The present prospective study included 2156 women and investigated the effect of gene variants in the vitamin D (VitD) metabolic and glucose pathways and their interaction with VitD levels during pregnancy on gestational diabetes mellitus (GDM). Plasma 25(OH)D concentrations were measured at the first and second trimesters. GDM subtype 1 was defined as those with isolated elevated fasting plasma glucose; GDM subtype 2 were those with isolated elevated postprandial glucose at 1 h and/or 2 h; and GDM subtype 3 were those with both elevated fasting plasma glucose and postprandial glucose. Six Gc isoforms were categorized based on two GC gene variants rs4588 and rs7041, including 1s/1s, 1s/2, 1s/1f, 2/2, 1f/2 and 1f/1f. VDR-rs10783219 and MTNR1B-rs10830962 were associated with increased risks of GDM and GDM subtype 2; interactions between each other as well as with CDKAL1-rs7754840 were observed ($P_{\text{interaction}} < 0.05$). Compared with the 1f/1f isoform, the risk of GDM subtype 2 among women with 1f/2, 2/2, 1s/1f, 1s/2 and 1s/1s isoforms and with prepregnancy body mass index $\geq 24$ kg/m$^2$ increased by 5.11, 10.01, 10.14, 23.45 times, respectively. Gene variants in VitD pathway interacts with VitD deficiency at the first trimester on the risk of GDM and GDM subtype 2.

**Keywords:** gestational diabetes mellitus; subtypes; gene polymorphism; vitamin D

1. Introduction

Gestational diabetes mellitus (GDM) is a growing public health problem [1,2] and associated with adverse perinatal and neonatal outcomes, including increased risks of gestational hypertension [3], preterm birth [4] and cardiovascular diseases [5]. Although a few risk factors of GDM have been identified, the etiology has not fully been elucidated [6].

Some research has focused on the genetic susceptibility of GDM. Moen et al. [7] found that MAP3K1-rs116745876, PRKCE-rs11682804 and NUAK1-rs11112715 were associated with higher fasting glucose levels at the first trimester and higher 2 h post-prandial glucose levels at the second trimester in pregnant women. Other two single nucleotide polymorphisms (SNPs) CDKAL1-rs7754840 and MTNR1B-rs10830962 identified from a genome-wide association study of GDM were found to be highly correlated with GDM, and another one, IGF2BP2-rs1470579, was relatively weakly correlated [8]. On the other
hand, genetic variants in the vitamin D (VitD) metabolic pathway were also found to be involved in the pathogenesis of insulin resistance and GDM [9–11]. The main circulating metabolite is 25(OH)D, a biomarker of VitD status. VitD metabolism is highly regulated, and variation in the expression or activity of key proteins may modify its level or effects. Key metabolic enzymes include: 25-hydroxylase (CYP3A4), which converts VitD to 25(OH)D; 1-hydroxylase (CYP27B1), which activates 25(OH)D to 1,25(OH)₂D; 24-hydroxylase (CYP24A1), which inactivates 25(OH)D and 1,25(OH)₂D; and megalin (LRP2), which reabsorbs 25(OH)D through endocytosis in the renal tubules. Other key components include vitamin D-binding protein (GC), which transports circulating metabolites, and the VitD receptor (VDR), which binds 1,25(OH)₂D to activate gene transcription and regulates VitD metabolism [12]. Compared to pregnant women with the CC genotype at VDR-rs1544410, the risk of GDM in pregnant women with the CT genotype was approximately doubled; compared to AA genotype at VDR-rs731236, the risk of GDM in pregnant women with the GA genotype was 1.42 times higher [13]. In addition, two SNPs, rs4588 and rs7041 on the GC gene, can form three allelic combinations (Gc1f, Gc1s and Gc2) and six different Gc isoforms, namely, 1s/1s, 1s/2, 1s/1f, 2/2, 1f/2 and 1f/1f [14,15]. According to the free hormone hypothesis, only free 25(OH)D and free 1,25(OH)₂D can directly exert biological functions [16,17], the proportion of which in blood were mostly influenced by the binding affinity of different Gc isoforms [18]. The polymorphism of VitD metabolic pathway genes, especially on the GC genes, may be good candidates to better understand how VitD levels are involved in the pathogenesis of GDM.

Most previous studies have regarded GDM as a homogenous disease, and little attention has been paid to GDM subtypes on the basis of the different time-point glucose levels of the oral glucose tolerance test (OGTT) [8,13]. Studies in non-pregnant women found that both isolated impaired fasting glucose (IFG) and isolated impaired glucose tolerance (IGT) patients were insulin resistance (IR) factors, but the target organs or tissues of IR were different [19–22]. Individuals with isolated IFG primarily manifest hepatic IR and relatively normal muscle IR. Otherwise, individuals with isolated IGT have normal to subtle hepatic IR and moderate to severe muscle IR. Thus, individuals with both IFG and IGT have both hepatic and muscle IR [19]. The different pathophysiological mechanisms of fasting and post-glycemic abnormalities result from distinct insulin sensitivity characteristics of the liver and muscle, respectively [20,21]. In addition, our previous population-based study found that VitD was associated with the occurrence of GDM with abnormal fasting glucose, especially among overweight/obese pregnant women, but not the occurrence of abnormal post-load glucose [23]. However, previous studies principally treated GDM as a dichotomous outcome when investigating the effects of gene variants on the VitD metabolic and glucose pathways on GDM, ignoring the different pathophysiological mechanisms of fasting and post-load glycemic abnormality [24].

