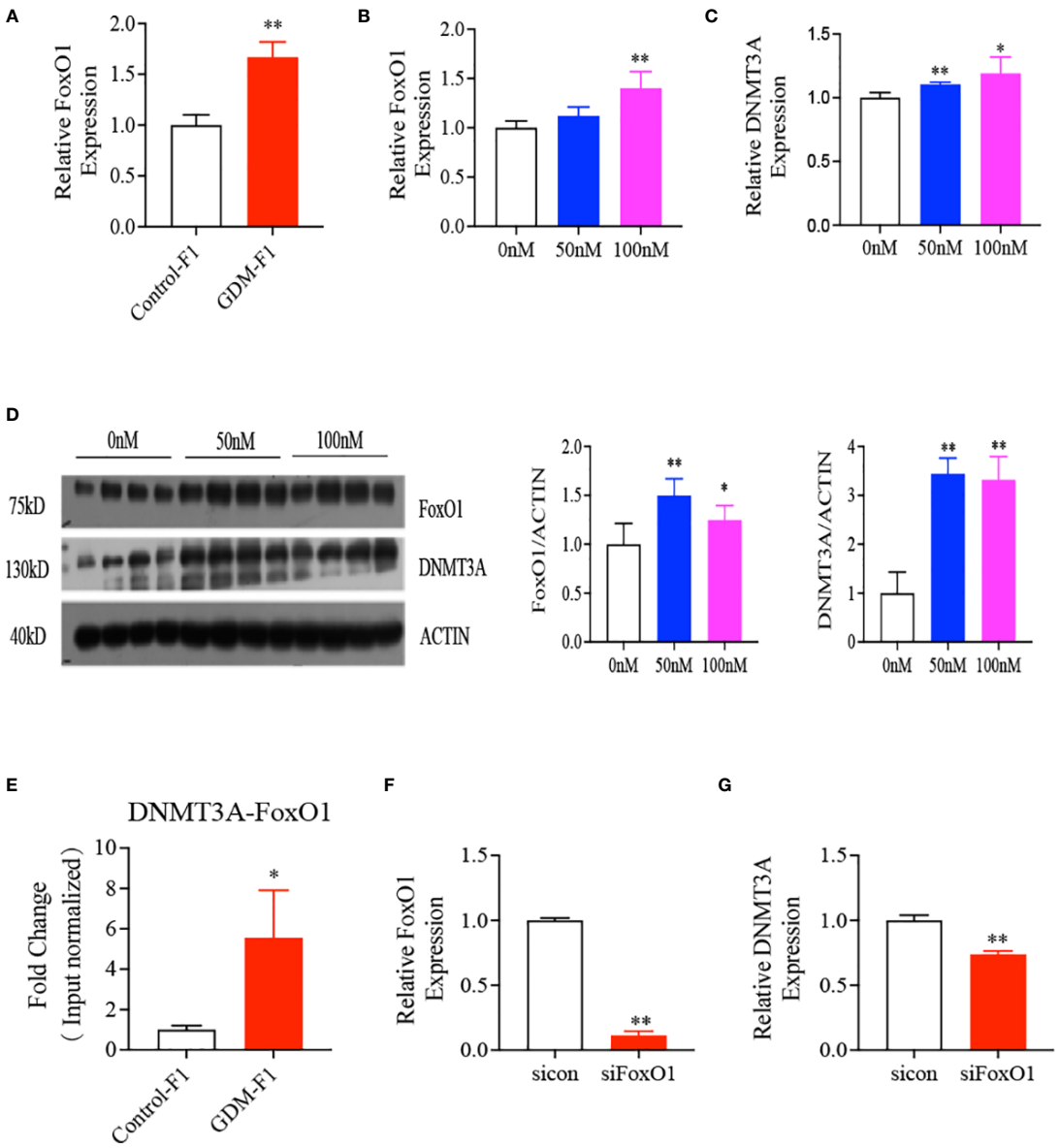

FIGURE 5 | Interaction between FoxO1 and DNMT3A. **(A)** Representative mRNA levels of FoxO1 in the liver of 8-week-old mice (every group contains 10 mice); **(B–D)** FoxO1, DNMT3A gene expression in HepG2 cells treated with 0, 50, 100 nmol/L insulin concentration after 24 hours by RT-qPCR or Western Blot (n = 4 replicates/group in at least three independent isolations); **(E)** chip-qPCR analysis to detect binding between FoxO1 and DNMT3A. IgG was as negative control. **(F, G)** mRNA level of FoxO1 and DNMT3A after silence interfere FoxO1 on HepG2 cells (n = 4 replicates/group in at least three independent isolations); For RT-qPCR, Data were analyzed with the Eq. 2-ΔΔCT, where ΔΔCT = ΔCT (treatment group) - ΔCT (control group), and ΔCT = ΔCT (sample) - ΔCT (internal control). The values were normalized to ACTIN mRNA levels, ACTIN was an internal control. In all panels, data are presented as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$. Significance was determined by Student *t* test.

level was significantly decreased in HepG2 cells following injection of si-FoxO1 (**Figures 5F, G**). These results indicated that elevated expression of FoxO1 increased the DNMT3A level through binding to its promoter region, which might be responsible for hypermethylation of the IGF2/H19 DMR region.

DISCUSSION

In this study, we demonstrated that the male offspring exposed to GDM environment developed significantly exacerbated glucose intolerance and insulin resistance as compared with the females. Similar observations have been made in a previous study (22). Downregulated IGF2/H19 gene expression in the liver of GDM male offspring can be explained by abnormal DNA methylation due to increased expression of DNMT3A. The IGF2/H19 epigenetic signature was already present in sperm samples from the paternal line (10). In turn, transcription factor FoxO1 binding in the promoter of DNMT3A resulted in altered DNMT3A expression. *In vitro* insulin stimulation experiment also verified the interaction between DNMT3A and FoxO1. Hence, our data strongly addressed the effect of hyperinsulinemia environment on GDM offspring.

The Diabetes in Pregnancy Study in Chicago revealed that offspring of diabetic mothers (age ranging from 10 to 16 years) had a significantly higher prevalence of IGT than the sex- and age-matched control groups (32). Boerschmann et al. (33) recruited 232 offspring of mothers with GDM (OGDM) and 431 offspring of non-diabetic mothers (ONDM), and they found the HOMA-IR was significantly increased in the OGDM group compared with those with ONDM mothers (33). This evidence demonstrates that maternal hyperglycemia in GDM confers a major risk of diabetes in exposed offspring (34). In addition, a number of diabetes-related animal models confirmed the phenomenon observed in clinical practice (10, 22). In our study, although there was no difference in the extent of fasting glucose in the offspring of GDM, blood glucose levels significantly increased at 30 or 60 min after injection in GDM offspring, especially for male mice, which indicated that GDM male mice demonstrated glucose intolerance in their adulthood. Moreover, after insulin injection, glucose levels in the male offspring exposed to GDM exhibited the tendency of slow declination and fast rising. This evidence strongly shows that male mice exposed to GDM have a manifestation of insulin resistance, and those glucose disturbances have a marked sex difference, in accordance with a previous study (10).

The liver is a critical organ during insulin-mediated modulation of metabolism, in particular glucose and lipid homeostasis. Insulin-like growth factor II (IGF2) is confirmed to be involved in stimulating glycogen synthesis in fetal hepatocytes through the IGF2-deficient mouse model (35). Furthermore, injecting IGF2 into hypophysectomized rats would induce decreased blood glucose and increased hepatic glycogen synthesis (36, 37). H19 locates downstream 90 kb of IGF2 on mouse chromosome 7 and is reciprocally imprinted. Downregulation of H19 stimulates an increase in the expression of gluconeogenic genes *via* FoxO1, subsequently resulting in increased glucose output (38, 39). In addition, decreased levels of H19 have been documented in insulin-resistant mice, which inhibit the expression of key metabolic genes by increasing the bioavailability of let-7 (40). Considering the important regulation of IGF2/H19 on the hepatic glucose metabolism, we detected the expression of IGF2/H19 on mouse livers. We found downregulation of IGF2/H19 expression in GDM-F1 mice compared with control mice, together with increased gluconeogenesis by PTT. Meanwhile, *in vitro* culture experiments confirmed the effect of insulin stimulation on IGF2/H19 in HepG2 cells, providing a potential explanation for intrauterine hyperinsulinemia directly affecting IGF2/H19 and subsequent impaired glucose metabolism.

Dabelea et al. (3) enrolled nuclear families in which at least one sibling was born before and one after the mother was diagnosed with type 2 diabetes and found that the sibling born after their mother displayed diabetes had a 3.7-fold higher risk of diabetes (3). The results emphasized the effects of abnormal *in utero* environments on fetuses beyond direct genetic transmission. DNA methylation without involving alteration of DNA sequences is susceptible to environmental stimuli such as toxic substances and thereby plays a critical role in elaborating the potential mechanisms of impaired glucose metabolism (41). The mouse IGF2 and H19 genes were regulated by allele-specific methylation at four DMRs: IGF2-DMR0, IGF2-DMR1, IGF2-DMR, and H19-DMR (42). Since glucose disturbance appeared much more prominent in male offspring, we examined all of the IGF2/H19 DMRs in the liver of GDM-F1 male mice. Our study indicated that *in utero* exposure to GDM could induce hypermethylation at IGF2-DMR0 and IGF2-DMR1 in GDM-F1 male offspring.

The transcriptional factor FoxO1 is an important regulator of key gluconeogenesis enzymes in the liver (43). In the fed state, insulin signaling stimulates Akt phosphorylation, subsequently activating FoxO1 expression, consequently increasing transcriptional induction of two gluconeogenic enzymes, glucose-6-phosphatase catalytic subunit (G6Pc) and phosphoenolpyruvate carboxykinase (PEPCK) (15, 44). Therefore, we detected the FoxO1 expression in our diabetes mouse model and found that GDM-F1 male mice showed increased expression of FoxO1, and *in vitro* experiments confirmed that exogenous insulin stimulation produced a similar tendency of FoxO1 as those *in vivo*. In addition, FoxO1 mediates targeted genes by binding to the FoxO-binding element as a transcriptional factor, such as uncoupling protein 1 (Ucp1) (45), peroxisome proliferator-activated receptor-alpha (PPARα) (46). However, by far, few studies have focused on the relationship between FoxO1 and DNMTs, which is a prerequisite for DNA methylation. In our study, through Chip-qPCR and *in vitro* knock-down experiments, we found that FoxO1 might regulate the expression of DNMT3A, subsequently inducing epigenetic alterations in the GDM-F1 male mice.

In conclusion, our study demonstrated that intrauterine hyperinsulinemia increased hepatic FoxO1 levels and contributed to the upregulation of DNMT3A, subsequently inducing the downregulation of IGF2/H19 expression in the liver of GDM-F1 mice. These findings provide insights into the

molecular mechanisms responsible for glucose intolerance and insulin resistance in first-generation males of GDM mice.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Zhejiang University Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

YJ contributed to the collection, analysis, and interpretation of data as well as manuscript preparation. HZ contributed to the animal model establishment. ZC contributed to the molecular experiments. X-HG, Y-CY, and YC contributed to the data collection and analysis. M-MY, B-WC, and DX contributed to the interpretation of data. MS contributed to the language editing. B-HZ and QL contributed to the study design, data interpretation, and manuscript preparation. QL is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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.2022.844707/full#supplementary-material

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Genetic Etiology of Neonatal Diabetes Mellitus in Vietnamese Infants and Characteristics of Those With *INS* Gene Mutations

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Background: Neonatal diabetes mellitus (NDM) is a rare (1:90,000 newborns) but potentially devastating metabolic disorder characterized by hyperglycemia combined with low levels of insulin. Dominantly-acting insulin (*INS*) gene mutations cause permanent NDM through single amino acid changes in the protein sequence leading to protein misfolding, which is retained within the endoplasmic reticulum (ER), causing ER stress and β-cell apoptosis. Over 90 dominantly-acting *INS* gene mutations have been identified in individuals with permanent NDM.

Patients and Methods: The study included 70 infants diagnosed with NDM in the first year of life between May 2008 and May 2021 at the Vietnam National Children's Hospital. Sequencing analysis of all the genes known to cause NDM was performed at the Exeter Genomic Laboratory, UK. Clinical characteristics, molecular genetics, and annual data relating to glycemic control (HbA1c) and severe hypoglycemia of those with *INS* mutations were collected. The main outcomes of interest were HbA1c, daily insulin dose, growth, and cognitive/motor development.

Results: Fifty-five of 70 infants (78.5%) with NDM harbored a mutation in a known disease-causing gene and of these, 10 had six different *de novo* heterozygous *INS* mutations. Mean gestational age was 38.1 ± 2.5 weeks and mean birth weight was 2.8 ± 0.5 g. They presented with NDM at 20 ± 17 weeks of age; 6/10 had diabetic ketoacidosis with pH 7.13 ± 0.26; plasma glucose level 32.6 ± 14.3 mmol/l and HbA1C 81 ± 15% mmol/mol. After 5.5 ± 4.8 years of insulin treatment, 9/10 have normal development with a developmental quotient of 80-100% and HbA1C 64 ± 7.3 mmol/mol, 9/10 have normal height, weight, and BMI on follow-up.

Conclusions: We report a series of Vietnamese NDM cases with dominant *INS* mutations. *INS* mutations are the third commonest cause of permanent NDM. We recommend screening of the *INS* gene in all children diagnosed with diabetes in the first year of life.

Keywords: diabetes mellitus in infants, INS mutations, neonatal diabetes mellitus, neonatal diabetes mellitus in Vietnamese infants, outcomes in infants with INS gene mutations

INTRODUCTION

Neonatal Diabetes mellitus (NDM) is defined by the presence of severe hyperglycemia associated with insufficient or no circulating insulin, occurring mainly before 6 months of age and rarely between 6 months and 1 year (1). The most frequent genetic causes of NDM resulting in abnormal β-cell function are methylation abnormalities of the chromosome 6q24 locus and mutations of the *ABCC8* or *KCNJ11* genes encoding the pancreatic β-cell potassium channel. Mutations in other NDM genes cause disease through a failure in pancreatic or β-cell development or as a result of the destruction of β-cells.

The human insulin gene (*INS*, OMIM # 176730) is located on the short arm of chromosome 11 (11p15.5) and encodes a single chain 110 amino acid peptide, preproinsulin, which is the insulin precursor (2, 3). Insulin biosynthesis begins by preproinsulin translocation from the cytoplasm into the endoplasmic reticulum (ER). Preproinsulin contains a signal peptide, B-chain, C-peptide, and A-chain (4). Preproinsulin is converted to proinsulin by removing the signal peptide. In proinsulin, the A chain links to B chain by C-peptide and proinsulin folds in the ER of the pancreatic β-cells. Only properly folded proinsulin molecules can transport to the Golgi apparatus and are converted to mature insulin by cleaving the C-peptide. The A-chain and B-chain of the mature insulin are crosslinked by two interchain disulfide bonds, namely, A7-B7, and A20-B19. The A-chain contains an internal disulfide bond between A6-A11.

Mutations in *INS* may affect the structure of preproinsulin, resulting in its abnormal processing to proinsulin or proinsulin misfolding (5). The abnormal or misfolded proinsulin gets retained in the ER, leading to severe ER stress and β-cell apoptosis. This process has been described in mouse models (6) and in humans (7, 8). Recent evidence suggests that *INS* mutations do not necessarily lead to β-cell death but rather the chronic ER stress interferes with β-cell growth and development (9). Mutations in *INS* cause permanent NDM (10), maturity-onset diabetes of the young (MODY10) (11), and hyperproinsulinemia (12). The first 10 heterozygous *INS* mutations causing NDM were reported in 2007 (13). Since then, over 50 dominantly-acting *INS* mutations have been reported. Autosomal recessive inheritance has also been described (14). In 2021, Støy et al. (15) reported 124 *INS* mutations in 389 diabetes cases referred to the Exeter Genomics laboratory, UK and suggested six mutation hotspots in *INS*, including A24, G32, F48, R89, C96 and c.188-31. The researchers also highlighted clinical heterogeneity in patients with mutations at these positions.

The phenotype of patients with diabetes due to a homozygous (or compound heterozygous) mutation in *INS* is characterized by severe intrauterine growth retardation (birth weight, <1 percentile) and neonatal diabetes, most likely reflecting severe insulin deficiency in the pre- and postnatal life, respectively. The majority of patients are diagnosed with diabetes in the first days or weeks of life and do not present with extrapancreatic manifestations (16). The phenotype usually resembles type 1 diabetes with requirement for insulin treatment. Insulin is a peptide hormone produced by beta cells of the pancreatic islets. It regulates the metabolism of carbohydrates, fats, and protein by promoting the absorption of glucose from the blood into the liver, fat, and skeletal muscle cells. In these tissues, the absorbed glucose is converted into either glycogen *via* glycogenesis or fats (triglycerides) *via* lipogenesis, or, in the case of the liver, into both. Insulin is secreted when blood sugar is high (as after a meal) and stops when blood sugar is low, and the liver releases glucose into the blood (15).

In our previous studies, we showed that dominant mutations in both subunits of the K_{ATP} channels resulted in a phenotype ranging from mild transient hyperglycemia to PND in our cohort. These children were diagnosed at the mean age of 8.7 ± 5.1 weeks and 51% had low birth weight (below 3rd percentile) (17). Hence, in this article, we report the mutation spectrum of NDM in individuals treated at the Vietnam National Children's Hospital and focus on the outcomes in those with *INS* mutations.

RESEARCH DESIGN AND METHODS

Individuals

The present study included 70 individuals who met the following criteria: (1) age at onset of diabetes <12 months; (2) hyperglycemia with fasting blood glucose ≥ 126 mg/dl (7.0 mmol/L) or random plasma glucose concentration ≥ 11.1 mmol/l and sustained for ≥ 2 weeks. Fasting was defined as no caloric intake for at least 4 h in children aged 0–1 years; (3) insulin dependence; and (4) exclusion of hyperglycemia caused by stress and infection and drug therapies.

Molecular Genetic Analyses

Genomic DNA was extracted from peripheral blood using phenol/chloroform methods at Vietnam National Children's Hospital. Mutation analysis was performed at the Exeter Genomic Laboratory, UK. Analysis of the coding regions and conserved splice sites of the *KCNJ11, ABCC8, INS, INSR, EIF2AK3, FOXP3, GATA4, GATA6, GCK, GLIS3, HNF1B, IER3IP1, PDX1, PTF1A, NEUROD1, NEUROG3, RFX6, SLC2A2, SLC19A2, WFS1*, and *ZFP57* genes was performed by a combination of Sanger

sequencing and targeted next generation sequencing as previously described (18). Methylation–specific MLPA was performed to investigate methylation abnormalities on chromosome 6q24. Individuals without a mutation in a known NDM gene were considered for whole genome sequencing analysis to detect novel genetic causes.

