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Gestational diabetes mellitus risk assessment, screening, diagnosis, and management before, during and after pregnancy

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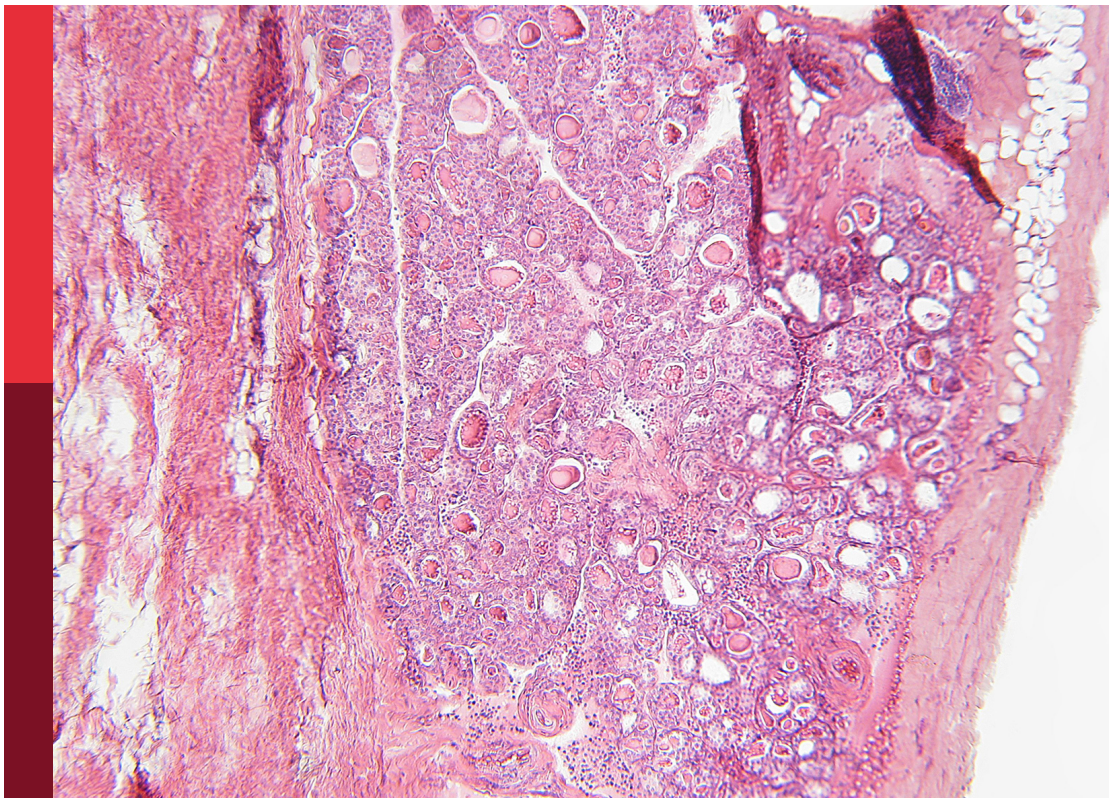

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The prevalence of gestational diabetes mellitus before and after the implementation of the universal two-child policy in China

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Background: After the universal two-child policy has been fully implemented, challenges regarding pregnancy complications seemed to be more severe in China. This study aimed to evaluate the prevalence of gestational diabetes mellitus (GDM) and the main risk factors for GDM before and after the implementation of the universal two-child policy in China.

Methods: A retrospective study was performed with 128,270 pregnant women who delivered at Ningbo Women & Children's Hospital from January 2010 to December 2020. Univariate and multivariate logistic regression analysis was applied to estimate the risk factors associated with GDM prevalence. Segmented regression analyses of interrupted time series (ITS) were conducted to assess the effect of the universal two-child policy on the trends of GDM.

Results: The prevalence of GDM increased remarkably from 4% in 2010 to 21% in 2020. ITS analysis presented that the prevalence of GDM increased by 0.190% (β1) per month from 2010 to 2016 (P<0.05), and by 0.044% (β1+β3) per month after the implementation of the universal two-child policy; the rate of elevation of GDM slowed down significantly (β3=-0.146, P=0.004). Advanced maternal age (>30 years), multigravidity, multiparity, multiple gestation and gestational hypertension were significantly associated with GDM. Advanced age remained an independent risk factor for GDM even after cross stratification with gravidity and parity. The proportion of women with advanced maternal age (>30 years) increased by 0.161% per month before the implementation of the universal two-child policy and increased by 5.25% during the policy took effect month, and gradually increased by 0.124% (β1+β3) per month after then.

Conclusions: The prevalence of GDM has sharply increased in the past decade. The growth rate of GDM slowed down after the implementation of the universal two-child policy in China, but the rate would maintain at a high plateau. The rise in the proportion of older pregnant women could increase the GDM rate. We recommend having children at a relatively optimal reproductive age when encouraging childbearing.

KEYWORDS

advanced maternal age, gestational diabetes mellitus, universal two-child policy, risk factor, interrupted time series

Background

Gestational diabetes mellitus (GDM) is defined as impaired glucose tolerance (IGT) that first occurs or is first detected during pregnancy (1). It is an emerging epidemic (2, 3), and approximately one in six live births is associated with hyperglycemia exposure *in utero* according to the International Diabetes Federation (IDF) 2019 (4). The incidence of GDM ranges from 25% in South-East Asia to 17.5% in the Middle East and North Africa, 12.6% in Europe and 10.4% in North America and the Caribbean region (5).

Concurrently, the incidence of GDM has risen dramatically and has caused tremendous increases in medical expenditures in China in recent decades (6, 7). A recent systematic analysis of 79,064 pregnant women in 21 regions of mainland China reported a pooled GDM prevalence of 14.8% using the International Association of Diabetes and Pregnancy Study Group (IADPSG) diagnostic criteria (8). Of note, the incidence of GDM in China displayed substantial differences across cities and regions. For example, in the Tongzhou district of Beijing, the overall prevalence of GDM was 24.24%, and the trend increased from 21.63% to 25.49% during 2013-2018 (9). In the same period, a population-based study in Xiamen reported that the rate of GDM ranged from 15.5% to 19.9% (10). Another retrospective study conducted Guangzhou, China from 2011 through 2017 estimated a total GDM rate of approximately 22.94% (11). Similarly, 15.8% of pregnant women from four maternity hospitals in Chengdu, Western China, were diagnosed with GDM in 2015 (12). However, in some remote areas, such as Xinjiang, the estimated prevalence of GDM is only 5.12% (8), which might be due to the relatively lower rates of GDM screening during pregnancy.

Several reports proposed that the GDM incidence was increasing and accompanied by a high proportion of pregnant women with advanced maternal age after the implementation of the two-child policy (6, 13). In recent decades, China has gradually loosened fertility restrictions from the one-child policy to the partial two-child policy, universal two-child policy and even the three-child policy. The one-child policy was implemented in China for 36 years after its promulgation in 1979 (14), and the strategy was strictly enforced, particularly among urban residents; however, beginning in 1984, couples in rural areas were permitted to have a second baby if their first child was a girl, the so-called "1.5-child policy" (15). By 2007, all provinces in China started to pilot the two-child policy for couples in which both partners were only children, except for in Henan, where piloting began in 2011. Then, in 2013, couples in which at least one person was an only child were allowed to have two children (15). Later, in October 2015, the Chinese government encouraged all couples to have two children, which marked the official end of the one-child policy and the beginning of the new, universal two-child policy (15). The universal two-child policy targeted approximately 90 million reproductive-aged women who had delivered one baby; however, nearly 60% of the target women were over 35 years old, and 50% were over 40 (16). Thus, the projected increase in pregnant women with advanced maternal age and the consequent rise in pregnancy complications, such as GDM, after the implementation of the universal two-child policy have caused extensive concern.

Currently, data assessing trends in the prevalence of GDM in China before and after the implementation of the universal two-child policy are relatively scant. Our study aimed to identify the changes in GDM and related characteristics of pregnancy or delivery following the implementation of the universal two-child policy among 128,270 pregnant women who delivered at Ningbo Women & Children's Hospital from January 2010 to December 2020.

Materials and methods

Study design and participants

This retrospective study was conducted to assess the temporal trend in GDM prevalence in women who delivered

babies at Ningbo Women & Children's Hospital from January 2010 through December 2020. A total of 140,676 pregnant women delivered their babies during this period. The exclusion criteria included pregestational diabetes mellitus (PGDM), duplicate records, and a missing diagnosis of GDM. A total of 128,270 eligible participants were eventually enrolled in the analysis. The data used in this study were extracted from electronic medical records.

Data source

The data included sociodemographic characteristics, maternal disease status, obstetric history, pregnancy complications, mode of delivery, and maternal and neonatal delivery outcomes.

Diagnosis of GDM

The fasting plasma glucose (FPG) test was carried out during the first trimester of gestation to leave out the pre-pregnancy diabetes, and the overt diabetes mellitus (DM) of FPG>7.0 mmol/L was excluded. GDM status was confirmed by two-step 50-g glucose challenge test (GCT) or one-step 75-g oral glucose tolerance test (OGTT) at 24-28 weeks of gestation. The 1-hour glucose value of 50-g GCT ≥11.2 mmol/L or 1-h glucose value ≥ 7.8 but subsequent 75-g OGTT had two or more items reached the standard criterions: FPG ≥ 5.6, 1h value ≥10.3, 2h value ≥8.6 or 3h value ≥ 6.7 was identified as GDM. The glucose value of one-step 75-g OGTT meeting or exceeding one of the following criteria: 0 h value ≥ 5.1 mmol/L, 1 h value ≥10.0 mmol/L, or 2 h value ≥ 8.5 mmol/L was diagnosed as GDM.

Ethics approval

This study was approved by the Ningbo University Medical Science Research Ethics Committee.

Statistical analysis

The major maternal and neonatal health characteristics are presented as the means ± standard deviations (SDs) or absolute frequencies (n) and relative frequencies (%) and were used to assess temporal trends from 2010 to 2020.

Segmented regression analyses of interrupted time series (ITS) were conducted to assess the effect of the two-child policy on the trend in GDM prevalence. The Durbin-Waston test was used to detect first-order autocorrelation (17), and the autocorrelated errors would be adjusted by the generalized least square estimator (GLSE) based on Prais-Winsten estimation. The ITS model was $Y_t = \beta0 + \beta1*time_t + \beta2*two\text{-}child\ policy_t + \beta3*time\ after\ two\text{-}child\ policy_t$, where Y_t is the GDM rate per month t, and time t is a continuous variable representing the months since the start of the study. Two-child $policy_t$ is a binary variable indicating the time before or after the implementation of the policy (coded as 0 for months between 2010 and 2016 and 1 for months thereafter). Time after two-child policy t is a continuous variable counting the number of months after the implementation of policy at time t. β0 is interpreted as the baseline level when T=0, and β1 indicates the preintervention slope. β2 and β3 present the change in the GDM rate after the intervention in the short- and long-term, respectively. The sum of β1 and β3 was used to evaluate the postintervention slope. The sensitivity analysis was conducted to estimate the robustness of ITS model using different month lags as the taking effect time after implementation of the universal two-chlid policy.

Univariate and multivariate logistic regression analysis were applied to estimate the potential risk factors of GDM, including reproductive age, gravidity, parity, the number of foetus and gestational hypertension (GH). Data analysis was carried out using SPSS software version 24.0 (SPSS Inc., Chicago, IL, USA) and R software (version 4.1.0). *P* values <0.05 for a two-tailed test were considered to be statistically significant.

Results

A total of 128,270 eligible participants who delivered at Ningbo Women & Children's Hospital between January 2010 and December 2020 were included, and the baseline characteristics and pregnancy outcomes are described in Table 1.

The mean age at delivery increased from 27 years (2010) to 30 years (2020). In the age stratification, pregnant women aged 25-29 years accounted for the major proportion during the examined years. The proportion of women aged 20-24 years decreased from 28% to 10%, while women aged 30-34 years and ≥35 years (advanced maternal age) increased from 19% to 34% and 9% to 15%, respectively. In addition, the proportions of primiparas and multiparas have gradually become comparable since 2017 (Table 1).

Univariate logistic regression analysis found associations of advanced maternal age [30-34 years, *OR* 1.64 (1.58-1.70); 35-39 years, *OR* 2.54 (2.42-2.66); 40-50 years, *OR* 3.26 (2.99-3.54)], multigravidity [two times, *OR* 1.18 (1.13-1.23); ≥3 times, *OR* 1.48 (1.43-1.54)], multipara [*OR* 1.27 (1.23-1.31)], multiple gestation [*OR* 1.46 (1.35-1.57)] and GH [*OR* 1.69 (1.58-1.81)] with GDM (Table 2). These variables were all included in the multivariate logistic regression model, and the age, multigravidity, multiple gestation and GH remained the significant risk factors for GDM, but the multipara became negative correlation wuth GDM

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Table 1 Characteristics of the participants according to delivery year.

Characteristics		2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
	N	9572	11,041	11,022	10,344	11,779	11,416	13,931	13,485	11,911	12,916	10,853
Age (year)		27±5	27±5	28±5	28±5	28±5	29±5	29±5	30±5	30±5	30±5	30±5
	<20	488 (5)	530 (5)	442 (4)	393 (4)	331 (3)	300 (3)	226 (2)	200 (2)	164 (1)	130 (1)	101 (1)
	20-	2667 (28)	2804 (25)	2560 (23)	2199 (21)	2048 (17)	1768 (16)	1756 (13)	1551 (12)	1362 (11)	1291 (10)	1043 (10)
	25-	3553 (37)	4450 (40)	4650 (42)	4371 (42)	5349 (45)	4971 (44)	6213 (45)	5409 (40)	4684 (39)	5176 (40.1)	4091 (38)
	30-	1844 (19)	2098 (19)	2371 (22)	2400 (23)	2914 (25)	3032 (27)	3888 (28)	3928 (29)	3591 (30)	4160 (32.2)	3724 (34)
	35-	854 (9)	905 (8)	798 (7)	789 (8)	950 (8)	1145 (10)	1581 (11)	1984 (15)	1751 (15)	1801 (13.9)	1596 (15)
	40-50	166 (2)	254 (2)	201 (2)	192 (2)	187 (2)	200 (2)	267 (2)	413 (3)	359 (3)	358 (2.8)	298 (3)
Gravidity (%)	One	3528 (37)	4442 (40)	4455 (40)	4261 (41)	4830 (41)	4177 (37)	5123 (37)	4346 (32)	4100 (34)	4692 (36.3)	3873 (36)
	Two	2656 (28)	2988 (27)	3006 (27)	2699 (26)	3129 (27)	3109 (27)	3873 (28)	3854 (29)	3269 (27)	3624 (28.1)	3012 (28)
	Three or more	3388 (35)	3611 (33)	3561 (32)	3384 (33)	3820 (32)	4130 (36)	4935 (35)	5285 (39)	4542 (38)	4600 (35.6)	3968 (37)
Parity (%)	Primipara	5839 (61)	7160 (65)	7198 (65)	6768 (65)	7613 (65)	6714 (59)	7910 (57)	6783 (50)	6244 (52)	6885 (53.3)	5836 (54)
	Multipara	3653 (38)	3756 (34)	3736 (34)	3524 (34)	4166 (35)	4702 (41)	6021 (43)	6702 (50)	5667 (48)	6031 (46.7)	5017 (46)
	NA	80 (1)	125 (1)	88 (1)	52 (1)	/	/	/	/	/	/	/
GDM (%)	Yes	337 (4)	429 (4)	878 (8)	1241 (12)	1772 (15)	1674 (15)	2285 (16)	2491 (19)	2088 (18)	2340 (18.1)	2271 (21)
	No	9235 (97)	10612 (96)	10144 (92)	9103 (88)	10007 (85)	9742 (85)	11646 (84)	10994 (82)	9823 (83)	10576 (81.9)	8582 (79)
GH (%)	Yes	496 (5)	515 (5)	532 (5)	458 (4)	527 (5)	455 (4)	434 (3)	430 (3)	421 (4)	578 (4.5)	574 (5)
	No	9076 (95)	10526 (95)	10490 (95)	9886 (96)	11252 (96)	10961 (96)	13497 (97)	13055 (97)	11490 (97)	12338 (95.5)	10279 (95)
Preeclampsia (%)	Yes	355 (4)	473 (4)	364 (3)	320 (3)	3 (0)	3 (0)	1 (0)	2 (0)	1 (0)	1 (0)	124 (1)
	No	9217 (96)	10568 (96)	10658 (97)	10024 (97)	11776 (100)	11413 (100)	13930 (100)	13483 (100)	11910 (100)	12915 (100)	10729 (99)
Polyhydramnios (%)	Yes	288 (3)	305 (3)	244 (2)	236 (2)	186 (2)	163 (1)	231 (2)	226 (2)	220 (2)	313 (2.4)	306 (3)
	No	9284 (97)	10736 (97)	10778 (98)	10108 (98)	11593 (98)	11253 (99)	13700 (98)	13259 (98)	11691 (98)	12603 (97.6)	10547 (97)
Oligohydramnios (%)	Yes	557 (6)	550 (5)	559 (5)	537 (5)	747 (6)	642 (6)	767 (6)	817 (6)	876 (7)	961 (7.4)	829 (8)
	No	9015 (94)	10491 (95)	10463 (95)	9807 (95)	11032 (94)	10774 (94)	13164 (95)	12668 (94)	11035 (93)	11955 (92.6)	10024 (92)
PROM (%)	Yes	1714 (18)	2159 (20)	2340 (21)	1982 (19)	2593 (22)	2381 (21)	3001 (22)	2816 (21)	2379 (20)	2624 (20.3)	2122 (20)
	No	7858 (82)	8882 (80)	8682 (79)	8362 (81)	9186 (78)	9035 (79)	10930 (79)	10669 (79)	9532 (80)	10292 (79.7)	8731 (80)
Placenta previa (%)	Yes	389 (4)	311 (3)	342 (3)	310 (3)	523 (4)	548 (5)	583 (4)	452 (3)	354 (3)	346 (2.7)	228 (2)
	No	9183 (96)	10730 (97)	10680 (97)	10034 (97)	11256 (96)	10868 (95)	13348 (96)	13033 (97)	11557 (97)	12570 (97.3)	10625 (98)
Number of foetus (%)	Single birth	9322 (97)	10663 (97)	10680 (97)	10021 (97)	11407 (97)	11016 (97)	13419 (96)	12968 (96)	11420 (96)	12398 (96)	10454 (96)
	Multiple births	250 (3)	378 (3)	342 (3)	323 (3)	372 (3)	400 (4)	512 (4)	517 (4)	491 (4)	518 (4)	399 (4)
Infant sex (%)	Male	5158 (54)	5802 (53)	5821 (53)	5490 (53)	6241 (53)	6062 (53)	7344 (53)	7196 (53)	6178 (52)	6842 (53)	5704 (53)

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Continued

Characteristics		2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
	Female	4333 (45)	5114 (46)	5110 (46)	4802 (46)	5537 (47)	5353 (47)	6586 (47)	6287 (47)	5729 (48)	6069 (47)	5144 (47)
	NA	81 (1)	125 (1)	91 (1)	52 (1)	1 (0)	1 (0)	1 (0)	2 (0)	4 (0)	5 (0)	5 (0)
Mode of delivery (%)	Vaginal birth	4303 (45)	4748 (43)	4869 (44)	4910 (48)	6041 (51)	5957 (52)	7215 (52)	7124 (53)	6272 (53)	6468 (50.1)	5248 (48)
	Cesarean	5007 (52)	5959 (54)	5772 (52)	5138 (50)	5470 (46)	5188 (45)	6296 (45)	6125 (45)	5486 (46)	6133 (47.5)	5110 (47)
	NA	262 (3)	334 (3)	381 (4)	296 (3)	268 (2)	271 (2)	420 (3)	236 (2)	153 (1)	315 (2.4)	495 (5)
Full-term birth (%)	Yes	8431 (88)	9500 (86)	9364 (85)	8970 (87)	10180 (86)	9975 (87)	12176 (87)	11846 (88)	10518 (88)	11352 (87.9)	9532 (88)
	No	1141 (12)	1541 (14)	1658 (15)	1374 (13)	1599 (14)	1441 (13)	1755 (13)	1639 (12)	1393 (12)	1564 (12.1)	1321 (12)
Pregnancy outcomes (%)	Livebirth	9530 (100)	10995 (100)	10985 (100)	10267 (99)	11673 (99)	11326 (99)	13857 (100)	13409 (99)	11853 (100)	12837 (99.4)	10815 (100)
	Malformation	8 (0)	14 (0)	2 (0)	19 (0)	8 (0)	10 (0)	4 (0)	2 (0)	7 (0)	18 (0.1)	9 (0)
	Stillbirth	34 (0)	32 (0)	35 (0)	46 (0)	90 (1)	73 (1)	70 (1)	70 (1)	50 (0)	57 (0.4)	29 (0)
	NA	/	/	/	12 (0)	8 (0)	7 (0)	/	4 (0)	1 (0)	4 (0)	/
Macrosomia (%)	Yes	250 (3)	208 (2)	206 (2)	193 (2)	660 (6)	712 (6)	869 (6)	830 (6)	646 (5)	700 (5.4)	567 (5)
	No	9322 (97)	10833 (98)	10816 (98)	10151 (98)	11119 (94)	10704 (94)	13062 (94)	12655 (94)	11265 (95)	12216 (94.6)	10286 (95)

NA, not available; GDM, Gestational diabetes mellitus; GH, Gestational hypertension; PROM, premature rupture of membranes.

Table 2 Univariate and multivariate logistic regression analysis of factors associated with GDM.

Characteristics	Group	GDM	non-GDM	*OR* (95%*CI*)	*P*
Univariate logistic regression analysis					
Age	25-29	6105 (34)	46812 (42)	Reference	
	<20	98 (1)	3207 (3)	0.23 (0.19–0.29)	<0.001
	20-24	1242 (7)	19807 (18)	0.48 (0.45–0.51)	<0.001
	30-34	5976 (34)	27974 (25)	1.64 (1.58–1.70)	<0.001
	35-39	3522 (20)	10632 (10)	2.54 (2.42–2.66)	<0.001
	40-50	863 (5)	2032 (2)	3.26 (2.99–3.54)	<0.001
Gravidity	One	5605 (32)	42222 (38)	Reference	
	Two	4754 (27)	30465 (28)	1.18 (1.13–1.23)	<0.001
	Three or more	7447 (42)	37777 (34)	1.48 (1.43–1.54)	<0.001
Parity	Primipara	9530 (54)	65420 (59)	Reference	
	Multipara	8267 (46)	44708 (41)	1.27 (1.23–1.31)	<0.001
Multiple gestation	No	16960 (95)	106808 (97)	Reference	
	Yes	846 (5)	3656 (3)	1.46 (1.35–1.57)	<0.001
GH	No	16670 (94)	106180 (96)	Reference	
	Yes	1136 (6)	4284 (4)	1.69 (1.58–1.81)	<0.001
Multivariate logistic regression					
Age				1.12 (1.11–1.12)	<0.001
Gravidity				1.07 (1.02–1.12)	0.004
Multipara				0.80 (0.77–0.84)	<0.001
Multiple gestation				1.35 (1.25–1.46)	<0.001
GH				1.46 (1.36–1.56)	<0.001

(Table 2). The proportion of advanced maternal age (≥30 years) was highly distributed in women with two or more pregnancy (51%) or multipara (61%) compared with first pregnancy (20%) or primipara (24%) (Table 3). Subgroup analysis found that age (≥30 years) remained the strongest risk factor for GDM even after stratification with gravidity or parity (Table 3).

The prevalence of GDM increased considerably from 4% in 2010 to 21% in 2020 (Table 1). Figure 1A displays a sharply increased GDM rate from 2010 to 2017, a relatively slow growth trend maintained after 2017. ITS analysis was applied to assess the change of the increased trend of GDM after the universal two-child policy, and the results illustrated that the prevalence of GDM increased by 0.190% (β1) per month from 2010 to 2016 (P<0.05), but by 0.044% (β1+β3) per month after the implementation of the universal two-child policy since 2017; the change of the trend was statistically significant (β3=-0.146, P=0.004) (Supplementary Table 1). Moreover, sensitive analysis were performed by setting different time lags after the implementation of policy, and ITS analysis at different month lags showed the same effect of the universal two-child policy on the change of GDM (Supplementary Table 2). We further assessed the effect of the universal two-child policy on the change of reproductive age. The results showed that the proportion of women with advanced maternal age (>30 years) increased by 0.161% per month before the implementation of the universal two-child policy, increased by 5.25% during the policy took effect month (set in January 2017), and then gradually increased by 0.124% (β1+β3) per month after then(Figure 1B and Supplementary Table 1).

Figures 2A, B presented the increased trend of GDM in different age groups, the women aged older than 30 years had a higher prevalence of GDM. Similarly, Figures 3A–D showed a higher GDM rate in women with multigravidity, multiparity, multiply gestations or GH. The rate of elevation of GDM slowed down in all groups after the two-chlid policy implemented in 2017 (Supplementary Table 1).

Discussion

The current study showed that the prevalence of GDM increased rapidly from 4% in 2010 to 21% in 2020 based on data from 128,270 pregnant women who delivered at Ningbo Women & Children's Hospital. The prevalence of GDM sharply increased, particularly from 2010 to 2016, and then maintained a slow growth trend since 2017. ITS analysis also demonstrated that the rate of elevation of GDM slowed down after the implementation of the universal two-child policy. Furthermore, our study found that the risk of GDM was markedly increased with advanced maternal age. It was noted that the maternal age at childbirth increased by an average of 3 years from 2010 to 2020. Moreover, the proportion of pregnant women older than 30 years increased, ranging from 29.9% (2010) to 51.7% (2020), and the growth trend in reproductive age (>30 years) still increased by 0.124% per month after the implementation of the universal two-child policy since 2017. These results indicated that the rising proportion of older pregnant women resulting from the implementation of the universal two-child policy might be an independent risk factor for GDM.

Our findings were generally consistent with several large population-based studies that demonstrated a strong relationship between advanced maternal age and GDM (18-20). Two large multicenter cohort investigations of singleton pregnancies reported that the GDM rate increased with maternal age (18, 19), and the rate reached a high plateau at approximately 40 years of age (18). Another prospective study based on national registry data also proposed that women aged 40-44 years and those aged 45 years or older had nearly three- and fourfold increased risks of developing GDM, respectively (20). Moreover, Chantal Mathieu et al. indicated that screening GDM by a maternal age ≥30 years and/or a BMI ≥25 could identify 81% of cases based on the 2013 WHO criteria (21). The underlying mechanism could be explained by the association of

Table 3 Stratification analysis by age and gravidity/parity for GDM.

Subgroups	Age>30y stratification (%)	GDM	non-GDM	*OR* (95%*CI*)	*P*
Gravidity & Age					
<30 & one pregnancy	38163 (80)	3660 (21)	34503 (31)	Reference	
≥30 & one pregnancy	9664 (20)	1945 (11)	7719 (7)	2.38 (2.24-2.52)	<0.001
<30 & two or more pregnancy	39108 (49)	3785 (21)	35323 (32)	1.01 (0.96-1.06)	0.68
≥30 & two or more pregnancy	41335 (51)	8416 (47)	32919 (30)	2.41 (2.31-2.51)	<0.001
Parity & Age					
<30 & Primipara	56685 (76)	5623 (32)	51062 (46)	Reference	
≥30 & Primipara	18265 (24)	3907 (22)	14358 (13)	2.47 (2.36–2.58)	<0.001
<30 & Multipara	20444 (39)	1817 (10)	18627 (17)	0.89 (0.84–0.94)	<0.001
>30 & Multipara	32531 (61)	6450 (36)	26081 (24)	2.25 (2.16–2.33)	<0.001

GDM; Gestational diabetes mellitus.