Thus, the aim of this study was to explore the effect of gene variants in the VitD and glucose metabolic-pathway-related genes, and their interactions with 25(OH)D concentrations on the development of GDM and GDM subtypes.

2. Materials and Methods

2.1. Study Design and Participants

This prospective cohort study was based on the data of Zhoushan Pregnant Women Cohort (ZPWC) from August 2011 to May 2018, which is an ongoing prospective cohort conducted in Zhoushan Maternal and Child Health Care Hospital, Zhejiang. Pregnant women were invited to participate in the cohort at their first prenatal visit. A more detailed description of the inclusion and exclusion criteria has previously been described in detail [23]. Briefly, pregnant women aged between 18 and 45 years without serious physical, mental health disease, threatened abortion or fetal malformation, and who received OGTT were included in the study. Informed consent was obtained from all participants before the investigation.
2.2. Collection of Data and Blood Sample

A structured questionnaire was administrated face-to-face by an interviewer to collect information on socio-demographic, lifestyle, and health behavior at the first trimester (T1: 8th–14th gestational week), second trimester (T2: 24th–28th gestational week), third trimester (T3: 32nd–36th gestational week) and 42nd day postpartum. OGTT was conducted during T2 according to a conventional pregnant care program. A 5 mL fasting venous blood sample was drawn at each visit and centrifuged under 4 °C; then, the plasma and white blood cells were divided and stored under −80 °C until use. The results of the OGTT were extracted from the electronic medical records system.

2.3. Measurement of 25(OH)D Concentrations

Liquid chromatography–tandem mass spectrometry (API 3200MD (Applied Biosystems/MDS Sciex, Framingham, MA, USA)) was used to measure plasma 25(OH)D$_2$ and 25(OH)D$_3$ concentrations. The plasma 25(OH)D concentrations were reported in ng/mL, and the lowest sensitivity of the measurement was 2 ng/mL for 25(OH)D$_2$ and 5 ng/mL for 25(OH)D$_3$. The intra-assay coefficient variance values were 1.47–7.24% and 2.50–7.59% for 25(OH)D$_2$ and 25(OH)D$_3$, respectively. The inter-assay coefficients variances were 4.48–6.74% and 4.44–6.76% for 25(OH)D$_2$ and 25(OH)D$_3$, respectively [23]. The 25(OH)D concentrations were the sum of 25(OH)D$_2$ and 25(OH)D$_3$. The laboratory located in Hangzhou, Zhejiang Province, is CAP-accredited and annually participates in CAP Proficiency Tests and China NCCL Trueness Verification Plan of 25(OH)D Assays, for which satisfactory results in these PT or EQA tests have been obtained in consecutive years.

2.4. Covariates Assessment

Plasma 25(OH)D < 20 ng/mL (50 nmol/L) was defined as VitD deficiency according to Endocrine Society clinical practice guidelines [25], and 25(OH)D concentrations ≥ 20 ng/mL as VitD non-deficiency. Body mass index (BMI) = weight (kg)/height$^2$ (m$^2$). Prepregnancy BMI was divided into four categories based on the Working Group on Obesity in China [26]: underweight, BMI < 18.5 kg/m$^2$; normal, BMI 18.5–23.9 kg/m$^2$; overweight, BMI 24.0–27.9 kg/m$^2$; obesity, BMI ≥ 28 kg/m$^2$. VitD supplementation was categorized as “Yes”, “No” and “Unknown”. According to the sunshine intensity and duration in different months [27], the seasons of blood sampling were divided as follows: spring (March to May), summer (June to August), fall (September to November) and winter (December to February).

2.5. GDM and Its Subtypes Classification

GDM screening has become a routine examination among pregnant women in China. OGTT was conducted between the 24th and 28th weeks of gestation. After an overnight fast (at least 8 h), 75 g glucose resolved in 300 mL water was given and drunk within 5 min the next morning. Venous blood samples were taken at 0 h, 1 h and 2 h during OGTT for measuring plasma glucose levels. Plasma glucose levels were immediately measured by the hexokinase method with commercially available kits (Beckman AU5800, Beckman Coulter Inc., Brea, CA, USA). Using criteria proposed by the International Association of the Diabetes and Pregnancy Study Group [28], GDM was diagnosed if any one of the following criteria were met: fasting plasma glucose (FBG) at 0 h ≥ 5.1 mmol/L, postprandial glucose at 1 h (PG1H) ≥ 10 mmol/L, or postprandial glucose at 2 h (PG2H) ≥ 8.5 mmol/L. In addition, according to different types of insulin resistance represented by the blood glucose level at the three time-point glucose levels examined by OGTT [22–24], GDM was further categorized into the following three subtypes: GDM subtype 1, with isolated FBG ≥ 5.1 mmol/L; GDM subtype 2, with isolated PG1H ≥ 10 mmol/L and/or PG2H ≥ 8.5 mmol/L; and GDM subtype 3, with both elevated FBG (≥ 5.1 mmol/L) and post-load plasma glucose (PG1H ≥ 10 mmol/L and/or PG2H ≥ 8.5 mmol/L).
2.6. SNP Selection and Genotyping

GDM-related SNP selection: to verify the previous findings by Kwak et al. [8] in Korean pregnant women and Moen et al. [7] among pregnant women in Norway, 3 SNPs (CDKAL1-rs7754840, MTNR1B-rs10830962 and IGF2BP2-rs1470579) related to GDM [8] and 3 SNPs (MAP3K1-rs116745876, PRKCE-rs11682804 and NUAK1-rs1112715) related to blood glucose during pregnancy were selected [7]. According to the minor allele frequency ≥ 10 of each SNP in the Chinese population from the 1000 Genomes Project database, 4 GDM-related SNPs, CDKAL1-rs7754840, MTNR1B-rs10830962, IGF2BP2-rs1470579 and PRKCE-rs11682804, were finally included.