The *INS* sequence was compared with the published reference sequence NM_000207.3 using Mutation Surveyor software version 2.61. When a novel variant was identified in the *INS* gene its frequency in population controls was assessed using the Genome Aggregation Database (gnomAD). The variants were crosschecked in variant databases, ClinVar, Leiden Open Variation Database (LOVD), and Human Gene Mutation Database (HGMD). The likely effect of the variant was predicted using the algorithms provided by VarSome tool (19). The variants were classified using the American College of Medical Genetics and Genomics (ACMG) best practice guidelines (20). Amino acid sequence of preproinsulin was adapted from Støy et al. (15). The three-dimensional structure 2KQP (21) of human proinsulin was used to visualize the predicted wild-type and mutant proinsulin using Swiss-PdbViewer (22). The intronic variant was analysed using the splice site prediction tools, Splice Site Prediction (https://www.fruitfly.org/seq_tools/splice.html) and Netgene2 (https://services.healthtech.dtu.dk/service.php?NetGene2-2.42).

Clinical Phenotype and Biochemical Analyses

Clinical phenotype and biochemical tests of children with *INS* mutations were collected. The symptoms at onset and laboratory reports were obtained from medical records including sex, date of birth, gestational age, birth weight, age of diagnosis, and characteristics at diagnosis such as weight, height, presence of diabetic ketoacidosis (DKA), and neurological symptoms. Insulin and C-peptide were measured using immunoassay chemiluminescent technology by automated biochemistry Hitachi 704. Clinical follow-up started at 3 to 6 months intervals following diagnosis. Height and weight were measured in the last visit and BMI calculated, with SDS determined using WHO standards (23). The self-reported frequency of severe hypoglycemia was recorded.

HbA1c was measured at every visit using the automated Beckman Coulter AU2700/AU680 system. Blood glucose level and HbA1C targets were determined according to the International Society of Pediatrics and Adolescent Diabetes (ISPAD) 2018 guidelines (24). Serum ICA was determined by indirect immune-fluorescence and histochemical methods employing frozen unfixed human/primate or rodent pancreatic section as substrates. Autoantibodies to protein tyrosine phosphatase IA2, Zinc Transporter 8 (ZnT8A) were determined by enzyme immunoassay. GAD autoantibody was determined by an *in vitro* qualitative ELISA test.

This study was approved by the Ethics Committee of Vietnam National Children's Hospital and all parents signed the informed consent.

Statistical Analysis

Data analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL). Data are reported as frequency (%), mean ± standard deviation (SD), or median and range, as appropriate.

RESULTS

Spectrum of NDM in Vietnam National Children's Hospital

Fifty-five of 70 infants (78.5%) with diabetes diagnosed before 12 months harbored a mutation in a known disease-causing gene. This including 54 individuals who were reported in our previous study (17) and one who was recently identified. Of these 28/55 (51%) had activating heterozygous *ABCC8* or *KCNJ11* mutations; 10 had heterozygous *INS* mutations; 11 had methylation abnormalities at chromosome 6q24; two had homozygous *EIF2AK3* mutations, one had a heterozygous *EIF2B1* mutation; one had a hemizygous *FOXP3* mutation; one had a compound heterozygous *GLIS3* mutation; and one had a compound heterozygous mutation in *IL2RA* (**Figure 1**). The ten infants with heterozygous *INS* mutations are described in detail in this study.

Clinical Characteristics of Patients With *INS* Gene Mutations

Clinical characteristics are provided for all probands diagnosed under 1 year of age in whom an *INS* gene mutation was identified (**Table 1** and **Supplementary Table 1**). The mean age at diagnosis was 20 ± 17 weeks with 7/10 probands (70%) diagnosed with diabetes before 6 months of age (**Table 1** and **Supplementary Table 1**). Three of the 10 infants with *INS* mutations were diagnosed between 6 and 12 months. The presentation was either with symptomatic hyperglycemia (30%) or DKA (70%). The majority (9/10) were born appropriate for gestational age with the mean birth weight of 2.8 ± 0.6 kg corresponding from 10^{th} to 50^{th} percentile, only one case was small for gestational age with a corrected birth weight on the 8^{th} percentile (**Table 1**).

By comparing the clinical features of children with an *INS* mutation with those with early-onset diabetes due to KATP channel mutations in our cohort we found that those with *INS* mutations presented with diabetes later (mean age 20 + 17 weeks vs 8.7 + 5.1, p=0.012) (**Table 2**).

Five children with a heterozygous *INS* mutation were screened for β-cell auto-antibodies (anti-GAD, ICA, ZnT8); all five children were negative. The majority had no residual β-cell function as evidenced by low or undetectable basal or stimulated C-peptide levels.

Molecular Genetic Analyses

Six *INS* mutations, p.L30V (c.88C>G), p.G32S (c.94G>A), p.C43S (c.127T>A), p.? (c.188-31G>A), p.R89C (c.265C>T), and p.C96R (c.286T>C) were identified in the 10 NDM patients (**Tables 3** and **4**). Of these, two mutations were each detected in three unrelated individuals [p.? (c.188-31G>A), and

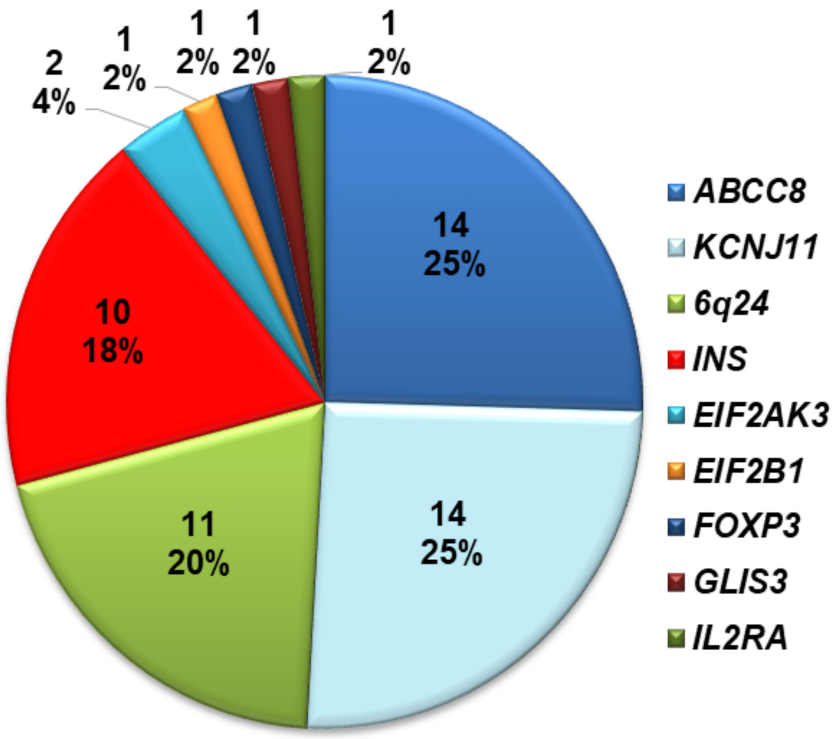

FIGURE 1 | Distribution of mutations identified in 70 Vietnamese patients with diabetes diagnosed before 1 year of age.

p.R89C (c.265C>T)], (**Table 4**). All variants were confirmed to have arisen *de novo* in the proband, were not presented in gnomAD and were classified as pathogenic variants in the ClinVar database (**Table 3**). All 5 missense variants were predicted to be deleterious by Mutation Taster, Sift, PolyPhen-2, Combined Annotation Dependent Depletion (CADD) and SNP & GO (**Supplementary Table 2**). The c.188-31>A mutation is located in intron 2 and predicted to create a new acceptor splice site with the introduction of 29 bp of intron 2 in the reading frame of exon 3 with a score of 0.62 and 0.44 in the Splice Site Prediction and Nextgene2, respectively (**Supplementary Figure 1**). According to the ACMG guidelines, all six *INS* variants were classified as pathogenic (**Table 3**).

Three mutations, p.L30V, p. G32S, and p.C43S are located in the B-chain of preproinsulin, and p.C96R is located in the A-chain of preproinsulin (**Figure 2**). The mature insulin includes three disulfide bonds, including A6-A11 (corresponding to C95-C100 in preproinsulin chain), B7-A7 (corresponding to C31-C96 in preproinsulin chain), and B19-A20 (corresponding to C43-C109 in preproinsulin chain). Therefore, the two mutations

TABLE 1 | Clinical characteristics of probands at diagnosis of diabetes resulting from an *INS* gene mutation.

	Age at diagnosis		All subjects
	0-6 months	> 6 – 12 months	
n	7	3	10
Sex (% male)	5 (71)	1 (33)	6 (60)
Birth weight (kg)	2.77 ± 0.68	3.0± 0.36	2.8 ± 0.59
Gestation age (week)	37.4± 2.7	39.6 ± 0.5	38.1± 2.5
Corrected birth weight (centile)	10-50	8-50	8-50
Age at diagnosis (months)	2.5± 2.0	9.7 ± 1.9	4.6 ± 3.9
C-peptide (nmol/l)	0.16 (0.036-1.09)	0.0003-0.8	0.4 ± 0.4
	Median (min-max)	Min - max	Mean ± SD
HbA1c (mmol/mol)	78.2 ± 45.4	89.3 ± 19.3	81.6 ± 38.6
Antibody status (Negative/Positive/NA)	4/0/3	1/0/2	5/0/5
DKA (%)	4 (57)	3 (100)	7 (70)

NA, not available; DKA, Diabetes Ketoacidosis.

TABLE 2 | Comparison of clinical characteristics of children with diabetes caused by a mutation in *INS*, *KCNJ11*, or *ABCC8*.

	INS	*KCNJ11*	*ABCC8*	p*
n	10	14	14	
Age at diagnosis (weeks)	19.9 ± 17.0	8.5 ± 5.7	8.2 ± 5.0	0.015
Gestational age (weeks)	38.1± 2.5	38.8 ± 1.2	39.5 ± 1.1	
Birth weight (kg)	2.8 ± 0.59	2.7 ± 0.5	2.8 ± 0.3	
Birth weight < 3rd (n)	0	7	7	
DKA	7	11	12	

**Differences between groups calculated using T- tests. DKA, Diabetes Ketoacidosis.*

p.C43S and p.C96R are predicted to result in a loss of disulfide bonds C43-C109 and C31-C96, respectively (**Supplementary Figure 2A, B**). In addition, a change to arginine at codon 96 might be forming clash bonds of R96 with C31 (**Supplementary Figure 2B**).

Treatment

All children with an *INS* mutation were treated with insulin. They have been followed up for 5.5 ± 4.8 years. The mean HbA1c at the last visit was 64.7 ± 7.3 mmol/mol (**Table 4**). Except for case 1, the height, weight and BMI were normal on follow-up. We screened for but did not detect chronic complications such as: retinopathy, albuminuria in cases 1, 3, 4, 5. Neurodevelopment was normal in all cases.

There were no episodes of severe hypoglycemia, defined as losing consciousness or having seizures (22), reported over the course of the follow-up in all patients (18).

DISCUSSION

In our comprehensive mutation analysis of a large cohort of 70 infants with diabetes diagnosed before 12 months of age enrolled at Vietnam National Children's Hospital, we identified gene mutations in 55 infants (78.5%) as previously described (17). The mutation pick-up rate in our study was similar to that in Slovakian infants (27), but lower than that in Ukrainian (28) and Chinese infants (29). In our cohort, mutations in the *INS* gene were the third most common genetic etiology after KATP channel mutations and chromosome 6q24 methylation abnormalities (**Figure 1**). This difference may be due to ethnicity, race, or study sample size.

Eight of the ten individuals had pathogenic variants located in a mutation hotspot highlighted by Støy et al. (15) namely p.G32S, p.R89C (n=3), p.C96R, and c.188-31G>A (n=3). Three of these, p.G32S, p.R89C, and p.C96R have been commonly reported across different ethnic groups (30–32).

Mutation at the B19 (C43) has been shown to result in completely retained of the *INS* protein in ER in *in vitro* experiments (33). Therefore, C43S disrupts the B19-A20 disulfide bond, leading to proinsulin accumulation which causes ER stress. The B7-A7 (C31-C96) is the second disulfide bond to create proinsulin folding. In our study, only patient 4 carried the p.C96R mutation. Balboa et al. (9) generated induced pluripotent stem cells (iPSCs) from a Finnish patient harboring the *INS* p.C96R mutation and CRIPSPR/SpCas9 corrected the iPSC lines, and differentiated them into β-like cells *in vitro*. The results showed that the mutation enhanced ER-stress and reduced proliferation in *INS*-mutant β-like cells without increasing apoptosis. Therefore, p.C96R leads to a defect of β-cell mass expansion, which is associated with diabetes development. Another mutation affecting C96, p.C96Y, has been studied in Akita mice (carrying the insulin 2 $Ins2^{+/C96Y}$ mutation) to improve

TABLE 3 | *INS* mutations identified in Vietnamese patients with neonatal diabetes mellitus.

Location	cDNA change (NM_000207.3)	Amino acid change (NP_000198.1)	Effect	LOVD	ClinVar	SNP id	HGMD	ACMG classification	Reference
Exon 2	c.88C>G	L30V	Missense	0000786601	–	–	CM081668	Pathogenic (PS2, PM1, PM2, PM5, PP3, and PP5)	(7)
Exon 2	c.94G>A	G32S	Missense	0000786583	VCV000021122 Pathogenic	rs80356664	CM074280	Pathogenic (PS1, PS2, PM1, PM2, PM5, PP3, and PP5)	(13, 25)
Exon 2	c.127T>A	C43S	Missense	–	–	–	CM154067	Pathogenic (PS2, PM1, PM2, PM5, and PP3)	(15)
Intron 2	c.188-31G>A	p.?	Splicing	0000473813	VCV000211186 pathogenic	rs797045623	CS120217	Pathogenic (PS2, PS3, PM2, PM4, PP3, and PP5)	(26)
Exon 3	c.265C>T	R89C	Missense	0000786591	VCV000021117 pathogenic	rs80356669	CM074283	Pathogenic (PS2, PM1, PM2, PM5, PP3, and PP5)	(13)
Exon 3	c.286T>C	C96R	Missense	–	VCV000918067 Pathogenic	rs1845839718	CM128900	Pathogenic (PS1, PS2, PM1, PM2, PM5, PP3, and PP5)	(13)

LOVD, Leiden Open Variation Database; HGMD, Human Genetic Mutation Database; ACMG, American College of Medical Genetics and Genomics.

TABLE 4 | Clinical and biochemical characteristics at last evaluation.

Patient	Current age (years)	Insulin requirement dose U/kg/day	HbA1c (mmol/mol)	Height at last evaluation cm/(SDS)	BMI (kg/m^2)/(SDS)	*INS* mutations
1	17	0.78	67	151.5 (-3.0)	16.7 (-2.1)	c.127T>A (p.C43S)
2	2.8	1.02	63	124 (-1.98)	14.4(-1.2)	c.188-31G>A (p.)?
3	8.1	0.56	65	117 (-1.94)	14.3 (-1.1)	c.188-31G>A (p.)?
4	8.5	0.77	67	135 (0.95)	16.7 (0.3)	c.286T>C (p.C96R)
5	6.8	1.1	62	123 (0.68)	15.2 (-0.07)	c.265C>T (p.R89C)
6	2.8	0.45	56	93.3 (-0.38)	16.0 (-0.03)	c.265C>T (p.R89C)
7	4.1	0.55	69	104 (-0.09)	13.4 (-2.4)	c.94G>A (p.G32S)
8	1.3	0.67	67	82 (0.66)	15.3	c.188-31G>A (p.)?
9	2.5	0.56	79	89.5 (-0.47)	15.3 (-0.82)	c.88C>G (p.L30V)
10	1.3	0.2	52	78 (0.18)	14.7	c.265C>T (p.R89C)
X ± SD	5.5 ± 4.8	0.6 ± 0.2	64.7± 7.3			

understanding of this mutation in diabetes pathogenesis, for example, misfolded proinsulin, ER stress, β-cell apoptosis or proliferation defect, or diabetes phenotype heterogeneity between male and female Akita mice (34–39).

The residue L30 (B6) could be involved in orienting the N-terminal region, maintaining local structure in the vicinity of the B7-A7 disulfide, or making contact with a receptor (40). The modeling of insulin also indicated L30 (B6) interacts with C95 (A6) *via* a hydrogen bond (distance = 2.06 Å) and the mutation p.L30V increases B6-A6 distance to 2.74 Å (**Supplementary Figure 3**). p.L30M and p.L30P increase Cys-Cys distance between B7-A7 to 7.53 and 5.98 Å, respectively, compared to 2.27 Å of wild-type L30 (8). Therefore, p.L30M and p.L30P disfavor disulfide bond formation and change the structure after disulfide bond formation. Functional assay showed that p.L30M and p.L30P perturbed proinsulin structure and lead to defective

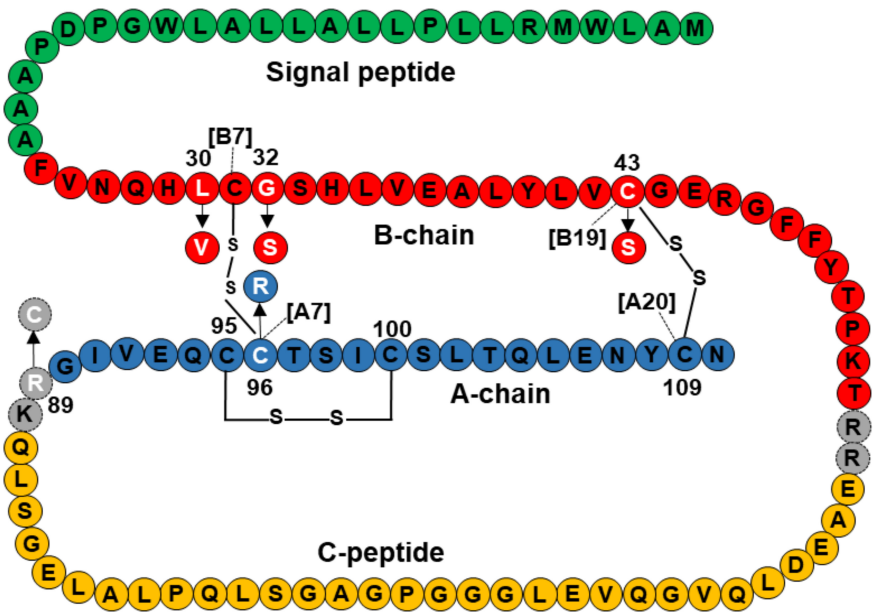

FIGURE 2 | Location of *INS* mutations identified in 10 Vietnamese patients in the preproinsulin. Green, red, orange, and blue color amino acids represent for signal peptide, B-chain, C-peptide, and A-chain, respectively. Mutations are marked in white, including L30V, G32S, C43S, R89C, and C96R. Amino acid sequence of preproinsulin was adapted from Støy et al. (15).

insulin secretion. The mutation p.L30V may act in the similar way to p.L30M and p.L30P, resulting a defect in proinsulin folding. The mutation p.L30V was first reported in a child with NDM (7) who showed a later onset (184 day-old versus 23 day-old) and a higher insulin dose (0.85 versus 0.56 U/kg/day) at the age of 30 months, compared to our case 9 (**Supplementary Table 1** and **Table 4**).