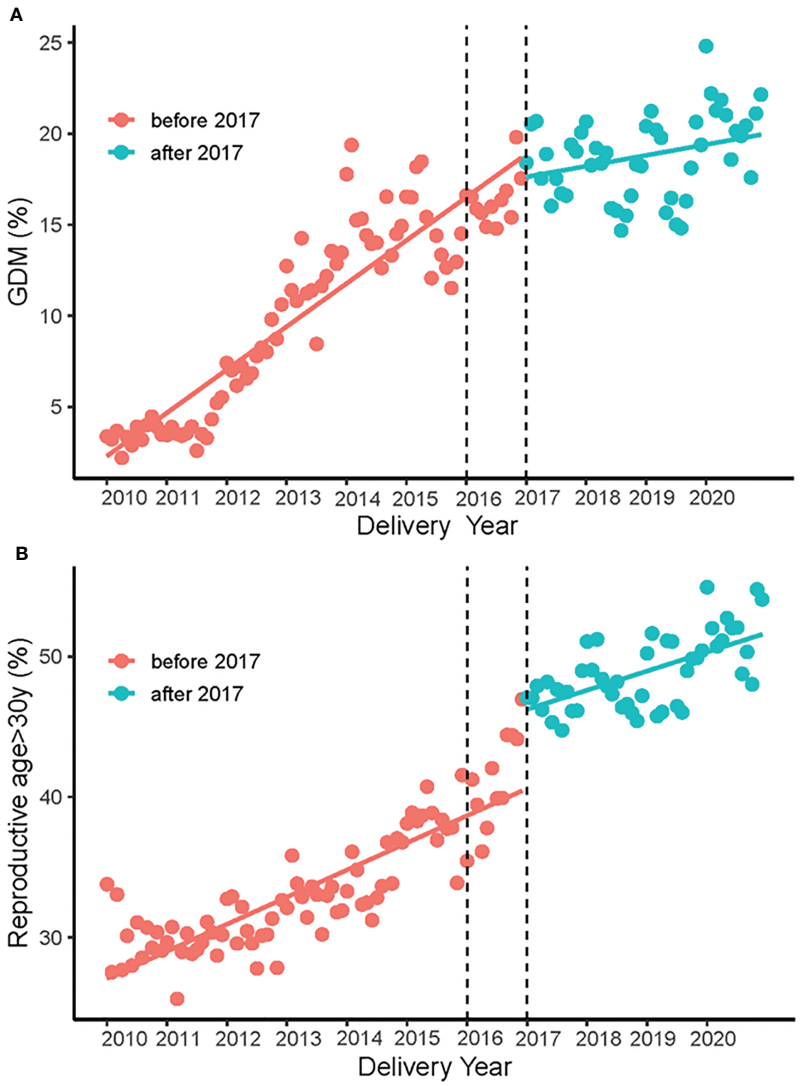

FIGURE 1
The increased trend of GDM rate and the proportion of women with advanced maternal age (>30 years) from 2010 to 2020 **(A)**. The growth trend of GDM before and after the implementation of the universal two-child policy **(B)**. The growth trend of the proportion of women with advanced maternal age (>30 years) before and after the implementation of the universal two-child policy.

aging with reduced insulin sensitivity, abnormal glucose tolerance, and impaired pancreatic B-cell function (18). However, the data from the Diabetes Surveillance System of the Ningbo Center for Disease Control and Prevention (CDC) showed that the age at GDM diagnosis was markedly increased after 2016 in Ningbo city, but the BMI was relatively steady. It seemed that delayed childbearing age was the most important risk factor for GDM after the implementation of the universal two-child policy.

In the current study, the rise in the proportion of older pregnant women might be maintained by the change in fertility policy. In particular, women aged 30-34 years accounted for the overwhelming majority among the increased proportion of older women (>30 years) and presented an increasing trend after the implementation of the two-child policy. However, the proportion of women aged 40 years or older remained lower than 3%. Accordingly, we speculate that eligible couples who desired to have children and were younger than 35 years old were more likely to apply for a second child after the implementation of the universal two-child policy, but the majority of women older than 40 years would express a lower desire for a second child given the potential for birth defects. In addition, several social factors, such as delayed marriage, the rising ratio of divorce and remarriage rates, increased desires for higher education and career achievement, the development of assisted reproductive technology and growing financial burdens,

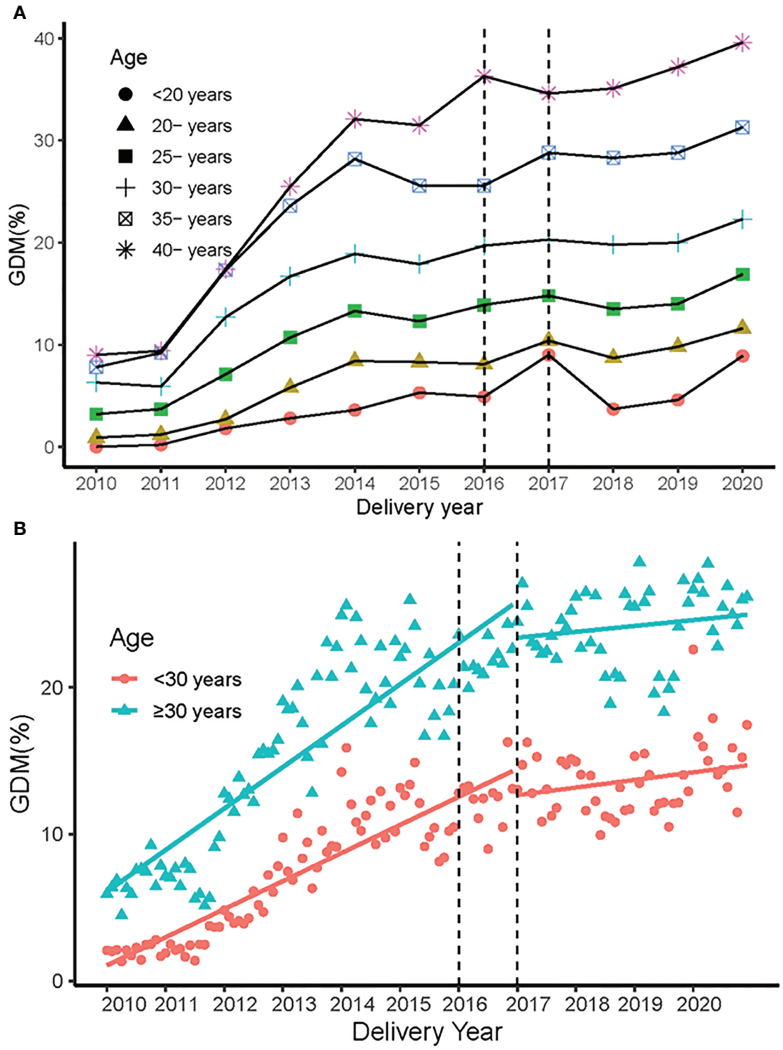

FIGURE 2
The prevalence of GDM in different age groups in 2010-2020. **(A)** The prevalence of GDM in different age groups from 2010 to 2020. **(B)** The change in growth trend of GDM before and after the universal two-child policy in different age groups.

could contribute to postponing childbearing in China. Therefore, we advocate for having children at a relatively optimal reproductive age when encouraging childbearing.

Although advanced maternal age was a strong independent risk factor for GDM, other physiological and socioeconomic factors should also be fully considered. Among our findings, multigravidity, multiparity, multiple gestation and gestational hypertension also contributed to GDM incidence, although these factors exerted less effects than age, and the trend in GDM exhibited slower growth in the above exposure groups after the implementation of the two-child policy in 2017. Previous research proposed an association between abortion and GDM (22). Our study found that the proportion of women with three or more pregnancies remained at approximately 35% in the past decade. It is not difficult to guess that most of these women had undergone induced abortions. The two-child policy could substantially reduce the number of abortions due to sex selection and unapproved second children, which would greatly improve maternal and infant adverse outcomes (15).

The trend of rapid growth in the prevalence of GDM during the past decade was also linked with elevated GDM screening, enhanced awareness of prenatal health care, improved medical service systems and modifiable lifestyle factors (23, 24). The diagnostic criteria of GDM using the IADPSG (2010) have been gradually recommended in China since 2011. The 2011 edition of the GDM health industry standards by the Ministry of Health of China, the 2013 edition of the Chinese Guidelines for the Diagnosis and Treatment of Diabetes Mellitus, and the

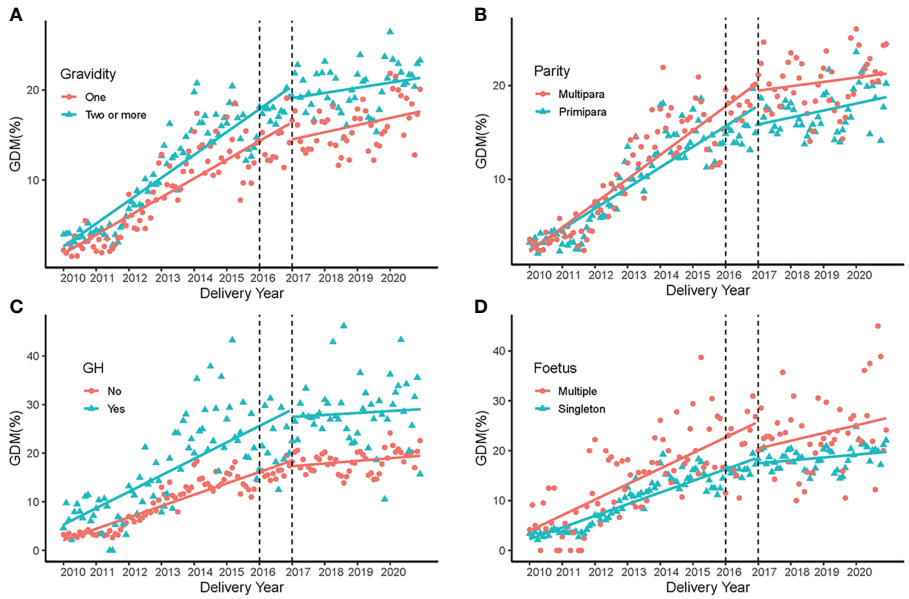

FIGURE 3
GDM rate in 2010-2020 in different subgroups. **(A)** Trends of GDM rate in different gravidity groups before and after the the universal two-child policy. **(B)** Trends of GDM rate in the primipara and multipara groups before and after the universal two-child policy. **(C)** Trends of GDM rate in women with and without gestational hypertension before and after the universal two-child policy. **(D)** Trends of GDM rate in the singleton and multiple fetus groups before and after the universal two-child policy.

Obstetrics and Gynecology Subcommittee of the Chinese Medical Association 2014 all adopted the IADPSG standard. These criteria recommend a "one-step" method (75 g OGTT) of testing at 24-28 weeks of gestation and have helped to identify more cases of GDM in pregnant women (25, 26). In our study, the one-step 75-g OGTT method almost completely replaced the two-step 50-g GCT method in Ningbo, Zhejiang province, China since 2012. Herein, the rapid rise in GDM in our study should be partially due to improved screening and diagnostic technology. Moreover, delayed childbearing age and the accompanying increase in assisted reproduction technology use can also exacerbate the incidence of GDM (27, 28). Other factors, such as genetic variations, obesity, inappropriate lifestyles during pregnancy and environmental exposures, are also associated with GDM (6, 29, 30). However, since the implementation of the policy to encourage childbirth, women of childbearing age have paid more attention to reproductive health and prenatal care, and improved health systems would provide more guidance and intervention before and during pregnancy, all of which might effectively reduce the occurrence of GDM. Taken together, the universal two-child policy might not aggravate the prevalence of GDM, but the GDM rate could remain at a relatively stable high level in the future.

In fact, most couples of childbearing age are cautious about the second-child and third-child policies. In November 2013, China launched the partial two-child policy allowing couples to have a second child when at least one member of the couple was an only child. However, by May 2015, only 13.2% (1.45/11 million) of eligible couples wanted to have a second child (15). Shortly afterward, the universal two-child policy was announced (October 2015). The number of live births reached 17.86 million and 17.23 million in 2016 and 2017 in China (31, 32), respectively, increasing by 7.9% and 4.1% compared with 2015 (Supplementary Figure 1); in particular, approximately 5.4 million births of multiparous women during the first eighteen months were due to the new policy (16). Surprisingly, China's fertility level did not increase continuously, as expected, and the numbers of live births in 2018 and 2019 were only 15.23 million and 14.65 million, respectively, which were even lower than the birth rates before the implementation of the universal two-child policy (Supplementary Figure 1) (33, 34). Consistently, our data also showed an apparent fertility peak in 2016 and 2017 and then a decline from the peak. Furthermore, the China Family Panel Studies (CFPS) 2018 reported that less than 10% of men and women aged 18-49 years present the desire to have a third child, of which 4.90% of those aged 18-24 years desire to have a third child, 6.92% of those aged 25-29 years desire to have a third child, and 16% of those aged 45-49 years desire to have a third child. Consequently, the lower fertility desire and higher proportion of pregnant women with advanced maternal age are still inevitable trends even after the three-child policy. Hence, the long-term impact of the fertility policy on fertility desire and childbearing age needs further observation in the future.

Our study focused on the prevalence of GDM and associated risk factors across the implementation of the universal two-child

policy from 2010 to 2020. This provided relatively reliable evidence of maintaining an upward trend in GDM with advanced reproductive age after the implementation of the universal two-child policy. Nevertheless, our data were limited to a single center: the Ningbo Women & Children's Hospital, Zhejiang Province. Hence, the results might vary in other districts in China due to different living habits, environmental exposures, and socioeconomic development.

Conclusions

In conclusion, the present study found that the prevalence of GDM has sharply increased in the past decade. Although, the growth rate of GDM slowed down after implementation of the universal two-child policy, the rate would maintain at a high plateau in the future. Notably, advanced maternal age was an independent risk factor for GDM, and the remarkable rise in the proportion of older pregnant women with the change in fertility policy seemed to be associated with the prevalence of GDM, as expected. In the future, large multicenter cohort studies are warranted to determine the long-term impact of the universal two-child policy on GDM.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ningbo University Medical Science Research Ethics Committee. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

LJ conceived the study with HZ and JX. JC, ZZ, YC, QZ, and LZ contributed to the acquisition of data, and HZ, ZZ, YC, LJ, and JX contributed to the analysis and interpretation of the data. HZ, LJ, and JX drafted the initial article, and all co-authors contributed to revising it for intellectual content. All co-authors have given final approval of the submitted version. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted. LJ is the guarantor. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.960877/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Total births in mainland China and total delivery volume in Ningbo Women & Children's Hospital from 2010 to 2020 The bar graph corresponds to the left ordinate value, and the line graph corresponds to the right ordinate value. The data for the total number of births and the total birth rate in mainland China from 2010-2020 were extracted from the National Economic and Social Development Statistical Bulletin of the People's Republic of China.

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Periconceptional diet quality is associated with gestational diabetes risk and glucose concentrations among nulliparous gravidas

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Background: Gestational diabetes mellitus (GDM) and elevated glucose concentrations below the threshold for GDM diagnosis have been associated with adverse pregnancy and offspring outcomes. Dietary interventions initiated during pregnancy have demonstrated inconsistent beneficial effects. Limited data exist regarding the effects of periconceptional diet on gestational glycemia.

Objective: To evaluate independent associations between periconceptional diet quality with GDM frequency and glucose concentrations from GDM screening and diagnostic tests among nulliparous gravidas.

Design: This is a secondary analysis of N=7997 participants from the NuMoM2b multicenter, prospective, observational cohort study of first pregnancies. The Alternative Healthy Eating Index (AHEI)-2010 was computed from food frequency questionnaires completed in early pregnancy (6-13 weeks), reporting usual dietary intake over the preceding 3 months. GDM screening was performed either by non-fasting 1-hour 50g glucose load (N=6845), followed by 3-hour 100g glucose tolerance test (GTT) for those with raised glucose concentrations (N=1116; at risk for GDM), or by a single 2-hour 75g GTT (N=569; all GDM risk levels). Logistic and linear regression were used to estimate the associations between the AHEI-2010 score with odds of GDM,

having raised blood glucose on the 1-hour screening test, and continuous glucose concentrations on screening and diagnostic tests. All models were adjusted for *a priori* covariates: maternal age, race/ethnicity, early-pregnancy body mass index, smoking habits, rate of gestational weight gain, energy intake, nausea and vomiting in early pregnancy, study site.

Results: Poorer periconceptional diet quality was observed among participants who were younger, with higher BMI, lower income levels, and of non-Hispanic Black or Hispanic ethnicity. The GDM rate was 4%. Each 1-point increase in AHEI-2010 score was associated with a 1% decrease in the odds of being diagnosed with GDM (beta=-0.015, p=0.022, OR=0.986, 95% CI 0.973 to 0.998). Diet quality was inversely associated with each post glucose load concentration on the non-fasting screening test and the 2-hour and 3-hour GTT.

Conclusion: Poor periconceptional diet quality is independently associated with an increased risk of GDM and with minor elevations in serum glucose concentrations on GDM screening and diagnostic tests, in a diverse cohort of nulliparas. Periconception intervention studies targeting diet quality are warranted.

KEYWORDS

periconception, pregnancy, alternative healthy eating index, diet quality, gestational diabetes mellitus, gestational glycemia, women's health

Introduction

Glycemic control during pregnancy is an imperative component of prenatal care. Gestational diabetes mellitus (GDM) is a common complication of pregnancy, currently estimated to affect 63.5 per 1000 live births in the United States, and its prevalence is increasing across all racial/ethnic groups (1). GDM carries significant maternal and/or perinatal morbidity as affected pregnant individuals are more likely to develop preeclampsia or undergo a cesarean delivery, while later in life they have up to 70% odds of developing type 2 diabetes (2). Neonates are at increased risk for large for gestational age birth weight, birth trauma, hypoglycemia, as well as obesity and diabetes later in life (2). Further, modestly elevated glucose concentrations on glucose tolerance tests (GTT), even in the absence of overt GDM, have been associated with an increased risk of adverse pregnancy outcomes (3, 4). Interventions to decrease the incidence of GDM and milder hyperglycemic cases are warranted.

Excess gestational weight gain (GWG) is a known, modifiable risk factor for GDM (5). Although several randomized controlled trials have shown improved adherence to GWG guidelines with lifestyle interventions (6), this has not consistently been associated with improvement in perinatal outcomes (7–9). The lack of evidence surrounding prenatal lifestyle interventions has led investigators to explore other modifiable nutrition parameters, including prenatal diet quality. Although no diet has been shown to be the single best choice for all pregnancies (10), benefit has been seen with the Mediterranean diet, the low glycemic-index diet, and diets that emphasize plant-based rather than animal-based protein (11–14).

As GDM is typically diagnosed in the late second or early third trimester, it has been suggested that implementing dietary changes at this stage is too late to maximally impact pregnancy and neonatal outcomes (15). Greater attention is now being paid to the preconception period as a potentially efficacious window for health behavior change to support improved pregnancy outcomes (16).

Preconceptional diets higher in red meat and processed foods have been previously shown to be associated with an increased incidence of GDM (11–13). In a prior analysis from the Nulliparous Pregnancy Outcomes Study: Monitoring Mothers-To-Be (NuMoM2b) cohort that the present study utilizes, periconceptional diet quality, measured by the Healthy Eating Index (HEI), was associated with numerous adverse pregnancy outcomes, but not with GDM incidence (17). However, the effect of periconceptional diet on glycemia across

a continuum, regardless of GDM diagnosis, has not yet been studied. Further, there is need for a more comprehensive characterization of periconceptional diet quality using an index that is strongly associated with chronic disease risk, such as the Alternative Healthy Eating Index (AHEI)-2010 (18, 19). The AHEI-2010 differs from the HEI in that it incorporates quantitative scoring for *qualitative* dietary guidelines (e.g., choose more fish, poultry, and whole grains, and if you drink alcohol, do so in moderation) that are specifically associated with reduced chronic disease risk, particularly diabetes and coronary heart disease (18).

The aim of this study was to determine the prospective association between periconceptional diet and frequency of GDM, as well as gestational glucose at the time of GDM screening and diagnostic testing, in a diverse cohort of nulliparas.

Materials and methods

This is a secondary analysis of maternal dietary and glycemic data from the NuMoM2b cohort, a large, multicenter, prospective observational study conducted at 8 U.S. medical centers from 2010 to 2013. Each site's local governing Institutional Review Board(s) approved the nuMoM2b protocol and procedures.

Individuals were eligible if they had a viable singleton pregnancy, had no prior pregnancy lasting ≥20 weeks' gestation, and were between 6 + 0 to 13 + 6 weeks' gestation at the time of enrollment. Exclusion criteria included age <13 years, history of ≥3 spontaneous abortions, likely fatal fetal malformation evident before enrollment, known fetal aneuploidy, assisted reproduction with a donor oocyte, multifetal reduction, or plan to terminate the pregnancy. Complete study protocol details have been previously published (20). Participants classified as having pre-gestational diabetes were excluded from the present analysis.

Diet was assessed by the validated modified Block 2005 Food Frequency Questionnaire (FFQ) at visit 1 (21, 22), when participants were between 6 + 0 to 13 + 6 weeks' gestation. The Block FFQ assesses energy intake, 52 nutrients and 35 food groups from approximately 120 food and beverage items and includes serial adjustment items to estimate portion size. Participants were asked to report usual dietary intake over the preceding 3 months, thereby reflecting the periconceptional period.

The AHEI-2010, a validated predictor of chronic disease risk (19), was computed as a summary score of overall diet quality. The AHEI-2010 is comprised of 11 food group or nutrient components: vegetables, fruit, wholegrains, sugary beverages and fruit juice, red and processed meat, nuts and legumes, long-chain omega-3 fats, trans fatty acids, polyunsaturated fatty acids, sodium, and alcohol. Individuals are assigned a score from 0-10 for each component, where higher scores indicate greater compliance to recommended intakes of that food group or nutrient. Component scores are summed to give a total AHEI-2010 score ranging from 0-110. The specific criterion for scoring each component has been previously described (18).

Nausea and vomiting of pregnancy commonly occurs in the first trimester and may influence dietary intake periconceptionally. The validated Pregnancy-Unique Quantification of Emesis (PUQE) scale was completed by participants at the first study visit to assess the degree of nausea and vomiting experienced. This scale produces a continuous score from 3-15, with higher scores representing more severe nausea and vomiting.

Outcome variables for this analysis were serum glucose concentrations on 50g glucose screening test, fasting and post glucose load serum glucose concentrations from GTTs performed as part of routine clinical practice, and presence or absence of GDM abstracted from medical records based on local diagnostic criteria (2-hour 75g GTT or 3-hour 100g GTT). Specifically, for the 2-hour GTT, a diagnosis of GDM is established when any single threshold value is met or exceeded (fasting, 92 mg/dL; 1-hour, 180 mg/dL; or 2-hour, 153 mg/dL) (23). For the 3-hour GTT, GDM is diagnosed when two or more threshold values are met or exceeded (fasting, 95 mg/dL; 1-hour, 180 mg/dL; 2-hour, 155 mg/dL; 3-hour, 140 mg/dL) (24). Having an elevated glucose result on the 50g non-fasting glucose screening test, using the threshold of ≥140 mg/dL as recommended by the American College of Obstetricians and Gynecologists (2), was also considered as a secondary outcome measure as this is a widely used indicator of GDM risk. Glucose concentrations from GTTs were considered separately in analyses according to 2-hour or 3-hour testing method. For participants who had multiple GTTs performed during pregnancy, the glycemic concentrations from the test conducted closest to 26-28 weeks' gestation were selected for this analysis, as this is the most widely accepted timepoint for routine GDM screening. Serum glucose concentrations were determined by enzymatic assay at each study site according to local protocols.

A priori covariates included in the analysis were selected based on their known association with GDM and impaired glycemia in pregnancy: maternal body mass index (BMI), age, self-reported race/ethnicity, self-reported smoking status within the prior 3 months (yes/no), rate of GWG until the approximate time of the GTT. Additionally we adjusted for energy intake and the PUQE score as these factors may influence the AHEI-2010 score. BMI was computed in early pregnancy using measured weight and height at enrollment, according to the formula weight (kg)/height $(m)^2$. Rate of GWG per week was computed as the difference in maternal measured weight between the enrollment visit and study visit 3 (at 22 + 0 to 29 + 6 weeks' gestation), divided by the number of weeks between measurement dates. Additionally, study site was entered to all models as a covariate to control for potential differences in

glycemic concentrations on GDM screening and diagnostic tests due to different assay kits and laboratory techniques.

Statistical analyses were performed using IBM SPSS Statistics version 26. The AHEI-2010 score was described continuously and categorically by quartiles. Descriptive statistics were used to describe maternal baseline characteristics, rate of GWG, incidence of GDM, and glycemic values. Differences in these variables were compared between participants with and without available dietary data by the independent sample t-test or chi-squared test. Among those with dietary data, differences in maternal characteristics, incidence of GDM, and glycemic concentrations across AHEI-2010 quartiles were determined by one-way ANOVA with *post-hoc* Tukey's test for continuous variables, and by chi-squared test for categorical variables. In the case of glycemic variables, AHEI-2010 quartiles were computed separately for each subset of participants with available glucose screening and/or tolerance testing data. The association between the continuous AHEI-2010 score and odds of developing GDM and having a raised glucose concentration (≥140 mg/dl) on the 50g glucose screening test were determined by logistic regression. A sensitivity analysis of the association between AHEI-2010 score and the odds of GDM was also performed separately for each group of subjects screened by the 1-step or 2-step methods. Associations between AHEI-2010 total score and component scores with glucose concentrations from the 50g glucose screening test, and with fasting and post glucose load concentrations on each GTT were analyzed by separate linear regression models. Missing data for covariates were handled by pairwise deletion in regression models. Results were considered statistically significant at $p<0.05$.

Results

There were 10,038 participants in the parent NuMoM2b study, 8259 of whom had dietary information from which AHEI-2010 scores were computed. Among these, 7997 had a documented outcome for GDM (presence or absence) and were included in the present study. Figure 1 describes the number of participants with available data according to each stage of GDM screening and/or diagnostic testing. Among those undergoing the 2-step testing method, 992 were at risk for GDM and underwent a 3-hour GTT. An additional 124 individuals underwent a 3-hour GTT who did not have the 50g screening test performed, presumably due to other risk factors (e.g., family history of diabetes, raised hemoglobin A1c value). A total of 325/7997 participants (4.1%) were documented as having a diagnosis of GDM. Of those, 42 did not have any recorded GTT data.

NuMoM2b participants with missing dietary data were, on average, of younger age, higher BMI, with a lower education and income level, and more likely to smoke periconceptionally and to be of Black or Hispanic versus White ethnicity (Supplemental Table 1). However, there were no significant differences in the incidence of GDM or having a raised blood glucose concentration on the screening test between those with missing and available dietary data.

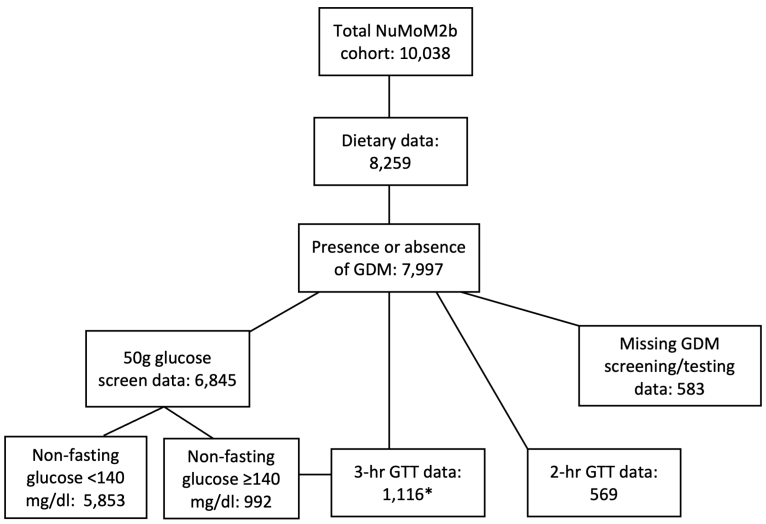

FIGURE 1
Flowchart of participants for the present analysis. Number of study participants with available data for presence or absence of GDM, diet quality, GDM screening, and GDM diagnostic testing. *3-hr GTT data available for 992 participants with a raised glucose concentration on the 50g screening test, plus 124 additional participants with other GDM risk factors who did not undergo a screening test. GDM, gestational diabetes mellitus; GTT, glucose tolerance test; NuMoM2b, Nulliparous Pregnancy Outcomes Study: Monitoring Mothers-To-Be.

Descriptive data for the study population stratified by AHEI-2010 quartile are presented in Table 1. All maternal demographic characteristics were significantly associated with periconceptional diet quality. Specifically, women of younger age, higher early-pregnancy BMI, lower educational attainment, periconceptional smoking habits, and below the federal poverty threshold were more likely to have an AHEI-2010 score in the lower quartiles (Table 1). Women from minority groups (Black and Hispanic participants) were also more likely to have a poorer quality diet compared to women of Non-Hispanic White or Asian race/ethnicity. Although the rate of GWG per week was significantly higher in those with AHEI-2010 scores in the highest versus lowest quartiles on ANOVA testing, this difference was no longer significant after adjusting for early-pregnancy BMI.

The overall rate of GDM was 4%, diagnosed at a mean gestational age of 28 ± 3 weeks among those undergoing the 3-hour 100g GTT, and 26 ± 3 weeks among those undergoing the 2-hour 75g GTT. The incidence of GDM and having an elevated glucose concentration on the 50g screening test did not differ across AHEI-2010 quartiles in the unadjusted analysis (Table 1). However, on logistic regression analysis adjusting for covariates, each 1-point increase in the AHEI-2010 score was associated with 0.01 reduced odds of having a GDM diagnosis (B=-0.014, p=0.023, aOR=0.986, 95% CI=0.974 to 0.998). This suggests that a 10-point increase in the AHEI-2010 score periconceptionally would correspond to a 10% reduction in the odds of developing GDM. For example, a 10-point score increase could be achieved by increasing vegetable consumption from two to five portions per day plus increasing fruit consumption from two to four portions. Consuming zero sugar sweetened beverages per day

TABLE 1 Descriptive statistics of study population stratified by periconceptional diet quality.