VitD-related SNP selection: the selection conditions of the VitD-related SNP in the study were as follows (satisfy any one) [15]: (1) a positive association between SNP and 25(OH)D concentration reported in the literature, and the minimum allele frequency (Minor allele frequency, MAF) ≥ 10%; (2) SNPs displayed in the functional region in the NCBI database: exon region, intron splicing point, 5′ end and 3′ end regulatory regions, and MAF ≥ 10%; (3) HapMap Chinese database, including gene regions, SNPs within 1500 bp at the 5′ end and 3′ end, using HaploView to select SNPs, and the conditions are: MAF ≥ 10%; R^2 ≥ 0.8 [15]. In addition, VDR is closely related to insulin secretion [29,30], and VDR-rs11568820 is a functional SNP of the VDR gene. Previous studies found that rs10783219 and rs11568820 on VDR have high LD (r^2 = 0.98). Therefore, the rs10783219 was selected as the surrogate SNP of rs11568820 [15]. Finally, a total of 13 SNPs related to 25(OH)D concentration in the VDR metabolic pathway were selected (CYP24A1: rs2209314, CYP3A4: rs2242480, GC: rs1155563, rs16846876, rs17467825, rs2282679, rs2298849, rs2298850, rs3755967, rs4588, rs7041, LRP2: rs10210408 and VDR: rs10783219).

Gc isoforms: based on two SNPs, rs4588 and rs7041, on the GC gene, the Gc isoform was categorized into six different isoforms, including 1s/1s, 1s/2, 1s/1f, 2/2, 1f/2 and 1f/1f, of which the proportions of free 25(OH)D were successively reduced. The 1f/1f isoform with the highest proportion of free 25(OH)D was used as the reference group.

The conventional phenol–chloroform extraction method was used to extract DNA from the peripheral blood leukocytes, which was then stored in TE-buffer at −80 °C. For SNP analysis, DNA was then diluted to 10 ng/µL using a Nanodrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, NC, USA). A Sequenom MassARRAY iPLEX Gold platform (Sequenom, San Diego, CA, USA) was used for SNP genotyping. In total, 17 SNPs were available for further analysis. The call rate of these SNPs was over 98%, which conformed to the Hardy–Weinberg equilibrium.

2.7. Statistical Analysis

t-tests and Wilcoxon signed-rank tests were used to compare the characteristics between GDM and non-GDM groups for continuous variables. Variance analysis was used to compare the characteristics between different GDM subtypes for continuous variables, and chi-squared tests were used for categorical variables between groups. Multiple linear regression models were used to analyze the association of SNPs in VitD and glucose metabolic-pathway-related genes, and their interactions with 25(OH)D concentrations at T1 and T2 with the blood glucose levels of each OGTT timepoint in a co-dominant genetic model. Multiple logistic regression models were used to analyze the relationship of SNPs, Gc isoforms and their interaction with 25(OH)D concentration at T1 and T2 with GDM as well as its subtypes in a co-dominant genetic model. Furthermore, stratification analysis by prepregnancy BMI was carried out to investigate the association between Gc isoforms and the risk of GDM and its subtypes [23]. To investigate the interaction between VDR-rs10783219, CDKAL1-rs7754840 and MTNR1B-rs10830962 on the risk of GDM and its subtypes, stratification analysis was carried out. In addition, to investigate the joint association of VitD status at T1 or T2 with Gc isoforms on the risk of GDM and its subtypes, we classified Gc isoforms into three groups—1f/1f and 1f/2; 2/2 and 1s/1f; and 1s/2 and 1s/1s—and crossover analysis was carried out. The hierarchical analysis was used to investigate the interaction between each SNP and 25(OH)D concentration on the
risk of GDM, and the $p$-value of the interaction term was calculated. To investigate whether there was a dose–effect relationship between Gc isoforms and subtypes of GDM, a trend test was applied in the multiple logistic regression model and Gc isoforms were treated as continuous variables for different isoforms (1s/1s, 1s/2, 1s/1f, 2/2, 1f/2 and 1f/1f), of which the proportion of free 25(OH)D was successively reduced. The above multi-factor models were all adjusted for possible confounding, including maternal age, prepregnancy BMI, OGTT season, etc. All test results were considered statistically significant at a value of $p < 0.05$. All analyses were performed using SAS (version 9.2, SAS Institute).

Sample size calculation: in the present study, the risks of GDM subtype 2 of GG genotype in MTNR1B-rs10830962 were 1.85 times greater than compared with the CC genotype. The prevalence of GDM in this study was 23.8%; among them, 58.5% were GDM subtype 2. We hypothesized that $\alpha = 0.05$, power = 80%, OR$_{gene}$ = 1.85, and the genotype frequency for SNP was 18%. Through QUANTO software, it was determined that the minimum case number for the GDM subtype 2 was 118, and the minimum case number for GDM was 202, which is lower than the number of GDM cases in this study (n = 513). Therefore, the sample size was large enough for the analysis of different GDM subtypes.