Støy et al. (13) suggested that G32 (B8) plays an important structural role by participating in a β-turn adjoining the central α-helix that lies in the proximity to the B7-A7 (C31-C96) disulfide bond. The substitution of L-serine for glycine at B8 (p.G32S) reorients the B8 conformation, resulting in rotation of B1-B8, turning the B7 cysteine away from its partner at A7. The p.G32S was proved to partially retain the protein in the ER and recruit to granules, and decrease co-transfected wild-type secretion (33). Such results explain the mechanism through which p.G32S causes diabetes.

A previous study investigating the mRNA of an individual heterozygous for the c.188-31G>A mutation detected aberrant transcripts with an insertion of 29 bp of intron 2 to exon 3 (26). This splice site mutation has been observed in the Caucasian population in Spain (26), United States (41), Czech Republic (42), and Japan (43) as causing NDM or maturity-onset diabetes of the young. Our study is the second report of this mutation in Asian individuals. Panova et al. (44) have successfully generated a neonatal diabetes-specific iPSC line harboring c.188-31G>A which may be used to investigate how the mutation affects insulin accumulation in the β- cells.

The p.R89C mutation creates an additional unpaired cysteine, which may disrupt disulfide bond formation in proinsulin. p.R89C is located at a CpG dinucleotide, a hotspot for pathogenic mutations in the human genome. This mutation hinders normal folding, leading to a reduction of properly folded protein. Rajan et al. (33) showed that p.R89C resulted in aberrant processing of proinsulin to insulin, however, no significant depletion of wild-type insulin secretion was observed.

In our study, seven children with *INS* gene mutations had diabetes onset before 6 months of age whilst three were diagnosed between 6 months and 12 months. The median age at diagnosis of the *INS* gene mutation carriers was 20 ± 17 weeks later than in Edghill's study (25), in which heterozygous *INS* mutations were found in 33/141 (23%) probands diagnosed before 6 months, 2/86 (2%) between 6 and 12 months, and none of 58 diagnosed between 12–24 months of age. In our cohort, 3 with *INS* mutations were diagnosed after the age of 6 months. Taken together, these data suggest that *INS* mutations are more common in infants presenting with NDM at a younger age, however further large cohort studies are needed to establish the prevalence of *INS* mutations in patients presenting at a range of ages, especially in those who do not have autoantibodies to islet cell proteins. A recent paper by Støy et al. reported that in a cohort of 274 individuals with *INS* dominant mutations, 65% were diagnosed before 6 months of age and 18% of patients between 6 and 12 months of age. Thus by testing individuals diagnosed with diabetes before 12 months for the *INS* gene, one would expect to pick up >80% of cases caused by pathogenic dominant variants in this gene (15) We therefore recommend that the *INS* gene should be screened in all children diagnosed with diabetes in the first year of life and not just before the age of 6 months, as well as those at an older age with features of monogenic diabetes including absent autoantibodies, in keeping with the ISPAD guidelines (45).

The majority of other NDM genetic subtypes result in intrauterine growth restriction (IUGR) or low birth weight, described as small for gestational age (SGA), which is due to insulin deficiency *in utero* (46). This is however not the case for dominant *INS* mutations as confirmed in our cohort where the mean birth weight was normal (2.8 ± 0.5kg, range 1.5–3.6) adjusted for gestational age, corresponding from the 8th percentile to 50th percentile.

Management of diabetes in infants and adolescents is a challenge. According to the ISPAD guidelines (24), the target HbA1c is < 7.0% for children, adolescents and young adults who have access to comprehensive care. In our study, only one case achieved the target HbA1c. While our cohort is small, this suggests that achieving the target glucose control in this population is challenging. Use of continuous glucose monitoring and insulin pump therapy may improve glycemic control, but universal funding for these technologies is not currently available in Vietnam.

Neurological dysfunction is a key feature of the phenotype of some patients with PNDM due to mutations in *KCNJ11* or *ABCC8* (47) but patients with *INS* gene mutations in our study did not have other associated extra-pancreatic features, including neurological dysfunction. This is consistent with previous studies (16).

A limitation of our study is the small sample size; however our data add to the limited published longitudinal data on children with *INS* mutations. It will be important to follow this cohort further to determine their risk of vascular complications compared with other forms of NDM and type 1 diabetes.

CONCLUSION

We report and characterise a series of children with *INS* gene mutations. These mutations are the third most common cause of diabetes in the first year of life. We recommend that the *INS* gene should be screened for mutations in all children diagnosed with diabetes before 12 months of age who are antibody negative. Long term follow-up of these children is important to inform clinical care and monitoring for complications.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: All disease-causing variants identified by next generation sequencing in this project

were uploaded onto the DECIPHER database (https://decipher.sanger.ac.uk/).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Vietnam National Children's Hospital IRB#1. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CN and VD conceptualized, designed the study, and wrote and reviewed the manuscript. CN, VD, BT, and NK provided patients' clinical information. MC reviewed/edited the manuscript. NL, EF, SF, TD, NH, and MC analyzed data, and wrote and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.866573/full#supplementary-material

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Inter-ankle Systolic Blood Pressure Difference Is a Marker of Increased Fasting Blood-Glucose in Asian Pregnant Women

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Objective: This cross-sectional study aimed to determine the relationship between clinical blood pressures and blood pressures measured using Doppler with blood glucose in pregnancy by ethnicity.

Methods: We recruited 179 (52% White European, 48% Asian) pregnant women at 24-28 weeks of gestation who underwent a glucose tolerance test in an antenatal clinic in Bradford Royal Infirmary, the UK, from 2012 to 2013. Systolic blood pressures in the arm (left and right brachial) and ankle [left and right posterior tibial (PT) and dorsalis pedalis (DP)] blood pressures were measured using a Doppler probe. The inter-arm (brachial) and inter-ankle (PT and DP) systolic blood pressure differences were obtained. A multivariate linear regression model adjusted for age, body mass index, and diabetes risk was used to assess the relationship between blood pressures and blood glucose.

Results: Asian pregnant women had higher blood glucose but lower ankle blood pressures than White Europeans. In White Europeans, brachial blood pressures and clinical blood pressures were positively associated with fasting blood glucose (FBG), but brachial blood pressures did not perform better as an indicator of FBG than clinical blood pressures. In Asians, increased inter-ankle blood pressure difference was associated with increased FBG. For each 10 mmHg increase in the inter-ankle blood pressure difference, FBG increased by 0.12 mmol/L (Beta=0.12, 95%CI: 0.01-0.23).

Conclusion: The relationship between blood pressures with blood glucose differed by ethnicity. In Asians, inter-ankle systolic blood pressure difference was positively associated with blood glucose. This is first ever report on ankle blood pressures with blood glucose in pregnancy which suggests future potential as a non-invasive gestational diabetes risk screening tool.

Keywords: ankle blood pressure, doppler, clinical blood pressure, glucose metabolism, OGTT (oral glucose tolerance test), pregnancy, race/ethnic differences

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INTRODUCTION

At the National Health Service Health Check in the UK, thresholds of body mass index (BMI) and blood pressures are used to screen for increased risk of cardio-metabolic diseases. However, blood pressure is not considered to calculate the risk of gestational diabetes mellitus (GDM). The current screening for the risk of GDM mainly depends on known risk factors such as age and BMI (1). Moreover, the current screening is applied to the general population and is not ethnicity-specific.

Asian pregnant women have a higher prevalence of GDM than White Europeans (2). They also have higher weight-related disease risks such as cardiovascular diseases at lower BMIs (3, 4). In this context, the method of screening high-risk populations for GDM based on known factors such as BMI may exclude Asian populations who have a lower BMI (5) and are not in the regular risk group. Therefore, it is crucial to develop an ethnicity-specific method to screen for GDM high-risk women.

We have previously demonstrated that the increased ankle blood pressures were associated with diabetes more than brachial blood pressures in non-pregnant primary care practice attendees (6). In addition, inter-ankle blood pressure difference has been demonstrated to better predict cardiovascular events in elderly patients compared with inter-arm blood pressure (7). So far, there is no study on the relationship between ankle blood pressure and blood glucose during pregnancy. Thus, this is the first study that aimed to determine the relationship between ankle blood pressure and its differences with blood glucose in pregnancy in Asians and White Europeans.

MATERIALS AND METHODS

Study Population

In this cross-sectional study 184 pregnant women at 24-28 weeks of gestation were recruited at an antenatal clinic in Bradford Royal Infirmary, the UK, in 2012-2013. Consecutive pregnant women were recruited without selection bias. All pregnant women were screened for GDM with a 75 g oral glucose tolerance test (OGTT). GDM was diagnosed according to the recommendations of the National Institute for Health and Care Excellence criteria: fasting blood glucose (FBG) ≥ 5.6 mmol/L or 2-hour blood glucose (2h-BG) ≥ 7.8 mmol/L (8). Pregnant women older than 18 years who provided written informed consent were included. The exclusion criteria included: 1) pre-pregnancy type 1 or type 2 diabetes, 2) FBG≥7.0 mmol/L (n=1) (9), 3) refusal to consent, and 4) missing values for FBG and 2h-BG (n=4). Finally, 179 pregnant women were included. GDM diagnosis and testing criteria were the same during the study period. Pregnant women were approached only once, with glucose tolerance test and the measurements of blood pressure performed on the same day. This cross-sectional study followed the principles set by the Declaration of Helsinki, according to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines and was approved by the National Research Ethics Committee (approval number: 10/H1302/28).

Blood Pressure Measurement and Blood Pressure Differences

On the day of OGTT test, arm (left and right brachial) and ankle [left and right posterior tibial (PT) and dorsalis pedalis (DP)] systolic blood pressures were recorded using a Doppler probe (Huntleigh Super Dopplex II, Huntleigh Healthcare, Cardiff, UK) as we did previously (6, 10). The left ankle blood pressure was calculated as the average of left PT and DP, and the right ankle blood pressure was calculated in the same way.

Blood pressure and blood pressure differences were obtained Inter-arm (brachial), inter-PT, and inter-DP systolic blood pressure differences were calculated as the absolute value of the differences in brachial, PT and DP systolic blood pressure on the left and right sides, respectively. Inter-ankle systolic blood pressure difference was calculated as the absolute value of the differences in left and right ankle blood pressure.

Data Collection

Patient clinical parameters collected from the medical records included age (years), ethnicity [White European, Asian (Indian, Pakistani, Bangladeshi, other Asian)], pregnancy week (weeks), BMI (kg/m^2), diabetes family history, previous abnormal fasting glucose, abnormal glucose tolerance or GDM, clinical blood pressure (systolic and diastolic, mmHg). Pulse pressure (mmHg) was calculated as the difference between systolic and diastolic blood pressure. Hypertension was defined as systolic/diastolic BP≥140/90 mm Hg or treatment for hypertension.

Statistical Analysis

Continuous variables were depicted as means and standard deviation and compared by *t*-test. Categorical variables were expressed as number (%) and compared by Chi-square test. Missing data were excluded from the analysis. We used linear regression to explore the relationship between blood pressure and blood glucose after adjusting for age, BMI and high diabetes risk (defined as family history of diabetes, previous abnormal fasting glucose, abnormal glucose tolerance or GDM). The statistical analyses were performed using the SPSS (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). A two-tailed $p<0.05$ was recognized statistically significant.

Sensitivity Analysis

As GDM is more frequent in Asian population compared with White Europeans (11), it may influence the relationship of ankle and brachial blood pressure with blood glucose. Therefore, analyses were repeated following exclusion of GDM-positive women.

RESULTS

The participants included 179 pregnant women, 93 White Europeans and 86 Asians. The Asian group was comprised of 53 (61.6%) Pakistani, 24 (27.9%) Bangladeshi, 4 (4.6%) Indian and 5 (5.8%) women of other Asian origins. This group

represents the Asian population in Bradford, the UK (12). Both groups had a similar gestational week, BMI, percentage of women with high diabetes risk (**Table 1**).

Ethnicity-Based Differences in Glycemic Control and Blood Pressures

There were significantly more GDM-positive cases among Asian pregnant women than White European pregnant women. Moreover, Asian participants were older, had higher FBG and 2h-BG. As a higher percentage of GDM-positive women among Asians may contribute to the observed differences in blood glucose concentrations, we excluded GDM patients and repeated the analysis. Asian pregnant women had a higher FBG, but not 2h-BG, than White European pregnant women (**Table 1**). These data suggest that White European and Asian pregnant women have different glucose metabolism as observed previously (13).

No difference was observed for routine clinically measured blood pressures. Two White European and one Asian women had hypertension. One Asian women was suffering from pre-eclampsia. Arm and ankle blood pressures (left PT, DP and ankle in general; right PT and ankle in general) measured using a Doppler probe were significantly higher in White European than Asian pregnant women (**Table 1**), similarly to what we observed previously in the non-pregnant populations (6, 10). These data suggest that Doppler examination of blood pressure in the ankle and arm arteries may be a more sensitive way to assess blood pressure status in pregnancy in different ethnicities.

As for the blood pressure difference, we observed that the lower the blood pressure measurement point in the body, the greater the difference in blood pressure between the left and right limbs. (Inter-DP > inter-PT > inter-brachial systolic blood pressure difference) in both groups. In terms of the comparison of the blood pressure difference between ethnic groups, Asians had higher inter-arm (brachial) and inter-ankle (PT, DP and overall) systolic blood pressure differences than White Europeans, but the differences did not reach significance.

TABLE 1 | Characteristics of study participants[a].

	Entire population (n=179)			Population excluding GDM-positive women (n=163)		
	White Europeans (n=93)	Asians (n=86)	*P* [b]	White Europeans (n=90)	Asians (n=73)	*P* [b]
Age (years)	27.4 ± 5.0	28.9 ± 4.7	**0.045**	27.2 ± 4.8	28.2 ± 4.1	0.135
Gestational Weeks	26.4 ± 1.2	25.7 ± 2.9	0.171	26.3 ± 1.2	25.7 ± 2.4	0.120
Body mass index (kg/m^2)	29.1 ± 5.5	28.5 ± 5.2	0.440	28.9 ± 5.3	28.3 ± 5.3	0.501
High Diabetes Risk [c]	3 (3.2%)	4 (4.7%)	0.623	2 (2.2%)	2 (2.7%)	0.823
GDM cases	3 (3.2%)	13 (15.1%)	**0.005**	–	–	
Blood glucose						
Fasting blood glucose (mmol/L)	4.21 ± 0.45	4.46 ± 0.50	**0.001**	4.18 ± 0.38	4.37 ± 0.39	**0.002**
2-hour blood glucose (mmol/L)	5.26 ± 1.13	5.80 ± 1.65	**0.012**	5.17 ± 1.02	5.32 ± 1.03	0.379
Systolic Blood pressure (Doppler)						
Arm						
Left brachial (mmHg)	107.1 ± 12.0	102.6 ± 13.7	**0.024**	106.9 ± 12.1	102.0 ± 13.9	**0.020**
Right brachial (mmHg)	111.2 ± 12.7	106.6 ± 12.1	**0.014**	111.1 ± 12.8	106.7 ± 12.1	**0.032**
Ankle						
Left PT (mmHg)	128.1 ± 17.7	120.2 ± 19.0	**0.005**	127.7 ± 17.7	119.1 ± 19.8	**0.004**
Left DP (mmHg)	123.5 ± 22.7	114.7 ± 19.3	**0.007**	123.1 ± 22.9	113.3 ± 20.0	**0.005**
Left ankle (mmHg)	125.3 ± 18.7	117.3 ± 18.4	**0.005**	124.9 ± 18.8	116.0 ± 19.0	**0.004**
Right PT (mmHg)	127.7 ± 22.1	119.9 ± 19.4	**0.013**	127.5 ± 22.4	119.1 ± 19.9	**0.014**
Right DP (mmHg)	122.7 ± 23.0	116.7 ± 22.0	0.077	122.3 ± 23.2	115.5 ± 21.9	0.058
Right ankle (mmHg)	125.4 ± 20.5	118.4 ± 19.4	**0.022**	125.1 ± 20.7	117.4 ± 19.5	**0.019**
Blood pressure difference						
Inter-brachial difference (mmHg)	7.1 ± 7.4	8.1 ± 9.7	0.440	7.2 ± 7.5	8.2 ± 9.3	0.461
Inter-PT difference (mmHg)	9.8 ± 11.3	10.2 ± 10.4	0.804	9.9 ± 11.4	10.5 ± 11.0	0.721
Inter-DP difference (mmHg)	10.6 ± 13.4	11.5 ± 14.0	0.650	10.7 ± 13.6	11.4 ± 14.6	0.775
Inter-ankle difference (mmHg)	7.7 ± 7.8	10.0 ± 9.5	0.098	7.9 ± 7.9	10.2 ± 10.0	0.124
Blood pressure (Clinics)						
SBP (mmHg)	111.5 ± 13.8	109.1 ± 11.6	0.206	111.2 ± 13.9	108.9 ± 11.7	0.253
DBP (mmHg)	64.7 ± 11.3	62.3 ± 10.3	0.150	64.6 ± 11.4	62.7 ± 10.5	0.289
Pulse pressure (mmHg)	47.2 ± 13.3	46.8 ± 11.6	0.831	47.0 ± 13.4	46.2 ± 11.9	0.681

All (100%) of the records included information related to maternal age, ethnicity and high diabetes risk; 184 (99.5%) to body mass index, 183 (98.9%) to systolic and right dorsalis pedalis blood pressure; 182 (98.4%) to right brachial, left and right posterior tibial blood pressure; 180 (97.3%) to diastolic, left brachial, left dorsalis pedalis blood pressure, and 2-hour blood glucose; 174 (94.1%) to fasting blood glucose.

[a]Data are represented as means ± standard deviation;

[b]Student t-test was used to assess statistical significance;

[c]High diabetes risk was defined as a family history of diabetes or previous abnormal fasting glucose or abnormal glucose tolerance or GDM;

DBP, diastolic blood pressure; DP, dorsalis pedalis; GDM, gestational diabetes mellitus; n, number; PT, posterior tibial; n, number; SBP, systolic blood pressure.

Significantly different p values are marked in bold.