Maternal characteristic	N with available data	Study population	AHEI Q1	AHEI Q2	AHEI Q3	AHEI Q4	P-value
AHEI-2010 score (range given in parentheses)	7997	55.1 ± 12.5 (22.4 - 96.9)	39.7 ± 4.5[a] (22.4 - 45.7)	50.2 ± 2.5[b] (45.8 - 54.5)	58.9 ± 2.6[c] (54.6 - 63.6)	71.7 ± 6.2[d] (63.7 - 96.9)	<0.001
Energy intake (Kcal)	7997	1715.2 ± 954.2	1947.8 ± 939.8	1692.6 ± 1010.5	1660.0 ± 1139.7	1561.5 ± 600.0	<0.001
Gestational age at enrolment (weeks)	7944	12.5 ± 2.7	12.5 ± 2.8	12.5 ± 1.9	12.6 ± 2.7	12.6 ± 3.2	0.615
Maternal age (years)	7995	27.3 ± 5.5	23.7 ± 5.1[a]	26.2 ± 5.3[b]	28.6 ± 5.0[c]	30.6 ± 4.3[d]	<0.001
Early pregnancy BMI (kg/m^2)	7890	26.2 ± 6.2	27.2 ± 7.2[a]	26.8 ± 6.4[a]	26.0 ± 5.8[b]	24.8 ± 4.9[c]	<0.001
BMI category	7890						
Underweight		179 (2.2)	70 (3.6)	43 (2.2)	30 (1.5)	36 (1.8)	<0.001
Normal weight		4066 (50.5)	880 (44.6)	903 (46.1)	1058 (53.5)	1225 (61.8)	
Overweight		1957 (24.5)	460 (23.3)	533 (27.2)	495 (25.0)	469 (23.7)	
Obese class I		922 (11.5)	268 (13.6)	267 (13.6)	231 (11.7)	156 (7.9)	
Obese class II or higher		766 (9.6)	293 (14.9)	214 (10.9)	164 (8.3)	95 (4.8)	
Race/ethnicity	7995						
Non-Hispanic White		5037 (63.2)	965 (48.4)	1168 (58.5)	1384 (69.1)	1540 (76.8)	<0.001
Non-Hispanic Black		906 (11.3)	479 (24.0)	249 (12.5)	122 (6.1)	56 (2.8)	
Hispanic		1317 (16.5)	398 (20.0)	440 (22.0)	289 (14.4)	190 (9.5)	
Asian		336 (4.2)	20 (1.0)	49 (2.5)	131 (6.5)	136 (6.8)	
Other		379 (4.7)	131 (6.6)	90 (4.5)	76 (3.8)	82 (4.1)	
Highest education received	7994						
Some or completed high school		1412 (17.7)	782 (39.2)	407 (20.4)	182 (9.1)	41 (2.0)	<0.001
Some or completed college		4626 (57.8)	1115 (54.9)	1327 (64.5)	1287 (60.6)	1030 (65.0)	
Postgraduate education		1956 (24.5)	116 (5.8)	302 (15.1)	606 (30.3)	932 (46.5)	
Below federal poverty threshold	6647	955 (11.9)	471 (34.2)	267 (16.9)	154 (8.6)	63 (3.3)	<0.001
Smoked tobacco prior to pregnancy	7991	1336 (16.7)	601 (30.2)	366 (18.4)	236 (11.8)	133 (6.6)	<0.001
Rate of GWG (kg/week)	6292	1.1 ± 0.5	1.0 ± 0.6[a]	1.1 ± 0.5	1.1 ± 0.6	1.1 ± 0.4[b]	0.024
Incidence of GDM	7997	326 (4.1)	89 (4.5)	82 (4.1)	85 (4.2)	70 (3.5)	0.447
Elevated 1-hr glucose concentration on 50g screening test	6845	992 (12.7)	261 (15.3)	222 (13.0)	258 (15.1)	251 (14.7)	0.214

Data are presented as mean ± SD for continuous variables or N (%) for categorical variables, where percentage values represent the incidence of a given characteristic within each AHEI quartile. P < 0.05 indicates significant differences across AHEI quartiles, computed by one-way ANOVA for continuous variables or chi-squared test for categorical variables. Different superscript letters indicate significant difference between specific AHEI quartiles for continuous variables, assessed by post-hoc Tukey's test. AHEI, Alternative Healthy Eating Index; GDM, gestational diabetes mellitus; GWG, gestational weight gain.

(versus any) or consuming at least one portion of nuts or legumes would also confer a 10-point increase in AHEI-2010 score. In the sensitivity analysis, the significant association between diet quality and odds of GDM among those tested by the 3-hour GTT remained (B=-0.018, p=0.029, aOR=0.983, 95% CI=0.967 to 0.998), while the association among those tested by the 2-hour GTT was not significant (B=-0.015, p=0.473, aOR=0.985, CI=0.946 to 1.026). The association between AHEI-2010 and having a blood glucose concentration ≥140 mg/dl on the screening test did not reach significance (B=-0.006, p=0.092, aOR=0.996, 95% CI=0.986 to 1.001).

Of those considered at risk for GDM who underwent the 3-hour 100g GTT, fasting glucose concentrations were significantly different across AHEI-2010 quartiles (Table 2), such that those in Q3 and Q4 (highest diet quality scores) had slightly lower fasting glucose concentrations than those in Q1 (Figure 2). There was no significant difference in mean post glucose load blood glucose concentrations according to AHEI-2010 quartiles on the 3-hour 100g GTT. Among those of all GDM risk levels undergoing the 2-hour 75g GTT, fasting glucose was not significantly different across AHEI-2010 quartiles, although small differences were observed in the 2-hour post glucose load concentrations (Table 2).

In the unadjusted linear regression analysis, the total AHEI-2010 score was significantly inversely associated with fasting glucose among those at risk for GDM (B=-0.094, p=0.002), as well as among those of all GDM risk levels undergoing the 2-hour GTT (B=-0.063, p=0.022). Diet quality was not associated with glucose concentrations following the 50g glucose screening test (B=-0.068, p=0.634), but was inversely associated with the 3-hour glucose concentration on the 3-hour GTT (B=-0.156, p=0.035), and with the 1-hour and 2-hour glucose concentrations from the 2-hour GTT (B=-0.224, p=0.021 and B=-0.181, p=0.034, respectively). After adjusting for covariates, all post-glucose load glucose concentrations were significantly inversely associated with the total AHEI-2010 score among those at GDM risk, and the association with fasting glucose trended towards significance (Table 3). Among those at all GDM risk levels, diet quality was significantly inversely associated with 1-hour glucose on the 50g screening test, and with 1-hour and 2-hour glucose on the 2-hour GTT, but not with fasting glucose (Table 3).

We explored the associations between AHEI-2010 component scores and glucose results on GDM screening and diagnostic tests, adjusting for confounding factors (Supplemental Table 2). Higher intake of long-chain omega-3 fats, primarily found in oily fish, was significantly inversely associated with 1-hour post-glucose load glucose concentration on the non-fasting screening test (B=-0.274, p=0.028), and with fasting (B=-0.265, p=0.036) and 3-hour post glucose load concentration (B=-0.788, p=0.014) on the 3-hour 100g GTT. A 1-point increase in the AHEI-2010 component score for long-chain omega-3 fats can be achieved by a 25 mg increase of fish oil intake per day, which equates to approximately 0.05 oz of wild salmon. Intake of sugary beverages was also independently associated with fasting glucose concentrations on the 3-hour 100g GTT (B=-0.239, p=0.028), such that greater adherence to guidelines to consume zero sugary beverages per day was associated with lower fasting glucose. In general, greater compliance to dietary recommendations for intakes of whole fruit and nuts and legumes was associated with lower post-glucose load glucose concentrations on the 3-hour GTT, while greater compliance to recommended intakes of whole fruit and vegetables was associated with lower post-glucose load concentrations on the 2-hour GTT.

Discussion

This study presents a detailed analysis of the association between periconceptional diet quality and maternal glycemia in the late second or early third trimester of pregnancy among a large, nationally representative cohort of nulliparous individuals in the

TABLE 2 Blood glucose concentrations from GDM screening and diagnostic tests stratified by AHEI-2010 quartile*.

Test type	Glucose (mg/dl)	AHEI Q1	AHEI Q2	AHEI Q3	AHEI Q4	P-value
50g glucose challenge test (N = 6845)						
1-hr blood glucose	110.8 ± 29.1	110.9 ± 27.5	109.3 ± 27.5	111.9 ± 28.1	111.0 ± 30.6	0.075
3-hr 100g GTT (N = 1116)						
Fasting blood glucose	81.0 ± 12.4	82.9 ± 13.7[a]	81.3 ± 11.2	79.8 ± 11.9[b]	79.8 ± 12.5[b]	0.009
1-hr blood glucose	155.5 ± 31.8	158.1 ± 13.7	153.8 ± 31.0	157.6 ± 31.3	152.6 ± 30.6	0.105
2-hr blood glucose	136.5 ± 31.8	137.5 ± 33.3	136.9 ± 31.2	139.1 ± 31.7	132.6 ± 30.8	0.100
3-hr blood glucose	106.3 ± 30.8	107.8 ± 31.3	107.4 ± 30.1	106.8 ± 29.4	103.3 ± 32.1	0.287
2-hr 75g GTT (N = 569)						
Fasting blood glucose	75.4 ± 7.7	76.4 ± 7.5	75.4 ± 7.4	75.5 ± 8.5	74.4 ± 7.1	0.178
1-hr blood glucose	118.8 ± 29.6	121.5 ± 28.9	121.6 ± 30.0	118.9 ± 28.8	113.4 ± 30.0	0.065
2-hr blood glucose	102.3 ± 23.9	104.1 ± 24.0	104.5 ± 24.6[a]	97.8 ± 22.0[b]	98.7 ± 24.2	0.027

*AHEI-2010 quartiles computed separately for each subset of participants according to GDM screening or diagnostic testing method. Different superscript letters indicate significant difference between specific AHEI quartiles, assessed by post-hoc Tukey's test. AHEI, Alternative Healthy Eating Index; GTT, glucose tolerance test.

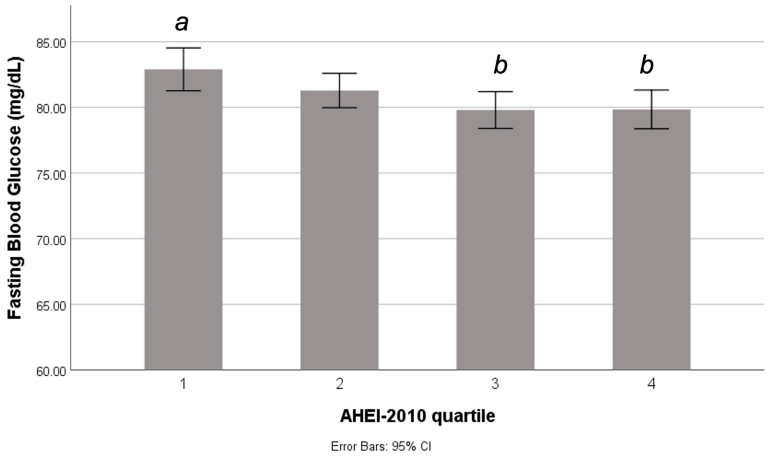

FIGURE 2
Fasting blood glucose according to periconceptional diet quality among pregnant women at risk for GDM. Difference in fasting blood glucose concentrations across quartiles of the AHEI-2010 score among individuals who underwent a 3-hour glucose tolerance test. Different letters indicate significant difference between quartiles at p<0.05. AHEI, Alternative Healthy Eating Index.

U.S. The results indicate that poorer diet quality is associated with increased odds of receiving a GDM diagnosis among those deemed to be at elevated risk of GDM (following the 2-step testing method), as well as having slightly higher glucose concentrations on GDM screening and diagnostic tests among those at all levels of GDM risk. Specifically, each 10-point increase in the AHEI-score, which can be practically achieved through simple dietary modifications such as cutting out sugar sweetened beverages or adding a daily portion of nuts or legumes, could confer a 10% reduced odds of developing GDM. Importantly, these results are independent of established GDM risk factors such as maternal BMI and rate of GWG. Higher intake of sugary beverages and lower intakes of oily fish were most prominently associated with higher glycemic results on the GTT among those at risk for GDM. Women of lower socioeconomic status and with higher BMI were identified as having the lowest periconceptional diet quality, which may contribute to increased risk for impaired gestational glycemia in these maternal populations. However, the NuMoM2b cohort was missing dietary data from some of the most demographically vulnerable women enrolled in the study, which limits the generalizability of these findings.

Few published observational studies exist that report prospective associations of periconceptional diet quality with GDM risk. Consistent with our findings, Tobias et al. reported a significantly reduced risk of GDM with higher AHEI-2010 scores among 15,254 participants of the Nurse's Health Study II in the U.S (25).. Conversely, in a smaller U.S cohort (N=1733) from the Project Viva study, dietary patterns, glycemic load, and intakes of

TABLE 3 Associations of periconceptional diet quality (total AHEI-2010 scores) with gestational glucose concentrations on GDM screening and diagnostic testing.

Test type	Beta	Std. Error	P-value	95% CI		Adj R^2
50g glucose challenge test						
1-hr blood glucose	-0.108	0.034	0.001	-0.174	-0.041	0.054
3-hr 100g GTT						
Fasting blood glucose	-0.062	0.034	0.069	-0.130	0.005	0.112
1-hr blood glucose	-0.247	0.090	0.006	-0.424	-0.071	0.027
2-hr blood glucose	-0.243	0.090	0.007	-0.420	-0.065	0.025
3-hr blood glucose	-0.228	0.087	0.009	-0.400	-0.056	0.015
2-hr 75g GTT						
Fasting blood glucose	-0.072	0.050	0.147	-0.170	0.026	0.092
1-hr blood glucose	-0.374	0.153	0.015	-0.675	-0.073	0.082
2-hr blood glucose	-0.354	0.137	0.010	-0.625	-0.084	0.097

Adjusted for covariates: maternal age, race/ethnicity, smoking status, early pregnancy body mass index, rate of gestational weight gain, energy intake, nausea and vomiting of pregnancy (PUQE score), study site. AHEI, Alternative Healthy Eating Index; GTT, glucose tolerance test.

specific nutrients or food groups in early pregnancy were not associated with GDM or impaired glucose tolerance, with the exception of an unexpected increased risk of GDM with higher omega-3 fatty acid intakes (26). The authors of the Project Viva study concluded that maternal pre-pregnancy BMI was a more significant driver of glycemia than diet. However, in that study a comprehensive diet quality score such as the AHEI-2010 was not utilized and therefore, the smaller sample size may have been insufficient to detect effects of specific dietary components on GDM incidence. Although Yee et al. previously reported no association of periconceptional diet quality measured by the HEI-2010 on GDM risk in the nuMoM2b cohort (17), the AHEI-2010 is a stronger predictor of diabetes risk in non-pregnant adults which may explain this discrepancy (18, 27). The previous study also did not investigate the association of diet quality with glucose concentrations on GDM screening and diagnostic tests, yet elevated glycemic concentrations that do not meet diagnostic criteria for GDM have been associated with an increased risk of adverse pregnancy outcomes. Although we identified some statistically significant differences in glucose concentrations across AHEI-2010 quartiles, these differences were small and may not translate to clinically meaningful perinatal outcomes beyond that of GDM incidence. Regardless, the current study contributes to the existing literature by considering the association of periconceptional diet quality with glycemic concentrations on a continuous spectrum irrespective of GDM diagnosis, and exclusively among a nulliparous cohort who had no prior GDM exposure.

There is increasing recognition that prenatal dietary and lifestyle interventions that are typically initiated around 12-16 weeks' gestation, are likely too late to exert significant metabolic change or reduced risk of GDM (15, 16). This is particularly relevant among those with pre-pregnancy overweight and obesity who may experience low-grade insulin resistance prior to conception (28). Yet, there is a paucity of published research reporting preconception dietary interventions with follow up across pregnancy, although these are plausible pathways by which a dietary intervention may help to improve gestational glycemia. For example, a few studies of preconception lifestyle interventions involving caloric restriction, physical activity, and behavior modification to achieve weight loss goals among women with obesity and fertility issues have reported beneficial effects on cardiometabolic health, including reductions in BMI, insulin resistance, and the metabolic syndrome (29–31).

Nearly fifty percent of pregnancies in the U.S. are unplanned, leaving many women without time to consider the importance of preconception diet quality (32). Thus, targeted public health initiatives and distribution of resources to support improved diet among non-pregnant women of reproductive age are warranted to help optimize maternal glycemia in future potential pregnancies and reduce the burden of GDM (15, 16, 33). Although women may be more receptive to health behavior change during pregnancy (34), it takes time to establish and maintain the optimal dietary changes that are required to beneficially impact glucose-insulin metabolism. Thus, starting this process around the second trimester is likely too late to substantially benefit gestational metabolic health. In contrast, implementing healthy lifestyle behaviors that help to optimize diet quality and weight status prior to conception may set the stage for easier maintenance of higher quality diet throughout pregnancy.

Results of our study among nulliparous individuals also highlight the population demographics that are most at risk for poor dietary quality. Allocating resources that support healthy dietary behaviors in younger women of reproductive age, who may not even be considering pregnancy in the immediate term, could benefit the health of future maternal populations (15, 35). Clinical interactions with younger, nulliparous women for contraceptive counselling or at well-women visits represents a window of opportunity to initiate conversations around healthy lifestyles for the long-term health benefits for them and for potential future children. Such interactions may require a multidisciplinary approach that includes primary care providers, pediatricians, gynecologists, and registered dietitians. Targeted nutrition education and messaging in schools and colleges could also reach a wide audience for promotion of preventative women's health. Simple, consistent nutrition messaging is required, such as encouraging avoidance of sugar sweetened beverages, consuming 1-2 portions of oily fish per week, and consuming at least 2.5 cups of vegetables, to support improvements in diet quality for all individuals who could become or currently are pregnant. Whether widespread achievement of these healthy dietary practices could translate to reduced population incidence of GDM remains to be determined.

Nutrition services that support socioeconomically disadvantaged groups are particularly warranted. In the U.S., programs such as Women Infants and Children offer food access and nutrition education only to women who are already pregnant or have young children. Therefore, underserved, nulliparous women who would be eligible for such programs but are not yet pregnant may fall through the gaps. This is a missed opportunity to support the health and wellbeing of our future prenatal populations and their offspring.

Future research directions should include well-designed clinical trials of pre-pregnancy diet and lifestyle interventions to test the effects on gestational glycemia, GDM risk, and other pregnancy complications. The optimal content and mode of delivery for such interventions remains to be determined, but utilizing behavior change theories in the study design, addressing the social determinants of health, and use of multicomponent interventions is recommended.

While our study results may not be generalizable to multiparas, this study is strengthened by its large sample size and diversity in maternal characteristics. Data on the outcome measures, gestational glycemia and GDM diagnosis, were abstracted from medical records by certified chart abstractors at each site. However, there was no harmonization of glucose assay methods across sites for standard GTTs which may be a

limitation, although we included study site as a covariate in analyses. Diet was assessed early in gestation using a validated FFQ that captured the periconceptional period, which is frequently not assessed in prenatal studies. As with all retrospective nutrition assessments, the FFQ method is subject to recall bias and misreporting. The absence of dietary assessment later in gestation may be considered a limitation of the study, as it is possible that diet quality scores remain consistent from periconception throughout pregnancy, and diet quality measured in the second trimester may be more strongly associated with odds of GDM and glucose concentrations. However, prenatal dietary interventions initiated in the early second trimester demonstrate inconsistent and low quality evidence for a reduced risk of GDM (36). Given that we found the odds of GDM is already associated with diet quality at the time of periconception, it stands to reason that earlier intervention may help establish healthy glucose tolerance in pregnancy to potentially lessen the likelihood of later GDM development. Another strength is the use of the AHEI-2010, which is considered a more comprehensive tool than the standard HEI to characterize diet quality and its relation to chronic disease risk (18, 19). Lastly, we considered key covariates in our analyses including race/ethnicity, early-pregnancy BMI and rate of GWG, which are recognized as among the most important risk factors for GDM.

In conclusion, a poorer periconceptional diet is independently associated with increased odds of GDM and slightly higher fasting and post glucose load blood glucose concentrations at the time of GDM screening and diagnostic testing in nulliparous individuals. Periconception intervention studies targeting diet quality with prospective follow-up across pregnancy are warranted.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: NICHD Data and Specimen Hub: https://dash.nichd.nih.gov/explore/study?q=numom2b&filters=[]&page=1&sortBy=relevance&asc=true&size=50.

Ethics statement

The studies involving human participants were reviewed and approved by each study site's local governing Institutional Review Board(s) approved the nuMoM2b protocol and procedures: Case Western University; Columbia University; Indiana University; University of Pittsburgh; Northwestern University; University of California at Irvine; University of Pennsylvania; and University of Utah. The patients/participants provided their written informed consent to participate in this study.

Author contributions

WG, DH, BM, HS, GS, RS, and JC designed research; WG, DH, BM, HS, GS, RS, conducted research; KL analyzed the data; KL and GM wrote the paper; KL and JC had primary responsibility for final content. All authors read and approved the final manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.940870/full#supplementary-material

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Specific gut bacterial and fungal microbiota pattern in the first half of pregnancy is linked to the development of gestational *diabetes mellitus* in the cohort including obese women

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Aims: Gestation is linked to changes in gut microbiota composition and function. Since gestational *diabetes mellitus* (GDM) can develop at any time of the pregnancy, we stratified the women into four groups according to the time and test used for the diagnosis. We focused on the gut microbiota pattern in early pregnancy to detect changes which could be linked to later GDM development.

Methods: We collected stool samples from 104 pregnant women including obese individuals (first trimester body mass index median was 26.73). We divided the women into four groups according to routine screening of fasting plasma glucose (FPG) levels and oral glucose tolerance test (oGTT) in the first and third trimesters, respectively. We processed the stool samples for bacterial 16S rRNA and fungal ITS1 genes sequencing by Illumina MiSeq approach and correlated the gut microbiota composition with plasma short-chain fatty acid levels (SCFA).

Results: We found that gut bacterial microbiota in the first trimester significantly differs among groups with different GDM onset based on unweighted UniFrac distances (p=0.003). Normoglycemic women had gut microbiota associated with higher abundance of family Prevotellaceae, and order Fusobacteriales, and genus *Sutterella*. Women diagnosed later during

pregnancy either by FGP levels or by oGTT had higher abundances of genera *Enterococcus*, or *Erysipelotrichaceae UCG-003*, respectively. We observed significant enrichment of fungal genus *Mucor* in healthy pregnant women whereas *Candida* was more abundant in the group of pregnant women with impaired oGTT. Using correlation analysis, we found that *Holdemanella* negatively correlated with *Blautia* and *Candida* abundances and that *Escherichia/Shigella* abundance positively correlated and *Subdoligranulum* negatively correlated with plasma lipid levels. *Coprococcus*, *Akkermansia*, *Methanobrevibacter*, *Phascolarctobacterium* and *Alistipes* positively correlated with acetate, valerate, 2-hydroxybutyrate and 2-methylbutyrate levels, respectively, in women with GDM.

Conclusions: We conclude that there are significant differences in the gut microbiota composition between pregnant women with and without GDM already at the early stage of pregnancy in our cohort that included also overweight and obese individuals. Specific microbial pattern associated with GDM development during early pregnancy and its correlation to plasma lipid or SCFA levels could help to identify women in higher risk of GDM development.

KEYWORDS

microbiome, mycobiome, early diagnosis, plasma metabolites, short-chain fatty acids, correlation

Introduction

Gestational *diabetes mellitus* (GDM) is the most common medical complication of pregnancy that affects more than 14% of women worldwide (1). It is described as any degree of glucose intolerance that appears during pregnancy (2). GDM is associated with many health complications affecting woman and the offspring, including gestational hypertension, preeclampsia, or preterm birth and fetal macrosomia, hypoglycemia, respiratory distress syndrome or cardiomyopathy (3, 4). In addition, women with GDM have about 40% higher risk of developing type 2 *diabetes mellitus* (T2DM) in the next 10 – 15 years (5, 6). The offspring of women with GDM are in increased risk for developing diabetes and obesity as well (5, 7). Nevertheless, several studies have described that infants breastfed by women diagnosed with GDM may have reduced risk of obesity or T2DM development later in their life (8, 9). Factors transferred by milk from mother to offspring modulate its microbiome, immune system tuning or metabolic activity which are tightly associated with obesity or T2DM (10–12). Intrinsic and extrinsic factors accompanying the metabolic and immunological changes during pregnancy, especially increased insulin resistance, gestational weight gain, family history of diabetes, obesity and immune tolerance against the fetus and placenta, are the prerequisite for the development of GDM (13, 14). These changes are also associated with alterations in the energy metabolism of pregnant women. The beginning of pregnancy is strongly related with the storage of energy. However, in the third trimester, the energy metabolism pathways are activated, which results in the release of glucose and fatty acids into the bloodstream (15). As a consequence of these significant changes in the metabolism, predisposed pregnant women are prone to develop GDM. Based on the diagnostic criteria, two main subtypes of GDM may be distinguished. The first one is characterized by women with repeatedly increased fasting plasma glucose (FPG; FPG ≥ 5.1 mmol/l) while the second one is detected postprandially after oral glucose tolerance test (oGTT; plasma glucose ≥ 10.0 mmol/l at 1h and/or ≥ 8.5 mmol/l at 2h during oGTT). These two subtypes differ in their pathophysiological mechanisms and also in the severity of health complications associated with GDM (16). This means the earlier GDM develops the more severe complications it brings. Therefore, prompt diagnosis is crucial for early dietary intervention and mitigation of the consequences.

The composition and metabolic activity of the gut microbiota have been described as factors that can influence glucose metabolism. For instance, a specific gut microbiota pattern has been observed in subjects with obesity, prediabetes or T2DM (17–19). Moreover, microbial diversity and its function in the gut are altered during pregnancy. In the first trimester, the gut microbiome of a pregnant woman is mostly similar to a healthy non-pregnant woman, while in the third trimester, a high degree of dysbiosis is observed, especially in the

decrease of short-chain fatty acids (SCFA)-producing bacteria and in the increase in Actinobacteria and Proteobacteria (20, 21). Although the relative abundance of the four dominant phyla (Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria) differs among mildly underweight pregnant women, pregnant women with normal body mass index (BMI), overweight, and obese pregnant women (22), any of these women can develop GDM. To date, few studies have focused on microbial or microbiota-associated metabolic changes in GDM development, and none of them have aimed at the differences in the gut microbiota composition among subtypes of GDM determined by FPG levels or oGTT.

The microbiota can also modify metabolic processes in the body through their metabolites, such as SCFA, branched-chain fatty acids or bile acids. SCFA are produced by gut microbiota through anaerobic fermentation of non-digestible carbohydrates. Major types of SCFA are acetate, propionate and butyrate that modulate energy metabolism and that are involved in the maintaining of glucose homeostasis (23). However, type and amount of SCFA depends on diet that affects the gut microbiota composition and function (24). Recently, the impaired insulin sensitivity in pregnancy and the development of GDM have been linked to diet as a source of substrates that are further processed by the gut microbiota, resulting in the formation of metabolites, such as SCFA (25, 26).

In our study, we focused on the early gut microbiota pattern in pregnant women in order to identify changes which could predict later GDM development. For this purpose, we sequenced gut bacterial and fungal microbiota in 104 pregnant women, representing a common Czech population of women with low, normal and high BMI. The women were divided into four subgroups according to their FPG levels and oGTT as follows: healthy pregnant women, pregnant women with impaired FPG in the first trimester, pregnant women with impaired FPG in the third trimester and pregnant women with impaired oGTT in the third trimester. Moreover, we correlated the microbiota changes with basic biochemical parameters and SCFA levels in plasma. Our data could help to determine early pregnancy microbial patterns that are associated with GDM development later during pregnancy and thus could help with its early detection.

Subjects, materials and methods

Study subjects and sampling

For this study, 104 pregnant women were enrolled during regular appointments at the Third Department of Internal Medicine – Nephrology, Rheumatology and Endocrinology, Olomouc University Hospital. The exclusion criteria for enrolment comprised of recent antibiotic treatment (at least three months before sampling) and a history of intestinal disease or major intestinal resection. Enrolled women were tested for GDM according to the recommendation of International Association of Diabetes and Pregnancy Study Groups (27). The detection and diagnosis of hyperglycemic disorders in pregnancy involves two phases. The first test is performed during an initial prenatal visit (usually in the first trimester) to reveal women with overt diabetes who have not been diagnosed before pregnancy. If the results are not sufficient for the diagnosis of overt diabetes but are abnormal (FPG ≥ 5.1 mmol/L but < 7.0 mmol/L), early GDM is suspected. Therefore, if overt diabetes is excluded, it is recommended to classify as GDM also the FPG values ≥ 5.1 mmol/L in early pregnancy. The second phase includes the 75g oGTT in 24th – 28th week of gestation in all women who had not previously been diagnosed with overt diabetes or GDM to detect GDM in this period. Clinical and biochemical parameters were collected in the first trimester of pregnancy from the patients' registry and the SCFA levels were extracted from a publication by Ivanovova et al. (2021) which describes the same cohort of women with GDM (28). Samples of feces were collected at two time points during the first and the third trimesters of pregnancy. Samples were frozen within 5h after collection and stored at -20°C until the DNA extraction.