3. Results

3.1. Subject Characteristics

A total of 2156 pregnant women were included in this study, and the characteristics of the participants are shown in Table 1. Of these, 513 (23.8%) women were diagnosed with GDM. The mean age and prepregnancy BMI of participants were 28.8 years old and 20.7 kg/m$^2$, respectively. Compared with non-GDM women, women with GDM had higher prepregnancy BMI, lower 25(OH)D concentrations at T2 and lower educational levels. As shown in Supplementary Table S1, compared with participants with GDM subtype 1, those with GDM subtype 2 and 3 were older and had higher VitD levels at T1 and T2.

Table 1. Baseline characteristics of pregnant women.

| Variables                  | Total     | non-GDM   | GDM       | $p$   |
|----------------------------|-----------|-----------|-----------|-------|
| Age, years                 | n = 2156  | n = 1643  | n = 513   | <0.0001 |
| Prepregnancy BMI (kg/m$^2$)| 28.8 (3.7)| 28.5 (3.5)| 29.6 (4.0)|       |
| 25(OH)D at T1 (ng/mL) *    | 18.9 (8.7)| 18.7 (8.7)| 19.5 (8.6)| 0.0884 |
| 25(OH)D$_3$                | 18.1 (8.6)| 17.9 (8.6)| 18.7 (8.6)| 0.0530 |
| 25(OH)D$_2$                | 0.6 (0.5) | 0.6 (0.5) | 0.5 (0.4) | 0.9761 |
| 25(OH)D at T2 (ng/mL) †    | 25.6 (11.5)| 26.0 (11.7)| 24.1 (10.7)| 0.0149 |
| 25(OH)D$_3$                | 24.6 (11.5)| 24.9 (11.7)| 23.3 (10.8)| 0.0310 |
| 25(OH)D$_2$                | 0.7 (0.7) | 0.6 (0.7) | 0.7 (0.6) | 0.6255 |
| VitD deficiency at T1 *    | 1281 (62.3%)| 983 (62.8%)| 298 (60.7%)| 0.3979 |
| VitD deficiency at T2 †     | 499 (36.4%)| 374 (34.8%)| 125 (42.2%)| 0.0180 |
| GDM rate                   | 513 (23.8%)| —         | —         | 0.0920 |
| OGTT season                |           |           |           |       |
| Summer/fall                | 1045 (48.5%)| 813 (49.5%)| 232 (45.2%)|       |
| Winter/spring              | 1111 (51.5%)| 830 (50.5%)| 281 (54.8%)|       |
| Educational level          |           |           |           | 0.0179 |
| ≤High school               | 589 (27.3%)| 428 (26.0%)| 161 (31.4%)|       |
| ≥High school               | 1567 (72.7%)| 1215 (74.0%)| 352 (68.6%)|       |
| Income per capita, RMB     |           |           |           | 0.3659 |
| <30,000                    | 191 (8.9%) | 143 (8.7%) | 48 (9.4%)  |       |
| ≥30,000                    | 1647 (76.4%)| 1269 (77.2%)| 378 (73.7%)|       |
| Not sure                   | 180 (8.3%) | 132 (8.0%) | 48 (9.4%)  |       |
| Unknown                    | 138 (6.4%) | 99 (6.0%)  | 39 (7.6%)  |       |
| Planned pregnancy          |           |           |           | 0.0411 |
| No                         | 709 (32.9%)| 563 (34.3%)| 146 (28.5%)|       |
| Yes                        | 1313 (60.9%)| 983 (59.8%)| 330 (64.3%)|       |
| Unknown                    | 134 (6.2%) | 97 (5.9%)  | 37 (7.2%)  |       |
### 3.2. Associations of SNPs and Its Interaction with VitD on GDM and GDM Subtypes

Compared with the wild-type genotype, the PG1H and/or PG2H levels of mutant genotypes were lower for LRP2-rs10210408, and higher for VDR-rs10783219, CDKAL1-rs7754840 and MTNR1B-rs10830962. Interactions between 25(OH)D concentrations at T1 and the CT genotype in CYP3A4-rs2242480, GA genotype in GC-rs2298849 and CC genotype in CDKAL1-rs7754840 on PG1H level, and the CT genotype in CYP24A1-rs2209314, TT genotype in GC-rs16846876 and GA genotype in GC-rs2298849 on PG2H level were observed (Supplementary Table S2, all $P_{\text{interaction}} < 0.05$). The risks of GDM and GDM subtype 2 of TA genotype in VDR-rs10783219 were 1.26 and 1.33 times greater compared with the AA genotype (Table 2). Compared with the CC genotype, GG genotypes in MTNR1B-rs10830962 were at higher risk of GDM (Table 2, $OR = 2.08$, 95% CI: 1.46–2.97), GDM subtype 1 (Table 2, $OR = 3.26$, 95% CI: 1.62–6.59) and subtype 2 (Table 2, $OR = 1.85$, 95% CI: 1.22–2.81). Compared with the wild-type genotypes, interactions between 25(OH)D concentrations at T1 and the CT genotype in CYP3A4-rs2242480, and the TT genotype in LRP2-rs10210408 on the risk of GDM and GDM subtype 2 were found (Table 2). However, interactions between SNPs and 25(OH)D concentrations at T2 on FBG, PG1H and PG2H levels of OGTT as well as GDM and its subtypes were not observed.