Relationship Between Blood Glucose and Blood Pressure in Pregnancy

We studied whether blood pressures might be related to blood glucose concentrations in pregnancy. Multiple linear regression analysis was conducted with blood pressure and blood glucose as continuous variables. Left brachial blood pressure (measured with Doppler) and systolic and diastolic blood pressure (routine clinical parameters) showed a positive association with FBG in White European pregnant women, also after excluding GDM patients. However, such a relationship was not observed in Asian pregnant women (**Table 2**).

In Asians, fasting blood glucose was only related to the inter-ankle blood pressure difference, specifically inter-DP blood pressure difference. In general, each 10 mmHg rise in inter-ankle blood pressure difference, FBG increased by 0.12 mmol/L (95%CI: 0.01-0.23), and 0.13 mmol/L (95%CI: 0.04-0.22) after excluding GDM patients. Such a relationship was not found in White European study participants. These data indicate that inter-ankle systolic blood pressure difference can be developed as a surrogate for a non-invasive measure of glucose intolerance in Asian pregnant women.

No association between blood pressure and 2-h BG were found in Asians or White Europeans (**Supplementary Table**).

DISCUSSION

Our Findings

We found that Asian pregnant women had higher blood glucose but lower ankle and brachial blood pressures (measured with Doppler) than White European pregnant women. In White European, left brachial and routine clinical blood pressure (SBP and DBP) were positively associated with FBG. While in Asian study participants, inter-ankle blood pressure difference was positively associated with FBG.

Comparisons With Other Studies

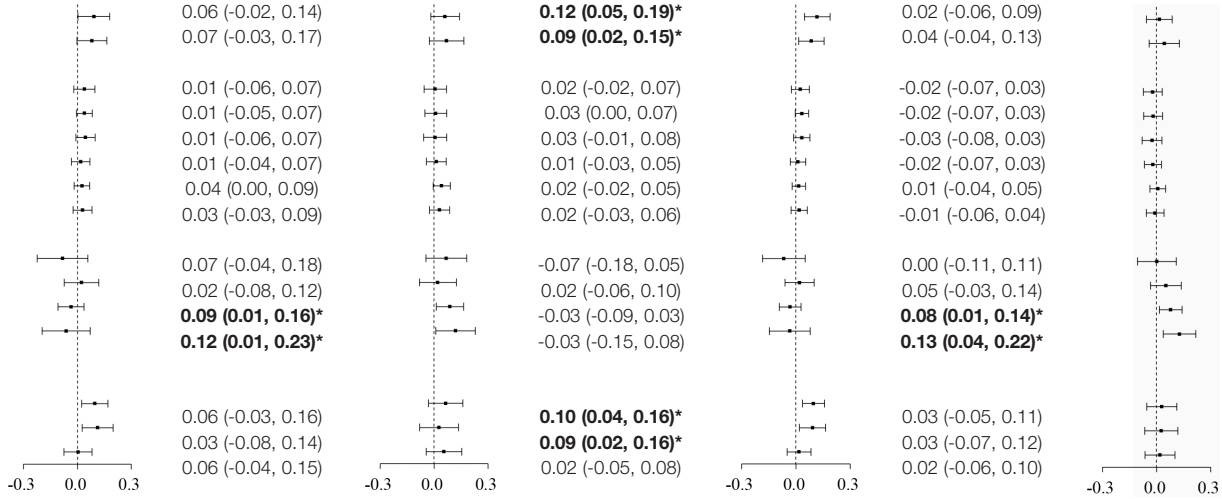
The relationship between blood pressure/hypertension and GDM, which was characterized as increased blood glucose, has been reported in previous studies. Pregnancy with hypertension is a risk factor for GDM (14). Higher systolic and diastolic blood pressure (15) but similar mean arterial blood pressure (16) were reported in GDM-positive compared with GDM-negative women. Moreover, increased blood pressure in the first trimester increased the risk of GDM (17). Overall, we found that blood pressure was associated with FBG in Asians and White Europeans, but the association varied by ethnicity. In White Europeans, the association between brachial blood pressure and blood glucose was similar to that between clinical blood pressure and blood glucose, indicating that brachial blood pressure did not perform better as an indicator of blood glucose.

In Asians, the association between fasting blood pressure and blood glucose was only reflected in inter-ankle systolic blood pressure difference. Ankle blood pressure was reported to be a better discriminator than brachial blood pressure for diabetes, independent of age and sex (6). Furthermore, inter-ankle blood pressure difference was a more useful blood pressure parameter. Compared to inter-arm blood pressure difference, inter-ankle blood pressure difference could better predict cardiovascular events and all-cause mortality in elderly patients (7). Higher inter-ankle blood pressure difference was also crucial in predicting rapid renal progression and progression to renal endpoints in patients with chronic kidney disease (18). In this study, we found that the difference in blood pressure between DP was larger than the difference between the brachial and PT in all participants. Inter-ankle systolic blood pressure difference ≥ 10 mmHg was considered abnormal and not uncommon in Asians (19). We found that Asian pregnant women had higher inter-ankle systolic blood pressure difference than White Europeans; however, the differences did not reach significance due to the relatively small sample size. After adjusting for age, BMI and diabetes risk, we found that as the inter-ankle blood pressure difference (specifically DP difference) increased, the FBG concentrations increased in Asian pregnant women. Fasting blood glucose is the glucose concentration after an overnight fast that reveals mostly endogenous glucose production and indicates insulin resistance. Postprandial blood glucose represents a sharp increase in glucose in response to exogenous glucose, and 2-h BG glucose mainly indicates glucose clearance due to action of insulin (20). Therefore, ankle blood pressure could be indicative of pathological insulin resistance in pregnancy. The possible mechanism may be that a high inter-ankle blood pressure difference is a result of disproportional reduction in elasticity of arterioles between the right and the left lower limb due to insulin resistance (21), which precedes the increase in fasting blood glucose (22). When detected and treated early, these changes could be reversed with lifestyle modifications or insulin sensitizers. Moreover, blood glucose concentrations have been demonstrated as an independent positive factor for inter-ankle blood pressure differences (19).

The ethnic difference in the association between inter-ankle systolic blood pressure difference and blood glucose may be caused by genetic background. For example, our study found that Asian pregnant women had a higher blood glucose concentrations than White Europeans which is in concordance with reports of a higher prevalence of GDM (2). One possible reason is that Asians have higher visceral body fat than White Europeans of the same BMI (23, 24), which could result from a genetic predisposition for higher insulin resistance along with sedentary lifestyle. Insulin resistance causes changes to the structure and function of the arterioles and capillary systems, in the lower limbs (25–28) and hence, independent of systemic blood pressure. Although Asians have a higher risk of GDM, the current screening is done for the general population and is not ethnicity-specific. Our study provides evidence of the significance of ankle systolic blood pressure measurement as a surrogate sensitive marker of blood glucose in populations with a higher incidence of GDM and insulin resistance.

TABLE 2 | The association between blood pressures and fasting blood glucose by ethnicity.

Blood pressures	Entire population (n=179)		Population excluding GDM-positive women (n=163)	
	White Europeans (n=93)	Asians (n=86)	White Europeans (n=90)	Asians (n=73)
	Beta (95%CI) [a]	Beta (95%CI) [a]	Beta (95%CI) [a]	Beta (95%CI) [a]
Systolic Blood pressure (Doppler)				
Arm				
Left brachial (/10mmHg)	**0.09 (0.00, 0.18)***	0.06 (-0.02, 0.14)	**0.12 (0.05, 0.19)***	0.02 (-0.06, 0.09)
Right brachial (/10mmHg)	0.08 (0.00, 0.16)	0.07 (-0.03, 0.17)	**0.09 (0.02, 0.15)***	0.04 (-0.04, 0.13)
Ankle				
Left PT (/10mmHg)	0.04 (-0.02, 0.10)	0.01 (-0.06, 0.07)	0.02 (-0.02, 0.07)	-0.02 (-0.07, 0.03)
Left DP (/10mmHg)	0.04 (-0.01, 0.08)	0.01 (-0.05, 0.07)	0.03 (0.00, 0.07)	-0.02 (-0.07, 0.03)
Left ankle (/10mmHg)	0.05 (-0.01, 0.10)	0.01 (-0.06, 0.07)	0.03 (-0.01, 0.08)	-0.03 (-0.08, 0.03)
Right PT (/10mmHg)	0.02 (-0.03, 0.07)	0.01 (-0.04, 0.07)	0.01 (-0.03, 0.05)	-0.02 (-0.07, 0.03)
Right DP (/10mmHg)	0.02 (-0.02, 0.07)	0.04 (0.00, 0.09)	0.02 (-0.02, 0.05)	0.01 (-0.04, 0.05)
Right ankle (/10mmHg)	0.03 (-0.03, 0.08)	0.03 (-0.03, 0.09)	0.02 (-0.03, 0.06)	-0.01 (-0.06, 0.04)
Blood pressure differences				
Inter-brachial difference (/10mmHg)	-0.08 (-0.22, 0.06)	0.07 (-0.04, 0.18)	-0.07 (-0.18, 0.05)	0.00 (-0.11, 0.11)
Inter-PT difference (/10mmHg)	0.02 (-0.07, 0.12)	0.02 (-0.08, 0.12)	0.02 (-0.06, 0.10)	0.05 (-0.03, 0.14)
Inter-DP difference (/10mmHg)	-0.04 (-0.11, 0.04)	**0.09 (0.01, 0.16)***	-0.03 (-0.09, 0.03)	**0.08 (0.01, 0.14)***
Inter-ankle difference (/10mmHg)	-0.06 (-0.20, 0.07)	**0.12 (0.01, 0.23)***	-0.03 (-0.15, 0.08)	**0.13 (0.04, 0.22)***
Blood pressure (Clinics)				
SBP(/10mmHg)	**0.10 (0.02, 0.17)***	0.06 (-0.03, 0.16)	**0.10 (0.04, 0.16)***	0.03 (-0.05, 0.11)
DBP (/10mmHg)	**0.11 (0.03, 0.20)***	0.03 (-0.08, 0.14)	**0.09 (0.02, 0.16)***	0.03 (-0.07, 0.12)
Pulse pressure (/10mmHg)	0.00 (-0.07, 0.08)	0.06 (-0.04, 0.15)	0.02 (-0.05, 0.08)	0.02 (-0.06, 0.10)



[a]*Models were adjusted for age, body mass index and high diabetes risk (family history of diabetes or previous abnormal fasting glucose or abnormal glucose tolerance or GDM);*
*Statistical significance is indicated as follows: * P<0.05.*
CI, confidence interval; DBP, diastolic blood pressure; DP, dorsalis pedalis; GDM, gestational diabetes mellitus; n, number; PT, posterior tibial; n, number; SBP, systolic blood pressure.
Values with statistically significant p values are marked in bold.

Implications

Due to cost, OGTT is done only in high GDM risk populations in some regions (29). Current GDM risk screening is based on known risk factors such as age, BMI, diabetes family history but does not include blood pressure (1). Such selective GDM risk screening may exclude Asians, who have a lower BMI (5), so are not in the regular risk group. Therefore, it is crucial to develop an ethnicity-specific measures to screen for GDM high-risk pregnant women. Recently, abdominal visceral adipose tissue depth (VAD) measured by ultrasound in early pregnancy was proposed to predict GDM better than pre-pregnancy BMI and thus used in GDM screening (30). However, although VAD measurement is non-invasive, it is time-consuming and needs specialist training. On the contrary, ankle systolic blood pressure can be done easily by a community health care assistant, especially with automated machines. We propose that inter-ankle systolic blood pressure difference may serve as an early indicator of changes of increased insulin resistance and blood glucose in pregnancy and be developed as a sensitive, convenient and affordable method for GDM risk screening. Inter-ankle systolic blood pressure difference may also be developed to screen post-partum diabetes in GDM patients. Moreover, our findings can be further investigated in other ethnicities.

Future Study

Brachial blood pressure changes dynamically during the various trimesters of pregnancy (31, 32). Whether similar changes can be observed in ankle blood pressure and what factors modulate these changes need elucidation. Thus, prospective studies starting in the first trimester with a larger group and different ethnicities need to be conducted to understand better the relationship between ankle blood pressure, its differences and blood glucose concentrations in pregnancy.

Strengths and Limitations

pt?>To the best of our knowledge, we are the first to report that inter-ankle blood pressure difference is a marker of blood glucose in Asian pregnant women. Our study has several strengths. First, we included blood pressure measured by Doppler device and clinical blood pressure and obtained inter-arm and inter-ankle systolic blood pressure differences. The inclusion of multiple blood pressure indicators enables us to comprehensively explore the association between blood pressures and blood glucose during pregnancy. The second strength is the blind study design; experimentalists were unaware of the study subjects' blood glucose concentrations. Third, GDM screening and testing criteria were the same during the study period; including measurements of blood pressure and blood glucose concentrations. Nevertheless, our study has some limitations. First, due to the cross-sectional study design, we cannot find a causal relationship between blood pressure and blood glucose. Second, the sample size is small, and analysis was done at a single center. Thus, we could not access the relationship between blood glucose and hypertension/preeclampsia. However, a significant association between inter-ankle blood pressure difference and blood glucose was found in this sample size, suggesting that our findings are sound.

CONCLUSION

The relationship between blood pressure and blood glucose differed by ethnicity. Inter-ankle blood pressure difference was positively associated with fasting blood glucose concentrations independently of age, BMI and high diabetes risk in Asian pregnant women. Thus, inter-ankle systolic blood pressure difference may allow prediction or early detection of insulin-resistance-related changes in pregnancy, especially in non-White Europeans that are at an increased risk of developing GDM. Therefore, inter-ankle systolic blood pressure difference, together with known risk factors such as age and BMI, should be further developed as the early, convenient, non-expensive and non-invasive method to identify the high-risk of GDM for preventive interventions.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are not publicly available since patient permission was not sought for the sharing of data, at the time of recruitment. Requests to access the datasets should be directed to k.kain@nhs.net.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Research Ethics Committee, the UK (REC reference number 10/H1302/28). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

The research conceptualization was by KK. KK designed research and supervised data acquisition; RZ, KK, and MG developed the analysis protocol and RZ analyzed the data; CC reviewed data analysis and discussed the project; MG, RZ and HV wrote the manuscript with the input from all the authors; all authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.842254/full#supplementary-material

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Sequential Screening Strategy in Early, Middle, and Late Pregnancy in Women at High Risk of Hyperglycemia

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Background: Hyperglycaemia in pregnancy (HIP) is closely associated with short- and long-term adverse fetal and maternal outcomes. However, the screening and diagnostic strategies for pregnant women with risk factors for HIP are not set. This prospective study aimed to explore a screening strategy for women at high risk for HIP.

Methods: A total of 610 pregnant women were divided into experimental (n=305) and control (n=305) groups. Pregnant women underwent a 75-g OGTT in early (<20 weeks), middle (24–28 weeks), and late pregnancy (32–34 weeks) in the experimental group and only in middle pregnancy in the control group. The general conditions, HIP diagnosis, and perinatal outcomes of the two groups were compared.

Results: In the experimental group, HIP was diagnosed in 29.51% (90/305), 13.44% (41/305), and 10.49% (32/305) of patient in early, middle, and late pregnancy, respectively. The total HIP diagnosis rate was significantly higher in the experimental group (53.44% vs. 35.74%, P<0.001). Multivariate logistic regression analysis revealed that previous gestational diabetes mellitus (GDM) (odds ratio, OR=9.676, P<0.001), pre-pregnancy body mass index (BMI) ≥23 kg/m^2 (OR=4.273, P<0.001), and maternal age ≥35 years (OR=2.377, P=0.010) were risk factors for HIP diagnosis in early pregnancy. Previous GDM (OR=8.713, P=0.002) was a risk factor for HIP diagnosis in late pregnancy. No significant differences in perinatal clinical data were observed between the experimental and control groups. The gestational age at delivery was significantly earlier in the experimental subgroup with early-HIP than in the experimental and control subgroups with normal blood glucose (NBG). The weight gain during pregnancy was lower in the experimental early-HIP, middle-HIP, and control NBG subgroups.

Conclusions: We recommend sequential screening in early and middle pregnancy for high-risk pregnant women with maternal age ≥35 years or pre-pregnancy BMI ≥23 kg/m^2, and in early, middle, and late pregnancy for high-risk pregnant women with a previous history of GDM.

Trial Registration: This study was registered in the Chinese Clinical Trial Registry (no. ChiCTR2000041278).

Keywords: pregnancy, hyperglycaemia, screening strategy, risk factor, oral glucose tolerance

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INTRODUCTION

Hyperglycaemia in pregnancy (HIP) is a common complication during pregnancy and is closely associated with short- and long-term adverse fetal and maternal outcomes. Hyperglycaemia first identified in pregnancy may be categorized into gestational diabetes mellitus (GDM) and Diabetes in pregnancy (DIP). The global incidence of GDM has increased due to a considerable increase in the incidence of type 2 DM consequent to poor lifestyle choices, an update to the diagnostic criteria for GDM, and emphasis on GDM screening (1–3). Screening, diagnosing, and treating HIP are therefore particularly important. Nonetheless, despite the continuous progress of HIP research in recent years worldwide, scholars have yet to reach a consensus on various aspects.

In 2010, the International Association of Diabetes and Pregnancy Study Groups (IADPSG) proposed a new standard for diagnosing GDM based on the results of the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study (4). There are multiple screening schemes and diagnostic standards, and there are controversies concerning the diagnostic threshold and the merits and demerits of the one-step and two-step methods. The screening and diagnostic strategies for pregnant women without risk factors for HIP are not set, and no conclusion has been drawn for high-risk pregnant women. In view of the compromise between the diagnostic time for GDM and the potential for subsequent treatment and risk control, guidelines usually recommend that HIP screening should be conducted between 24 and 28 weeks of pregnancy, and that high-risk women should be screened during their first antenatal visit or early pregnancy. These early screening procedures are mainly intended to detect women with prediabetes or diabetes before pregnancy early on, rather than to predict those who will develop GDM in late pregnancy. Nevertheless, the available evidence suggests that GDM may be present before the recommended time for screening, particularly among high-risk women such as those with prior GDM or obesity (5, 6). When screening high-risk pregnant women in early pregnancy, the blood glucose levels sometimes fall between the diagnostic criteria for routine screening in early pregnancy and those for non-pregnant adults with diabetes, which has been referred to as early GDM (7). There is no consensus on the diagnostic threshold for early GDM. The World Health Organization (WHO) and the International Federation of Gynecology and Obstetrics (8) advocate that the same blood glucose threshold may be used to diagnose GDM at any stage of pregnancy. However, most guidelines do not seem to agree, and it appears that some guidelines deliberately avoid this.