This study was approved by the Ethics Committee at Olomouc University Hospital (approval no. 120/17). An informed consent was obtained from all subjects before enrolment.

DNA extraction from stool samples and sequencing

Total DNA was extracted using ZymoBIOMICS DNA Miniprep Kit (ZYMO Research, Irvine, CA, USA)according to the manufacture´s protocol with repeated bead-beating using FastPrep homogenizer (MP Biomedicals, Santa Ana, CA, USA). PCR targeting V3 and V4 regions of bacterial 16S was conducted using Kapa HiFi HotStart Ready mix (Roche, Penzberg, Germany) using 341F (5′-CCTACGGGNGGCWGCAG- 3′) and 806R (5′-GGACTACHVGGGTWTCTAAT- 3′) primers (Generi Biotech, Hradec Kralove, CZ). Cycling conditions consisted of initial denaturation (95°C, 4 min) followed by 30 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), extension (72°C, 30 s) and final extension (72°C, 5 min). PCR targeting of fungal ITS1 region was performed also with Kapa Hifi HotStart Ready mix (Roche) using primers with barcodes ITS1-5.8Sfw (5′-AAGTTCAAAGAYTCGATGATTCAC-3′) and ITS1-5.8Srv (5′-AAGTTCAAAGAYTCGATGATTCAC-3′). Cycling conditions consisted of initial denaturation (95°C, 4 min) and 35 cycles of denaturation (95°C, 30 s), annealing (60°C, 30 s), extension (72°C, 30 s) and final extension (72°C, 5 min). PCR triplicates were pooled and purified by SequalPrep

Normalization Plate Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples within library were pooled, concentrated (Eppendorf centrifugal vacuum concentrator), purified with DNA Clean&Concentrator kit (ZYMO Research)and sequencing adaptors were ligated using Kapa HyperPrep kit (Roche). Ligated libraries were quantified with KAPA Library Quantification Kit (Kapa Biosystems) and sequenced on MiSeq Illumina Platform using Miseq Reagent Kit v3 (Illumina) at The Genomics Core Facility, CEITEC (Brno, Czech Republic). Sequencing data were processed using QIIME version 1.9.1 (29). Raw reads were demultiplexed and quality filtered, allowing no N characters, a maximum of three consecutive low-quality base calls, a maximum unacceptable Phred quality of Q20, and a maximum of 1.5 barcode errors. Chimeric reads were detected and discarded using USEARCH algorithms (30). Fungal reads were in addition extracted for ITS1 region using ITSx package (31). Identification of representative sequences was done using RPD classifier (32) against bacterial GREENGENES database 13.8 (33) and fungal UNITE database 7.2 (UNITE Community (2017): UNITE QIIME release. Version 01.12.2017. UNITE Community. https://doi.org/10.15156/BIO/587481). Finally, OTU table was produced. The data are available in the Sequence Read Archive (SRA) https://www.ncbi.nlm.nih.gov/bioproject/PRJNA833950.

Briefly, for microbiota analysis, the number of observed OTUs (operational taxonomic unit) and Chao1, Shannon, Simpson and Faith Phylogenetic Diversity indexes were used to describe alpha diversity and Principle Coordinate Analysis (PCoA) based on weighted and unweighted UniFrac distance for bacteria and Bray-Curtis and Jaccard distance for fungi were used to characterize beta diversity. The permutational multivariate analysis of variance (PERMANOVA) was used for the determination of statistical differences among groups. Furthermore, Linear discriminant analysis effect size (LEfSe; RRID: SCR_014609) was used to determine the features discriminating communities in each group (29, 34). Functional potential of a bacterial metagenome was predicted by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) tool, using the 16S rRNA amplicon data (35).

Statistics

Data were analyzed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Statistical differences between two groups were calculated by nonparametric Mann-Whitney U test. In the case of more groups, nonparametric Kruskal-Wallis test with Dunn´s *post-hoc* testing were used. Data were expressed as medians with first and third quartiles. Values of $p < 0.05$ were considered significantly different. Covariations of gut microbiota with other factors were calculated by Spearman´s correlation analysis with Bonferroni ´s adjustment for multiple comparisons.

Results

Clinical data and blood samples analyses

For basic group differentiation, we compared clinical and biochemical parameters of healthy pregnant women (HC) and pregnant women with diabetes who had impaired FPG in the first (GDM1) or in the third trimester (GDM2), and with impaired oGTT in the third trimester (GMD3; Table 1). We found a significant increase in body weight (GDM2 $p<0.01$; GDM3 $p<0.05$) and BMI (GDM1 $p<0.01$; GDM2 $p<0.001$; GDM3 $p<0.01$) in women with GDM compared to healthy pregnant women mainly due to obese women inclusion. The GDM2 and GDM3 groups showed significant increase in cholesterol (GDM2 $p<0.001$; GDM3 $p<0.01$), triglycerides (GDM2 $p<0.001$; GDM3 $p<0.001$), low-density lipoprotein (LDL; GMD2 $p<0.01$; GDM3 $p<0.05$), non-high-density lipoprotein (nonHDL; GDM2 $p<0.001$; GDM3 $p<0.01$) and 3-hydroxybutyrate (GDM $p<0.05$; GDM $p<0.001$) compared to healthy pregnant women. The highest FPG levels were determined in the GDM1 ($p<0.001$) and GDM2 women ($p<0.001$).

Gut bacterial microbiota composition differs between pregnant women with and without diabetes in the first trimester

To characterize differences in gut microbiota between pregnant women with and without diabetes, we collected fecal samples in the first trimester and processed them for sequencing analysis. We found mostly non-significant reduction in all alpha diversity indexes in the samples from women with GDM, except for the Simpson and Faith phylogenetic diversity indexes which describe the richness of the samples. Women diagnosed later during pregnancy either by FPG levels or by oGTT had significantly lower diversity compared to healthy controls (Figure 1A). We observed significantly different composition of gut microbiota in normoglycemic pregnant women and pregnant women with GDM measured by PERMANOVA based on unweighted and weighted UniFrac with values $p=3x10^{-3}$ and $p=6x10^{-3}$, respectively (Figures 1B–E). Subsequent LEfSe analysis identified bacteria significantly different among groups based on their relative abundances (Figure 1F). Gut microbiota of normoglycemic pregnancies was associated with increased abundance of family Prevotellaceae, order Fusobacteriales and genus *Sutterella*. The women who developed impaired insulin resistance later in

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TABLE 1 First trimester clinical and biochemical data of healthy pregnant women and pregnant women with impaired FPG or oGTT.

Group [n]	HC (22)	GDM 1 (29)	GDM 2 (31)	GDM 3 (22)
Definition	Healthy pregnant women with normal FPG (FPG < 5.0 mmol/L)	Pregnant women with impaired FPG (FPG ≥ 5.1 mmol/L) in the first trimester	Pregnant women with impaired FPG (FPG ≥ 5.1 mmol/L) in the third trimester	Pregnant women with impaired oGTT in the third trimester
Age [y]	23 – 36 30 (28; 32)	21 – 46 31 (28;34)	24 – 44 32 (27;36)	25 – 40 32 (28;35)
Body height [cm]	158 – 179.5 172 (165.5; 176.5)	155 – 183 168 (162; 172)	151 – 177 166 (162; 172)	155 – 176 168 (163; 172)
Body weight [kg]	55 – 111 67.5 (62.3; 74.9)	52 – 126 78 (68; 84)	59 – 118 81.5 (70; 90)**	51 – 110 80 (68; 97)*
BMI [kg/m^2]	19.55 – 38.41 23.5 (20.6; 25.7)	17.58 – 37.91 27.4 (24.4; 32.1)**	22.76 – 44.53 28.7 (24.8; 31.4)***	18.29 – 44.12 28.9 (24; 33.8)**
Obese [%]	4.5	41	32	45
Waist [cm]	68 – 102 83 (77; 95.8)	73 – 121 93 (89.8; 98.8)*	82 – 116 101 (95; 109.3)***	60 – 127 96.5 (89.5; 113.3)**
Systolic BP [mmHg]	107 – 140 121 (114.5; 127)	97 – 148 122 (110; 132)	102 – 158 120 (113; 126)	107 – 150 125 (112; 139)
Diastolic BP [mmHg]	66 – 90 78.5 (70.3; 81)	64 – 94 75 (69; 82)	60 – 91 73 (66; 81)	62 – 100 76 (67; 86)
Pulse [BPM]	64 – 96 83 (76.5; 88)	67 – 114 85 (79; 93)	61 – 112 89 (80; 94)	63 – 115 86 (79; 97)
Cholesterol [mmol/L]	4.16 – 6.03 4.96 (4.6; 5.54)	4.27 – 7.97 5.42 (4.6; 6.5)	4.6 – 9.1 6.24 (5.5; 7.1)***	4.47 – 8.78 6.01 (5.5; 6.4)**
Triglycerides [mmol/L]	0.88 – 2.09 1.2 (1.07; 1.32)	0.69 – 3.85 1.58 (1.21; 1.9)	0.84 – 3.68 2.3 (1.8; 3.1)***	0.58 – 3.81 2.3 (1.7; 3)***
HDL [mmol/L]	1.36 – 2.97 1.96 (1.8; 2.3)	1.22 – 3.07 1.8 (1.6; 2.2)	1.28 – 2.96 1.87 (1.6; 2.2)	1.08 – 2.97 1.73 (1.6; 2.2)
LDL [mmol/L]	1.69 – 7.93 2.35 (2.3; 2.8)	1.63 – 4.1 2.94 (2.3; 3.8)	1.49 – 5.76 3.3 (2.7; 4)**	1.78 – 4.8 3.03 (2.7; 3.7)*
nonHDL [mmol/L]	2.3 – 8.9 2.9 (2.7; 3.5)	2.0 – 5.8 3.5 (3; 4.1)	2.48 – 7.3 4.2 (3.7; 5)***	2.3 – 6.5 4 (3.6; 4.9)**
FPG [mmol/L]	3.7 – 5.1 4.25 (4.1; 4.5)	4.2 – 6.0 5.1 (4.9; 5.3)***	4.1 – 5.5 4.8 (4.5; 5)***	4.0 – 5.3 4.4 (4.2; 4.6)
C-peptide [pmol/L]	337.0 – 1262.0 658 (557; 888)	228.0 – 1619.0 681.5 (545; 961)	304.0 – 1716.0 696.5 (593; 880)	321.0 – 1705.0 600 (478; 798)
CP-RI [ng/mg]	3.45 – 14.47 6.65 (4.9; 9)	2.13 – 17.9 5.5 (4.1; 7.7)	2.2 – 13.43 6.17 (4.8; 7.1)	2.73 – 14.49 6.94 (5.6; 9.8)
HbA1c [% (mmol/mol)]	4.4 (25) – 5.3 (34) 4.9 (30) (4.7 (28); 5.1 (32.75))	4.5 (26) – 6.2 (44) 5.1 (32) (5.0 (31); 5.4 (35))**	4.4 (25) – 5.4 (36) 5.1 (32) (5.0 (31); 5.2 (33.25))	4.3 (24) – 5.7 (39) 5.1 (32) (4.8 (29); 5.2 (33))
Acetate [μmol/L]	0.97 – 23.05 9.42 (4.9; 13.8)	2.18 – 39.98 9.92 (5.5; 13)	1.99 – 42.69 9.4 (6; 14)	2.43 – 30.38 8.99 (5.14; 15)
Propionate [μmol/L]	0.01 – 1.62 0.78 (0.24; 1.3)	0.15 – 1.82 0.76 (0.4; 1.19)	0.11 – 2.48 0.75 (0.43; 1)	0.03 – 1.46 0.74 (0.38; 1.06)
Butyrate [μmol/L]	0.12 – 1.56 0.35 (0.22; 0.54)	0.12 – 1.07 0.31 (0.22; 0.4)	0.1 – 1.11 0.29 (0.19; 0.46)	0.11 – 0.6 0.32 (0.17; 0.45)
Valerate [μmol/L]	0.01 – 0.1 0.06 (0.033; 0.07)	0.01 – 0.12 0.05 (0.04; 0.07)	0.02 – 0.18 0.05 (0.03; 0.07)	0.03 – 0.3 0.05 (0.04; 0.08)
Hexanoate [μmol/L]	0.05 – 0.43 0.18 (0.13; 0.24)	0.08 – 0.43 0.17 (0.13; 0.23)	0.06 – 0.5 0.19 (0.13; 0.24)	0.09 – 0.43 0.2 (0.15; 0.24)
3-hydroxybutyrate [μmol/L]	16.32 – 55.79 31.84 (22.2; 42.9)	9.29 – 211.7 42.46 (25.8; 76.7)	12.8 – 196.1 43.83 (29.9; 74.6)*	27.7 – 299.4 91.2 (49.4; 137.2)***
2-hydroxybutyrate [μmol/L]	6.7 – 37.61 22.58 (15.9; 27.4)	11.3 – 69.76 23.17 (18.5; 28.2)	6.72 – 44.02 24.48 (18.4; 29.9)	14.43 – 71.31 26.12 (20.4; 38.1)
isobutyrate [μmol/L]	0.15 – 2.71 0.64 (0.35; 0.97)	0.14 – 1.54 0.72 (0.42; 0.99)	0.25 – 1.92 0.69 (0.42; 0.96)	0.2 – 1.32 0.56 (0.28; 0.93)

(Continued)

TABLE 1 Continued

Group [n]	HC (22)	GDM 1 (29)	GDM 2 (31)	GDM 3 (22)
isovalerate [µmol/L]	0.23 – 0.73 0.37 (0.31; 0.41)	0.24 – 0.72 0.38 (0.32; 0.48)	0.1 – 0.69 0.33 (0.3; 0.43)	0.21 – 2.21 0.35 (0.3; 0.49)
2-methylbutyrate [µmol/L]	0.1 – 5.99 1.98 (0.64; 2.89)	0.15 – 15.31 1 (0.41; 3.48)	0.15 – 15.65 0.61 (0.36; 1.99)	0.27 – 52.05 1.23 (0.51; 2.66)
4-methylvalerate [µmol/L]	0.07 – 0.39 0.21 (0.12; 0.33)	0.06 – 0.66 0.21 (0.13; 0.31)	0.1 – 0.43 0.2 (0.17; 0.25)	0.14 – 0.5 0.26 (0.22; 0.32)

*p<0.05, **p<0.01, ***p<0.001 measured by Kruskal-Wallis test with Dunn´s post-hoc testing. The data are presented as medians with first and third quartiles in parentheses. FPG, fasting plasma glucose; oGTT, oral glucose tolerance test; BMI, body mass index; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA1c, glycated hemoglobin.

pregnancy (GDM2) had higher abundance of genera *Enterococcus* and *Erysipelotrichaceae UCG-003*.

Prediction of metabolic pathways associated with the abundance of gut bacteria showed that most of them were linked to energy metabolism or active cell division, especially pathways producing components of cell membranes and cell walls (Figure S1).

Gut fungal microbiota composition shows moderate changes between pregnant women with and without diabetes at the first trimester

To characterize the gut mycobiota, we sequenced the ITS region in the samples from healthy pregnant women and

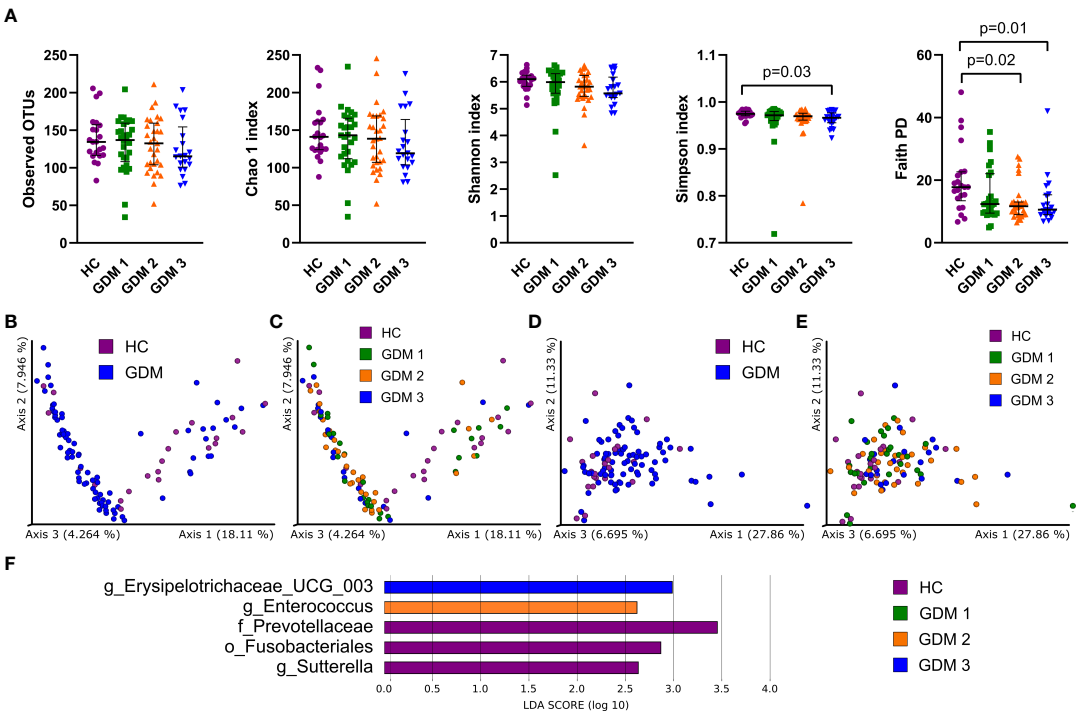

FIGURE 1
Gut bacterial community composition in the first trimester of pregnancy. **(A)** Alpha diversity indexes. Beta diversity of gut bacteria on unweighted **(B, C)** and weighted **(D, E)** UniFrac distance metric-based PCoA graphs show comparison of control healthy pregnant women (HC) with those with gestational diabetes mellitus (GDM) diagnosed at any time during the pregnancy. **(F)** LEfSe analysis of significantly different strains among groups. Statistically significant differences were measured by Kruskal-Wallis test with Dunn´s *post-hoc* testing. The data are presented as medians with interquartile range. OTUs, operational taxonomic units; PD, phylogenetic diversity.

pregnant women with GDM. Fungal alpha diversity indexes showed significant reduction in GDM pregnant women as described by species richness (observed OTUs) and by species abundance (Chao 1 index, Figure 2A). We did not observe any significant clustering of groups in neither Bray-Curtis dissimilarity plot based on fungal abundance nor in Jaccard distance plot comparing fungal composition among samples (Figures 2B–E). Using LEfSe analysis, we found that there was significant enrichment in genus *Mucor* in healthy pregnant women and that genus *Candida* was more abundant in the group of pregnant women with impaired oGTT in the third trimester (Figure 2F).

GDM leads to different types of dysbiosis at the class level

Comparison of microbiota relative abundances in the samples collected during the first and third trimester showed significantly different patterns that distinguished healthy pregnant women and women with GDM. In the first trimester (V1), normoglycemic women were associated with higher abundance of bacterial classes Bacteroidia and γ-Proteobacteria, archeal class Methanobacteria, and fungal classes Mucuromycetes, Eurotiomycetes, Microbotryomycetes and Malasseziomycetes compared with pregnant women with GDM (Figures 3A, B). Interestingly, the differences in these classes were more or less narrowed later in the pregnancy. In the third trimester (V3), pregnant women with GDM showed significant increase in classes Negativicutes and Clostridia, especially of the family Oscillospiraceae, and lower abundance of classes Desulfovibrionea and Bacilli compared to normoglycemic women (Figure 3C). These two later classes included significantly more abundant genera *Bilophila*, *Leuconostoc*, *Streptococcus* and *Erysipelotrichaceae UCG-003* in healthy women (Figure S2). Although the analysis of fungal community showed no differences at class level during the third trimester, we found significant enrichment of family Debaryomycetaceae and genus *Rhodotorula* in women with GDM (Figure S3).

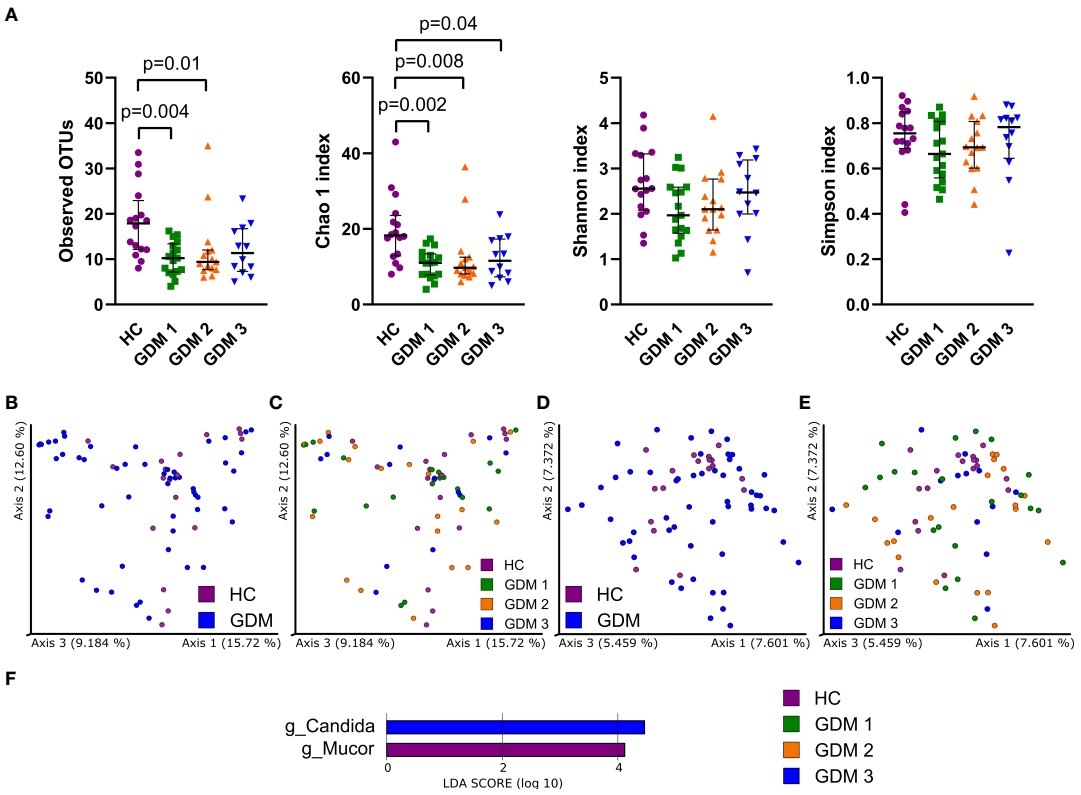

FIGURE 2
Gut fungal microbiota composition in the first trimester of pregnancy. **(A)** Alpha diversity indexes. Beta diversity of gut fungi on Bray-Curtis **(B, C)** and Jaccard **(D, E)** distance metric-based PCoA graphs show comparison of control healthy pregnancies (HC) with those with gestational diabetes mellitus (GDM) diagnosed at any time during the pregnancy. **(F)** LEfSe analysis of significantly different strains among groups. Statistically significant differences were measured by Kruskal-Wallis test with Dunn´s *post-hoc* testing. The data are presented as medians with interquartile range. OTUs,ndash; operational taxonomic units.

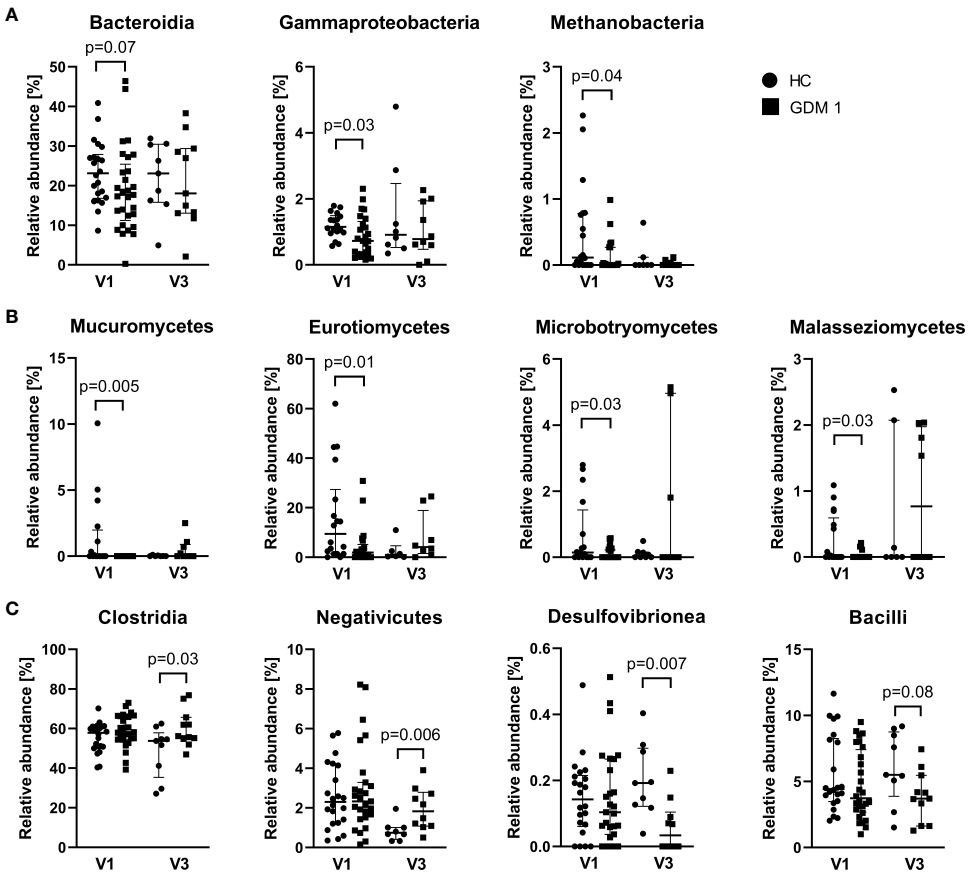

FIGURE 3
Differently abundant classes of bacteria and fungi between normoglycemic women (HC) and women with early-diagnosed GDM (GDM 1) at the first trimester – V1 **(A, B)** and the bacterial classes in the third trimester – V3 **(C)**. Only significantly different classes are shown. The data are presented as medians with interquartile range. The abundances were compared by Mann-Whitney U test and $p < 0.05$ was considered statistically significant.

Different intra- and inter-kingdom associations are linked to the GDM

Using Spearman correlation analysis, we found several significant associations among gut microbiota. Normoglycemic women (HC) showed a strong positive correlation of genera *Bacteroides* and *Roseburia* (r=0.75; $p=6x10^{-5}$) and negative associations of genera *Dialister* with *Phascolarctobacterium* (r=-0.66; $p=9x10^{-4}$) and *Parabacteriodes* with *Romboutsia* (r=-0.65; $p=9x10^{-4}$) (Figure S4). Pregnant women with impaired FPG (GDM 1) had positive correlation of bacterial genus *Prevotella* with fungal *Cladosporium* (r=0.59; $p=9x10^{-4}$) (Figure S5). Pregnant women with impaired FPG in the third trimester (GDM 2) showed strong negative correlations of genera *Dialister* with *Phascolarctobacterium* (r=-0.71; $p=9x10^{-6}$) and *Holdemanella* with *Blautia* (r=-0.67; $p=4x10^{-5}$). The GDM 2 group also showed several positive correlations, including the associations of genera *Fusicatenibacter* with *Agathobacter* (r=0.65; $p=8x10^{-5}$), *Bifidobacterium* and *Collinsella* (r=0.64; $p=10^{-4}$) and bacterial genus *Phascolarctobacterium* with archeal *Methanobrevibacter* (r=0.63; $p=10^{-4}$) (Figure S6). Women with impaired oGTT in the third trimester (GDM 3) did not show any significant associations; the strongest one was negative correlation of bacterial genus *Holdemanella* with yeast *Candida* (r=0.62; $p=4x10^{-3}$) (Figure S7).

Correlation of bacterial strains with biochemical parameters and SCFA levels

To observe early associations between bacteria and plasma parameters measured in the first trimester, Spearman correlation analysis was used (Figure 4A). In normoglycemic women, we found very strong negative correlation of genus *Subdoligranulum* with plasma levels of LDL (r=-0.75; $p=9x10^{-5}$), nonHDL (r=-0.74; $p=10^{-4}$) and cholesterol (r=-0.68; $p=7x10^{-4}$) and genus

Holdemanella which was also negatively associated with the level of CP-RI (r=-0.70; $p=10^{-3}$). Interestingly, these correlations were not detected in early diagnosed group of pregnant women with GDM (GDM 1). The pregnant women with later onset of the GDM showed different associations, including negative correlation of genus *Prevotella* with cholesterol (r=-0.57; $p=10^{-3}$) and genus *Collinsella* with CP-RI (r=-0.58; $p=10^{-3}$) and positive correlation of genus *Anaerostipes* with CP-RI (r=0.61; $p=5x10^{-4}$) and *Escherichia/Shigella* group with nonHDL (r=0.82; $p=3x10^{-6}$), LDL (r=0.70; $p=3x10^{-4}$) and triglycerides (r=0.67; $p=6x10^{-4}$) levels.