As shown in Table 3, significant interactions between CDKAL1-rs7754840 and VDR-rs10783219 on the risk of GDM and GDM subtype 2 ($P_{\text{interaction}}: 0.0121$ and $0.0432$) as well as interactions between CDKAL1-rs7754840 and MTNR1B-rs10830962 on the risk of GDM and GDM subtype 1 ($P_{\text{interaction}}: 0.0082$ and $0.0071$) were found.

### 3.3. Associations of Gc Isoforms and VitD with GDM and GDM Subtypes

Compared to women with Gc isoforms of 1f/1f, those with Gc isoforms of 1f/2 and 1s/2 had higher levels of PG1H and PG2H among women with prepregnancy BMI ≥ 24 kg/m² (Supplementary Table S3). In addition, after adjusting for potential confounders, dose–effect relationships of Gc isoforms with GDM and GDM subtype 2 ($P_{\text{trend}}: 0.0046$ and 0.0011, Supplementary Table S4) were observed among women with prepregnancy BMI ≥ 24 kg/m². Compared to women with Gc isoforms of 1f/1f and 1f/2 and VitD non-deficiency at T1 and T2, those with Gc isoforms of 1s/2 and 1s/1s had increased risk of GDM and GDM subtype 2 (OR = 2.21, 95% CI: 1.14–4.30; OR = 2.79, 95% CI: 1.20–6.49, Table 4). However, combined effect of 25(OH)D concentrations at T1 or T2 with Gc isoforms on the risk of GDM and GDM subtypes were not observed (Table 4).
### Table 2. Relationship of SNPs in VitD and glucose metabolic pathway and its interaction with 25(OH)D concentrations at T1 and T2 with GDM and GDM subtypes.

| SNPs       | Genotype | GDM 1 Case (%) | GDM 1 OR (95% CI) Case (%) | GDM Subtype 2 Case (%) | GDM Subtype 2 OR (95% CI) Case (%) | GDM Subtype 3 Case (%) | GDM Subtype 3 OR (95% CI) Case (%) |
|------------|----------|----------------|-----------------------------|------------------------|------------------------------------|------------------------|------------------------------------|
|            |          |                |                            |                        |                                    |                        |                                    |
| CYP24A1    | rs2209314 | TT 193 (25.1)  | Ref 62 (8.1)                | Ref 106 (13.8)         | Ref 25 (3.2)                        | Ref 106 (13.8)         | Ref 25 (3.2)                        |
|            | rs2242480 | CC 129 (23.8)  | Ref 85 (6.9)                | Ref 170 (13.8)         | Ref 37 (3.0)                        | Ref 170 (13.8)         | Ref 37 (3.0)                        |
|            | rs1155563 | TT 761 (22.2)  | Ref 47 (6.2)                | Ref 95 (12.5)          | Ref 27 (3.5)                        | Ref 95 (12.5)          | Ref 27 (3.5)                        |
|            | rs11682804| GC 839 (18.8)  | Ref 30 (3.6)                | Ref 106 (12.6)         | Ref 22 (2.6)                        | Ref 106 (12.6)         | Ref 22 (2.6)                        |
|            | rs2298849 | AA 809 (21.4)  | Ref 51 (6.3)                | Ref 100 (12.4)         | Ref 22 (2.7)                        | Ref 100 (12.4)         | Ref 22 (2.7)                        |
|            | rs1155563 | TT 761 (22.2)  | Ref 47 (6.2)                | Ref 95 (12.5)          | Ref 27 (3.5)                        | Ref 95 (12.5)          | Ref 27 (3.5)                        |
|            | rs11682804| GC 839 (18.8)  | Ref 30 (3.6)                | Ref 106 (12.6)         | Ref 22 (2.6)                        | Ref 106 (12.6)         | Ref 22 (2.6)                        |
| CYP3A4     | rs2209314 | TT 193 (25.1)  | Ref 62 (8.1)                | Ref 106 (13.8)         | Ref 25 (3.2)                        | Ref 106 (13.8)         | Ref 25 (3.2)                        |
|            | rs2242480 | CC 129 (23.8)  | Ref 85 (6.9)                | Ref 170 (13.8)         | Ref 37 (3.0)                        | Ref 170 (13.8)         | Ref 37 (3.0)                        |
|            | rs1155563 | TT 761 (22.2)  | Ref 47 (6.2)                | Ref 95 (12.5)          | Ref 27 (3.5)                        | Ref 95 (12.5)          | Ref 27 (3.5)                        |
|            | rs11682804| GC 839 (18.8)  | Ref 30 (3.6)                | Ref 106 (12.6)         | Ref 22 (2.6)                        | Ref 106 (12.6)         | Ref 22 (2.6)                        |
|            | rs2298849 | AA 809 (21.4)  | Ref 51 (6.3)                | Ref 100 (12.4)         | Ref 22 (2.7)                        | Ref 100 (12.4)         | Ref 22 (2.7)                        |
|            | rs1155563 | TT 761 (22.2)  | Ref 47 (6.2)                | Ref 95 (12.5)          | Ref 27 (3.5)                        | Ref 95 (12.5)          | Ref 27 (3.5)                        |
|            | rs11682804| GC 839 (18.8)  | Ref 30 (3.6)                | Ref 106 (12.6)         | Ref 22 (2.6)                        | Ref 106 (12.6)         | Ref 22 (2.6)                        |
|            | rs2298849 | AA 809 (21.4)  | Ref 51 (6.3)                | Ref 100 (12.4)         | Ref 22 (2.7)                        | Ref 100 (12.4)         | Ref 22 (2.7)                        |
|            | rs1155563 | TT 761 (22.2)  | Ref 47 (6.2)                | Ref 95 (12.5)          | Ref 27 (3.5)                        | Ref 95 (12.5)          | Ref 27 (3.5)                        |
|            | rs11682804| GC 839 (18.8)  | Ref 30 (3.6)                | Ref 106 (12.6)         | Ref 22 (2.6)                        | Ref 106 (12.6)         | Ref 22 (2.6)                        |