In middle and late pregnancy, insulin-antagonistic substances such as tumor necrosis factor, placental prolactin, and leptin increase among pregnant women; hence, insulin sensitivity in pregnant women decreases with an increase in gestational age. In pregnant women with limited insulin secretion, this physiological change cannot be compensated, resulting in high blood glucose levels (9). This suggests that pregnant women with normal blood glucose levels during routine screening in middle pregnancy may still have high blood glucose levels in late pregnancy. Nonetheless, the current guidelines have few relevant recommendations for HIP screening after 28 weeks of pregnancy, and relevant research is lacking.

Maternal age ≥35 years, being overweight or obese before pregnancy, history of GDM, and polycystic ovary syndrome (PCOS) are considered high-risk factors for HIP. However, there are few study investigated the incidence of HIP and perinatal outcomes in high-risk pregnant women, particularly in the last 10 years after the release of the new IADPSG standard. Research on HIP screening programs for high-risk pregnant women only focuses on increasing early screening in early pregnancy, and studies on supplementary screening in late pregnancy are scarce. Moreover, studies in which sequential HIP screening is conducted in early, middle, and late pregnancy are even scarcer.

Our hospital is a critical care centre in southwest China and caters to multiple pregnant women with high-risk factors, including maternal age ≥35 years, being overweight, and obesity. Over the last 5 years, the incidence of HIP in our hospital's obstetrics department was 25.28% (13893/54952). In the study, we aimed to find a HIP screening strategy (sequential screening in early, middle, and late pregnancy) for high-risk pregnant women taking advantage of regional (i.e., high incidence of HIP) and hospital resources (i.e., high proportion of high-risk pregnant women). Therefore, we analyzed the diagnosis rate of HIP between two screening strategies, the diagnostic basis of HIP diagnosis in different screening periods, and factors contributing to a diagnosis of HIP during early and late pregnancy, and comparison of perinatal outcomes between two screening strategies.

MATERIALS AND METHODS

Study Subjects

A total of 610 pregnant women who had undergone antenatal examination and delivery at the West China Second University Hospital of Sichuan University from July 2017 to June 2019 were alternately divided into the experimental (n=305) and control (n=305) groups according to the chronological order of the first antenatal visit. The inclusion criteria were one or more of the following factors: (1) family history of diabetes (first-degree relatives with type 2 DM); (2) pre-pregnancy body mass index (BMI) ≥23 kg/m^2; (3) maternal age ≥35 years; (4) PCOS before pregnancy; (5) impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) before pregnancy; (6) history of GDM in previous pregnancy; and (7) history of macrosomia. The exclusion criteria were: (1) diagnosis of type 1 or 2 DM or other glucose and lipid metabolism diseases before pregnancy; (2) combination with serious internal and surgical diseases; (3) need for the intake of drugs that could affect glucose and lipid metabolism during pregnancy; (4) failure to regularly complete prenatal care or delivery at our hospital, resulting in failure to

Abbreviations: DIP, diabetes in pregnancy; FPG, fasting plasma glucose; GDM, gestational diabetes mellitus; HAPO, Hyperglycemia and Adverse Pregnancy Outcome; HIP, hyperglycaemia in pregnancy; IADPSG, International Association of Diabetes and Pregnancy Study Groups; IGT, impaired glucose tolerance; LGA, large for gestational age; NBG, normal blood glucose; PCOS, polycystic ovary syndrome.

obtain complete medical records; and (5) gestational age ≥20 weeks at the first antenatal visit. The pregnant women provided written informed consent prior to enrolment. The study protocol was approved by the Ethics Committee of West China Second Hospital of Sichuan University, which follows the principles of the Declaration of Helsinki (2020[073]). This study was registered with the Chinese Clinical Trial Registry (registration number ChiCTR2000041278).

Research Methods

The HIP screening methods and diagnostic criteria published by the WHO (10) were adopted. Screening was carried out with the 75-g oral glucose tolerance test (OGTT) as follows (11): Prior to the test, a 3-day continuous normal diet was adopted, followed by fasting for ≥ 8 hours before the examination, during which 300 mL of liquid containing 75 g glucose was ingested within 5 minutes. For blood glucose measurement, venous blood was extracted before, and at 1 and 2 hours after sugar intake (counting the time from drinking glucose water). During the examination, the subjects sat quietly and were not permitted to smoke.

GDM was diagnosed if at least one of the following criteria were met (10): (1) FPG ≥5.1 mmol/L but <7.0 mmol/L; (2) 1-hour plasma glucose ≥10.0 mmol/L following a 75-g oral glucose load; and (3) 2-hour plasma glucose ≥8.5 mmol/L but <11.1 mmol/L following a 75-g oral glucose load. DIP was diagnosed if at least one of the following criteria were met: (1) FPG ≥7.0 mmol/L and (2) 2-hour plasma glucose ≥11.1 mmol/L following a 75-g oral glucose load.

Prospective longitudinal sequential studies were only conducted in the experimental group. The pregnant women in the experimental group underwent a 75-g OGTT during their first antenatal visit (early pregnancy, <20 weeks) and in middle (24–28 weeks) and late pregnancy (32–34 weeks); however, those who were diagnosed with HIP did not undergo any subsequent 75-g OGTT. The pregnant women in the control group underwent a 75-g OGTT in middle pregnancy only (24–28 weeks). General information (age, height, pre-pregnancy BMI, gestational age at the first antenatal visit, maternal history, family history, and history of diseases, among others) and perinatal outcome data (gestational age at delivery, birth method, shoulder dystocia, indications for caesarean section, insulin use during pregnancy, pregnancy complications [e.g., hypertensive disorders of pregnancy, premature birth], neonatal weight, Apgar score, incidence of neonatal hypoglycemia, neonatal respiratory distress syndrome, and others) were prospectively collected from the two groups (**Figure 1**).

Statistical Analysis

Statistical analysis of the data was performed using SPSS 24.0 (IBM Corp., Armonk, NY, USA). Continuous data with a normal distribution and homogeneity of variance were presented as the arithmetic mean ± standard deviation (x ± s). An unpaired *t*-test was used for comparisons between the two groups. Multiple comparisons were conducted using an analysis of variance and were adjusted for *via* the Bonferroni *t*-test. Continuous data without a normal distribution and homogeneity of variance were presented as the median (interquartile range) [M (P25–P75)], and non-parametric tests were used. Categorical variables are presented as counts and percentages, and comparisons between the two groups were performed using the Chi-square test. The Chi-square test for the $R \times C$ table and the Bonferroni method were employed to adjust for multiple comparisons. Multivariate logistic regression models were used to identify risk factors for diagnosis during different periods. Two-sided p-values <0.05 were considered statistically significant.

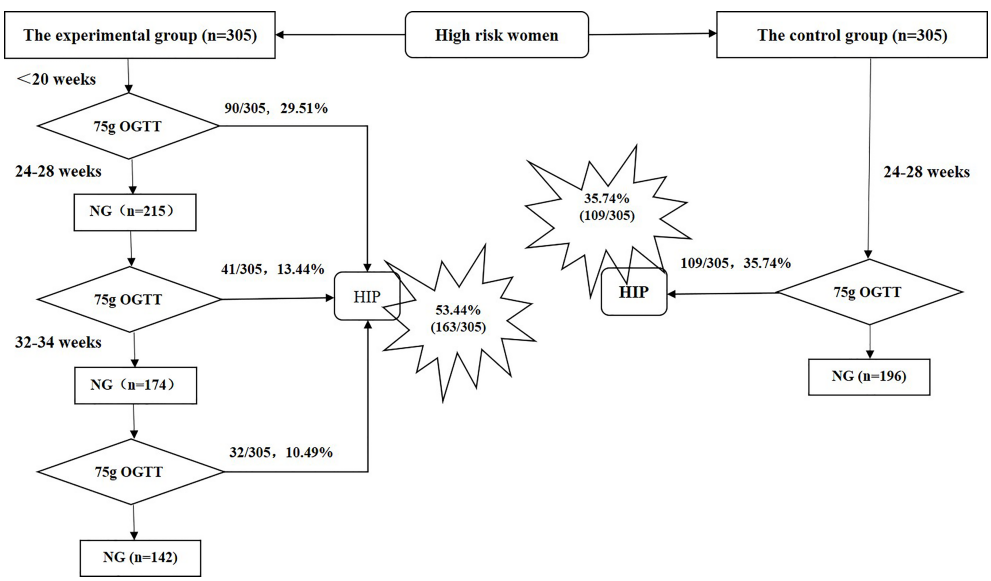

FIGURE 1 | Screening period and hyperglycemia in pregnancy diagnosis in the experimental and control groups. NG, normal glucose; OGTT, oral glucose tolerance test; HIP, hyperglycemia in pregnancy.

RESULTS

Comparison of Risk Factors Between the Experimental and Control Groups

Differences in the rates of high-risk factors, such as maternal age ≥35 years, family history of diabetes, pre-pregnancy BMI ≥23 kg/m^2, PCOS, pre-pregnancy IFG or IGT, previous GDM, and history of macrosomia, were not significant between the experimental and control groups (**Table 1**).

HIP Diagnosis in the Experimental and Control Groups

In the experimental group, 163 (53.44%) of 305 women were diagnosed with HIP, among whom 156 (95.71%) had GDM and 7 (4.29%) had DIP. In the control group, 109 (35.74%) of 305 women were diagnosed with HIP, among whom 100 (91.74%) had GDM and 9 (8.26%) had DIP. **Figure 1** shows the numbers of women in the experimental group diagnosed with HIP during early, middle, and late pregnancy. In the experimental group, the overall rate of HIP diagnosis during the early, middle, and late screening periods was significantly higher than that of the control group's single screening in mid-pregnancy (53.44% vs. 35.74%, P<0.001). The rate of HIP diagnosis in the experimental group for the early- and mid-pregnancy screening was higher but not significantly different (42.95% vs. 35.74%, P=0.068; **Figure 1**).

A total of 272 women were diagnosed with HIP in the experimental and control groups. Of these, 37 (13.60%) had abnormal FPG levels, 203 (74.63%) had abnormal glucose levels following a 75-g oral glucose load, and 32 (11.76%) had both abnormal FPG levels and abnormal glucose levels following a 75-g oral glucose load. The distributions of the diagnostic basis for HIP during each screening period in the experimental group and during mid-pregnancy screening in the control group were similar to the overall distribution. No significant difference in the distributions of diagnostic basis was observed between the groups (P=0.217; **Table 2**).

Analysis of Factors Contributing to a Diagnosis of HIP During Early and Late Pregnancy in the Experimental Group

For the experimental group, multivariate logistic regression analysis was used to examine factors influencing the diagnosis of HIP outside the routine screening period (i.e., early and late pregnancy). Data in **Table 3** indicate that previous GDM (odds ratio, OR=9.676, 95% confidence interval [CI]: 4.202–22.279, P<0001), pre-pregnancy BMI ≥23 kg/m^2 (OR=4.273, 95% CI: 2.349–7.773, P<0.001), and maternal age ≥35 years (OR=2.377, 95% CI: 1.231–4.590, P=0.010) were risk factors for HIP diagnosis in early pregnancy. Previous GDM (OR=8.713, 95% CI: 2.164–35.081, P=0.002) was a risk factor for HIP diagnosis in late pregnancy (**Table 4**)Among the 305 subjects in the experimental group, 40 women with previous GDM were identified, among whom 35 (87.50%) were diagnosed with HIP; the diagnosis rates in early, middle, and late pregnancy were 68.57% (24/35), 14.29% (5/35), and 17.14% (6/35), respectively. A total of 118 women had a pre-pregnancy BMI ≥23 kg/m^2, among whom 64.41% (76/118) were diagnosed with HIP; the diagnosis rates in early, middle, and late pregnancy were 64.47% (49/76), 22.37% (17/76), and 13.16% (10/76), respectively. Among 156 women with maternal age ≥35 years, 53.21% (83/156) were diagnosed with HIP; the diagnosis rates in early, middle, and late pregnancy were 55.42% (46/83), 22.89% (19/83), and 21.69% (18/83), respectively.

Comparison of Perinatal Outcomes

There were no significant differences between the experimental and control groups with respect to perinatal clinical data, including gestational age at delivery, weight gain during pregnancy, neonatal birth weight, or in terms of rates of insulin use, shoulder dystocia, preterm birth, hypertensive disorders of pregnancy, macrosomia, large for gestational age (LGA), low-birth-weight infants, small for gestational age, neonatal respiratory distress syndrome, and neonatal hypoglycemia (**Table 5**).

The experimental group was thus divided into four subgroups—namely, experimental NBG (n=142), experimental early-HIP (n=90), experimental middle-HIP (n=41), and experimental late-HIP (n=32) subgroups. The control group was divided into two subgroups, namely the control NBG (n=196) and control middle-HIP (n=109) subgroups. The perinatal clinical data of the six subgroups were compared, and the results indicated significant differences in gestational age at delivery (P=0.026), weight gain during pregnancy (P<0.001), rate of insulin use (P=0.031), and incidence of low-birth-weight infants (P=0.007) (**Table 6**). We conducted multiple comparisons of these four variables. The gestational age at delivery was significantly earlier in the experimental early-HIP subgroup than in the experimental NBG (38.45 ± 1.61 vs. 38.96 ± 1.30 weeks, P=0.04) and control NBG (38.45 ± 1.61 vs. 38.99 ± 1.35 weeks, P=0.01) subgroups. Weight gain during pregnancy was significantly lower in the experimental

TABLE 1 | Comparison of high-risk factors between the experimental and control groups.

High-risk factors	Experimental group (n=305)	Control group (n=305)	χ^2	P
Maternal age ≥35 years, n (%)	156 (51.15)	149 (48.85)	0.321	0.571
Family history of diabetes, n (%)	98 (32.13)	92 (30.16)	0.275	0.600
Pre-pregnancy BMI* ≥23 kg/m^2, n (%)	118 (38.69)	128 (41.97)	0.681	0.409
PCOS†, n (%)	36 (11.80)	36 (11.80)	0.000	1.000
Pre-pregnancy IFG‡ or IGT§, n (%)	5 (1.64)	1 (0.33)	1.515	0.218
Previous GDM‖, n (%)	40 (13.11)	27 (8.85)	2.834	0.092
History of macrosomia, n (%)	19 (6.23)	16 (5.25)	0.273	0.601

BMI, body mass index; †PCOS, polycystic ovary syndrome; ‡IFG, impaired fasting glucose; §IGT, impaired glucose tolerance; ‖GDM, gestational diabetes mellitus.

TABLE 2 | Diagnostic basis of HIP diagnosis in different screening periods.

Group	Screening period	A*n (%)	B†n (%)	A+Bn (%)	χ^2	P
Experimental group	Early pregnancy (n=90)	16 (17.78)	60 (66.67)	14 (15.56)		
	Middle pregnancy (n=41)	8 (19.51)	31 (75.61)	2 (4.88)		
	Late pregnancy (n=32)	3 (9.38)	25 (78.13)	4 (12.50)		
Control group	Middle pregnancy (n=109)	10 (9.17)	87 (79.82)	12 (11.01)		
Total (n=272)		37 (13.60)	203 (74.63)	32 (11.76)	8.168	0.217

*Abnormal FPG levels.
†Abnormal glucose levels following a 75-g oral glucose load.

early-HIP subgroup than in the experimental late-HIP (9.65 ± 4.78 vs. 12.09 ± 3.61 kg, *P*=0.003), experimental NBG (9.65 ± 4.78 vs. 13.11 ± 3.87 kg, *P*<0.001), and control NBG (9.65 ± 4.78 vs. 12.52 ± 3.77 kg, *P*<0.001) subgroups. Furthermore, weight gain during pregnancy was significantly lower in the experimental middle-HIP subgroup than in the experimental late-HIP (10.21 ± 4.18 vs. 12.09 ± 3.61 kg, *P*<0.001), experimental NBG (10.21 ± 4.18 vs. 13.11 ± 3.87 kg, *P*=0.048), and control NBG (10.21 ± 4.18 vs. 12.52 ± 3.77 kg, *P*=0.001) subgroups. Weight gain during pregnancy was also significantly lower in the control middle-HIP subgroup than in the experimental late-HIP (10.15 ± 4.01 vs. 12.09 ± 3.61 kg, *P*=0.017), experimental NBG (10.15 ± 4.01 vs. 13.11 ± 3.87 kg, *P*<0.001), and control NBG (10.15 ± 4.01 vs. 12.52 ± 3.77 kg, *P*<0.001) subgroups. However, no significant differences in the rates of insulin use and low-birth-weight infants were noted among these six subgroups after multiple comparisons.

DISCUSSION

Diagnosis of HIP in High-Risk Pregnant Women

Few studies have conducted sequential screening in early, middle, and late pregnancy for women with high-risk factors for HIP. In our experimental group, the HIP diagnosis rate was approximately 54% (163/305); among these patients, almost 30% were diagnosed early at the first antenatal visit, and a further 10% were diagnosed at the conventional screening point in mid-pregnancy. More importantly, the screening strategy identified an additional 10% of HIP cases in late pregnancy. The percentages of pregnant women diagnosed with HIP were 55.21%, 25.15%, and 19.63% in the three screening periods, respectively, confirming the value of additional screening in early and late pregnancy. The study similar to ours was a multi-country, multicenter study from Europe that included only pregnant women with pre-pregnancy obesity and analyzed HIP diagnosis at <20, 24–28, and 35–37 weeks. In that study, the overall incidence was approximately 39% (395/1023), whereas the incidence at <20, 24–28, and 35–37 gestational weeks was 23.66% (242/1023), 9.19% (94/1023), and 5.77% (59/1023), respectively. Among 395 subjects diagnosed with HIP, the rates of diagnosis were 61.27%, 23.80%, and 14.94% in early, middle, and late pregnancy, respectively (5). The screening method employed and distribution of overall diagnoses in that study were similar to those in ours, although it only involved one risk factor and had partial loss to follow-up in middle and late pregnancy.

Most related studies have focused on comparing early to routine screening. A meta-analysis (12) revealed that 15–70% of high-risk pregnant women were diagnosed with GDM during early screening. Bianchi et al. (13) recruited 290 pregnant women with prior GDM, pre-pregnancy BMI ≥30 kg/m^2, or FPG levels of 5.55–6.94 mmol/L at the first prenatal visit; half had undergone sequential screening in early and middle pregnancy (experimental group), whereas the other half had been conventionally screened in middle pregnancy (control group). They reported similar overall HIP diagnosis rates between the experimental and control groups, but in the experimental group, 42.7% of high-risk women had been diagnosed in early pregnancy, and 31.3% of women with normal 75-g OGTT results in early pregnancy had been diagnosed in their second trimester. Thus, the overall diagnosis rate of sequential screening in early and middle pregnancy was consistent with that of routine screening in middle pregnancy; nevertheless, more than half of high-risk pregnant women had hyperglycaemia prior to middle pregnancy and had been diagnosed in early pregnancy, which is similar to our results. Bozkurt et al. (14) investigated the pathophysiological characteristics of pregnant women with GDM and reported that pregnant women with GDM diagnosed in early pregnancy had lower insulin sensitivity and abnormal cell function than those

TABLE 3 | Multivariate logistic regression analysis of factors influencing HIP diagnosis in early pregnancy.