Comparison of the associations of bacterial relative abundance with the levels of SCFA showed no specific pattern in normoglycemic women whereas GDM promoted some covariations (Figure 4B). In the GDM 1 group, genus *Akkermansia* positively correlated with the levels of valerate (r=0.58; $p=10^{-3}$) and genus *Streptococcus* showed strong negative correlation with the levels of 4-methylvalerate (r=-0.67; $p=8x10^{-4}$). In the GDM 2 group, archeal genus *Methanobrevibacter* positively correlated with the levels of valerate (r=0.61; $p=4x10^{-4}$) and bacterial genus *Phascolarctobacterium* with 2-hydroxybutyrate levels (r=0.62; $p=3x10^{-4}$). In the GDM 3 group, genera *Coprococcus* and *Alistipes* positively correlated with the levels of acetate (r=0.67; $p=10^{-3}$) and 2-methylbutyrate (r=0.69; $p=5x10^{-4}$), respectively.

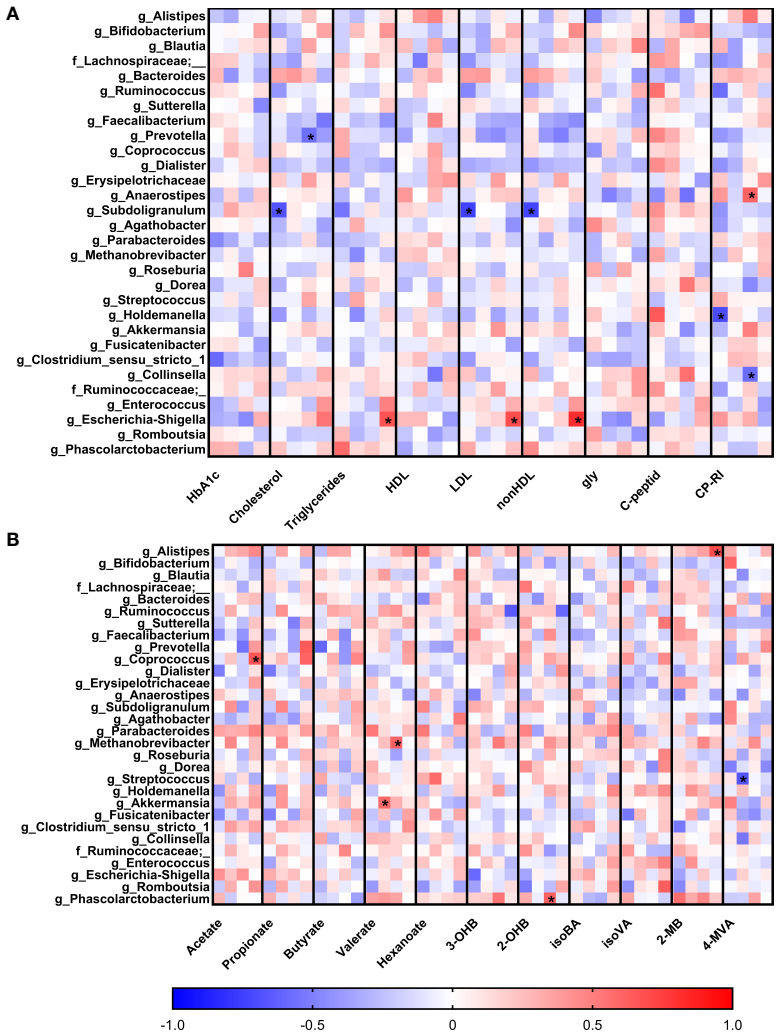

FIGURE 4
Correlations of serum biochemical parameters **(A)** and levels of short-chain fatty acids **(B)** with bacterial abundances. Within each column, the subcolumns are in order: healthy pregnant women, GDM1, GDM2 and GDM3. The strength and polarity of correlation is color-coded, e.g. negative correlation in shades of blue. All p-values were adjusted for multiple comparisons, p<0.001 was considered statistically significant and significant correlations were marked with the asterisks.

Discussion

In this study, we examined gut microbiome pattern of women in early stage of pregnancy to identify changes that are associated with GDM development. Systematic reviews have shown that although most of the studies observed an association between GDM and gut microbiota dysbiosis, no GDM-specific gut microbiota was identified (36, 37). Moreover, the contribution of gut mycobiome is often neglected. Though, there is a presumption that gut microbiota composition and function may contribute to the development of GDM (36). For this purpose, we focused on the composition of gut microbiota in early pregnancies.

In healthy population, gut microbiome contains six bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia, with the dominance of the first two (38). Previous studies showed significant microbiota changes in normoglycemic women during pregnancy (20, 39, 40). Whether these changes contribute to or are a consequence of the development of GDM is a debated question. Several studies have reported increased abundance of Firmicutes or Actinobacteria and Proteobacteria in women with GDM (20, 21, 40, 41). In addition, enrichment of genera *Parabacteroides*, *Ruminococcus*, *Eubacterium*, *Prevotella*, *Collinsella*, *Rothia*, and *Desulfovibrio* has been also observed in pregnant women with GDM compared to normoglycemic controls (21, 42–44). On the contrary, increased abundance of Bacteroidetes and Actinobacteria as well as enrichment of *Faecalibacterium*, *Methanobrevibacter*, *Alistipes*, *Bifidobacterium* or *Eubacterium* has been described in normoglycemic pregnant women (40, 42, 43, 45). Most of these studies focused on the microbiota composition at the third trimester, i.e. after the onset of GDM. Therefore, we aimed more on the microbiota pattern in early pregnancy that could predict development of GDM. In our cohort of pregnant women, gut microbiota of normoglycemic women was associated with increased abundance of family Prevotellaceae, order Fusobacteriales and genus *Sutterella*. Interestingly, Wang *et al.* (2020) identified a significant decrease of the family Alcaligenaceae (including genus *Sutterella*) in the ascending colon of patients with T2DM. Subsequent experimental study showed increased abundance of *Sutterella* in the cecum of T2DM rats that underwent Roux-en-Y gastric bypass surgery (46). Thus, suggesting that this genus may beneficially affect glucose metabolism. Furthermore, we found that women who developed impaired insulin resistance later in pregnancy had higher abundance of genera *Enterococcus* or *Erysipelotrichaceae UCG-003*. This is in agreement with Ferrocino et al. (2018) who found that insulin resistance positively correlated with class Erysipelotrichia (45). Meanwhile, Crusell et al. (2018) observed reduction of *Erysipelotrichaceae* in women with GDM (21). Though, our results are supported by another study that found higher levels of *Erysipelotrichaceae* also in obese individuals (47). Since our study included obese individuals our results may be affected by this fact as well. Individuals with obesity have different profile of the gut microbiota in comparison to non-obese individuals (48). Moreover, obesity and GDM can influence many maternal and neonatal processes, includingthe breast milk microbiota and simultaneously the offspring gut microbiota. For example, compared to control samples, colostrum of women with either obesity or GDM was enriched in genera *Staphylococcus* or *Prevotella*, respectively (44).

Our study is one of the first to investigate the association between gut fungi and the GDM. Fungal communities in the gut constitute a minor component of the entire gut microbes thus are still poorly understood. According to recent shotgun metagenomic sequencing analysis, fungi represent approximately 0.1% of the total gut microbes (49). In our study, we found a significant enrichment of genus *Mucor* in healthy pregnant women. Members of this genus have been negatively correlated with obesity suggesting their association with microbiota of healthy lean individuals (50). Indeed, our cohort of normoglycemic pregnant women included only 4.5% of obese individuals. Recently, genus *Penicillium* has been associated with the gut mycobiota of healthy pregnant women (51) but we did not observe higher levels of this genus in our groups. In the group of pregnant women with impaired oGTT, we observed increased abundance of genus *Candida* in the third trimester. This is in agreement with very recent study by Ferrocino et al. (2022) who observed an increasing abundance of *Candida* between the second and third trimesters (52). *Candida albicans* inhabits the gastrointestinal tract, mouth and vaginal mucosa in 40 – 60% of healthy adults as a commensal organism, but it may cause disease in immunocompromised individuals (53, 54). Several studies have already reported increased abundance of *Candida albicans* in obese individuals and in patients with type 1 *diabetes mellitus* (T1DM) and T2DM (50, 55–57). Moreover, it is generally assumed that pregnant woman with GDM are more prone to *Candida* vaginal infection (58–60).

Decreased abundance of *Roseburia* and *Bacteroides* was observed in the GDM women compared to healthy pregnant women (42). In accordance, we determined positive correlation of these two bacteria in healthy women but not in the GDM group. In the groups of pregnant women with impaired FPG/oGTT in the third trimester, we found negative correlation of *Holdemanella* with *Blautia* and with yeast *Candida*, respectively. Romaní-Pérez et al. (2021) showed that *Holdemanella*, an

intestinal bacterium isolated from metabolically healthy individuals, had anti-diabetic effect through glucagon-like peptide 1 signaling pathway and its supplementation improved glucose tolerance in a diet-induced obese mouse model (61). Increased abundance of *Collinsella* and reduced abundance of *Bifidobacterium* have been reported in pregnant women with GDM compared to healthy controls (21, 43). Nevertheless, we found positive correlation of *Collinsella* with *Bifidobacterium* in pregnant women with impaired FPG in the third trimester. In the same group, we also observed a positive correlation of *Methanobrevibacter smithii* and *Phascolartobacterium*. On the other hand, *Phascolartobacterium* negatively correlated with genus *Dialister* in the GDM2 and normoglycemic groups. Increased abundance of *Dialister* and reduced abundance of *Phascolartobacterium* have been related to impaired insulin sensitivity in obese individuals (62).

Healthy pregnancy is characterized by complex metabolic and hormonal changes. Plasma lipid concentrations change during pregnancy due to increasing insulin resistance. Serum levels of high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), total cholesterol, and to lesser extent triglycerides (TG) are elevated throughout the pregnancy (63–65). In our study, we observed significantly higher levels of cholesterol, LDL-C, and TG in women with GDM compared to healthy women which is consistent with other studies (66–69). Moreover, it has been shown that gut microbiota can influence the levels of blood lipids (70, 71). Here, we found that lipid levels were linked to specific gut microbiota. In normoglycemic women, we found very strong negative correlation of genus *Subdoligranulum* with serum levels of LDL, nonHDL, and cholesterol. In contrast, in women with later onset of GDM, we found positive correlation of *Escherichia/Shigella* group with LDL, nonHDL, and triglycerides and negative correlation of genus *Prevotella* with total cholesterol. While the SCFA-producing genus *Subdoligranulum* has been connected with health promoting effects, the family Enterobacteriaceae has been enriched in GDM and has already been linked to T2DM and obesity (19, 43, 72).

In contrast to a well described role of lipids, the role of SCFA in pregnancy is still poorly understood. SCFA are derived from fermentation of carbohydrates and proteins by the gut microorganisms (73). They provide energy to colonocytes and maintain intestinal homeostasis by acting as signaling molecules that transmit messages between microbiota and host organs (74). We found positive correlations of valerate with genus *Akkermansia* and archaeon *Methanobrevibacter* in pregnant women with impaired FPG in the first and third trimester, respectively. In the GDM2 group, genus *Phascolarctobacterium* positively correlated with 2-hydroxybutyrate levels. In the study of Dudzik *et al.* (2017), an increase in 2-hydroxybutyrate in patients with diagnosed GDM in the second trimester of pregnancy was detected. Moreover, 2-hydroxybutyrate levels were significantly higher in GDM women that developed T2DM after parturition. Therefore, 2-hydroxybutyrate may serve as a prognostic tool for the prediction of early onset of the complications related to diabetes in women with GDM after delivery (75).

Overall, our study revealed significant differences in gut bacterial and fungal microbiota composition between healthy pregnant women and women who develop GDM in the first half of pregnancy. Furthermore, we identified correlations between individual microorganisms and plasma biochemical parameters, including SCFA levels. We found several microbial patterns that could be used in specific diagnostic test in the first trimester to identify women in higher risk of GDM. Nevertheless, our results need to be validated by further studies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA833950.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee at University Hospital Olomouc, Olomouc, Czechia (approval no. 120/17). The patients/participants provided their written informed consent to participate in this study.

Author contributions

KK and DK designed the study. OK and DK collected clinical samples and biochemical data. NG and MV processed the samples for microbiota sequencing. MK processed sequencing data and bioinformatics. EI, DF and JF processed fatty acids analysis. MV, RR, ZZ, DF, MH and KK drafted the manuscript. All the authors read and approved the final version of the manuscript.

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Conflict of interest

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

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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.2022.970825/full#supplementary-material

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Perspectives from metabolomics in the early diagnosis and prognosis of gestational diabetes mellitus

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Gestational diabetes mellitus (GDM) is one of the most common metabolic disorders in pregnant women. The early detection of GDM provides an opportunity for the effective treatment of hyperglycemia in pregnancy, thus decreasing the risk of adverse perinatal outcomes for mothers and newborns. Metabolomics, an emerging technique, offers a novel point of view in understanding the onset and development of diseases and has been repeatedly used in various gestational periods in recent studies of GDM. Moreover, metabolomics provides varied opportunities in the different diagnoses of GDM from prediabetes or predisposition to diabetes, the diagnosis of GDM at a gestational age several weeks earlier than that used in the traditional method, and the assessment of prognosis considering the physiologic subtypes of GDM and clinical indexes. Longitudinal metabolomics truly facilitates the dynamic monitoring of metabolic alterations over the course of pregnancy. Herein, we review recent advancements in metabolomics and summarize evidence from studies on the application of metabolomics in GDM, highlighting the aspects of the diagnosis and differential diagnoses of GDM in an early stage. We also discuss future study directions concerning the physiologic subtypes, prognosis, and limitations of metabolomics.

KEYWORDS

gestational diabetes mellitus (GDM), metabolomics, newborn infant, diagnosis and prediction, type 2 diabetes

Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is a common metabolic disorder that is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (1). GDM affects approximately 14% of pregnancies worldwide, representing approximately 18 million births annually (2). Being overweight, being of advanced maternal age, having micronutrient deficiencies, and having a family history of insulin resistance and/or

diabetes are risk factors for GDM (3). Meanwhile, the risk of GDM is increased in case of disturbances in the metabolism of the three nutrients, namely, carbohydrates, fat, and protein (4).

In clinical practice, the diagnosis of GDM is accompanied by several challenges. It is challenging to differentiate GDM from prediabetes or predisposition to diabetes in some cases; moreover, there is a possibility of heterogeneity of physiologic processes underlying hyperglycemia in women with GDM. Hyperglycemia in pregnancy is associated with adverse maternal and prenatal outcomes; however, there is a lack of international consensus regarding the timing of the screening method and optimal cutoff points for the diagnosis and intervention of GDM (5). Routine screening of the general population, including pregnant women, helps in identifying patients with prediabetes or predisposition to diabetes (5). Furthermore, based on the metabolic abnormality in insulin sensitivity or deficient insulin secretion, patients with GDM can be classified as cases with predominant insulin sensitivity defects, predominant insulin secretion defects, or normal glucose tolerance (6).

The metabolism of a pregnant woman undergoes constant alterations once the pregnancy starts to support fetal development. Increased serum insulin secretion and insulin resistance are the most obvious maternal metabolic changes (Figure 1). During pregnancy, the amount of insulin secreted by pancreatic β cells steadily increases until the peak in the third trimester and returns to the normal level after delivery (7, 8). Along with increased insulin secretion, there is a decrease in maternal insulin sensitivity at the end of the first trimester, which continues until before delivery (9, 10). The insulin receptor signal is affected by increased placental lactogen, placenta-derived human growth hormone, progesterone, cortisol, prolactin, and other hormones, leading to GDM (11). Pathophysiologically, GDM occurs when there is an imbalance in insulin sensitivity and secretion during pregnancy. In detail, the level of insulin secreted by pancreatic β cells is unable to keep up with the increasing insulin resistance (12).

GDM develops among women with normal glucose before pregnancy in a more occult way throughout trimesters. Women with GDM are usually more likely to experience pregnancy-related complications, including high blood pressure and large birth weight (2), which are improved by effective glycemic control. Thus, timely detection and control of GDM are dispensable for the decrease in pregnancy-related complications (13, 14). Furthermore, children born to mothers with GDM are at high risk of suffering from type 2 diabetes mellitus and obesity at an early age (15–17). Therefore, it is necessary to put greater efforts into exploring GDM, particularly with respect to early diagnosis and prognosis.

There are alterations in metabolism during pregnancy, and hyperglycemia is a metabolic disorder. In this review, we discuss updates in metabolomics and summarize studies on the application of metabolomics in GDM, highlighting aspects of the diagnosis and differential diagnoses of GDM in an early stage. We also mention future study directions concerning the physiologic subtypes, prognosis, and limitations of metabolomics.

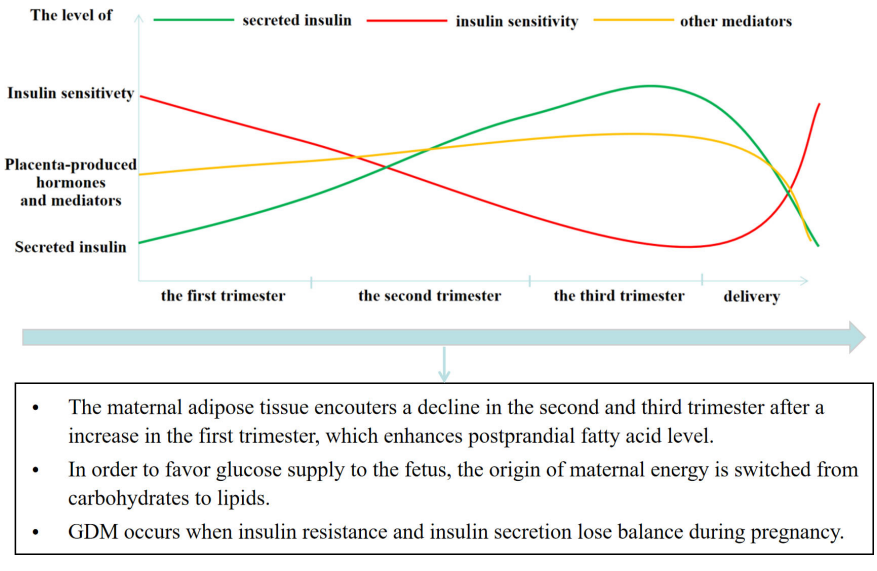

FIGURE 1
The glucose metabolism alteration and its influences in pregnancy.

Metabolomics in gestational diabetes mellitus studies

The composition of the metabolome, the complete set of metabolites and lipids in a biological system, directly reflects the physiological status, gene expression, and environmental stimuli of the biological system. Changes in the concentration or rate of transformation of metabolites under pathophysiological processes, such as aging and diseases, can be used as biomarkers for the diagnosis and prediction of clinical outcomes. Metabolomics has the advantage of recording disease-relevant metabolic changes and recognizing new biomarkers of disease processes (18). After further verification, these important metabolites can be used for disease diagnosis, therapeutic response assessment, or even predicting susceptibility to diseases (19).

Metabolomics has been successfully used to distinguish many disease-associated metabolite types in cancer, inflammatory bowel disease, asthma, diabetes, traumatic brain injury, metabolic syndrome, Parkinson's disease, and so on (20–20). In a series of studies, the changes in metabolites were analyzed in the specific stages of pregnancy in women with pregnancy-associated complications, such as preeclampsia and GDM (20–22). The results of a metabolomics study interpreted the disease after the integration of multiple factors, including disease process, environmental exposures, demographic variations, and dietary habits, which are also the origin of study heterogeneity (23). Therefore, a successful metabolomics study calls for considerate preparations, which include consideration of confounding variables, powerful calculation for sample size, and standard sample extraction and storage (24). In metabolomics studies of GDM, the known confounding variables include ethnicity, maternal age, pregravid body mass index, family history of diabetes, history of GDM, and newborn sex (24). Furthermore, statistical power analysis should be performed to form an appropriate sample size (25). Metabolomics, as an important part of the biological system, mainly analyzes blood, urine, and feces and then studies the small-molecule metabolites of various metabolic pathway matrices and products (26). In the studies of GDM or other gestation-associated disorders, the serum in the umbilical cord and amniotic fluid is also collected for analysis. In rare cases, placenta or mothers' hair is collected for analysis. In general, samples should be stored at −80°C for short-term periods. Usually, complex and time-consuming sample preparation procedures are not used, except for the collection of samples of the placenta or mothers' hair.

In the process of metabolomics, proton nuclear magnetic resonance (NMR) and mass spectrometry (MS) are effective tools for analyzing the molecular composition of a sample. Liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE) are used for metabolite separation. LC, GC, or CE combined with MS or NMR spectroscopy are the most commonly used metabolomics platforms (27). Proton NMR is widely used in metabolomics studies due to its nondestructive nature and ability to simultaneously measure many organic compounds present in biological samples. However, the low sensitivity of proton NMR, which permits the detection of metabolites only at the micromolar level, is the major limitation of NMR as a comprehensive technique (28). Conversely, MS-based methods provide increased sensitivity and the ability to assay a diverse range of cellular metabolites over a varied polarity range. As such, in clinical metabolomics, NMR has a trend to be superseded by the evolved MS-based methods (23). Untargeted and targeted approaches are the two analytical strategies commonly used in metabolomics (29). The untargeted approach detects metabolites without an *a priori* hypothesis and is more suitable for studies focused on assessing potential biomarkers or metabolic mechanisms for diseases (29–32). The targeted approach analyzes the specific kind of metabolites and the relative metabolic pathways with *a priori* information and is used for biomarker validation and studying a specific biological pathway (29–32). Data generated by untargeted approaches are extremely complex, and the majority of peaks in the profile are not identifiable. Furthermore, the concept of fingerprinting in an untargeted approach was initially developed for microbiology to classify microbial species but is not useful in clinical applications (33). Currently, metabolomics datasets for annotating the spectral features from the untagged approach are not available (34). Conversely, in clinical applications, data processing and normalization are critical in untargeted metabolomics studies. Profile clustering may be used for the diagnosis of patients. Targeted metabolomics is an important workflow because of the higher sensitivity and selectivity and the validation and expansion of results from the untargeted analysis (35).

In addition to high efficiency, ease of interpretation, and acceptable cost, clinical practice poses an additional requirement for metabolomics, and superior reproducibility, particularly in the case of disease prediction. However, metabolomics usually generates a long list of metabolites, which could not be directly used in clinical practice. Advanced algorithms are needed to define and integrate metabolites with the utmost potential. Traditionally, univariate analysis and logistic regression are performed. Recently, machine learning, a data analysis technique that develops algorithms for predicting outcomes by "learning" from data, has been increasingly highlighted as a competitive alternative to regression analysis. Machine learning has been mainly classified into supervised and unsupervised. Hierarchical clustering, principal component analysis, and self-organizing maps are the unsupervised methods that have been used in analyzing metabolomics data. Supervised methods include support vector machines, partial least squares, analysis of variance, k-nearest neighbors, and discriminant function analysis (36). Machine learning outperforms conventional regression in terms of its ability to capture nonlinearities and

complex interactions among multiple predictive variables (37). A few studies on the prediction of GDM have been conducted for comparing the performances of machine learning and logistic regression. Liu et al. (38) developed a machine learning-based prediction model for GDM in women within early pregnancy and compared it with a traditional logistic model. The machine learning method with extreme gradient boosting had similar performance in validation but was better in calibration. Meanwhile, the results of studies comparing machine learning with logistic regression should be critically interpreted. Ye et al. (39) and Wu et al. (40) used a series of machine learning methods to select candidate predictors and build predictive models for GDM. As a result, not all methods in machine learning outperformed logistic regression. For instance, in a study by Ye et al. (39), only three out of eight machine learning methods (AdaBoost, Vote, and LGB) invariably outperformed logistic regression in both external validation and calibration. In a study by Wu et al. (40), the machine learning algorithms had an inferior balance for sensitivity and specificity (Youden index) than the traditional logistic regressions, except for deep neural networks. Furthermore, the models from machine learning algorithms were inclined to have high specificity but low sensitivity (40). Meanwhile, the sample size and the number of variables are another concern when using machine learning.

Comparison of metabolic profiles between gestational diabetes mellitus and type 2 diabetes mellitus by metabolomics

The diagnostic paradigm of GDM is a problem across different guidelines throughout the world. The American Diabetes Association (ADA) formally classifies GDM as "diabetes first diagnosed in the second or third trimester of pregnancy that is not overt (preexisting type 1 or type 2) diabetes" (13). GDM is typically diagnosed using an oral glucose tolerance test between 24 and 28 weeks of gestation. However, the International Association of Diabetes and Pregnancy Study Group also recommends screening for overt diabetes at the first antenatal visit. The ADA standard might have difficulties in distinguishing patients with true GDM from those patients with either prediabetes or predisposition to diabetes. There is a lack of international consensus on the screening and diagnosis of GDM; furthermore, the intentions of early diagnosis of GDM and differentiation from prediabetes or predisposition to diabetes have not been obtained yet. Therefore, metabolomics is rendered to have great expectations in the discernment of GDM.

Several investigations of metabolite profiles have facilitated the identification of potential mechanistic pathways for both diabetes and GDM, thus helping detect their similarities and disparities. Protein metabolism reflected by changes in plasma amino acid concentrations is reported with high frequencies (41). Branched-chain amino acids (BCAAs), including valine, leucine, and isoleucine, are repeatedly reported to be associated with risk factors for diabetes (42). In contrast, elevated levels of BCAAs in women with GDM compared with controls have not been observed in all circumstances. The pioneering study by Metzger et al. (43) observed elevated levels of BCAAs in women with GDM at 30–39 weeks of gestation, which was also later confirmed by Butte et al. (44). In another study, fasting maternal plasma carnitine (total, free, and acyl-carnitine), beta-hydroxybutyrate, free fatty acids, glycosylated hemoglobin, and 21 amino acids were assayed at 30–33 weeks of gestation. Of the 21 amino acids, only methionine, glycine, alanine, citrulline, and ornithine levels were found to be significantly higher in the study group than those in the control group. Meanwhile, Pappa et al. (45) delineated that in GDM, ketogenic amino acids and the branched-chain amino acid isoleucine are released at low rates from the skeletal muscles and mostly catabolized in the liver rather than in the peripheral tissues. Along with BCAAs, alterations in the metabolic by-products of protein, including aromatic amino acids, sulfur-containing amino acids, and asymmetric dimethylarginine, contribute to the development of diabetes and insulin resistance (46). However, inconsistent results have been drawn in various studies on GDM (46, 47). Further study in larger populations is required for explaining the interactions between GDM and the metabolism of proteins. The major components of triacylglycerols, non-esterified fatty acids (NEFAs), are the energy source for many body tissues. Increased circulating levels of NEFAs have been well described in studies on insulin resistance and type 2 diabetes (48, 49). Similarly, upregulated levels of NEFAs in women with GDM were detected in the third trimester of pregnancy, which might be aggravated by an increase in dietary intake of polyunsaturated and saturated fatty acids during pregnancy (50, 51). However, few metabolomics studies are conducting head-to-head comparisons between GDM and diabetes. Moreover, it is important to match the confounding factors including gestational time, techniques used in metabolomics, and other metabolic disorders when applying metabolomics in patients with GDM and diabetes.

Early diagnosis of gestational diabetes mellitus by metabolomics

According to Clarke et al. (52), the early diagnosis of GDM and timely treatment at an average of 17 weeks of gestation minimized neonatal adverse events. However, the traditional methods based on the oral glucose tolerance test often detect GDM at 24–28 weeks of gestation, thus leaving patients with GDM untreated for weeks and causing deleterious effects on the fetus. Hence, there is a need for examining novel diagnostic biomarkers for GDM to facilitate early detection and treatment.

According to metabolomics, abnormal metabolism occurs before the GDM attack (53). Generally, GDM is a multifaceted

condition that involves changes in various metabolic pathways including amino acids, carbohydrates, lipids, and purines (47). A series of studies have attempted to determine biomarkers in urine, amniotic fluid, or plasma for diagnosing GDM at 14–25 weeks of gestation. Pinto et al. (54) performed NMR spectroscopy to identify alterations in metabolites in maternal plasma and lipids extracted at 2–21 weeks of gestation. Compared with those who did not develop GDM, the potential patients with GDM had increases in plasma valine and pyruvate, with decreases in proline, urea, and 1,5-anhydroglucitol. In the study by Hou et al. (55), liquid chromatography-mass spectrometry (LC-MS), GC, and NMR were performed on maternal serum from pregnant women with GDM and normal glucose tolerance. The results showed that the changes in free fatty acids, BCAAs, lipids, and organooxygen compounds differentiated the GDM groups from the healthy group. Furthermore, Hou et al. (55) built models for the risk prediction of GDM based on data from metabolomics and key clinical parameters. In addition, increases in acetate, creatine, creatinine, choline, 3-hydroxyisovalerate, and hydroxyisobutyrate and decreases in trimethylamine N-oxide and betaine in the first trimester are also considered potential signs of developing GDM (56, 57).