**Abbreviations:** GDM, gestational diabetes mellitus; VitD, vitamin D; subtype 1, elevated fasting glucose and normal post-load glucose; subtype 2, normal fasting glucose and elevated post-load glucose; subtype 3, elevated fasting and post-load glucose. * Adjusted for maternal age, prepregnancy BMI, parity, educational level, income, physical exercise and OGTT season. † Binomial logistic regression model; ‡ multinomial logistic regression model. § p < 0.05; †† p-value of the interaction term SNPs * 25(OH)D concentration at the first trimester < 0.05.
Table 3. Interactions between CDKAL1, MTNR1B and VDR on risk of GDM and GDM subtypes *.

| SNPs          | Risk Allele of GDM | n  | GDM * | OR (95% CI) | GDM Subtype 1 * | OR (95% CI) | GDM Subtype 2 * | OR (95% CI) | GDM Subtype 3 * | OR (95% CI) |
|---------------|--------------------|----|-------|-------------|-----------------|-------------|-----------------|-------------|-----------------|-------------|
|               |                    |    | Case (%) |              | Case (%) |              | Case (%) |              | Case (%) |              |
| CDKAL1-rs7754840 | VDR-rs10783219       |    |          |             |             |             |             |             |             |
| GG            | T                  | 633| 128 (20.2) | 0.81 (0.60–1.09) | 24 (3.8) | 0.80 (0.43–1.50) | 85 (13.4) | 0.76 (0.53–1.08) | 19 (3.0) | 1.05 (0.52–2.10) |
| GC            | T                  | 819| 164 (20.0) | 1.35 (1.05–1.75) | 31 (3.8) | 1.55 (0.91–2.63) | 111 (13.6) | 1.34 (1.00–1.81) | 22 (2.7) | 1.14 (0.61–2.13) |
| CC            | T                  | 264| 63 (23.8) | 1.36 (0.89–2.08) | 12 (4.6) | 1.36 (0.52–3.59) | 39 (14.8) | 1.16 (0.69–1.95) | 12 (4.6) | 2.82 (0.99–8.04) |
| CC/CC         | T                  | 1083| 227 (21.0) | 1.37 (1.10–1.70) | 43 (4.0) | 1.51 (0.95–2.38) | 150 (13.9) | 1.31 (1.02–2.70) | 34 (3.1) | 1.49 (0.90–2.44) |

MTNR1B-rs10830962 | CDKAL1-rs7754840       |    |          |             |             |             |             |             |             |
| CC            | T                  | 572| 91 (15.9) | 1.26 (0.91–1.76) | 17 (3.0) | 1.35 (0.64–2.67) | 62 (10.8) | 1.27 (0.85–1.88) | 12 (2.1) | 1.31 (0.54–3.18) |
| GC            | T                  | 848| 186 (21.9) | 0.90 (0.71–1.16) | 30 (3.5) | 0.72 (0.40–1.27) | 122 (14.4) | 0.89 (0.67–1.19) | 34 (4.0) | 1.15 (0.69–1.94) |
| GG            | T                  | 296| 78 (26.4) | 1.88 (1.20–2.94) | 20 (6.8) | 2.99 (1.34–6.68) | 51 (17.2) | 1.59 (0.94–2.69) | 7 (2.4) | 1.70 (0.50–5.76) |

| MTNR1B-rs10830962 | CDKAL1-rs7754840       |    |          |             |             |             |             |             |             |
| CC            | C                  | 572| 91 (15.9) | 0.89 (0.63–1.24) | 17 (3.0) | 0.37 (0.17–1.61) | 62 (10.8) | 0.97 (0.65–1.45) | 12 (2.1) | 0.74 (0.31–1.74) |
| GC            | C                  | 848| 186 (21.9) | 1.08 (0.84–1.38) | 30 (3.5) | 0.86 (0.48–1.53) | 122 (14.4) | 1.08 (0.81–1.44) | 34 (4.0) | 1.32 (0.78–2.24) |
| GG            | C                  | 297| 78 (26.3) | 1.89 (1.23–2.91) | 20 (6.7) | 3.06 (1.41–6.66) | 51 (17.2) | 1.48 (0.90–2.46) | 7 (2.4) | 3.66 (1.04–12.6) |

Abbreviations: GDM, gestational diabetes mellitus; VDR, vitamin D; T1, first trimester; T2, second trimester; subtype 1, elevated fasting glucose and normal post-load glucose; subtype 2, normal fasting glucose and elevated post-load glucose; subtype 3, elevated fasting and post-load glucose. * Adjusted for maternal age, prepregnancy BMI, parity, educational level, income, physical exercise and OGTT season. † Binomial logistic regression model; ‡ multinomial logistic regression model. p < 0.05.