Variables	B	Wald χ^2	P	OR	95% CI
Maternal age ≥35 years	0.866	6.654	0.010	2.377	1.231–4.590
Pre-pregnancy BMI ≥23 kg/m^2	1.452	22.624	<0.001	4.273	2.349–7.773
Family history of diabetes	0.485	2.300	0.129	1.625	0.868–3.042
PCOS*	0.640	1.856	0.173	1.897	0.775–4.767
Pre-pregnancy IFG† or IGT‡	1.125	1.245	0.264	3.079	0.427–22.193
Previous GDM§	2.270	28.448	<0.001	9.676	4.202–22.279
History of macrosomia	-0.119	0.036	0.850	0.888	0.257–3.064

*PCOS, polycystic ovary syndrome; †IFG, impaired fasting glucose; ‡IGT, impaired glucose tolerance; §GDM, gestational diabetes mellitus.

TABLE 4 | Multivariate logistic regression analysis of factors influencing HIP diagnosis in late pregnancy.

Variable	*B*	Wald χ^2	*P*	OR	95% CI
Maternal age ≥35 years	0.694	1.890	0.169	2.001	0.774–5.380
Pre-pregnancy BMI ≥23 kg/m^2	0.632	1.567	0.211	1.881	0.700–5.057
Family history of diabetes	0.555	1.235	0.266	1.743	0.654–4.642
PCOS*	-0.298	0.112	0.738	0.742	0.129–4.269
Pre-pregnancy IFG† or IGT‡	1.359	0.946	0.331	3.892	0.252–60.183
Previous GDM§	2.165	9.279	0.002	8.713	2.164–35.081
History of macrosomia	0.582	0.520	0.471	1.790	0.368–8.714

**PCOS, polycystic ovary syndrome; †IFG, impaired fasting glucose; ‡IGT, impaired glucose tolerance; §GDM, gestational diabetes mellitus.*

with GDM diagnosed in middle pregnancy or those with NBG levels. Immanuel J et al. (15), investigated the physiological composition of GDM in early gestation, they found that insulin resistance contributed relatively more than reduced insulin secretion to the development of early GDM in this mainly obese population and was associated with greater risk of adverse perinatal outcomes. Based on these studies, we speculate that women could benefit from screening for insulin resistance in early pregnancy, but there were no measures of insulin resistance and secretion in our study, which needs to be improved in subsequent studies.

Few studies have employed repeat OGTT in late pregnancy. A Dutch study (16) reported that 23.5% of pregnant women were diagnosed with GDM based on a second OGTT in late pregnancy. In our study, 174 pregnant women in the experimental group who had normal OGTT results in the first and middle periods underwent a repeat OGTT in late pregnancy, and 32 (18.39%) of the 174 pregnant women received a supplementary diagnosis, with the diagnosis rates being similar in middle and late pregnancy (13.44% vs 10.49%). However, considering the few reported findings and weak evidence of late-pregnancy screening, HIP diagnosis in late-pregnancy still requires further research.

High-Risk Factors and Screening Period

Domestic and international guidelines include numerous risk factors for HIP, such as specific race (e.g., Asian), advanced age, being overweight or obese before pregnancy, family history of diabetes, and previous GDM. Nevertheless, numerous studies have shown that these risk factors are not identical (17, 18). When early OGTT in high-risk pregnant women yields normal results, OGTT in subsequent middle pregnancy is required and may be repeated in the third trimester in some women. Considering the inconvenience and discomfort of OGTT, it is of great importance to analyze the correlation between risk factors and the diagnosis period to further narrow the target population and achieve "precise" screening. The European multicenter study (19) mentioned above also analyzed this issue and found clinical characteristics independently associated with GDM/overt diabetes differed by OGTT time point. The identified risk factors can help define the target population for future intervention trials.

A study (6) reported that previous GDM (OR=22.3, 95% CI: 12.2–40.7) and class-III obesity (OR=12.7, 95% CI: 10.3–15.6) were most strongly associated with early diagnosis. Other influencing factors included class-II obesity (OR=6.85, 95% CI: 5.64–8.33), class-I obesity (OR=3.3, 95% CI: 2.77–3.93), overweight (OR=1.35, 95% CI: 1.14–1.61), maternal age ≥35 years (OR=1.24, 95% CI: 1.06–1.46), and diabetes in a first-degree relative (OR=1.50, 95% CI: 1.31–40.7). Another European study (20) showed that GDM in early pregnancy was associated with previous GDM (OR=2.74, 95% CI: 1.66–4.50), history of delivering a large infant (OR=1.97, 95% CI: 1.03–3.98), and a higher pre-pregnancy BMI (OR=1.05, 95% CI: 1.00–1.10). Our results indicated that previous GDM, pre-pregnancy BMI ≥23 kg/m^2, and maternal age ≥35 years were risk factors for HIP diagnosis during the first prenatal visit and that previous GDM was also a risk factor for HIP diagnosis in late pregnancy.

TABLE 5 | Comparison of perinatal outcomes between the experimental and control groups.

Perinatal outcomes	Experimental group (*n*=305)	Control group (*n*=305)	t/χ^2	*P*
Gestational age at delivery (weeks)	38.77 ± 1.33	38.91 ± 1.30	-1.385	0.167
Weight gain during pregnancy (kg)	11.59 ± 4.45	11.67 ± 4.01	-0.239	0.811
Neonatal birth weight (g)	3284.49 ± 471.01	3281.41 ± 423.77	0.085	0.932
Insulin use, *n* (%)	25 (15.34)	11 (10.09)	0.000	0.994
Shoulder dystocia, *n* (%)	1 (0.33)	0 (0.00)	0.000	1.000
Preterm birth, *n* (%)	21 (6.89)	19 (6.23)	0.107	0.744
Hypertensive disorders of pregnancy, *n* (%)	12 (3.93)	14 (4.59)	0.161	0.689
Polyhydramnios, *n* (%)	5 (1.64)	9 (2.95)	1.170	0.279
Macrosomia, *n* (%)	15 (4.92)	11 (3.61)	0.643	0.423
Large for gestational age, *n* (%)	28 (9.18)	25 (8.20)	0.186	0.666
Low-birth-weight infants, *n* (%)	4 (1.31)	0 (0.00)	2.265	0.132
Small for gestational age, *n* (%)	17 (5.57)	8 (2.62)	3.378	0.660
Neonatal hypoglycemia, *n* (%)	3 (0.98)	3 (0.98)	0.000	1.000
Neonatal respiratory distress syndrome, *n* (%)	2 (0.66)	3 (0.98)	0.000	1.000

TABLE 6 | Comparison of perinatal outcomes among subgroups.

		Gestational age at delivery (week)	Weight gain during pregnancy (kg)	Low-birth-weight infants*n* (%)	Insulin use*n* (%)
	NBG subgroup	38.96 ± 1.30	13.11 ± 3.87	0 (0.00)	-
Experimental group	Early-HIP* subgroup	38.45 ± 1.61	9.65 ± 4.78	3 (2.11)	19 (13.38)
	Middle-HIP* subgroup	38.71 ± 1.03	10.21 ± 4.18	0 (0.00)	5 (3.52)
	Late-HIP* subgroup	38.84 ± 0.64	12.09 ± 3.61	1 (0.70)	1 (0.70)
Control group	NBG subgroup	38.99 ± 1.35	12.52 ± 3.77	0 (0.00)	-
	Middle-HIP* subgroup	38.77 ± 1.20	10.15 ± 4.01	0 (0.00)	11 (7.75)
t/χ^2		2.568	14.241	10.441	8.686
P		0.026	<0.001	0.007	0.031

**HIP, hyperglycemia in pregnancy.*

Several studies have shown the role of previous GDM in early HIP diagnosis. The recurrence of hyperglycaemia in subsequent pregnancies indicates the recurrence of GDM may even be due to abnormal glucose metabolism before subsequent pregnancies. In a meta-analysis (21) that included 18 studies enrolling 19,053 women, the overall recurrence rate was 48% for GDM, and the subgroup analysis indicated higher recurrence rates of 59% and 54% for Hispanic and Asian women, respectively. Here, the overall recurrence rate for GDM in 67 pregnant women with previous GDM was 74.63% (50/67) and was as high as 87.5% (35/40) in the experimental group. Furthermore, 24 (68.57%), 5 (14.28%), and 6 (17.14%) of 35 pregnant women with recurrent GDM in the experimental group were diagnosed in early, middle, and late pregnancy, respectively. This not only shows that sequential screening programs in early, middle, and late pregnancy may screen more women with recurrent GDM, but also indicates that more than two-thirds of women with recurrent GDM may be diagnosed early in the first prenatal test, and that nearly one-fifth of women may receive supplementary diagnosis in the third trimester. Additionally, this study found that previous GDM was a risk factor for HIP diagnosis in late pregnancy. Despite few relevant studies, the correlation between previous GDM and HIP diagnosis in late pregnancy (OR=2.6, 95% CI: 1.0–6.3) has been reported (16).

Different populations have different associations with BMI, body fat percentage, and body fat distribution. According to the WHO and evidence from studies conducted in several Asian countries, 23 kg/m^2 can be used as the cut-off point for overweight in the Asian population (22). The guidelines for GDM by the American College of Obstetricians and Gynecologists (23) regard overweight or obesity as a necessary condition, fully emphasizing the importance of BMI. These guidelines also note that Asian women with a BMI ≥23 kg/m^2 are considered overweight. Therefore, pre-pregnancy BMI ≥23 kg/m^2 was included as a high-risk factor in our study. Overweight or obesity is a common risk factor associated with early diagnosis (6, 20). In our study, HIP was diagnosed in 64.41% (76/118) of high-risk pregnant women with a pre-pregnancy BMI ≥23 kg/m^2 in the experimental group, and most were diagnosed at the first antenatal visit (64.47%, 49/76). Harreiter J et al. (20) reported that early GDM was significantly more common with higher pre-pregnancy BMI, the sum of skinfolds in early pregnancy, prior history of GDM, and previous macrosomia, and in a subanalysis of nulliparous women, multivariate logistic regression found that higher prepregnancy BMI was the only risk factor for an early GDM diagnosis. Early screening of overweight and obese pregnant women in early pregnancy may attract the attention of both doctors and patients, and weight and blood glucose management may be carried out in the early period.

We identified that maternal age ≥35 years was a risk factor for HIP diagnosis at the first antenatal visit, which has also been previously reported. Here, more than half of high-risk pregnant women aged ≥35 years were diagnosed with HIP, and 55% of them were diagnosed at the first antenatal visit. In the context of comprehensive implementation of the two-child policy in China, the number of older pregnant women is increasing, and we should, therefore, pay attention to early HIP screening in this group.

Selection of Screening Methods for High-Risk Pregnant Women

The global guidelines recommend HIP screening for pregnant women with risk factors at the first prenatal visit or in early pregnancy; however, recommendations for screening methods vary. The Society of Obstetricians and Gynecologists of Canada (24) consider that both a 50-g glucose challenge test and 75-g OGTT may be used for early pregnancy screening. The Canadian Diabetes Association (25) indicates that non-pregnancy screening tests (FPG or HbA1c) or 75-g OGTT may be used in early pregnancy. However, the National Institute for Health and Care Excellence (26) clearly suggests that 75-g OGTT should be used instead of FPG, random blood glucose, HbA1c, glucose challenge test, or urine sugar to test for HIP in women with risk factors. According to the Queensland guidelines (27), FPG may only reflect part of glucose metabolism, whereas HbA1c is only suitable for diagnostic testing in early pregnancy. Nonetheless, the principal value of HbA1c lies in the identification of women who likely have pre-existing glucose abnormalities rather than milder degrees of hyperglycaemia and known hemoglobinopathies; moreover, the effects on HbA1c results should be considered. Guidelines offer little advice on selecting appropriate screening methods for late pregnancy. Our results showed that less than one-fifth of pregnant women were diagnosed with HIP by a simple abnormality in FPG levels at different stages of pregnancy, and most of them presented with abnormal blood glucose levels following a 75-g oral

glucose load. Therefore, irrespective of whether the OGTT is conducted during the routine screening period or in early or late pregnancy, it is a reasonable and necessary choice because it may detect any degree of hyperglycaemia and reflects both fasting and post-load blood glucose levels. However, our result may not apply to all areas, because in one European research (20), fasting glucose alone identified 78.5% of women in early pregnancy, and 21.5% were diagnosed with elevated 1-h and/or 2-h glucose levels.

Influence of the Screening Strategy on Perinatal Outcomes

HIP is closely related to long- and short-term adverse maternal and infant outcomes (28), and effective management is essential for reducing adverse consequences. We look forward to early pregnancy screening to ensure earlier treatment as well as additional screening to avoid misdiagnosis to reduce adverse outcomes due to non-intervention. However, studies have drawn mixed conclusions concerning the relationship between these screening strategies and perinatal outcomes. A study (29) confirmed that early screening improved the primary composite outcome (emergency caesarean section, neonatal hypoglycemia, and macrosomia; OR=0.62, 95% CI: 0.43–0.91). Nevertheless, several studies have not arrived at that conclusion. Hosseini et al. (30) compared pregnant women with abnormal blood glucose levels to those with NBG levels during pregnancy. They reported that diagnosis in early pregnancy was associated with an increased risk of macrosomia, LGA, caesarean section, 1-minute Apgar score <7 among neonates, neonatal respiratory distress syndrome, and admission to the neonatal intensive care unit, whereas diagnosis in middle pregnancy was only associated with an increased risk of macrosomia, LGA, and caesarean section. Similarly, Harper et al. (31) conducted a trial involving 962 obese women and concluded that early screening did not reduce the incidence of the primary composite outcome (macrosomia, shoulder dystocia, neonatal hypoglycemia, neonatal hyperbilirubinemia). This may be related to the fact that diagnosis in early pregnancy denotes the more serious pathological status of these pregnant women (13). Our results indicated no significant difference in the incidence of adverse outcomes between the experimental and control groups. However, in a paired comparison of subgroups, we observed that the weight gain during pregnancy was lower in the experimental early-HIP, usual-middle-HIP, and control NBG subgroups than in the experimental late-HIP, experimental NBG, and control NBG subgroups, suggesting that early diagnosis aids in capturing the attention of both doctors and pregnant women, thus facilitating pregnancy weight management.

In summary, the impact of early screening on perinatal outcomes cannot be easily concluded, and further research is required. Furthermore, no study has reported the influence of early diagnosis on long-term maternal and pediatric outcomes; hence, this field also needs to be explored. Few studies investigating late pregnancy screening have been conducted. A study (16) reported that the rates of macrosomia (16.3% vs. 35.3%, P=0.011) and LGA (18.6% vs. 39.7%, P=0.018) were lower in individuals with abnormal blood glucose levels than in those with NBG levels in late pregnancy. We did not show that supplementary diagnosis in late pregnancy improved the short-term perinatal outcomes.

This is the first study on a sequential screening strategy in early, middle, and late pregnancy for women with HIP risk, which identified the role of sequential screening strategy in increasing HIP diagnosis rates in high-risk pregnant women and identified high-risk pregnant women who needed additional screening and tried to explore the impact of the new screening strategy on perinatal outcomes. Our study makes up for the gap in screening HIP in pregnant women at high risk and provides a reference value for obstetricians in clinical work. But our study is limited because we only explored the effects on maternal and infant perinatal outcomes. As we know, the effects of the HIP do not end with the end of pregnancy. Considering the influence of HIP on the mother and her offspring, further research is warranted to explore the long-term outcomes of high-risk pregnant women and their newborns, which is what our team is currently working on.

In conclusion, we showed that sequential screening in early, middle, and late pregnancy significantly increased the HIP diagnosis rate in high-risk pregnant women. The earlier the diagnosis, the more likely both doctors and patients would focus on weight gain control during pregnancy. Previous GDM, pre-pregnancy BMI ≥23 kg/m^2, and maternal age ≥35 years were risk factors for HIP diagnosis at the first antenatal visit, whereas previous GDM was a risk factor for HIP diagnosis in late pregnancy. Less than one-fifth of pregnant women were diagnosed with HIP by simple abnormality in FPG levels at different stages of pregnancy, and most presented with abnormal blood glucose levels following a 75-g oral glucose load. Therefore, 75-g OGTT is a reasonable and necessary choice for pregnancy screening in high-risk pregnant women. We did not observe beneficial effects of sequential screening in early, middle, and late pregnancy on perinatal outcomes other than weight control during pregnancy. Nonetheless, considering all treatment-related costs, it may represent a preventive investment against type 2 DM, cardiovascular disorders, and kidney diseases for the mother and against type 2 DM and metabolic syndrome for her offspring. Consequently, we recommend sequential screening in early and middle pregnancy for high-risk pregnant women with maternal age ≥35 years or pre-pregnancy BMI ≥23 kg/m^2 and in early, middle, and late pregnancy for high-risk pregnant women with previous GDM.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by West China Second University Hospital, Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LZ is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. YX drafted the manuscript and collected and analyzed the data. LZ conceived and designed the study, helped draft and critically revise the manuscript, and coordinated the data collection. QW contributed to the data collection and analysis and helped draft the manuscript. M-FD, Y-MW, and NH contributed to the data collection and analysis. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.829388/full#supplementary-material

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Jiangtang Tongmai Prescription Reduced Diabetic Lung Injury Through SnoN and TGF-β1/Smads Signaling Pathway

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By establishing a rat diabetes model in rats with intervening treatment by Jiangtang Tongmai Prescription (JTTMP), this study explored the restorative pairing effect of JTTMP on diabetic lung injury. The model of type II diabetes model was used to establish the rat diabetes model, using a high-fat diet and streptozotocin (STZ) induction. Different doses of JTTMP and metformin were administered as a therapeutic to intervene, and blood was collected to assess the blood glucose level of each group of rats. HE (Hematoxylin and eosin (H&E) staining was performed to detect the morphological changes in rat lung tissue and enzyme-linked immunoassay ELISA was used to detect and quantify the expression of interleukin (IL)-6, TNF tumor necrosis factor-αa, and IL-1β in serum and the lung tissue of each group of rats. The level expression of TGF-β1 [transforming growth factor (TGF)-β1), SnoN (transcriptional co-repressor Ski-N terminal (SnoN)], Smad2, Smad3, Smad7, and other signaling pathway proteins were assessed by Western blot. In comparison with the normal control (NC) group, rats in the diabetes model (DM) group lost weight and showed significantly increased blood sugar levels. The levels of TGF-β1 and Smad2/3 were increased in the DM group but Smad7 decreased. After 8 weeks of JTTMP intervention, the level of TGF-β1 and Smad2/3 decreased but Smad7 increased, blood sugar decreased significantly and the expression of inflammatory factors in lung tissue decreased. Therefore, JTTMP may activate SnoN and the downstream TGF-β1/Smads signaling pathway to repair diabetic lung injury, which suggests its application has potential for future clinical treatment of diabetes with lung injury.