Zhu et al. (58) explored metabolomics markers and developed a panel for the early diagnosis of GDM, which paved the way for clinical practice. Time-of-flight GC-MS was performed in cohorts from three population-based studies conducted by different centers, which included 168 patients with GDM and 622 normal controls. The general study cohort had uniform diagnostic criteria but heterogeneity in ethnicity. Ten-fold cross-validated Lasso regression was used to identify predictive metabolomics markers at 10–13 and 16–19 weeks of gestation for GDM. Purinone metabolites at both 10–13 and 16–19 weeks of gestation and amino acids, amino alcohols, hexoses, indoles, and pyrimidine metabolites at 16–19 weeks of gestation were positively associated with GDM risk. Finally, Zhu et al. (58) found that a 17-metabolite panel at 10–13 weeks of gestation and a 13-metabolite panel at 17–19 weeks of gestation outperformed the model using conventional risk factors, including fasting glycemia.

Longitudinal metabolomics in studies on gestational diabetes mellitus

The drawback of most of the published studies is measuring maternal metabolic profiles at only one time point during pregnancy or pooling metabolome data across trimesters. Metabolite alterations may occur in conjunction with substantial metabolic changes in the maternal body during different trimesters of pregnancy, highlighting the value of longitudinal metabolomics research at different pregnancy stages. Several studies have also been dedicated to determining the dynamic alterations in metabolites across different time points during pregnancy, which completely marked the metabolic profiles of GDM.

The pioneering study of GDM by longitudinal metabolomics was conducted by Law et al. (59). LC-MS untargeted metabolomics for maternal plasma was performed along with innovative sample preparation and multilevel statistical methods. All participants were scheduled for three antenatal visits at 11–14, 23–27, and 29–33 weeks of gestation. Compared with the healthy controls, the participants who developed GDM showed a reduction in polyunsaturated phospholipids in the first trimester, independent of the stage of gestation and steroid hormones. In, 2017, Law et al. (60) conducted another longitudinal metabolomics study on GDM. In this follow-up study, urine samples were collected at every antenatal visit during the three trimesters. LC-MS untargeted metabolomics was performed to assess the differences in the urinary metabolome of patients with GDM and healthy controls over the course of pregnancy. Accordingly, before placental hormones or the fetoplacental unit could have produced any physiological effect, the tryptophan–kynurenine pathway was activated in patients with GDM, ultimately leading to uric acid production. The results of Law et al. (60) supported the notion that GDM is a predisposed condition and can be predicted by urinary metabolome countering tryptophan and purine. The two studies by Law et al. (59, 60) set an important role of longitudinal metabolomics in the early diagnosis and prediction of GDM.

Zhao et al. (61) performed MS-based untargeted metabolomics in pregnant women with GDM and healthy controls in their first and second trimesters to investigate the trimester-specific alterations of metabolites related to GDM. In the first trimester, the GDM group had 31 significantly altered metabolites, which were mainly attributed to purine metabolism, fatty acid β-oxidation, and urea cycle and tricarboxylic acid cycle pathways. In the second trimester, significant changes in fold changes across trimesters were detected in six amino acids, lysophosphatidylcholine, and uric acid, which might have contributed to the occurrence and progression of GDM (61). The study by Zhao et al. (61) truly recognized the dynamic monitoring of metabolic alterations by metabolomics over the course of pregnancy.

Apart from GDM, obesity and hypertensive disorders are also common metabolic disorders in pregnancy. It has been suggested that the so-called metabolic disturbances caused by GDM are confused with other concurrent metabolic disorders. Kivelä et al. (62) explored the metabolic profiles of pregnant women suffering from all three metabolic complications. Proton NMR was performed on blood samples collected at a median of 13, 20, and 28 weeks of gestation. Across all three time points, women with obesity had significantly higher levels of very-low-density lipoprotein, fatty, and amino acids and more adverse metabolic profiles. Meanwhile, many of the adverse metabolic profiles associated with GDM were rendered nonsignificant after adjustment for body mass index (62).

Metabolic profiles of women with gestational diabetes mellitus in different physiologic subtypes

For women who are not pregnant, hyperglycemia results from a defect in either insulin secretion or insulin sensitivity (63), which supports the possibility of the physiologic heterogeneity of GDM. According to the metabolic abnormality in insulin sensitivity or deficient insulin secretion, GDM can be classified into three physiologic subtypes: insulin sensitivity defects, insulin secretion defects, and normal glucose tolerance (6). It is of clinical importance to classify GDM into physiologic subtypes, which are associated with risks of adverse perinatal outcomes (64). For instance, women with GDM with high insulin resistance have higher rates of preterm delivery, labor induction, Cesarean section, neonatal hypoglycemia, and neonatal intensive care unit admissions (64, 65). Several lines of evidence indicate the different metabolic profiles existing in patients with GDM with the three physiologic subtypes. Obesity-related factors, including pre-pregnancy overweight and elevated gestational weight gain in the first trimester, are specific to the insulin-resistance subtype (66). Layton et al. (67) measured lipid markers in fasting plasma collected during the second trimester for characterizing lipid profiles in women with different physiologic subtypes of GDM. Women with GDM characterized by a predominant insulin sensitivity defect had significantly higher triglycerides, lower high-density lipoprotein, and higher NEFA than those with GDM and normal glucose tolerance. Women with GDM characterized by a predominant insulin secretion defect had higher NEFA levels than those with GDM and normal glucose tolerance. Currently, no study has been conducted to determine the metabolic characteristics of GDM with different physiologic subtypes by metabolomics. The physiologic subtypes of GDM are closely associated with the prognosis of mothers and newborns; thus, it is essential to perform studies on the physiologic subtypes along with data of metabolomics and clinical indexes, which help detect indications of abnormal metabolism belonging to different subtypes of GDM.

Limitations of metabolomics in the clinical practice of gestational diabetes mellitus

There are some limitations of metabolomics in the clinical practice of GDM. First, the process of metabolomics needs higher efficiency. The early detection and control of GDM result in less adverse perinatal outcomes. It usually takes weeks to months before clinicians obtain the final outcomes of metabolomics. According to the ADA standard, the disparity between the oral glucose tolerance test and metabolomics is approximately 10 weeks. It is essential to enhance the efficiency in the process of metabolomics. Second, obvious heterogeneity and low reproducibility exist in the present studies of GDM concerning metabolomics, which is a complication for clinicians in setting a definite cutoff value for one type of metabolite. The differences in GDM diagnostic criteria used, variation in analytical platforms used, analysis of different types of specimens, and disparity in the inherent characteristics of the cohort population are the main sources of heterogeneity (62). Therefore, future multicenter metabolomics studies on GDM are proposed using unified diagnostic criteria, longitudinal supervision of metabolites, and efficient data processing methods to cater to clinical practice.

Conclusion

Metabolomics, an emerging technique, offers a new point of view in understanding the onset and development of diseases. In recent studies of GDM, metabolomics has been repeatedly used in various gestational periods. Metabolomics is rendered to have great expectations in the different and early diagnoses of GDM. Longitudinal metabolomics truly facilitates the dynamic monitoring of metabolic alterations over the course of pregnancy. Furthermore, patients with GDM with different physiologic subtypes have different prognoses and metabolic backgrounds. It would be of clinical importance to perform metabolomics in consideration of physiologic subtypes of GDM and clinical indexes. In conclusion, metabolomics requires further improvement in terms of efficiency and uniform standards in practice.

Author contributions

MZ and HY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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AST-to-ALT ratio in the first trimester and the risk of gestational diabetes mellitus

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Background: Aspartate aminotransferase-to-alanine transaminase ratio (AST/ALT) has been reported affect the risk of type 2 diabetes (T2DM), but it is uncertain if it has relationship with gestational diabetes mellitus (GDM).

Objectives: Our study aimed to investigate the association between AST/ALT ratio in the first trimester and the risk of subsequent development of GDM.

Method: This prospective cohort study enrolling 870 pregnant women, 204 pregnant women with missing data or liver diseases were excluded, 666 pregnant women were included in this study containing 94 GDM women. Blood samples were collected in the first trimester. Univariate analysis and multivariate logistic regression were used to evaluate the association between AST/ALT and GDM. Nomogram was established based on the results of multivariate logistic analysis. Receiver Operating Characteristic (ROC) curves and calibration curves were used to evaluate the predictive ability of this nomogram model for GDM. Decision curve analysis (DCA) was used to examine the clinical net benefit of predictive model.

Results: AST/ALT ratio (RR:0.228; 95% CI:0.107-0.488) was associated with lower risk of GDM after adjusting for confounding factors. Indicators used in nomogram including AST/ALT, maternal age, preBMI, waist circumference, glucose, triglycerides, high density lipoprotein cholesterol and parity. The area under the ROC curve (AUC) value of this predictive model was 0.778, 95% CI (0.724, 0.832). Calibration curves for GDM probabilities showed acceptable agreement between nomogram predictions and observations. The DCA curve demonstrated a good positive net benefit in the predictive model.

Conclusions: The early AST/ALT level of pregnant women negatively correlated with the risk of GDM. The nomogram including AST/ALT at early pregnancy shows good predictive ability for the occurrence of GDM.

KEYWORDS

gestational diabetes mellitus, alanine transaminase, aspartate aminotransferase, nomogram, predictive value

Introduction

For Gestational diabetes mellitus (GDM), the major pregnancy-related endocrinopathy, has been steadily increasing worldwide in many countries over recent decades (1). According to the International Diabetes Association in 2021, 51 studies from 41 countries reported that among pregnant women aged 20-49 years, the incidence of hyperglycemia was 16.7%, and 70%-90% of hyperglycemia cases were caused by GDM, 2021 (2). In China, approximately 17.6% of pregnant women suffered from GDM (3). GDM is associated not only with impaired glucose tolerance or type 2 diabetes mellitus (T2DM) after giving birth to women (4) but also has short- and long-term effects on children such as excessive fetal development, preterm delivery, increased incidence of T2DM, and obesity (5). Therefore, early detection of GDM through screening programs is essential to treat and prevent such diseases and to advance appropriate management. Currently, random blood glucose, fasting blood glucose and oral glucose tolerance tests (OGTT) are usually used to predict or identify GDM. However, such testing is expensive, time-consuming, and cannot anticipate or detect all cases (6); therefore, knowledge of new biomarkers for predicting GDM is crucial.

The liver, an organ essential for maintaining glucose homeostasis and insulin resistance, is important in the pathogenesis of metabolic syndrome (7). Liver transaminases such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are usually used to assess the health of the liver (8) especially enable to reflect the accumulation of fat in the liver (9). The serum AST/ALT ratio, a feature of viral hepatitis, was first proposed by Fernando De Ritis in 1957 (10). However, the AST/ALT ratio is not only used to assess liver disease but also cardiovascular disease (11), chronic kidney disease (12) and metabolic syndrome (13). A previous study found that AST/ALT can affect the risk of T2DM (14). The AST/ALT ratio independently has negative association with the risk of developing T2DM, and the relationship was non-linear (15). In addition, the measurement of these liver enzymes involves well-standardized, simple, inexpensive, and routine tests that do not require fasting before venipuncture, suggesting that they could be incorporated in scores to predict diabetes risk (16). Since the pathogenesis of GDM is similar to that of T2DM, it is suggested that AST/ALT ratio may correlate with the risk of GDM. To date, only one study has focused on the association between AST/ALT in early pregnancy and subsequent GDM (17), which reported that ALT/AST was independently associated with the incidence of GDM. However, some potential confounding factors were not adjusted in this study, and AST/ALT predictive value in GDM have not valued. Therefore, it is of interest to explore the association between AST/ALT levels and the occurrence of GDM and investigate its predictive value for GDM.

This study aimed to determine whether AST/ALT of pregnant women in the first trimester was associated with the risk of subsequent development of GDM and their potential predictive value for GDM.

Methods

Study population and design

The pregnant women in the first trimester were recruited in Hunan Maternal and Child Health Hospital from Mar. 2017 to Dec. 2018. The inclusion criteria were as follows: natural conception; no history of diabetes or GDM, hypertension, thyroid and cardiovascular and cerebrovascular diseases before pregnancy; cases with renal disease or collagen vascular diseases; no acute infection in the last 2 weeks and no antibiotics during pregnancy; not taking drugs that may affect glucose metabolism; planning to complete obstetric examination and delivery in Hunan Maternal and Child Health Hospital; pregnant women who voluntarily participate in the project at $10\text{-}13^{+6}$ weeks of pregnancy. The questionnaire data and blood samples of these participants were collected from the first trimester and followed up until the 2 h, 75-g OGTT (24-28 weeks) was performed to diagnose GDM. According to the purpose of this report, we excluded participants whose ALT, AST as well as the 3-point

OGTT results were missing and women with liver diseases. All participants provided written informed consent, and the study was approved by the Medical Ethics Committee of Hunan Maternal and Child Health Hospital (no. EC201624).

Ascertainment of outcome

The diagnostic criteria for GDM in this study were the 2011 IADPSG criteria (18). GDM was defined at 24–28 gestational weeks based on the results of the OGTT. Pregnant women were diagnosed with GDM when their applied glucose level was elevated in one or more of the following: fasting blood glucose (FBG) ≥ 5.1 mmol/L, 1 hour blood glucose (1-hBG) ≥ 10.0 mmol/L, 2 hours blood glucose (2-hBG) ≥ 8.5 mmol/L.

Measurement of liver enzymes and other clinical indicators

Blood samples were collected during the first trimester and stored at −80°C. ALT, AST, blood glucose and blood lipids containing triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) were tested in the first trimester. All assays were performed at the Hunan Provincial Maternal and Child Health Hospital Laboratory. All measurements were performed in duplicate and the results were reported as the mean.

Questionary data

Some questionnaire data were also collected during the first trimester, which included family history of diabetes, hyperlipidemia, and hypertension (yes, no), alcohol consumption (yes, no), the weight of pregnant women from pre-pregnancy to second trimester and height, maternal age, parity, waist circumference (WC), and exercise for more than 30 minutes before pregnancy (yes, no). Pre-pregnancy body mass index(preBMI) was calculated as pre-pregnancy weight/height^2 (kg/m^2). Gestational weight gain was calculated by first and second trimester weights.

Calculation of sample size

We calculated sample size using PASS 11.0. A meta-analysis in mainland China found that incidence of GDM in older pregnant women was 26.7% (P1 = 26.7%), whereas that in younger pregnant women was just 13.4% (P2 = 13.4%), α=0.05, β=0.1. The risk of developing GDM in those exposed to other suspicious risk factors (such as obesity, family history of diabetes, etc.) concerned in this study is mostly higher than 26.7% (19). According to the calculation, a sample size of at least 378 participants are required to develop the GDM prediction model.

Statistical analysis

The Kolmogorov–Smirnov equality of distributions test was used to check the normality of continuous variables, median and interquartile range (IQR) describing continuous variables that were not normally distributed, mean ± standard deviation (SD) was used to describe normally distributed continuous variables, and percentages were used to describe categorical variables. Differences between the GDM and non-GDM groups were analyzed using the t-test or Wilcoxon rank-sum test and the Chi-square test or Fisher's exact test for continuous and categorical variables, respectively. Multivariate logistic regression analysis adjusted for covariates which have significant differences in univariate analysis was performed to assess the association of AST/ALT with subsequent risk of GDM.A prediction nomogram was constructed based on the results of multivariate logistic regression. We plotted the receiver operating characteristic (ROC) curve to evaluate the nomogram, and a calibration curve (Hosmer-Lemeshow test) was used to assess the goodness of fit. To determine the clinical utility of the nomogram, decision curve analysis was applied to GDM patients by quantifying the net benefit at different threshold probabilities. We performed a correlation analysis of the diagnostic results of OGTT and AST/ALT. Statistical significance was set at P value < 0.05 (two-tailed). All analyses were conducted using SPSS22.0 and R 4.2.1 with R packages caret, rmda, rms, regplot, and pROC.

Results

Basic characteristics of cohort

A total of 870 pregnant women were enrolled and followed up until the outcome, 204 were excluded because ALT, AST, or 3-point OGTT results were incomplete, or because pregnant women had liver diseases. Finally, 666 participants were included in the study, of whom 94 were diagnosed with GDM, with an incidence of 14.1%.

The basic characteristics of the study cohort are shown in Table 1. The average age of pregnant women with GDM (31.93 ± 4.69 years) was significantly higher than that of normal women (29.15 ± 3.92 years). GDM cases had higher preBMI, WC, and percentage of parity≥1 than those in non-GDM women.

As for the clinical parameters in the first trimester (shown in Table 2), GDM women had significantly higher glucose (4.87 vs. 4.64), TG (1.55 vs. 1.33) and ALT (18.00 vs. 14.30) levels, but

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TABLE 1 Basic Characteristics of GDM and non-GDM pregnant women.

Characteristics	GDM (n = 94)	Non-GDM (n = 572)	*P*
Maternal age	31.93 ± 4.69	29.15 ± 3.92	<0.001
PreBMI(kg/m^2)	22.00 ± 2.82	20.44 ± 2.50	<0.001
WC (cm)	80.93 ± 8.24	77.86 ± 7.76	<0.001
Gestational weight gain	5.53 ± 3.79	5.44 ± 2.89	0.840
Parity			0.023
≧1	48 (51.1%)	220 (38.5%)	
0	46 (48.9%)	352 (61.5%)	
Family history of hypertension			0.35
yes	28 (29.8%)	200 (35.0%)	
no	66 (70.2%)	372 (65.0%)	
Family history of diabetes			0.099
yes	13 (13.8%)	51 (8.92%)	
no	81 (86.2%)	521 (91.1%)	
Alcohol consumption in first trimester			0.508
yes	4 (4.3%)	16 (2.8%)	
no	90 (95.7%)	556 (97.2%)	
Exercise more than 30 min pre-pregnancy			0.539
yes	20 (21.3%)	162 (28.3%)	
no	74 (78.7%)	410 (71.7%)	

a lower AST/ALT ratio (0.96 vs. 1.18) and HDL-C level (1.83 vs. 1.98).

Multivariable analysis and model construction

Multivariate logistic regression analysis was performed to determine whether AST/ALT ratio had association with the risk of GDM (Table 3). After adjusting for maternal age, preBMI, glucose, WC, TG, HDL-C, and parity (variables with significant differences in univariate analysis), the results demonstrated that with an increase in AST/ALT, the risk for the development of GDM will decrease (RR:0.228; 95% CI: 0.107-0.488).

Establishment and evaluation of a predictive nomogram

According to the results of the logistic regression analyses, a nomogram that could predict the occurrence of GDM was established (Figure 1). The area under the ROC curve (AUC) value of this model (Figure 2) was 0.778 (95% CI:0.724~0.832, P<0.001). Calibration curve shows good agreement between predicted and actual results (Figure 3), c-index was 0.778 and the Hosmer-Lemeshow test p value was 0.683. AST/ALT was significant correlated with the incidence of GDM(r =-0.177, p<0.001). Finally, DCA plot showed that the predictive nomogram model provided good net positive benefit for most threshold probabilities (Figure 4).

TABLE 2 Clinical parameters in GDM and non-GDM pregnant women in the first trimester.

Characteristics	GDM (n = 94)	Non-GDM (n = 572)	*P*
ALT(UI/L)	18.00 (13.10,25.93)	14.30 (10.30,18.60)	<0.001
AST(UI/L)	15.88 (17.90,21.90)	17.70 (14.10,20.10)	0.054
AST/ALT	0.96 (0.79,1.21)	1.18 (1.02,1.49)	<0.001
Glucose(mmol/L)	4.87 ± 0.41	4.64 ± 0.44	<0.001
TG(mmol/L)	1.55 (1.22,1.95)	1.33 (1.75,1.08)	<0.001
LDL-C(mmol/L)	2.59 ± 0.65	2.45 ± 0.67	0.066
TC(mmol/L)	4.63 ± 0.73	4.57 ± 0.79	0.454
HDL-C(mmol/L)	1.83 ± 0.43	1.98 ± 0.41	0.001

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TABLE 3 Results of multivariable logistic regression for GDM.

Model	B	RR (95%Confidence Interval)	*P*
AST/ALT	-1.476	0.228 (0.107~0.488)	<0.001
Maternal age	0.112	1.119 (1.038~1.195)	<0.001
PreBMI	0.085	1.088 (0.971~1.220)	0.195
WC	-0.013	0.987 (0.952~1.024)	0.494
Parity	-0.124	0.884 (0.499~1.626)	0.872
Glucose	0.987	2.682 (1.488~4.830)	0.001
TG	0.391	1.478 (0.984~2.219)	0.060
HDL-C	-0.410	0.664 (0.357~1.232)	0.194

Discussion

In this cohort study, we examined the relationship of AST-to -ALT ratio in early pregnancy and the incidence of GDM in Chinese pregnant women. The ratio of AST/ALT increased, and the risk of occurrence to GDM decreased. In addition, a predictive nomogram of GDM that included AST/ALT levels in early pregnancy was established, which showed good discrimination and clinical usability for predicting the development of GDM.

Liver transaminases, AST and ALT, are widely reported that has close relationship with the occurrence of T2DM (20–22). However, studies on the relationship between liver transaminases and GDM are limited and conflicting. Leng J et al. reported that elevated ALT levels in the first trimester, even within normal range, are associated with the risk of GDM (23). Other researchers found that liver transaminases, including ALT and AST, cannot predict GDM (24). AST/ALT as a liver marker has been recently reported is correlated with metabolic diseases. Elevated ALT levels and low AST/ALT ratios have been discovered to be associated with insulin resistance (IR) (25), and AST/ALT was considered as a surrogate marker for IR and hyperinsulinemia (26). Moreover, the AST/ALT ratio was found to be negatively associated with the incidence of T2DM and was

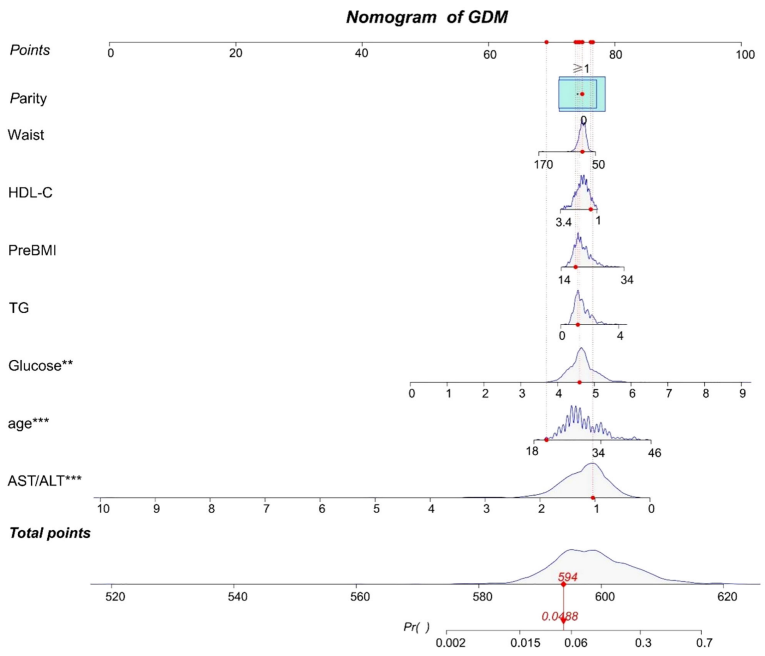

FIGURE 1
A nomogram to predict for the risk of GDM. Scores are calculated by aligning the dots on each numbered row with the dots of the"Points" row. The total score is obtained by adding up all the scores and plotted on the "Total points" line. The difference in the relative proportion of patients in parity (0, ≥1) is represented by the rectangular area. Participant 1 in our study is listed as an example (expressed in red). Her total score was 594, which indicating that her probability of GDM was 4.88%. ***P* value < 0.01 and ****P* value < 0.0001.

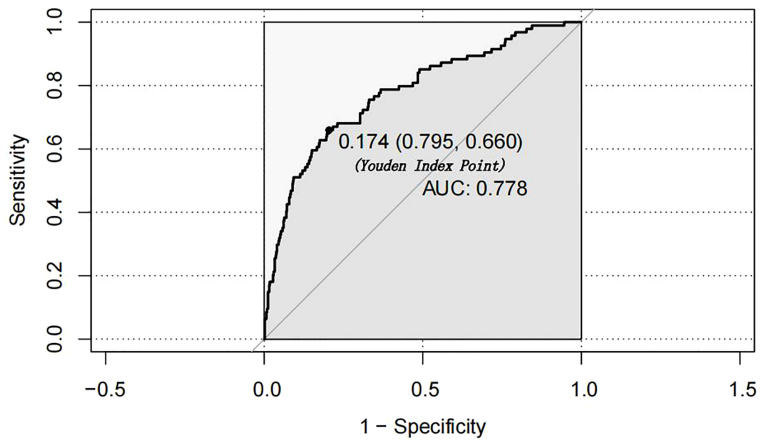

FIGURE 2
Receiver operating characteristic (ROC) curves of nomogram.

demonstrated to be one of the best predictors of metabolic syndrome and T2DM in the Asian population (27–29). However, only one study investigated the relationship between AST/ALT levels in the first trimester and GDM. Consistent with our study, they reported that lower AST/ALT in early pregnancy, even within the normal range, was an independent risk factor for GDM (17). Compared to this study, our study included and adjusted for more confounding variables to make the results more reliable.

Although the potential mechanism underlying the relationship between the AST/ALT ratio and GDM remains unclear, several speculations exist. The liver minimizes postprandial glucose fluctuations by absorbing and storing glucose (30). Liver damage can affect postprandial glucose, since 60-65% of the oral glucose load is processed by the liver (31). The level of ALT and AST can reflect fat accumulation in the liver (32) and decreased AST/ALT is considered a biomarker of nonalcoholic fatty liver diseases (NAFLD), even if its value is

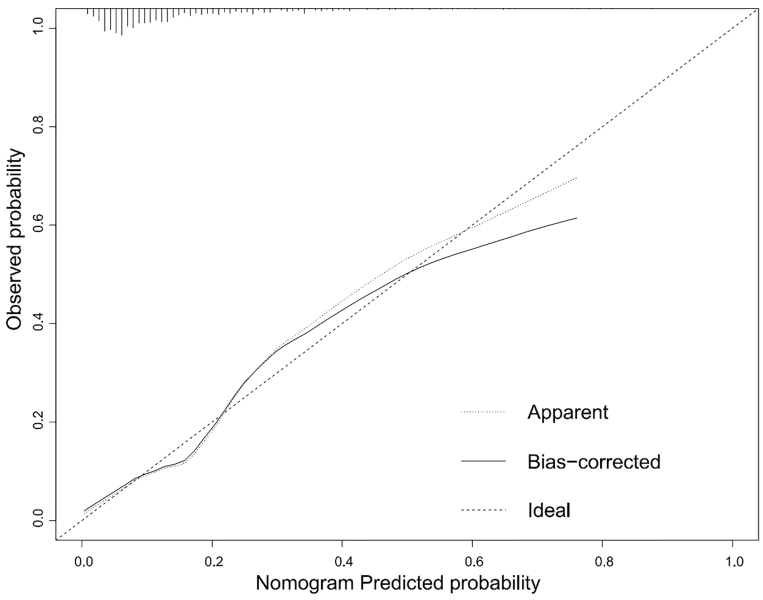

FIGURE 3
Calibration curves for the nomogram. The x-axis represents the predicted rate of GDM. The y-axis shows observed probability of GDM occurrence. The dashed diagonal line is the ideal line. The line adjacent to the ideal line represents the predictive accuracy.

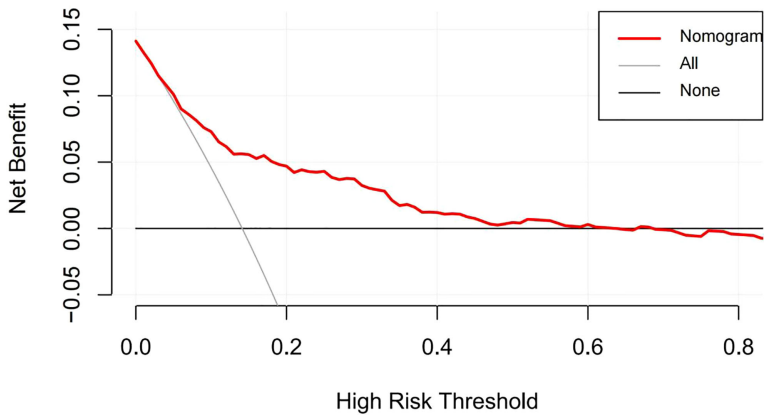

FIGURE 4
Decision curve analysis of the nomogram. The red line represents the clinical net benefits according to the threshold probabilities; The horizontal line assumes that net benefit when no one develops a GDM; the solid gray line indicates the net benefit that all cases suffer GDM.

within the normal range (33). NAFLD can lead to hepatic IR and is considered a feature of metabolic syndrome (34), which causes more production of glucose by the liver (20), affecting human blood sugar and the occurrence of GDM. On the other hand, another study also reported that a positive correlation existed between ALT and fasting glucagon levels which suggesting an interaction exists between the liver and α-cell function (35), which may affect the blood glucose metabolism and leads to GDM.