Table 4. The relationship of VitD status at T1 and T2, Gc isoforms with GDM and GDM subtypes *.

| VitD Deficiency | Gc Isoforms | n  | GDM * | OR (95% CI) | GDM Subtype 1 * | OR (95% CI) | GDM Subtype 2 * | OR (95% CI) | GDM Subtype 3 * | OR (95% CI) |
|----------------|-------------|----|-------|-------------|-----------------|-------------|-----------------|-------------|-----------------|-------------|
| T1            | T2          |    |       |             |                 |             |                 |             |                 |             |

Abbreviations: GDM, gestational diabetes mellitus; VitD, vitamin D; T1, first trimester; T2, second trimester; subtype 1, elevated fasting glucose and normal post-load glucose; subtype 2, normal fasting glucose and elevated post-load glucose; subtype 3, elevated fasting and post-load glucose. * Adjusted for maternal age, prepregnancy BMI, parity, educational level, income, physical exercise and OGTT season.

4. Discussion

The current study demonstrated significant associations of variant genotype of SNPs at VDR-rs10783219 and MTNR1B-rs10830962 with the risk of GDM and GDM subtype 2. Furthermore, CDKAL1-rs7754840 interacts with VDR-rs10783219 and MTNR1B-rs10830962 on GDM subtypes. In addition, among women with prepregnancy BMI ≥ 24 kg/m², a dose–effect relationship between Gc isoforms and GDM subtype 2 was observed.

The LRP2 gene plays an important role in the preservation of vitamin D metabolites and delivery of the precursor to the kidney for the generation of 1α,25(OH)2D₃ [15,31],

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polymorphisms of which were associated with increased risks of severe VitD deficiency and related bone disease [32]. Our study initially found that variation at LRP2-rs10210408 was related to higher postprandial glucose levels among pregnant women. In addition, interactions between LRP2-rs10210408 and VitD level at T1 on the risk of GDM and GDM subtype 2 were found, which indicated that variations of the A allele to T at LRP2-rs10210408 might influence glucose metabolism through VitD during pregnancy.

VDR-rs11568820 is a functional SNP and its variant may improve the islet activity of the calcium-sensing receptor, which further inhibits insulin secretion [33]. Only one study has reported that the variant at VDR-rs11568820 impairs the secretion of pancreatic islets and increases the risk of type 2 diabetes in the adult cohort and PG2H in children [30]. In the present study, we identified that the homozygous variant at VDR-rs10783219 in pregnant women was associated with higher PG1H (β = 0.24, p = 0.0212), and higher risks of GDM (TA/TT vs. AA: OR = 1.28) and GDM subtype 2 (TA/TT vs. AA: OR = 1.31). According to the high-linkage relationship between VDR-rs10783219 and VDR-rs11568820 in this population [15], we could speculate that it might be the highly interlinked VDR-rs11568820 that exhibits the biological functions. VDR-rs11568820 not only plays an important role in the development of type 2 diabetes, but also of GDM. Significant associations between CDKAL1-rs7754840 and PG2H, as well as GDM, were also observed in our study, which was consistent with the genome-wide association study reported by Kwak et al. [8]. Variants at CDKAL1-rs7754840 may affect the conversion process from proinsulin to insulin [34]. This study further confirmed that variants at CDKAL1-rs7754840 increased the risk of GDM in Chinese populations. Furthermore, we also found that for each additional G risk allele at MTNR1B-rs10830962, the risk of GDM increased by 52% and 108%, and GDM subtype 2 by 43% and 85%, respectively, which was consistent with previous studies [8,35]. The MTNR1B gene encodes melatonin receptor 2 (MTNR2), which could significantly inhibit the expression of 3′,5′-cyclic adenosine monophosphate in cells, and subsequently reduces insulin secretion [36,37]. Therefore, variants of the C allele to G at MTNR1B-rs10830962 are likely to inhibit the release of insulin in islet cells and increase the risk of GDM.

Meanwhile, we also identified a significant interaction between VDR-rs10783219 and CDKAL1-rs7754840 as well as MTNR1B-rs10830962 on GDM. Variants at VDR-rs10783219 increased the risk of GDM and GDM subtype 2 among women with a variant at CDKAL1-rs7754840, suggesting that the protective effect of VitD on GDM was more obvious in patients with abnormal islet cell functions. In addition, the T allele at VDR-rs10783219 and the C allele at CDKAL1-rs7754840 separately increased the risk of GDM subtype 1 among women with the GG genotype at MTNR1B-rs10830962 (OR = 2.99, 95%CI: 1.34–6.68; OR = 3.06, 95%CI: 1.41–6.66) (Pinteraction = 0.2611; Pinteraction = 0.0071). Given that the MTNR1B gene could reduce the secretion of insulin, the conversion obstacles of proinsulin to insulin mediated by the CDKAL1 gene might be strengthened with reduced insulin secretion. The above interaction between SNPs found in this study provides a new perspective for the study of the pathogenesis of GDM, but the specific biological mechanism still needs to be verified by further studies.