Keywords: JTTMP, TGF-β1/Smads signal, SnoN protein, diabetic lung injury, diabetes

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INTRODUCTION

Diabetes mellitus is a metabolic disease that presents with chronically increased glucose levels. Diabetes is clinically characterized by hyperglycemia, with symptoms such as polydipsia, polyuria, polyphagia, and weight loss. In addition to high blood sugar, it is often accompanied by a series of metabolic disorders related to protein, fat, water, and electrolyte processing. Without timely and

effective treatment it can cause disease to multiple systems of the body, including diabetic retinopathy, nephropathy, diabetic foot, diabetic nerve disease, and more severe cases such as diabetic ketoacidosis, diabetic lactic acidosis, and diabetic nonketotic hyperosmolar coma (1). Recently, studies have found that the lungs of diabetic patients also show fibrous tissue-like changes similar to the myocardium and kidney, which indicates that pulmonary fibrosis is one of the complications of diabetes (2, 3). The Jiangtang Tongmai Prescription (JTTMP) treatment can be used to treat diabetes as it effectively reduces blood sugar (4, 5), but whether it plays a role in the treatment of diabetic lung injury still needs to be explored. Considering this, we used streptozotocin (STZ) to establish a rat model of diabetes in which to observe the degree of lung injury (6, 7). This was then followed by an intervention with JTTMP to study the effect on diabetic lung injury and its targets and explore the mechanism of JTTMP against lung injury in the treatment of diabetes.

MATERIALS AND METHODS

Materials

The JTTMP Traditional Chinese medicine and STZ were freshly prepared in our laboratory. Metformin (M107827, Aladdin); blood glucose test strips for rat interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β factor enzyme-linked immunoassay (ELISA) detection kit (RA20607, RA20035, RA20020, Bioswamp); eosin, hematoxylin, neutral resin (E8090, G1140, G8590, Solarbio); Protein Marker, BCA protein concentration determination kit (XY-MY-0112, XY-MY-0096, Shanghai Xuanya); polyvinylidene fluoride membrane, enhanced chemiluminescence reagent (XF-P3360, ZDSJ140, Xinfan company); Tween-20 (PW0028, LEAGEN company); RIPA tissue cell rapid lysate (BL504A, biosharp); transforming growth factor (TGF)-β1, Smad2, Smad3, Smad7, p-Smad7, Ski-N terminal (SnoN), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody (PAB39276, PAB30712, PAB44700, PAB40077, PAB45936, PAB41857, PAB36269, Bioswamp); p-Smad2 antibody (ab188334, Abcam); p-Smad3 antibody (9520T, CST); Goat anti-Rabbit immunoglobulin G (SAB43714, bioswamp); MaxVision TM secondary antibody and horseradish peroxidase (HRP)-Polymer (Kit-5020, Maixin company).

The equipment used included: Electronic balance (JM-A3002, Zhuji Company); blood glucose monitor; surgical straight scissors, tissue forceps (J21070, J41050, Admiralty); inverted fluorescence microscope (DMILLED, Leica company); high-speed refrigerated centrifuge, microplate reader, plate washer (Icen-24R, AMR-100, APW-200, Hangzhou Aosheng Company); constant temperature oven (DHG-9023A, Shanghai Yiheng Company); automatic tissue dehydrator, stall Slice baking machine (TKD-TSF, TKD-TK, Hubei Kangqiang Company); paraffin slicer (RM2235, Leica Microsystems); biological tissue embedding machine-freezer (TB-718L, Thailand Technology); automatic chemiluminescence analyzer (HT8300/8500; Hongji Company); water bath (H.SWX-600BS, Shengke Company); and ultrapure water device (ULUPURE, MilliPORE France).

Construction and Identification of Rat Diabetes Model

The study included 36 rats, half male and half female, 8 weeks old, and weighing about 250 g each. The animals were obtained from Three Gorges University with laboratory animal license number: SYXK (E) 2018-0104, certificate No. 42010200003097. Rats were raised under SPF conditions. The housing conditions were: temperature of 22–26°C, relative humidity of 50–60%, artificial light and dark conditions for 12 h, and adaptive feeding for 1 week. 6 rats were selected as the normal control group, and the other 30 rats were the model group. They were given high-sugar and high-fat diet (10% sucrose, 10% lard, 10% egg yolk powder, 1.5% cholesterol 0.5% bile Sodium, 68% full feed) for 4 weeks (8). After 4 weeks, the model group was starved for more than 12 h, but with drinking, and then intraperitoneally injected with 35 mg/kg freshly prepared 1% STZ solution (6). After 72 h, rats were successfully modeled for diabetes with random blood glucose ≥16.7 mmol/L from the posterior tail vein.

Drug Intervention and Sample Collection

After the model was successfully generated, the rats were divided into 6 groups for drug intervention, each with 6 rats: ① Normal control group (NC): fed with ordinary feed; ② Diabetes model group (DM): equal volume of drinking water gavage. Gavaged once a day for 8 consecutive weeks and administered high-fat diet; ③ JTTMP low-dose group (DM+JTTMP 63 mg/kg): JTTMP 63 mg/mL suspension 1 ml/(kg·d) was given by intragastric administration for 8 weeks, and high-fat diet was administered; ④JTTMP middle-dose group (DM+JTTMP 126 mg/kg): JTTMP 126 mg/mL suspension 1 ml/(kg·d) was intra-gastrically administered for 8 weeks and the high-fat diet was continued; ⑤ JTTMP high-dose group (DM+JTTMP 252 mg/kg): JTTMP 252 mg/mL suspension 1 mL/(kg·d) was gavaged for 8 weeks, and high-fat diet was administered; and ⑥ Metformin group (DM+Met): 54.3 mg/ml Metformin aqueous solution of 1 ml/(kg·d) gavaged and administered for 8 weeks, and high-fat diet was administered.

After the drug treatments were completed, the rats in each group were sacrificed, and the serum was frozen for later use. The left lung tissue was fixed with 4% paraformaldehyde, and the right was stored at −80°C until analysis.

General Behavior Observation

During the experiment, the activity, eating, drinking, and excretion of the rats were observed, and the weight of the rats was recorded every day. The comparison of weight changes in each group was statistically analyzed.

Fasting Blood Glucose Test in Rats

Blood from the tail vein was collected in each group of rats before and after drug intervention, the fasting blood glucose was

measured by the test strip method, and the blood glucose value data was recorded.

The Detection of Inflammatory Factors IL-6, TNF-α, IL-1β in Serum and Lung Homogenate of Each Group of Rats by ELISA

The serum and lung tissue homogenate of rats from each group was collected, and ELISA was performed using 50 μL of each standard and sample solution, and phosphate-buffered saline as a negative control. An equal volume of enzyme-labeled IL-6 or TNF-α and IL-1β antibodies was added to the relevant sample and standard wells. Plates were sealed with film and incubated at 37°C for 30 min. Plates were spun and washed. The developer (50 μL) was added and incubated at 37°C for 10 min. Finally, 50 μL of stop solution was added and the absorbance (OD value) at a wavelength of 450 nm was measured. A standard curve was used to calculate the concentrations of IL-6, TNF-α, and IL-1β in the sample.

Observation of Morphological Changes of Rat Lung Tissue by Hematoxylin and Eosin Staining

The lung tissue of rats from each group was collected, fixed with 4% paraformaldehyde, and embedded in paraffin. As per routine practice, tissue sections were deparaffinized, stained with hematoxylin for 3–6 min, washed for 1–2 min, differentiated with 1% hydrochloric acid alcohol for 1–3 s, promoting liquid returned to blue for 5–10 s, washed with running water for 15–30 s, stained with 0.5% eosin solution for 2–3 min, washed with distilled water for 1–2 s, then 80% ethanol for 15–30 s, then 95% ethanol for 15–30 s, and finally anhydrous ethanol for 1–2 s. After drying, sections were sealed with neutral gum. The pathology of lung tissues such as the alveolar wall and inflammatory infiltration were analyzed under a microscope.

Western Blot Detection of TGF-β1, SnoN, Smad2, Smad3, Smad7, p-Smad2, p-Smad3, p-Smad7 Signal Protein in Rat Lung Tissue

Rat lung tissues were collected from each group, briefly homogenized, and protein extraction reagent at 5 μl/mg and protease inhibitor phenylmethylsulfonyl fluoride was added at a final concentration of 1 mM. Tissues were lysed for 15 min on ice and centrifuged at 4°C, 13800 r/min for 15 min. The protein concentration of the supernatant was quantified and adjusted. Protein samples had loading buffer added and were denatured at 100°C for 5 min. Electrophoresis separation was performed by 120 g/L sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the nitrocellulose membrane was transferred, and 5% skimmed milk powder was used to block for 2 h at room temperature, then TGF-β1 antibody (1:1000), Smad2 antibody (1:1000), Smad3 antibody (1:1000), Smad7 antibody (1:1000), p-Smad2 antibody (1:1000), p-Smad3 antibody (1:1000), p-Smad7 antibody (1:1000), and GAPDH antibody (1:1000) were incubated overnight on samples at 4°C. The membrane was washed with tris-buffered saline with 0.1% Tween® 20 Detergent (TBST) thrice and incubated with HRP-labeled secondary antibody (1:20000) for 2 h at room temperature. The membrane was again washed with TBST thrice. Finally, the enhanced chemiluminescence was analyzed after exposure in the darkroom.

Statistical Analysis

All data were analyzed by SPSS 21.0. The *t*-test for the comparison of two sample means was performed. Data were expressed as the $\bar{x} \pm s$, the comparison between multiple groups was determined by one-way analysis of variance, and $P<0.05$ indicates that the difference was statistically significant.

RESULTS

Changes in Body Weight of Rats in Each Group Over Time

After the generation of the model, the bodyweight of the DM group gradually decreased over time. After 4 weeks, the body weight in the DM group was significantly lower than that of the NC group at each time point ($P<0.01$). Compared with the DM group, the extent of weight loss in the DM+JTTMP 126 mg/kg group was reduced ($P<0.05$), and the weight loss in the DM+JTTMP 252 mg/kg and the DM+Met groups were significantly improved. In summary, the weight of the DM+Met group at each time point was much higher than that of the DM group ($P<0.05$, **Table 1**).

Effect of JTTMP on Blood Sugar of Diabetic Rats

After the model was successfully established, it was found that the glucose level of the DM group (20.15 ± 2.48 mmol/L) was higher than that of the NC group (5.68 ± 0.68 mmol/L, $P<0.001$). The glucose level in the DM group remained higher than in the NC group after 8 weeks ($P<0.001$). There was no obvious difference in blood glucose between the DM group and the treated group before the drug intervention; however, for 8 weeks after the drug intervention, the glucose in the DM+JTTMP 126 mg/kg group decreased to be lower than the DM group ($P<0.05$). The blood glucose level in the DM+JTTMP 252 mg/kg and DM+Met groups further decreased to be significantly lower than that of the DM group ($P<0.01$, **Table 2**).

Expression of Inflammatory Factors IL-6, TNF-α, IL-1β in Serum and Lung Homogenate of Rats

To clarify whether JTTMP plays a role in improving resistance to inflammatory injury in diabetes, we first detected the level of IL-6, TNF-α, and IL-1β in the serum and lung tissue in each group of rats by ELISA. The results showed that the expressions of IL-6, TNF-α, and IL-1β in serum and lung homogenate in the DM group were higher than those of the NC group ($P<0.001$). The level of inflammatory factors in the DM+JTTMP and DM+Met groups was significantly lower than those in the DM

TABLE 1 | The effect of JTTMP on the body weight of diabetic rats induced by STZ ($\bar{x} \pm s$, n=6).

Group	BW (g)				
	Before drug intervention	2 week	4 week	6 week	8 week
NC	490.18 ± 12.06	537.84 ± 7.53	563.32 ± 7.38	575.74 ± 12.91	580.48 ± 13.31
DM	560.58 ± 2.41	422.92 ± 10.16	342.76 ± 3.40**	274.38 ± 3.00**	216.32 ± 5.32**
DM+JTTMP 63 mg/.kg^{-1}	552.62 ± 6.28	415.32 ± 7.73	340.58 ± 4.06	285.44 ± 6.09	236.84 ± 5.58
DM+JTTMP 126 mg/.kg-1	555.40 ± 3.56	418.62 ± 2.86	366.68 ± 1.59$^{\#}$	316.62 ± 2.91$^{\#}$	271.2 ± 3.19$^{\#}$
DM+JTTMP 252 mg/.kg-1	552.98 ± 8.81	419.40 ± 4.46	380.70 ± 3.27$^{\#}$	344.72 ± 3.60$^{\#\#}$	309.42 ± 5.26$^{\#\#}$
DM+Met	552.48 ± 8.84	424.76 ± 13.04	386.48 ± 6.60$^{\#}$	361.98 ± 9.04$^{\#\#}$	321.36 ± 8.51$^{\#\#\#}$

*vs NC group, **P<0.01; vs DM group, #P<0.05, ##P<0.01, ###P<0.001.*

group (P<0.05), and as the dose of JTTMP increased, the expression of inflammatory factors gradually decreased. In the high-dose group with JTTMP 252 mg/kg, inflammatory factors were significantly reduced (P<0.001) to similar levels close to the normal level (**Figure 1**).

Changes in Lung Tissue Structure in Each Group of Diabetic Rats

To further define the role of JTTMP in the process of diabetic inflammatory injury, changes to the lung structure of rats were observed by H&E staining. The lung tissue in the NC group showed no obvious abnormality. In comparison with the NC group, the alveolar cavities in the DM group were reduced, the alveolar compartments were thickened, and some of the alveolar cavities were atrophied or collapsed with a large quantity of infiltrated inflammatory cells. In comparison with the DM group, lung tissue lesions in the DM+JTTMP and DM+Met groups were improved. As the dose of JTTMP increased, lung tissue lesions were significantly improved, inflammatory cell infiltration decreased, and alveolar spacing returned to normal (**Figure 2**).

JTTMP Reduced Diabetic Lung Injury Through SnoN Protein and Downstream TGF-β1/Smads Signaling Pathway

To explore the molecular mechanism of JTTMP in reducing diabetes lung injury, the key proteins in the TGF-β1/Smads pathway were assessed by Western blot. There were no significant changes in the expression of Smad2, Smad3, and Smad7 in each group, while phosphorylated Smad2 and Smad3 expressions were much higher in the DM group than in the NC group (P<0.01, **Figures 3A, B**). After treatment, the levels of p-Smad2 and p-Smad3 in the DM+JTTMP and DM+Met groups were much lower than in the DM group, and their expression level gradually decreased with the increase of the dose of JTTMP (P<0.05, **Figures 3A, B**). The level of p-Smad7 in the DM group was much lower, and p-Smad7 in the DM+JTTMP and DM+Met groups increased after treatment and also showed a dose-dependent effect of JTTMP (P<0.05, **Figures 3A, B**). To further clarify the targets of Smad protein interaction, we assessed the expression of SnoN and TGF-β1. The expression of SnoN in the DM group was significantly lower than in the NC group (P<0.01) but was increased in the DM+JTTMP and DM +Met groups after treatment (P<0.05, **Figures 3C, D**), which was consistent with the expression of p-Smad7. The expression of TGF-β was significantly increased in the DM group but decreased with the increase of JTTMP (P<0.05, **Figures 3C, D**).

DISCUSSION

Diabetes is very often a chronic disease. With the improvement of people's living standards, the incidence of diabetes is gradually increasing. According to statistics, in China, the incidence of diabetes has reached 2%, there are 40 million diagnosed diabetic patients, and this is increasing at a rate of 1 million per year. As it is predicted that by 2025, the number of diabetic patients in the world will reach 370 million, diabetes has become a serious threat to global public health (9, 10).

This study shows that an STZ-induced rat model of diabetes had a clear decrease in body weight and increase in blood glucose, while after 8 weeks of JTTMP drug intervention blood glucose level was significantly decreased, and the extent of weight loss was reduced. These results are consistent with those described in the literature (11).

TABLE 2 | Comparison of FPG among the different groups ($\bar{x} \pm s$, n=6).

Group	FDP (mmol/L)	
	Before drug intervention	8 week
NC	5.68 ± 0.68	5.65 ± 0.89
DM	20.15 ± 2.48***	19.38 ± 1.37***
DM+JTTMP 63 mg/.kg^{-1}	18.44 ± 1.29	17.02 ± 1.14
DM+JTTMP 126 mg/.kg-1	18.48 ± 1.35	14.95 ± 1.55$^{\#}$
DM+JTTMP 252 mg/.kg-1	20.74 ± 3.29	13.94 ± 0.96$^{\#\#}$
DM+Met	19.26 ± 2.23	11.76 ± 1.01$^{\#\#}$

*vs NC group, ***P<0.001;vs DM group, #P<0.05, ##P<0.01.*

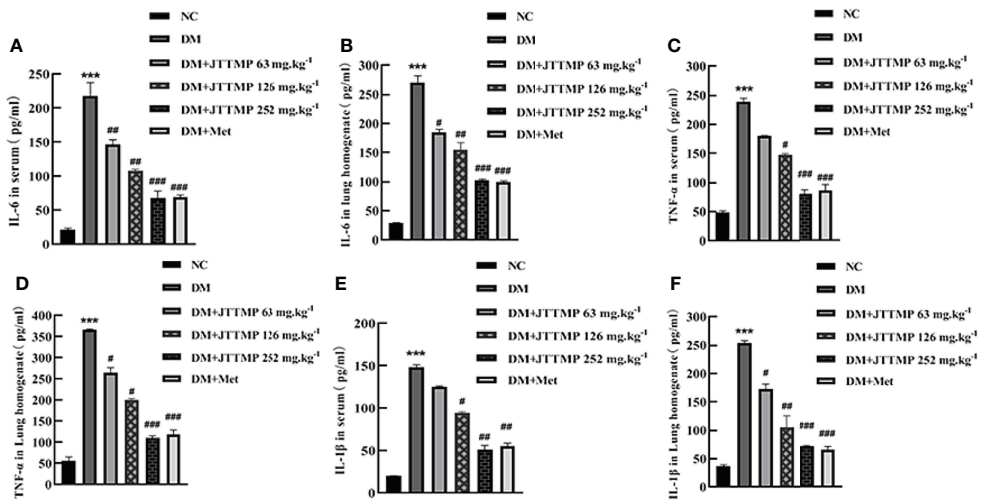

FIGURE 1 | Expression of inflammatory factors IL-6, TNF-α, and IL-1β in serum and lung homogenate of rats from different groups. **(A, B)** The concentration of IL-6 in serum and lung homogenate of rats from different groups. **(C, D)** The concentration of TNF-α in serum and lung homogenate of rats from different groups. **(E, F)** The concentration of IL-1β in serum and lung homogenate of rats from different groups. *vs* NC group, $^{**}P<0.001$; *vs* DM group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

Recent studies have shown that the lung is also damaged by diabetes (12, 13). Diabetic lung tissue has fibrotic changes similar to diabetic cardiomyopathy (14). Our results found that the alveolar cavity of the lung tissue of diabetic rats was reduced, inflammatory cells had infiltrated, the alveolar compartment was thickened, and a large number of collagen fibers had proliferated, showing pulmonary fibrosis. These results are consistent with previously reported results of diabetic lung damage (12–14). At the same time, we measured the levels of IL-6, TNF-α, and IL-1β in the serum and lung homogenate of diabetic rats. With the increase of the dose of JTTMP, the expression of inflammatory factors gradually decreased. Therefore, JTTMP was shown to alleviate diabetic lung injury and improve the prognosis of diabetic lung injury.