In our study, we first constructed a nomogram of GDM, which demonstrated that AST/ALT ratio in the first trimester could be used to predict GDM. The nomogram can be widely used as a personalized risk prediction tool with an intuitive digital interface and improved accuracy, which making it easy for clinicians and pregnant women to calculate the risk of GDM based on their own conditions. Various first-trimester nomograms for GDM have been proposed; however, most clinical indicators in these models include only blood glucose and lipids, and liver transaminases were not included. The predictive performance of these nomograms was criticized as having limited diagnostic accuracy, and the AUC of these models ranged from 0.690-0.770, which was less accurate than that of our model (36–38). Moreover, some prediction models containing a number of new biomarkers, such as high molecular weight adiponectin, omentin-1 (39), putrescine (40), and RNA (41), yet have high AUC values; however the detection technology is complex and expensive making them unsuitable for routine screening prediction. In our model, maternal age, preBMI, parity, WC, blood glucose, TG, HDL-C and AST/ALT could be easily measured in the first 3 months of pregnancy with good practicality. In addition, the results of the DCA curve also proved that our model had a positive effect confirming the clinical value of the model.

There are some strengths and weaknesses to our study. Our study was a prospective cohort study that could better explain the causal relationship between AST/ALT levels in the first trimester and GDM. In addition, we first established a nomogram including AST/ALT to predict GDM and demonstrated that model has better discrimination and predictive value. However, limitations also existed in our study. First, limited by actual survey results, although the prevalence of GDM in the cohort was close to the national level, the cases in our cohort was small, this may result in less statistically significant results for some factors with small associations, while increasing the confidence interval for the results. Second, our study involved a single-center cohort, which is not representative of the entire Chinese GDM population. Further evaluating external validity and updating the nomogram in large, multicenter study populations is imperative. Third, because of the robustness of the data, we did not monitor the dynamic changes in AST/ALT ratio from the first to the second trimester and were unable to explore the impact of these dynamic changes on the risk of GDM. Further studies could focus on this topic.

Conclusion

In conclusion, the AST/ALT ratio in the first trimester negatively correlated with the risk of GDM. The nomogram for GDM, including AST/ALT at early pregnancy, shows favorable discrimination and predictive value.

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 Medical Ethics Committee of Hunan Maternal and Child Health Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RA, HT and MC designed the idea and method of this study. Validation, SM, NZ, HL and TX organize and verify the data of this study. SM, RA and NZ analyzed the data. RA and SM wrote the manuscript. HT and MC supervised and supported the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Gestational diabetes is associated with alteration on pelvic floor muscle activation pattern during pregnancy and postpartum: Prospective cohort using electromyography assessment

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Background and objective: Gestational diabetes mellitus (GDM) is a comorbidity which may cause acute and lifelong disorders to mother and child. Alterations in muscular and connective tissues have been associated with GDM in translation studies, characterizing gestational diabetic myopathy. Pregnancy-specific urinary incontinence and sexual disabilities, disorders that depend on the pelvic floor muscle (PFM) integrity, are also associated with GDM both during and after pregnancy. The aim was to compare PFM activation patterns between GDM and non-GDM women from 24–30 gestational weeks to 18–24 months postpartum during a standard clinical test during gestation and postpartum.

Methods: We conducted a prospective three-time-point cohort study from gestation (24–30 weeks—T1, and 36–38 weeks—T2) to 18–24 months postpartum (T3). PFM electromyography was recorded in primigravida or primiparous women with one previous elective c-section with or without the diagnosis of GDM according to the American Diabetes Association criteria. A careful explanation of the muscle anatomy and functionality of the PFM was given to participants before EMG assessment. The outcome measures were PFM activation patterns assessed during pregnancy and postpartum, comparing intra and between groups. PFM activation patterns were assessed

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by normalized electromyography signal at rest and during 1-second (sec) phasic, 10-sec hold, and 60-sec sustained contractions.

Results: Demographic and obstetric data showed homogeneity between groups. The GDM group achieved peak PFM EMG amplitudes similarly to the non-GDM group, but they took longer to return to baseline levels during the ~1-sec contraction (flicks). During 10-sec hold contractions, the GDM group sustained lower levels of PFM activation than the non-GDM group at both 36–38 weeks of gestation and 18–24 months postpartum when compared to the non-GDM group.

Conclusion: The results suggest that GDM impaired PFM control mainly on 1-sec flicks and 10-sec hold contraction, which appears to develop during late pregnancy and extends long-term postpartum. This motor behavior may play a role on pelvic floor dysfunctions.

KEYWORDS

gestational diabetes, pelvic floor, pregnant, electromyography, postpartum

Introduction

Gestational diabetes mellitus (GDM) and gestational diabetic myopathy have been described as risk factors to pelvic floor muscle dysfunction (PFMD) during pregnancy and postpartum (1–10). Compromised PFM integrity may predispose women to PFMD such as pregnancy-specific urinary incontinence (PS-UI) (2) and postpartum urinary incontinence, which have substantial social and economic burden, in addition to high public health costs (11). More specifically, GDM has been associated with higher prevalence of both PS-UI and IU postpartum, with worsening of severity and quality of life during pregnancy and over the first year postpartum compared to non-GDM women (1–3, 5, 12). Taken together, current evidence indicates that PFM could be failing to perform contractions properly in women with GDM. A clinical triad composed of pelvic floor muscle (PFM) myopathy, PS-UI, and GDM is the focus of research. However, there is a lack of studies with longitudinal design assessing PFM function during and after pregnancy, especially in the GDM group (13).

Experimental studies in moderate diabetic rat models have shown that the periurethral and rectus abdominis muscles present deterioration, such as atrophy, thinning, disorganization, and co-localization of fast and slow fibers (7, 8, 10, 14). These data are consistent with those observed in rectus abdominis muscle tissues collected from pregnant women with GDM during C-section (6, 15), which suggests that GDM is indeed capable of damaging the muscular tissue causing a myopathic process (6, 15–17). Establishing a rational line by the morphological findings from urethral and rectus abdominis muscle of rats (7, 8, 10, 14) and rectus abdominis on pregnant women (6, 15), the PFM is also potentially impacted by the myopathic process (6). Due to the invasive nature of PFM biopsy, functional tests have been employed to evaluate the impact of GDM on its function. Electrophysiological tools (18–21) such as electromyography (EMG) have been used to understand PFM motor behavior during pregnancy, but fewer showed how GDM implies PFM function impairments when compared to non-diabetic pregnant women. In a study using electromyography (EMG), the amplitude of PFM signals during rest and hold contraction was decreased from the second to the third trimester. When three-dimensional ultrasonography (3D-US) was used, negative biometric changes, such as a low increase in the hiatal area, a decrease in the anteroposterior diameter, and a reduced levator ani muscle thickness, have also been observed between these two time points (16, 17).

Although previous studies have demonstrated impairments in PFM function associated with GDM, current evidence is still inconclusive in relation to the time frame in which these impairments evolve and whether women with GDM are capable of recovering PFM function after delivery (22). These are important clinical questions to understand the underlying pathophysiology of PFMD. Hence, the aim of this longitudinal study was to compare PFM activation patterns between GDM and non-GDM women from 24–30 gestational weeks to 18–24 months postpartum during a standard clinical test during gestation and postpartum.

Methods

Study design, participants, and group composition

This prospective cohort study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Ethical Committee of the Botucatu Medical School of Sao Paulo State University (Protocol Number CAAE 82225617.0.0000.5411). The STROBE checklist was applied on the study. Written informed consent was obtained from all participants after careful explanation of all research procedures.

The inclusion criteria were as follows: pregnant women between 24 and 30 weeks of gestation in the first assessment; singleton pregnancy; 18–40 years of age; ability to perform a palpable contraction of the PFM (23); had not received PFM training or any musculoskeletal PFM treatment previously or during pregnancy. The exclusion criteria were clinical diagnosis of diabetes (type I or II, or overt diabetes in previous pregnancy), history of urinary incontinence (UI), having had more than two pregnancies, previous vaginal delivery, previous prolapse or incontinence surgery, failure to understand or follow the command to contract PFM, history of neurological diseases, visible genital prolapse, cervical isthmus incompetence, smoking, preterm birth, abortion, and participants who withdrew their consent during cohort.

The diagnosis guidelines proposed by the American Diabetes Association were used to identify patients with GDM (24) using the 75-g oral glycemic tolerance test (75g-OGTT). The test was applied to all participants at 24 gestational weeks, and participants were assigned to the GDM group if they presented fasting glycemic levels ≥92 mg/dl or 1 h ≥180 mg/dl or 2 h ≥153 mg/dl. Conversely, participants who had lower glycemic levels were allocated to the non-GDM group.

Participant recruitment and assessment

Participants were evaluated at three time points: 24–30 weeks of gestation (T1), at 36–38 weeks of gestation (T2), and 18–24 months postpartum (T3). The same procedures were followed at each time point.

Eighty-two participants between 24 and 30 weeks of gestation who met the criteria were recruited from the Perinatal Diabetes Research Center (PDRC) of Botucatu Medical School/UNESP/Brazil, between 2017 and 2019. After giving their written consent, they were invited to answer a questionnaire with personal details; clinical and obstetric historic and anthropometric measures were taken.

Afterward, the PFM examination was explained and subsequently conducted by a single trained physiotherapist (CBP) with 4 years of experience in PFM evaluation. After emptying their bladder, participants were asked to lie down on the stretcher in supine position with their lower limbs flexed. Explanation about the anatomy and function of PFM was provided. To guarantee that participants understood the instructions, vaginal digital palpation was performed, and a PFM contraction was requested by giving the verbal instruction "squeeze the vaginal muscle and hold them as hard as possible, as if you were holding urine until I say to relax". Visual inspection was held to ensure an isolated PFM contraction was well executed, without unusual/excessive co-contraction of the adductor and gluteus, hip movements, or expulsion movements (16, 25, 26). Afterward, participants were asked to perform a short sequence of PFM contractions, in preparation to the Glazer protocol of clinical evaluation (27) that would be used for the PFM EMG assessment: three brief contractions of 1-sec (Flick) phasic contraction and three contractions sustained for 10-sec (Hold) sustained PFM contractions. Participants received strong verbal encouragement and during contractions, and digital palpation was used to confirm that they performed maximal voluntary contractions (MVCs) on every attempt. During the 5-min rest period before EMG recordings, additional instructions were given depending on the performance, any possible doubts were clarified addressed, and the instruction to contract the PFM as hard as they could before relaxing was reinforced.

EMG recordings and experimental protocol

The EMG signals were recorded using a two-channel device (Miotool 200 Uro; Porto Alegre, Brazil) with a gain of 1,000, a 14-bit A/D converter, an input impedance of 10 (10) Ohm/2 pF, and a common mode rejection ratio (CMRR) at 126 dB. Signals were sampled at 2,000 Hz. PFM EMG was recorded using only one channel and an intravaginal probe sensor (Figure 1A) with two opposite stainless-steel electrodes (85 × 25 mm) positioned on both sides of the vaginal sidewall, coupled to a differential sensor with a ring connection. A water-soluble gel was applied before introducing the probe into the vaginal canal. The reference electrode was placed on the ulna's styloid process following the SENIAM recommendations (28).

The Glazer clinical protocol (Figure 1A) was used to standardize PFM activation. The protocol consists of the following sequence: (i) a 60-sec rest (Baseline-pre); (ii) brief 1-sec phasic contractions (Flicks) repeated five times, followed by a 10-sec rest interval; (iii) 10-sec sustained contractions (Hold) repeated five times, with a 10-sec rest in between; (iv) a 60-sec sustained endurance contraction (Endurance); and (v) a 60-sec rest (Baseline-post) (27, 29) (Figure 1B). The following verbal instructions were given to all participants to explain the execution of each task: (i) "Please, stay relaxed as quite as possible, until I say to you

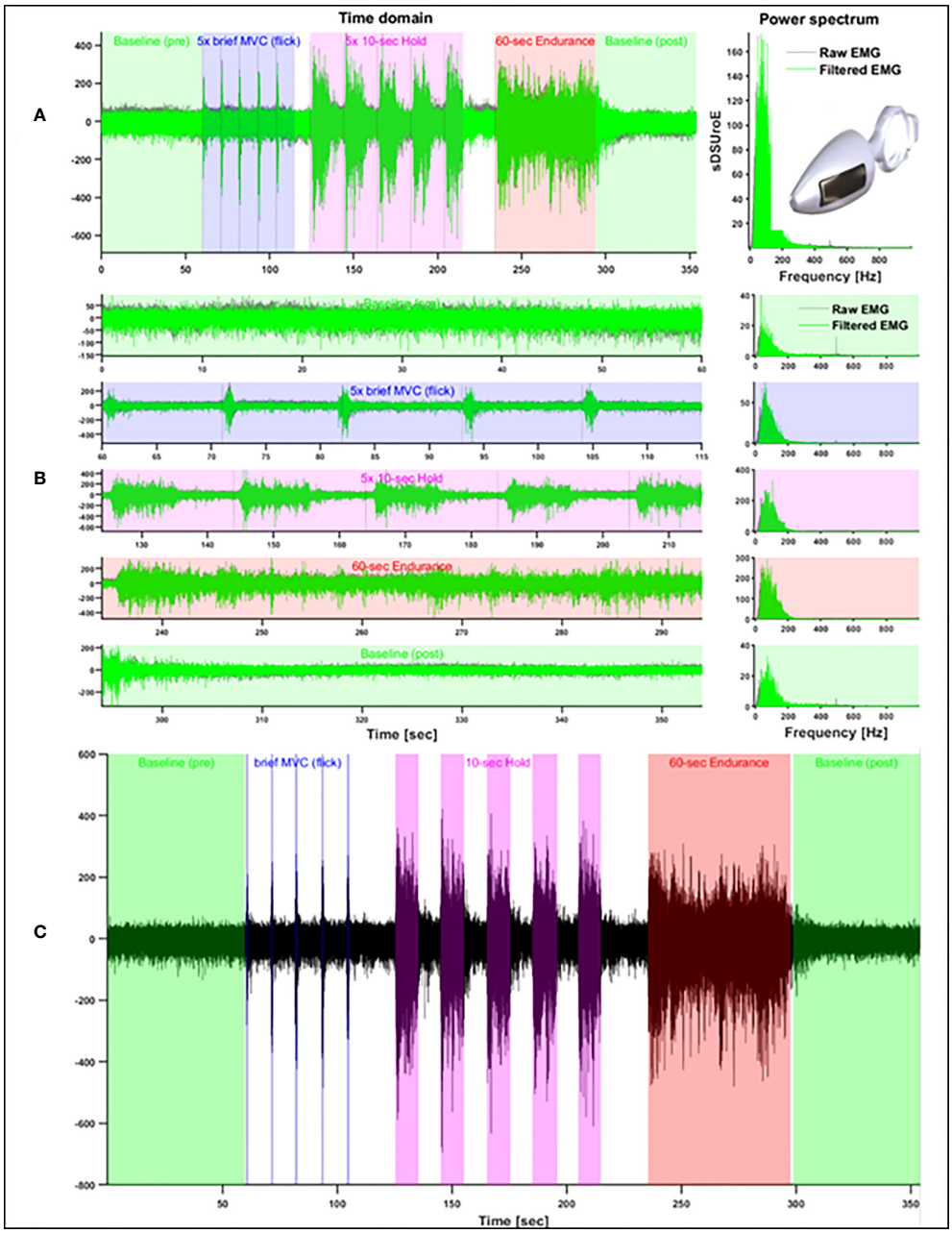

FIGURE 1
Glazer Protocol plots showing full signal and intravaginal probe image **(A)**, protocol segment tasks **(B)** and contraction time window with all performed tasks **(C)**.

to contract the PFM" (Baseline-pre); (ii) "Please, squeeze your vaginal and anus muscles as harder as possible and relax as soon as instruct you" (Flick); (iii) "Please, squeeze and hold your vagina and anus as harder and as long as possible until 10 seconds" (Hold). They were encouraged to sustain the MVC during 10 sec by the verbal instruction: "keep squeezing, keep going, keep going"; (iv) during the 60-sec endurance contraction, the same instruction used for the 10-sec Hold contraction was given, but the instruction to "keep squeezing, contract, keep going, contract as harder as possible, keep going" was continuously repeated during the 60 sec; (v) and in the last 60-sec rest period (baseline-post), participants were instructed to "Relax your vagina and anus as much as possible and stay relaxed for 60-sec".

Electromyographic signal processing

The EMG signals were processed offline using custom programs using a band-pass filter of 20–500 Hz implemented in MATLAB (2014b, The MathWorks, Inc., Natick, MA, USA). First, the quality of the signals from each data collection was evaluated based on visual inspection and signal-to-noise ratio (SNR). Recordings with a low SNR, where the EMG was not discernible from the background or contains excessive signal artifacts, were removed from the analyses (n = 13). Because we detected significant contamination from the power line (60 Hz), an adaptive least mean squares (LMS) filter was implemented, using MATLAB function dsp.LMSFilter, in order to selectively remove contamination at 60 Hz and higher harmonics. The central frequency of the filter was adjusted in each case, depending on the presence of contamination in each harmonic.

The EMG profiles were obtained by applying the root mean square (RMS) function to the entire signal using a sliding window of 200 msec. Consistent with previous studies using the same protocol, the RMS EMG profiles were then normalized by the highest peak detected across the five repetitions of the Flick task (30). Although the Glazer protocol defines fixed time windows for the execution of each task, we ensured the precise selection of time windows of each contraction task by using a single-threshold algorithm to automatically detect the EMG onset and offset of muscle activity (31), which were confirmed by visual inspection (see Figure 1). Rest periods (Baseline-pre and -post) were initially selected from the timing expected from the protocol and were also visually inspected, with adjustments when necessary.

To characterize the muscle activation patterns of each subject, we extracted the following parameters from the normalized RMS EMG profiles of each task: average and peak amplitudes, standard deviation of the amplitude, and coefficient of variation. For the Flick and Hold tasks, we also extracted the time from EMG onset to peak amplitude and the time from peak amplitude to EMG offset. Using time windows of 200 msec, we also estimated the slopes (%/sec) of the RMS EMG after EMG onset (i.e., "increase rate of activity") and before EMG offset (i.e., "decrease rate of activity"), as well as the slopes before and after the time of peak amplitude (Figure 2).

Finally, the full RMS EMG waveforms from the Flick and Hold tasks were compared between groups using the technique of wavelet-functional ANOVA (wfANOVA) (32, 33). As we were interested in both the phasic activation patterns and the rest amplitudes before and after each contraction, we selected time windows that included 3 sec before and after each contraction. Using Subject as a random effect, all task repetitions from each subject were included in the wfANOVA model. For each task, the RMS EMG waveforms were transformed into the wavelet domain, allowing temporally localized features to be represented by a small number of orthogonal (independent) wavelet coefficients. These coefficients were then statistically tested between groups using a one-way ANOVA at each time point to evaluate if there were differences in PFM activation patterns between groups. Significant *between-group* contrasts were identified and transformed back from the wavelet domain into the time domain for visualization.

Sample size estimation

Sample size calculation was performed *a priori* using G*Power. Calculations were performed considering a repeated measures design, a power of 0.80, a probability of error α 0.05, and an effect size of 0.25 calculated by the partial n^2 of 0.06. According to the study design, it was considered for the calculation two groups (GDM and non-GDM) and three measurements (i.e., three time points), an estimated correlation among repetition measures of 0.5, and non-sphericity correction of 1; the estimated sample size required was at least 28 participants (14 in each group).

Statistical methods

The software IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, N.Y., USA), was used for statistical analysis. The chi-square test or Fisher's exact test was applied to compare the nominal data between groups. The Mann–Whitney U test was applied to compare independent categories on table. The EMG parameters were tested using a two-way general linear model (GLM), with Group (GDM, non-GDM) and Time Point (1–3) as factors, with repeated measures on the time-point factor (i.e., within-subject). The hypothesis of sphericity was tested by the Mauchly test, and when the sphericity was rejected, the Greenhouse–Geisser correction was applied. When a significant main effect or interaction effect was found, pair-wise *post-hoc* tests were applied using Bonferroni correction and relative percentages were used to show the magnitude of differences on the statistical tests. Furthermore, as mentioned previously, the full RMS EMG waveforms from the Flick and Hold tasks were compared between groups using the technique of wfANOVA. Differences were considered statistically significant if $p < .05$.

Results

Flow of participants through the study

The flowchart in Figure 3 illustrates the number of women examined at each time point and the reasons for dropout. Among all initially included participants (n = 82), 48 women

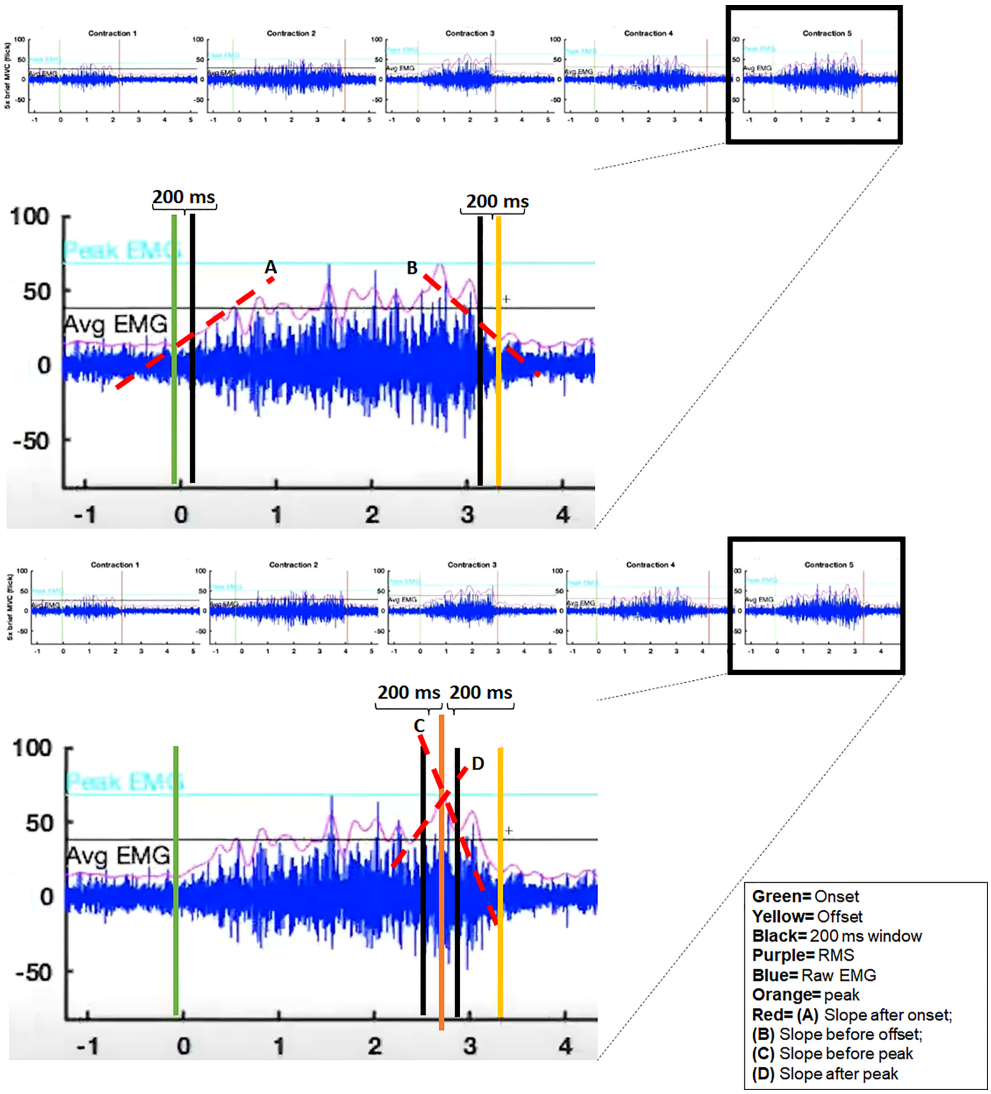

FIGURE 2
Example EMG recording of the Flick task from a representative subject, illustrating the EMG variables used in the analyses.

were allocated in the non-GDM group and 34 in the GDM group. Sixty-two participants remained on T2 (34 non-GDM and 28 GDM), and 46 returned to complete T3 on postpartum (26 non-GDM and 20 GDM). The reasons for dropout were not related with DMG complications. Due to technical failure related to an inappropriate signal-to-noise ratio (maybe attributed to probe movement, inherent equipment/ambient noise) and/or not detectable EMG burst (maybe due to intrinsic reasons), the inclusion of 13 participants was unfeasible. Therefore, the EMG analyses were proceeded with participants who had all time points completed and with good EMG signal quality (19 non-GDM and 14 GDM).

No significant group differences were found in participant characteristics during gestation or postpartum (Table 1). The glucose tolerance test values, as expected, showed marked group differences on fasting, 1 and 2 h after OGTT.

Table 2 shows the average (and standard deviation) of the parameters extracted from the RMS EMG divided across groups and time points, as well as the results from the GLM. The variables evaluated during the 60-sec pre-baseline did not differ between or within groups. During the 1-sec Flick contractions, there was an interaction between time points and groups on the average EMG amplitude [$F(1.619,43.759) = 4.568$; $p = .022$]. *Post-hoc* analyses revealed that the GDM group decreased (-11.0%) the activation levels from T1 to T3 ($p = .040$). Additionally, during T1, the GDM group showed a higher (+12.1%) slope after onset (increased rate of EMG activity) compared to the non-GDM group

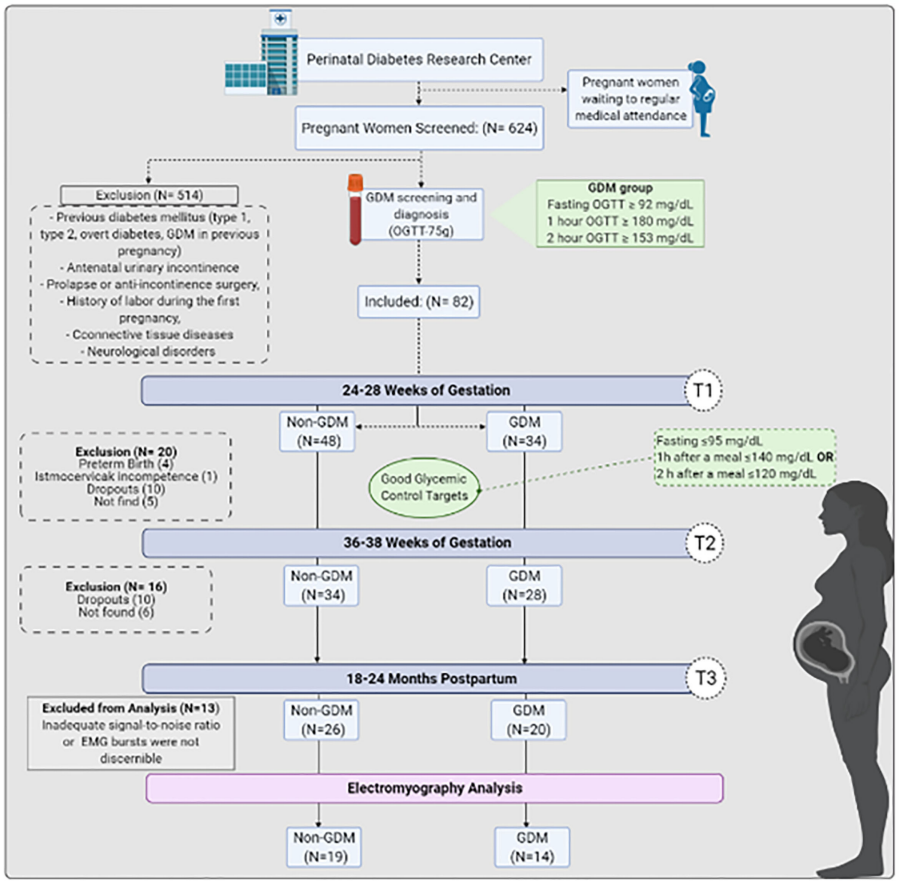

FIGURE 3
GDM women's screening, diagnosis, enrollment, follow-up analysis and reasons for signal exclusion from analysis.