Traditionally, 25(OH)D was thought to be taken up by cells of the kidney binding to vitamin D-binding protein through megalin/cubilin-mediated endocytosis. However, studies [38,39] have found that although the levels of both 25(OH)D and 1,25(OH)_{2}D in blood and urine were low in megalin knockout and vitamin D-binding protein knockout mice, vitamin D-binding protein knockout mice did not show symptoms of VitD deficiency, unlike megalin knockout mice. In addition, vitamin D-binding protein knockout mice would rapidly manifest symptoms of VitD deficiency when fed with a VitD-deficient diet. In 2019, the first case of the human homozygous deletion of a GC gene reported by Henderson et al. [17] confirmed that this mechanism found in animals also applies to humans. The above research indicates that free 25(OH)D or 1,25(OH)_{2}D is the main form to exert the biological VitD effects. Furthermore, the proportion of free 25(OH)D of individuals with different Gc isoforms is different: individuals with the 1f/1f isoform have the highest free 25(OH)D concentrations, and individuals with 1s/1s have the lowest, followed by 1f/2,
2/2, 1s/1f and 1s/2 [16]. This study initially reported that the associations of Gc isoforms with GDM and GDM subtypes during pregnancy were different in pregnant women with different prepregnancy BMI. Significant associations were only observed among women who were overweight or obese before pregnancy. The distribution of Gc isoforms was significantly different between blacks and whites along with the distribution of fat with the same BMI [40]. More than 90% of blacks were of Gc1f type, whereas the majority of whites are of Gc1s type; Asians were in between [41]. The accumulation of abdominal fat is a risk factor for insulin resistance and metabolic syndrome [42]. Given the strong association between BMI and insulin resistance [43], we speculated that overweight and obese pregnant women might have underlying insulin resistance before pregnancy, and the difference in insulin resistance among pregnant women with different Gc isoforms may be caused by the difference in body fat distribution. In this study, it was found that compared with the 1f/1f isoform, pregnant women with 1s/2 and 1s/1s isoforms had higher risk of GDM subtype 2, indicating higher visceral and liver fat content, and thus, higher muscle insulin resistance. However, the specific pathophysiological mechanism needs to be confirmed by further studies.

Our previous study [23] found that serum 25(OH)D only affected FBG and GDM subtypes with abnormal fasting glucose. However, this study found that free 25(OH)D (represented by Gc isoforms) mainly influences postprandial glucose levels and GDM subtype 2. The difference between serum 25(OH)D and free 25(OH)D on glucose and GDM risk indicates that the proportion of free 25(OH)D is mainly related to muscle insulin resistance or insulin secretion, and serum 25(OH)D in circulation is not mainly mediated by free 25(OH)D, which may be related to fasting gluconeogenesis levels in the liver, and plays its role in lowering glucose levels through megalin/cubilin-mediated endocytosis through the kidney or parathyroid cells [44]. However, combined effects of 25(OH)D concentrations at T1 or T2 with Gc isoforms on the risk of GDM and GDM subtypes were not observed.

Strengths of the current study included the prospective cohort design and the relatively large sample size, which may guarantee the authenticity of the research results and higher statistical test efficiency. Furthermore, we initially divided GDM into different subtypes based on the different mechanisms of insulin resistance. The risks of GDM and GDM subtypes in pregnant women with different Gc isoforms have been investigated for the first time, and the effect of prepregnancy BMI and longitudinal changes in VitD during pregnancy on the association between Gc isoforms and GDM as well as its subtypes was considered. However, there were several potential limitations in this study. Insulin levels, which could more accurately distinguish different types of insulin resistance in GDM, were not detected simultaneously during the OGTT examination in this study. In addition, the average prepregnancy BMI of the population in this study was low, and about 12% of the pregnant women were overweight (10.3%) or obese (2.1%). Furthermore, in this study, we investigated whether there was a VitD supplementation of participants during pregnancy, but did not consider the supplementation dose because the clinically recommended supplementation dose of VitD for pregnant women is between 400 and 600 IU. However, the type of VitD supplementation was unknown, which restricted the study to further explore how the SNP affected the response to VitD supplementation on serum 25(OH)D concentrations and its impact on GDM. Therefore, the results of this study may be limited when extrapolating to obese or severely obese pregnant women.

5. Conclusions

In conclusion, our results showed that variants of SNPs at VDR-rs10783219 and MTNR1B-rs10830962 significantly increased the risk of GDM and GDM subtypes with normal fasting glucose and elevated post-load glucose, and interactions were investigated between each other as well as with CDKAL1-rs7754840. With lower Gc isoforms, the proportions of free 25(OH)D were related to an increased risk of GDM with abnormal postprandial blood glucose in prepregnancy overweight and obese women. The present study explored whether gene variants in the VitD metabolic and glucose pathway would
affect the risk of GDM from a genetic point of view. In addition, the 25(OH)D concentration is very unstable and can easily be affected by exposure factors such as supplementation and sunlight exposure. Identifying the effect of gene variants in the VitD and glucose metabolic-pathway-related genes on the development of GDM and GDM subtypes could more objectively evaluate the relationship between VitD and GDM and provide standards for subsequent clinical applications.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/nu13124220/s1, Supplementary Table S1. Baseline characteristics of pregnant women with different GDM subtypes, Supplementary Table S2. Association of SNPs and its interaction with VitD level at T1 and T2 on three time-point plasma glucose levels of OGTT, Supplementary Table S3. Association of Gc isoforms and different time-point plasma glucose levels of OGTT, Supplementary Table S4. Association of Gc isoforms with GDM and GDM subtypes among women with different prepregnancy BMI values.

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**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available because they contain information that could compromise the privacy of research participants.

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**Conflicts of Interest:** The authors declare that they have no conflict of interest.

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