Smad, the downstream signaling molecule of the TGF-β family, is the sole substrate of TGF-β. Under the stimulation of a variety of related factors, TGF-β firstly binds to TGF-β receptor II on the cell membrane and activates the TGF-β receptor, directly phosphorylates Smad2 and Smad3, finally forms a trimer with Smad4 and transfers to the nucleus, thereby internally regulating the transcription of the corresponding target gene. Smad7 is an inhibitory Smad protein, which can reduce the expression of TGF-β through negative feedback regulation of the TGF-β signal transduction pathway, thereby playing a protective effect on diabetic lung tissue.

Studies have shown that there is a higher level of TGF-β1 in diabetic patients than normal. Among the related cytokine networks, TGF-β1 is recognized as the most closely related

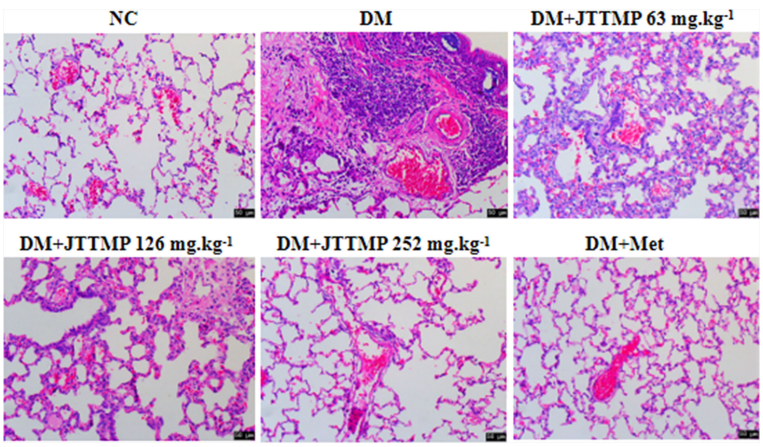

FIGURE 2 | Microphotographs showing structural changes with H&E staining in lung tissue of rats from different groups (200×).

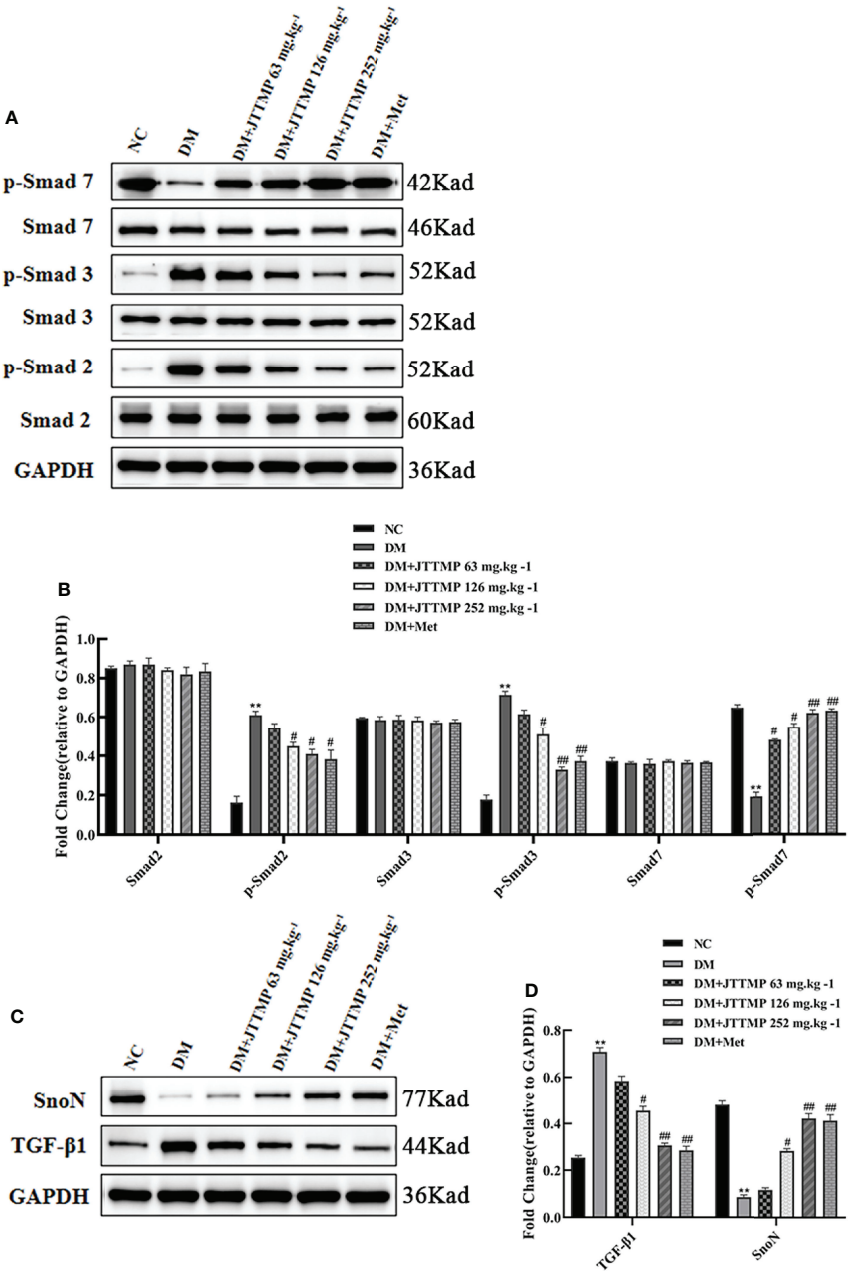

FIGURE 3 | Western blot analysis of TGF-β1/Smads expression in lung tissue of rats from different groups. **(A)** Smads protein family expression in the lung of rats from different groups; **(B)** Quantitative analysis of Smads protein family expression; **(C)** TGF-b1 and SnoN expression in lung tissue of rats from different groups; **(D)** Quantitative analysis of TGF-b1 and SnoN expression. $^{**}P<0.01$; *vs* DM group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

factor that mediates the formation, occurrence, and development of fibrosis (15). TGF-β1 can stimulate fibroblasts to divide and proliferate, promote their activation into myofibroblasts, and reduce their degradation. The Smads protein family is currently the only known type I receptor related to TGF-β1 that can mediate its signal from the cell membrane receptor to the corresponding cell nucleus (16, 17). Studies have shown that the Smads proteins family contains 9 members, and TGF-β1 can activate Smad2/3 and promote fibrosis. In addition, the main inhibitory regulatory protein Smad7 and the TGF-β1/Smads signaling pathway coordinate and cooperate to perform signal transduction (18–20). Our study shows that the level of TGF-β protein in the DM group was higher than that of the NC group, indicating that diabetic lung injury may be associated with the increase of TGF-β. The expression of Smad2/3 in the diabetic rats increased, while the expression of Smad7 decreased, suggesting that TGF-β participates in the formation of diabetic lung fibrosis by upregulating Smad2/3 and downregulating

Smad7 expression. Following JTTMP treatment, TGF-β can reduce the level of Smad2/3 and increase the level of Smad7. At the same time, blood sugar levels are reduced, and the effect may be more significant as treatment time is extended. These results support the theoretical explanation of the TGF-β1/Smads pathway in the previously mentioned literature (18–20).

The transcriptional co-repressor SnoN protein belongs to the Ski protein family. Its most important function is to negatively regulate TGF-β signal transduction by binding to the Smad protein (21). Ski and SnoN simultaneously interact with R-Smad (Smad2/3) through their N-terminal region and with co-Smad (Smad4) through a SAND-like domain (22). In this way, it prevents the function of the Smad complex and activates TGF-β target genes. In addition, the binding of SnoN can also stabilize the inactive Smad heteromers, to prevent the further binding of the active Smad complex (23). In this study, when diabetic lung injury occurred, the TGF-β1/Smads signaling pathway interacted with SnoN protein, thereby inhibiting the inflammatory signal and reducing the further occurrence of lung injury.

Diabetes is categorized as a "wasting-thirst" disorder in Traditional Chinese Medicine. The pathogenesis of diabetes in Traditional Chinese Medicine mainly includes yin deficiency and fire prosperity, and lung and kidney yin deficiency. The JTTMP treatment is composed of three Chinese medicines including Huanglian, Chuanqiong, and Ligustrum lucidum. The combination of various drugs has the effect of nourishing yin and removing blood stasis (24). JTTMP can effectively treat diabetes, but whether it is beneficial in the treatment of diabetes with lung injury still needs to be explored. This study used STZ induction to establish a rat diabetic model, and following intervention with JTTMP it was found to be effective at alleviating diabetic lung damage through SnoN protein and downstream TGF-β1/Smads pathway activity. Further research on the TGF-β/Smads signaling pathway will help clarify the mechanism of diabetic lung injury and provide new targets for the prevention and treatment of diabetic lung injury.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Hubei University of Chinese Medicine.All animal experimentation was conducted in accordance with accepted standards of humane animal care, as outlined in the Ethical Guidelines.

AUTHOR CONTRIBUTIONS

ND: Study design, contributed to statistical analysis, and manuscript drafting.; CZ: Contributed to data analysis and helped in results, discussion, and drafting. All authors critically reviewed and agreed on the final version of the manuscript.

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Research advances in gestational, neonatal diabetes mellitus and metabolic disorders

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Editorial on the Research Topic
Research advances in gestational, neonatal diabetes mellitus and metabolic disorders

Diabetes mellitus (DM) is among the most common disorders affecting people of all ages across the globe from neonates to seniors (1). Indeed, DM has emerged as one of the major health concerns of this century with a huge economic burden that continues to grow, especially in low and middle-income counties (2). In addition to type 1 and II diabetes (T2DM), gestational diabetes mellitus (GDM) (3) and neonatal diabetes mellitus (NDM) are now more frequently reported (4). GDM poses significant dangers not only for the mother but also for the developing fetus (5). Unlike the common forms of diabetes, the causes and associated complications of GDM and NDM are not yet completely understood. However, diabetes may also be associated with metabolic syndromes (MetS), a medical term used for a combination of diabetes, high blood pressure (hypertension), and obesity (6). Diabetes also creates a greater risk for coronary heart disease, stroke, and other conditions affecting blood vessels, and notably in pregnancy, a relationship has been established between maternal MetS, GDM, and pregnancy outcomes (7). The current Research Topic aimed to collect studies reporting advancements in clinical and basic research related to GDM, NDM, and associated metabolic disorders. After a rigorous process of selection and review, the current volume presents an authoritative collection of 20 research articles exploring new dimensions in this topic.

Firstly, introducing major players in metabolic disorders, Zhen et al. reviewed the role of kynurenic acid (KYNA) in the pathogenesis and development of several human endocrine and metabolic diseases. KYNA is a signaling molecule and a major player in metabolic diseases inclusive of diabetes and obesity. Their study promotes the notion that

KYNA enhances energy metabolism and prevents obesity while reducing insulin resistance and inflammation. And on this basis, the authors suggest KYNA as a potential therapeutic marker/target for metabolic diseases. Alzaim et al. studied the possible association of the vitamin D receptor (VDR) polymorphism (Folk1) with the metabolic syndrome in pregnant women of Arabian descent. Interestingly, they did not find a significant association of metabolic syndrome with Folk1 in VDR; however, carriers of the ff allele were at risk for full maternal MetS, and their study further suggested that the ff genotype in Folk1 could be used as a genetic marker for maternal MetS in pregnant Arab women.

A well-established fact is that type-2 diabetes is abruptly increasing among at-risk populations due to increasingly unhealthy lifestyles among other associated factors (8). Jia et al. studied the involvement of gut microbiota in the regulation of central obesity and type-2 diabetes in Chinese individuals. Notably, they observed a significantly higher percentage of sugar and amino acid metabolism-related gut microbiota in obese patients with T2DM. This suggests that the gut microbiota should be taken into consideration for planning appropriate therapeutic strategies.

Diabetic patients are also prone to develop other morbidities such as liver and kidney dysfunction and lung injury (9). Ding et al. describe a traditional Chinese medicine Jiangtang Tongmai Prescription (JTTMP) that activates SnoN and TGF-β1/Smads signaling pathways to promote diabetic lung injury repair. It is also common that diabetic patients to suffer from a variety of psychological problems. Therefore, considering the psychological care of diabetes patients represents an integral factor for maintenance and recovery regimens. Li et al. studied the effects of individualized nursing and health education (INHE) on patients with T2DM and hypertension. They reported that INHE effectively improves the psychological cognition of T2DM plus hypertension patients, helping to improve blood pressure and blood sugar control.

Raza et al. report the differential expression and association of genes including DMBX1, TAL1, ZFP161, NFIC, NR1H4, SRR, NFKB1, and PDE4B with the pathophysiological development of metabolic disorders including diabetes. Diabetes can affect people of all ages and risk factors may vary with the age group involved (1). Neonatal diabetes is an extremely rare disorder caused by genetic mutations in specific genes including dominantly acting insulin (INS) (10). Ngoc et al. performed a genetic analysis on 70 Vietnamese NDM cases from 2008 to 2021 and report causative mutations in genes known to be associated with NDM. In particular, 55 of the 70 infants (78.5%) harbored mutations in the disease-causing genes ABCC8 and KCNJ1 while 10 subjects showed six distinct heterozygous INS mutations, indicating that INS mutations are the third most common cause of NDM in Vietnam.

Gestational diabetes mellitus can significantly affect the course of pregnancy and its early diagnosis and care improve maternal health and pregnancy outcomes (11). Liu et al. presented a nested case-control study, in which they evaluated serum levels of putrescine in 47 women with GDM, specifically during weeks 8-12 of gestation. Putrescine serum levels were significantly higher in these women during their first trimester, indicating that putrescine can be used as a GDM marker during the first trimester. Adding to the early detection of GDM diagnosis, Zhang et al. compared ankle blood pressure with blood glucose in 179 (52% White European, 48% Asian) pregnant women at 24-28 weeks of gestation. Besides reporting ethnic differences, they suggest that higher ankle blood pressure could be associated with the risk of developing gestational diabetes. Serum uric acid level is a known potential risk factor for GDM and it is also known that hypertension produces adverse birth outcomes. Riis et al. examined the diurnal salivary uric acid (sUA) levels in 44 healthy women during early-mid and late pregnancy. According to their observations, sUA showed an association with maternal pre-pregnancy BMI, age, prior-night sleep duration, and fetal sex. Maternal blood pressure and gestational weight gain also showed significant associations with sUA levels across pregnancy.

Xu et al. enrolled 610 Han Chinese pregnant women to study the short- and long-term adverse fetal and maternal outcomes of hyperglycaemia in pregnancy (HIP). Multivariate logistic regression analysis revealed that previous gestational diabetes mellitus (GDM), pre-pregnancy body mass index (BMI) (≥23 kg/m^2), and maternal age (≥35 years) were risk factors for HIP in early pregnancy. Further regarding GDM diagnosis, Raets et al. performed glucose challenge tests (GCT) and oral glucose tolerance tests (OGTT) in 1804 pregnant women during weeks 24-28 of gestation. They advised a one-step screening strategy with an OGTT for women at higher risk for GDM, while women without these risk factors were suggested to have a two-step screening strategy with GCT. Mamun and Khan reviewed the available literature regarding COVID-19 infection in pregnant women and concluded that patients with GDM are more vulnerable to coronavirus infection specifically to the COVID-19 Delta Variant of Concern (VOC).

Juchnicka et al. analyzed the differential expression of circulating miRNAs in GDM women and reported that four miRNAs (miR-16-5p, miR-142-3p, miR-144-3p, and miR-320e) showed prominent expression in GDM. Chen et al. shed light on the differential expression and association of circRNAs in GDM patients, reporting the overall top 4 genes (CBLB, ITPR3, NFKBIA, and ICAM1) and corresponding circRNAs (circ-CBLB, circ-ITPR3, circ-NFKBIA, and circ-ICAM1) were related to T cell receptor signaling pathways. This introduces a novel concept where the upregulation of T cell receptor signaling may be involved in GDM development. Moreover, this suggests that monitoring T cell receptor signaling activation during early gestation could represent a novel diagnostic approach. Wang et al. performed GDM risk association studies for rs7747752 in CDKAL1 and GUDCA/DCA polymorphisms and found that

patients carrying the rs7747752 allele with low GUDCA/DCA have a significant risk of developing GDM.

While GDM definitively influences pregnancy outcomes, whether GDM has any relation with NDM, or postnatal diabetes was unknown. Jiang et al. created a GDM mouse model to investigate whether GDM causes diabetes in offspring. They concluded that the intrauterine hyperinsulinemia induced hepatic FoxO1 levels, subsequently increasing the expression of the epigenetic writer-reader DNMT3A resulting in differentially methylated regions in IGF2/H19. Their work pointed to a potential molecular mechanism underlying glucose intolerance and insulin resistance in the first male generation of GDM mice but whether GDM contributes to NDM remains unsolved.

Conclusions and perspectives

This volume contributed by twenty research groups with often different interests highlights many important aspects of diabetes/gestational diabetes. Different biomarkers for diagnosis and prognosis along with potential therapeutic targets were identified along with adding a variety of new information to the topic. Along with the potential diagnostic and therapeutic approaches for gestational diabetes, this includes the involvement of gut microbiota in diabetes along with genetic biomarkers for gestational diabetes and neonatal diabetes. Nevertheless, a major question regarding pregnancy outcomes especially GDM triggers NDM or postnatal diabetes remains unanswered. Together with increasing our biological understanding of different aspects of diabetes, it can be anticipated that these contributions will find broad applications, ranging from purely scientific endeavours to use as clinical guidelines for the treatment of diabetic patients.

Author contributions

All authors participated equally to this work. All authors contributed to the article and approved the submitted version.

Conflict of interest

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