[main effect of group, F (1,27) = 4.504; p = .043, *post-hoc* p = .043]. Moreover, the main effects of time point revealed that, independent of group, women took more time to reach peak EMG, during T1 compared to T3 (non-GDM: 22.2% and GDM: +11.1%) [F (2,54) = 8.354; p <.001, *post-hoc* p <.001]; the task duration was lower at T1 compared to T2 (non-GDM: -16.6% and GDM: -5.5%), and T3 (non-GDM and GDM: -16.6%) in both groups decreased from T1 to T2 [F (2,54) = 9.536; p <.001, *post-hoc* T1 to T2 p = .008 and T1 to T3 p <.001]; and the rate of EMG increase after onset was lower during T1 compared to T2 (non-GDM: +48.5% and GDM: +6.6%) [F (2,54) = 3.633; p = .033, *post-hoc* p = .041]. Finally, interactions between Group and Time Points revealed that the standard deviation of the EMG amplitude on the non-GDM group increased from T1 to T2 (+12.1%) [F (2,54) = 3.345; p = .043, *post-hoc* p = .031] and the EMG slope before offset (decrease rate of EMG activity) was less intense at T1 compared to T2 (-55.4%) and T3 (-47.4%) in the non-GDM group [F (2,54) = 4.812; p = .012 *post-hoc* T1 to T2 p = .005 and T1 to T3 p = .015].

During the 10-sec hold task, there was an interaction between Group and Time Point on the time from peak to EMG offset [F (2,62) = 5.068; p = .009], indicating that the GDM group took less time to return to baseline after the peak in T3 compared to T2 (-20%) (p = .023). The main effects of Time Point revealed that, independent of group, task duration was larger at T3 than both T1 (non-GDM: +2.8% and GDM: +1.9%) and T2 (non-GDM: +1.9% and GDM: +2.8%) [F(1.580,46.735) = 3.895; p = .026, *post-hoc* T1 to T3 p = .023 and T1 to T3 p = .023] and that the EMG slope before peak was greater at T3 compared to T1 (non-GDM: +16.7% and GDM: +37%) [F (2,62) = 3.335; p = .042, *post-hoc* p = .035].

During the last PFM contraction task of the protocol, the 60-sec hold, there were no significant main effects of interactions with

TABLE 1 Average participant characteristics for non-GDM and GDM groups along time points.

Variable		Non-GDM (n = 19)	GDM (n = 14)	p
Ethnicity	Caucasian	13 (68.4%)	7 (50%)	.472[$]
	Other	6 (31.6%)	7 (50%)	
Smoking in pregnancy		0 (0.0)	0 (0.0)	1.000[#]
Smoking postpartum		0 (0.0)	0 (0.0)	1.000[#]
Education level–min. high school		7 (36.8%)	4 (28.6%)	.453[$]
Diabetes postpartum		0 (0.0)	0 (0.0)	1.000[#]
Age (years)[1]		26 (18-39)	29 (18-40)	.529[$]
BMI (kg/m^2) pre-pregnancy		23.6 (19.1-30.7)	25.2 (18.5-34.7)	.900*
BMI (kg/m^2) at 24–30 weeks		26.4 (19.1-32.9)	25.9 (21.6-37.4)	.843*
BMI (kg/m^2) at 36–38 weeks		28.4 (21.2-34.0)	27.7 (22.8-38.7)	.928*
BMI (kg/m^2) postpartum[3]		24.6 (17.1-35.2)	24.2 (18.3-36.6)	.957*
Weeks of gestational[1]		26.0 (24.2-29.0)	27.0 (24.0-29.0)	.506*
Weeks of gestational[2]		36.0 (35.3-38.0)	36.0 (35.0-38.0)	.843*
Postpartum time		24.0 (18.1-24.0)	19.5 (18.0-24.0)	.123*
Delivery mode	C-Section	14 (73,3%)	11 (78,6%)	.746[$]
	Vaginal	5 (26,3%)	3 (21,4%)	
Newborn weight at birth (grams)		3100 (2205-4100)	3150 (2560-3935)	.577*
Blood glucose (mg/dL) [1]		84 (65-90)	88 (76-98)	.077*
OGTT (mg/dL)—fasting[1]		76.0 (71.7-90.0)	92.0 (76.0-124.0)	.000*
OGTT—1 h (mg/dL) [1]		122.0 (76.7-163.0)	152.0 (82.0-211.0)	.012*
OGTT—2 h (mg/dL) [1]		110.0 (64.6-148.0)	138.5 (72.0-179.0)	.019*

Non-GDM, non-gestational diabetes mellitus group; GDM, gestational diabetes mellitus group; BMI, body mass index; OGTT, oral glucose tolerance test. [1]Evaluation at 24–30 weeks of gestation. [2]Evaluation at 36–38 weeks of gestation. [3]Evaluation at 18–24 months postpartum. Data are presented in median (minimum–maximum) or absolute frequency (n) and percentage (%). p-values are based on *Mann–Whitney U, [$]chi-square, and [#]Fisher's exact. Significance $p < 0.05$. p-values represent the results from the relevant statistical tests.

Group or Time Point. During the 60-sec post-baseline rest period, there was a significant effect of Group on the standard deviation of EMG amplitude, and the GDM group showed a lower amplitude (-62.5%) compared to the non-GDM group [$F(1,10) = 5.319$; $p = .044$]. A main effect of Time Point revealed that the peak amplitude was greater at T3 than at T2 (non-GDM: +7% and GDM: +264.8%) [$F(2,20) = 4.152$; $p = .031$, $p = .023$] independent of group.

Figure 4 shows the results of the wfANOVA analysis, with the average EMG patterns of each group and the significant Group contrasts during the Flick and Hold PFM contraction tasks at each time point. The significant contrasts indicate that, during the Flick contractions, the GDM group generally had a greater PFM EMG amplitude than non-GDM after ~1 sec of contraction, suggesting to return from peak amplitude to baseline level contractions. During the 10-sec Hold contractions, the non-GDM group activated the PFM at higher contraction intensities than the GDM group at both time points T2 and T3, although the timing of the contrasts differed between time points: At T2, the GDM group had a lower initial peak amplitude during Hold but similar amplitudes after ~2 sec of contraction; at T3, the initial peaks from both groups had similar (normalized) amplitudes, after which the levels of PFM activation decreased faster for the GDM group, remaining lower than for the non-GDM group until near the end of the contraction.

Discussion

This study assessed PFM EMG patterns from pregnancy to long-term postpartum (18–24 months) in women with and without GDM. Using a well-established protocol for pelvic floor assessment, we reproduced a similar sequence of PFM contractions requested in clinical consultations, commonly used to identify the motor strategy during brief and sustained PFM tasks. No significant group differences were found during the Baseline-pre and Endurance tasks, and only minor differences during Baseline-post. During 1-sec Flick contractions, the EMG activation of all participants decreased on postpartum compared to T1. Wavelet analysis showed that, although the GDM group achieved peak PFM EMG amplitudes similar to the non-GDM, they took longer to return to baseline levels. During 10-sec Hold contractions, the GDM group sustained lower levels of PFM activation than the non-GDM group at both T2 and T3.

Our study was based on evidence of changes in physiological and anatomical factors in the female PFM demonstrated by morphological studies in pregnant rats and humans (6). A reduced ratio of fast to slow fibers and a co-localization of fast and slow fibers have been observed in striated urethral muscle of diabetic pregnant rats compared with non-diabetics and non-pregnant rats (8, 9). More recently, similar findings were found in rectus abdominis muscles of pregnant women with GDM,

TABLE 2 Group mean ± standard deviation (across subjects) of the parameters extracted from the EMG signals at each task of the Glazer protocol.

	Non-GDM (19)			GDM (14)			General linear model		
EMG variables	**T1 time point**	**T2 time point**	**T3 time point**	**T1 time point**	**T2 time point**	**T3 time point**	**p between groups**	**p Interaction group *vs.* time points**	**p Time points**
60-sec pre-baseline (rest)									
Average (%)	8.5 ± 8.0	7.7 ± 5.0	8.3 ± 6.2	6.2 ± 3.9	5.8 ± 3.2	8.3 ± 3.1	.461	.760	.664
Peak (%)	21.9 ± 19.4	16.8 ± 10.2	18.0 ± 11.8	17.1 ± 11.7	13.9 ± 6.1	24.4 ± 8.8	.907	.411	.415
Amplitude SD (%)	2.5 ± 1.8	2.3 ± 1.4	2.3 ± 1.4	2.1 ± 1.4	1.7 ± 0.6	3.4 ± 1.4	.951	.258	.264
Amplitude CV (%)	32.7 ± 6.2	30.0 ± 8.7	30.3 ± 6.8	33.6 ± 6.3	32.6 ± 10.3	42.0 ± 14.9	.096	.199	.310
Task duration	57.1 ± 4.9	59.7 ± 0.6	59.4 ± 0.5	58.6 ± 2.2	59.4 ± 0.4	58.5 ± 0.4	.854	.347	.201
SNR	1.5 ± 1.0	1.3 ± 0.6	1.3 ± 0.4	1.2 ± 0.4	1.5 ± 0.6	1.2 ± 0.2	.833	.466	.889
1-sec phasic (Flicks)									
Average (%)	50.0 ± 9.0	52.2 ± 4.7	51.9 ± 5.9	55.2 ± 4.7[a]	52.7 ± 5.3	49.1 ± 5.2[a]	.558	**.022**	.233
Peak (%)	83.6 ± 11.1	87.9 ± 5.6	86.8 ± 6.4	89.2 ± 5.0	86.9 ± 6.1	84.0 ± 5.7	.711	.063	.495
Amplitude SD (%)	20.5 ± 3.2[b]	23.0 ± 3.0[b]	22.2 ± 3.3	22.6 ± 2.4	22.1 ± 3.0	21.7 ± 2.9	.806	**.043**	.287
Amplitude CV (%)	42.1 ± 8.3	44.7 ± 7.0	43.6 ± 7.0	41.6 ± 6.3	42.8 ± 7.6	44.7 ± 7.3	.825	.626	.290
Time from onset to peak (sec)	0.9 ± 0.2[&]	0.8 ± 0.2	0.8 ± 0.2[&]	0.9 ± 0.3*	0.8 ± 0.3	0.7 ± 0.2*	.978	.146	**<.001**
Time from peak to offset (sec)	1.0 ± 0.3	0.7 ± 0.2	0.8 ± 0.3	0.9 ± 0.3	0.8 ± 0.3	0.9 ± 0.2	.442	.172	.052
Task duration	1.8 ± 0.4[&$]	1.5 ± 0.3[&]	1.5 ± 0.5[$]	1.8 ± 0.4*[£]	1.7 ± 0.3*	1.5 ± 0.2[£]	.650	.306	**<.001**
Slope after onset (%/sec)	111.7 ± 30.8[c&]	165.7 ± 52.3[&]	152.9 ± 55.5	174.5 ± 62.5c*	186.1 ± 64.2*	169.3 ± 67.9	**.043**	.117	**.033**
Slope before offset (%/sec)	-96.3 ± 64.6[de]	-149.7 ± 61.9[d]	-142.8 ± 63.4[e]	-130.5 ± 69.4	-124.7 ± 53.6	-115.4 ± 43.2	.741	**.012**	.110
Slope before peak (%/sec)	100.9 ± 57.1	125.3 ± 55.3	131.0 ± 38.1	118.2 ± 50.7	116.6 ± 67.0	120.8 ± 38.1	.971	.423	.367
Slope after peak (%/sec)	-116.8 ± 46.9	-148.4 ± 71.1	-114.9 ± 42.0	-99.5 ± 32.8	-116.1 ± 41.1	-129.0 ± 38.3	.254	.174	.162
SNR	21.1 ± 15.1	24.6 ± 13.6	28.4 ± 36.3	22.8 ± 16.8	30.9 ± 22.0	14.8 ± 10.8	.701	.157	.437
10-sec hold									
Average (%)	52.2 ± 15.5	56.4 ± 20.0	52.9 ± 16.6	48.0 ± 10.6	51.4 ± 17.3	46.7 ± 18.3	.241	.962	.437
Peak (%)	101.2 ± 27.9	106.3 ± 32.3	99.7 ± 21.5	95.2 ± 10.8	98.2 ± 18.6	95.9 ± 29.2	.268	.941	.729
Amplitude SD (%)	17.8 ± 5.6	19.0 ± 5.7	17.5 ± 4.5	17.6 ± 3.0	18.1 ± 3.4	16.4 ± 5.4	.491	.916	.383
Amplitude CV (%)	35.7 ± 9.1	35.6 ± 8.3	35.3 ± 9.5	38.1 ± 8.2	38.0 ± 11.0	38.2 ± 14.4	.361	.988	.996
Time from onset to peak (sec)	3.1 ± 1.8	2.4 ± 1.7	2.9 ± 2.0	2.1 ± 1.6	3.2 ± 2.3	2.2 ± 1.8	.548	.019	.715
Time from peak to offset (sec)	7.0 ± 1.8	7.8 ± 1.8	7.4 ± 2.0	8.2 ± 1.5	7.0 ± 2.2[f]	8.4 ± 1.7[f]	.408	**.009**	.313
Task duration	10.1 ± 0.5[&]	10.2 ± 0.4[$]	10.4 ± 0.3[&$]	10.3 ± 0.4*	10.2 ± 0.4[£]	10.5 ± 0.4*[£]	.189	.516	**.026**
Slope after onset (%/sec)	116.9 ± 57.4	144.7 ± 57.0	151.1 ± 50.3	144.1 ± 45.2	160.7 ± 62.4	147.2 ± 71.1	.397	.397	.126
Slope before offset (%/sec)	-81.7 ± 61.0	-83.6 ± 44.9	-97.4 ± 67.1	-60.0 ± 29.4	-80.0 ± 58.8	-75.1 ± 45.1	.266	.626	.356
Slope before peak (%/sec)	121.8 ± 47.3[&]	136.9 ± 59.8	142.2 ± 55.0[&]	125.4 ± 53.9*	124.9 ± 53.2	171.8 ± 76.6*	.588	.310	**.042**
Slope after peak (%/sec)	-129.1 ± 62.6	-123.7 ± 48.4	-130.9 ± 37.8	-105.1 ± 32.5	-107.6 ± 43.1	-123.9 ± 75.5	.190	.781	.577
SNR	25.8 ± 22.3	23.7 ± 17.4	26.1 ± 26.8	17.0 ± 13.2	23.8 ± 14.0	9.7 ± 4.4	.053	.186	.414
60-sec endurance									
Average (%)	38.7 ± 14.5	48.7 ± 28.3	39.9 ± 14.1	38.4 ± 11.7	37.3 ± 10.2	40.2 ± 36.5	.540	.602	.790

(Continued)

TABLE 2 Continued

EMG variables	Non-GDM (19)			GDM (14)			General linear model		
	T1 time point	T2 time point	T3 time point	T1 time point	T2 time point	T3 time point	p between groups	p Interaction group *vs.* time points	p Time points
Peak (%)	116.8 ± 50.0	114.9 ± 58.6	109.1 ± 12.8	92.7 ± 16.7	93.4 ± 22.4	111.8 ± 104.5	.337	.673	.915
Amplitude SD (%)	17.3 ± 6.6	18.4 ± 10.8	18.3 ± 4.5	13.4 ± 4.7	13.4 ± 3.1	14.8 ± 13.5	.091	.943	.894
Amplitude CV (%)	46.8 ± 13.9	38.8 ± 10.5	51.3 ± 20.5	36.1 ± 11.6	37.0 ± 9.2	42.8 ± 21.9	.249	.391	.030
Task duration	60.9 ± 1.0	60.4 ± 1.0	60.3 ± 1.4	59.5 ± 2.4	60.7 ± 1.6	59.2 ± 3.0	.174	.244	.333
SNR	14.0 ± 12.0	17.9 ± 12.7	13.0 ± 10.2	10.7 ± 6.5	16.4 ± 7.2	7.2 ± 4.4	.275	.698	.067
60-sec post-baseline (rest)									
Average (%)	10.0 ± 7.7	9.0 ± 3.3	8.4 ± 2.4	7.0 ± 0.6	3.5 ± 0.9	10.4 ± 4.5	.234	.137	.235
Peak (%)	25.0 ± 12.3	22.6 ± 7.8$^{\&}$	24.2 ± 12.5$^{\&}$	17.5 ± 3.7	9.1 ± 2.1*	33.2 ± 14.3*	.304	.054	**.031**
Amplitude SD (%)	3.4 ± 1.5	3.2 ± 1.0^{f}	3.3 ± 1.7	2.4 ± 0.4	1.2 ± 0.1^{f}	3.6 ± 1.4	**.044**	.176	.138
Amplitude CV (%)	46.8 ± 33.6	37.2 ± 10.1	37.9 ± 11.1	33.8 ± 6.2	34.8 ± 6.2	36.7 ± 11.2	.322	.753	.782
Task duration	56.2 ± 3.6	56.9 ± 1.6	57.9 ± 0.8	55.7 ± 0.3	57.3 ± 1.9	55.1 ± 1.7	.158	.269	.532
SNR	2.0 ± 1.4	1.4 ± 0.6	1.5 ± 0.9	1.3 ± 0.4	1.1 ± 0.2	1.4 ± 0.4	.273	.729	.622

Non-GDM, non-gestational diabetes mellitus group; GDM, gestational diabetes mellitus group; SD, standard deviation; CV, coefficient of variation; %, percentage; Sec, seconds. Same letters and symbols indicate differences detected by post-hoc (Bonferroni) contrasts test; p value < 0.05.
Results are presented from the two-way general linear model (GLM) using factors Group (non-GDM, GDM) and Time Point (T1: 24–30 weeks of gestation, T2: 36–38 weeks of gestation, T3: 18–24 months postpartum) as factors, with repeated measures on Time Point.

who showed a decreased cross-sectional area of both slow and fast muscle fibers, in addition to a decreased number of fast fibers and an increased number of slow fibers (6, 34).

It is reasonable that morphologic and metabolic changes in PFMs are likely to contribute to UI (30, 35, 36). Indeed, higher UI prevalence and severity have been associated with hyperglycemic disturbances not only during pregnancy (2, 3, 37) but also on prediabetes and clinical diabetes (38, 39). Three-dimensional ultrasonography during rest showed that there is a decrement of the thickness of the levator ani muscle (17) during pregnancy, which is consistent with previous morphological findings of a myopathic process on musculoskeletal tissue of GDM pregnant women (6, 15). However, conclusive evidence to support this relationship has not yet been assessed due to the lack of studies assessing pelvic floor function by direct measures (40–42), particularly on pregnancy until medium and long-term postpartum (13).

Autonomic neuropathic dysfunctions in the bladder are associated with hyperglycemia (43, 44). Besides it, findings on external anal sphincter using electrophysiological methods showed that diabetic polyneuropathy caused by clinical diabetes mellitus (DM) affects the pudendal nerve by an

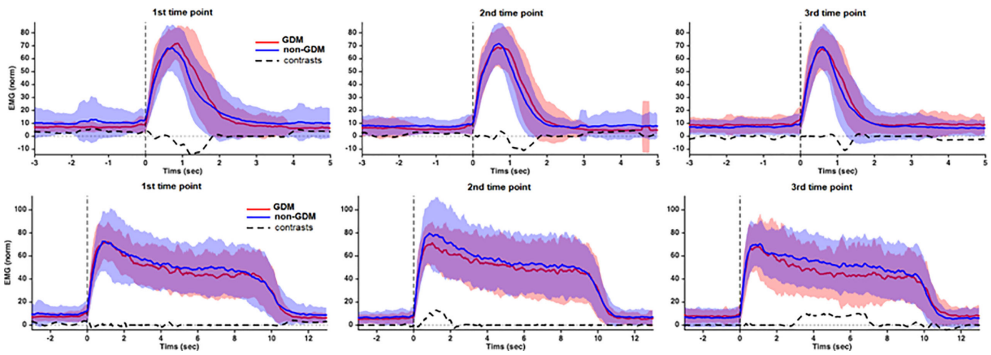

FIGURE 4
Group average and SD of the RMS EMG during the 1-sec Flick and 10-sec Hold PFM contraction tasks from Glazer protocol. Before averaging, the EMG patterns from each subject was expressed as percentage of the peak recorded during the 1-sec Flick contractions. Positive contrasts indicate that GDM < non-GDM. Source: Diamater Study Group.

increase in motor unit action potentials (MUPs), mean duration, mean amplitude, mean phases, satellite rate, and percentage of long-duration MUPs and polyphasic potentials (40, 45).

Previous studies in pregnant woman populations with GDM showed differences in PFM activation between GDM and non-GDM groups at 24–30 and 36–38 weeks of gestation, particularly during rest and hold contractions (16). Although different EMG processing and normalization methods hinder direct comparison with the present study, our results complement previous findings by demonstrating that, as they approach the end of pregnancy, women with GDM show reduced ability to perform brief PFM contractions and to sustain long PFM contractions at the same level as their non-GDM counterparts.

Motor control studies have shown that the reduction in EMG amplitude secondary to muscle weakness could not fully explain the UI, and they showed that the pre-activation of the PFM could have a great contribution on the continence mechanisms (30). Other mechanisms should be addressed to explain better the motor strategy used by the GDM group along (46). Thus, we decided to include, besides the amplitude and peak quantification; analyses of contraction oscillation; temporal analyses related to onset, peak, and offset; and rate of recruitment during the begin and end of the contraction and the peak.

The findings from the 1-sec Flick contractions showed that the GDM group decreased their levels of PFM activation from T1 to T3, whereas the non-GDM group maintained similar levels of activation along time points. We believe that the significant increase in amplitude standard deviation clinically implies about amplitude variability during the same task the non-GDM group could contribute to allow the non-GDM group to maintain the level of activation. The implications of low or high variability is still controversial in literature, but there is evidence that a higher variability may represent an adaptive mechanism to maintain the task performance (47).

The impairments in PFM function observed in women with GDM have been attributed to physiological and anatomical changes to the musculoskeletal system, namely, reduced cross-sectional area and reduced number of fast fiber type, in addition to impairments in ionic channels, as well as fat infiltration and proliferation of connective tissue in the PFM (48). Nevertheless, we cannot exclude other confounding factors, including the volitional component (i.e., choose not to activate) and technical aspects inherent to EMG acquisition which could affect both groups (49).

Both groups achieved peak quicker on T3 compared to T1 on the flicks task. As this characteristic was the same on the groups and no differences were found between T1 and T2, the pregnancy itself may have an implication on it. A quicker response of the pelvic floor is important mainly when intra-abdominal pressure is higher to promote continence. Other studies should consider exploring the latency of PFM onset to peak in comparing it with other structures involved on the modulation of intra-abdominal pressure (50).

Although our protocol had a standard task duration, we observed that both groups decreased duration in the flicks contractions on T2 and T3 compared to T1 to T2 in both groups and achieved peak on T3 quicker than T1. We believe that this is probably a result of a learning effect: as participants got familiar with the tasks, both groups were able to reach peak amplitude more quickly than before, increasing the rate of EMG activity (slope after onset). Additionally, we expected on T1 that the groups may have the same recruitment characteristics, but the GDM group activates PFM around 60% more per second compared to non-GDM.

Concerning the deactivation on the end of the task (slope before offset), the non-GDM decreased the rate of EMG activity intensely from T1 to T2 and from T1 to T3. The GDM group used the same strategy to relax pelvic floor muscle along time points. When comparing the full RMS EMG waveforms between groups, we found that, at all three time points, the GDM group took longer T1 to return from peak amplitude to baseline levels, as revealed by a higher EMG amplitude compared to non-GDM after peak EMG. This observation wave characteristic is corroborated by a recent study applying the same protocol to continent and incontinent women that found that the incontinent group took more time to relax after Flick contractions (51).

On the 10-sec Hold tasks, whereas traditional amplitude measurements were not able to identify major differences between groups or time points, the analyses in the wavelet domain found a reduced EMG amplitude in the GDM group compared to non-GDM at time points T2 and T3, which means that when events along the task are taken into consideration, different motor control patterns are found between groups along the task duration in each time point. During T1, the motor pattern was mostly similar between groups. It could be explained by the fact that this is the screening period to GDM, so it is the point that glycemia starts to get higher and maybe there is no drastic influence on muscle yet. Also, the discrete but significant differences on T2 could be explained by the fact that the cross-section area of slow fibers are decreased in the GDM group (6, 15). Although the capacity of the morphological recovery on postpartum is unknown, our findings suggest that PFM control continues to be impaired postpartum in the GDM group. Additionally, the GDM group took more time to return from peak to offset from T2 and T3. Although the task duration statistically increased from T1 to T2 and T3, it was less than 1 sec and may not be relevant clinically.

During post-baseline resting, there were differences related to the peak from T2 to T3 in both groups and the GDM group on T2 oscillated less during the final resting. Although significant, these two characteristics without an additional change on average amplitude, clinically, do not provide a valuable reflection about the task in general.

EMG is a valuable but challenging method to evaluate PFM function; hence, interpretation of the present results should be

made with caution to avoid mistaken conclusions (52). Although the findings from the present study may be partially explained by morpho-pathological processes involved in GDM, there are several concerns to consider: first, the test–retest reliability of PFM EMG amplitude along time points shows heterogeneity among studies in the literature (53, 54). Previous studies have suggested that this heterogeneity arises mainly due to electrode movement, which contaminates the signal with motion artifact and changes the population of motor units recorded, making it difficult to evaluate the same motor units across different time points (54). In addition, some studies have assessed raw EMG amplitudes, which turns the external validity and results comparisons unfeasible.

This novel cohort study evaluated PFM activity in pregnant women with and without GDM at three distinct time points during and after delivery. We argue that the strengths of the study were that (i) we only included continent pregnant women; (ii) we excluded from analysis participants who did not complete the cohort entirely; (iii) only high-quality EMG was included on the analysis, confirmed by high SNR and absence of signal artifacts; (iv) we assessed many different parameters, including traditional amplitude and timing parameters and the assessment of the full RMS EMG waveform, in an attempt to perform a comprehensive assessment of the motor strategies during PFM contractions; and (v) the EMG amplitude of each subject was normalized by the maximal voluntary activation to allow comparisons between groups and time points.

Nevertheless, we also acknowledge some limitations in our study, which should be taken into consideration in future studies. First, we had a relatively high dropout rate, which is a common problem in cohort studies and randomized controlled trials assessing pregnant women (22), probably underpinned by the major changes in women's life that accompany pregnancy and delivery. Second, the assessment of vaginal pressure or force, concomitant with EMG, would have been valuable to assess changes in force-generating capacity and allow more reliable estimates of maximal voluntary contractions (30). In addition, we also did not consider fatigue measurements, mainly because as shown by other authors we have a gap on literature about standardized protocols to assess PFM fatigue (55). Third is the employment of intravaginal high-density surface electromyography to allow others such as the number of motor unit action potentials by the decomposed signal (56). Finally, the use of vaginal probes with suction, designed to minimize movement artifacts and ensure optimal electrode alignment with the muscle fiber direction, is likely to enhance the technical quality of the EMG recordings.

Conclusion

Our findings show impaired PFM motor control strategies on pregnant women with GDM compared to non-GDM during execution of 1-sec Flick and 10-sec Hold contractions during pregnancy and 18–24 months postpartum. Taken together, these results suggest that differences on motor behavior of GDM women arise in late pregnancy and exacerbate on postpartum.

Research implications

To the best of our knowledge, this is the first study to provide information about PFM neuromuscular strategy of woman GDM in a long-term follow-up. Further studies should be necessary to investigate the influence of this strategy on PFM strength and pelvic floor dysfunctions. This additional information should be important to delineate preventive and therapeutic strategies on this population.

Data availability statement

The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper. Access to the data should be required to the corresponding author.

Ethics statement

This study was reviewed and approved by Institutional Ethical Committee of Botucatu Medical School of Sao Paulo State University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

CBP and SKN are first authors on this work. MVCR and AMPB are last authors on this work. These authors contributed equally to this work. CBP, SKN, FAP, CISF, CBP, AMPB, MVCR e Diamater Study Group contributed to conception and design of the study. CBP, SKN, FAP, CISF e Diamater Study Group were responsible for participants during the study. CBP, GTAN, SES organized the database. CBP e SES performed EMG analysis and statistical analysis CBP wrote the first draft of the manuscript. AMPB, MVCR, CRP, SES reviewed the first draft. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

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

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Comparison of insulin requirements across gestation in women with hyperglycemia in pregnancy: A prospective cohort study

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Objectives: The aim of this study is to explore the daily insulin dose and the percentage change in preprandial and basal insulin dosage of women with different types of hyperglycemia in pregnancy (HIP) during the whole gestation and postpartum period.

Methods: A total of 121 subjects with HIP requiring insulin therapy were enrolled from a prospective cohort consisted of 436 pregnant women with hyperglycemia. The subjects were divided into three groups: Group 1 [type 1 diabetes mellitus (T1DM) and maturity onset diabetes of the young (MODY)], Group 2 [type 1 diabetes mellitus (T2DM)], and Group 3 [gestation diabetes mellitus (GDM)]. The primary study measurements included daily dose and percentage of different types of exogenous insulin requirements across gestation in different groups.

Results: Insulin total daily dosage of Group 1 was highest among the three groups and increased significantly from the first to the second/third trimester. Percentage of preprandial insulin increased from 53.8% (46.7, 60.0) and 54.5% (42.3, 62.9) in the first trimester to 63.6% (54.9, 75.0) and 67.2% (51.8, 73.7) in the second/third trimester in Group 1 and Group 2. All subjects with T1DM and 18.6% of subjects with T2DM still required insulin administration after delivery, with a 26.9% (19.0, 46.0) and 36.7% (26.9, 52.6) decrease in total insulin dose, respectively, whereas subjects with GDM and MODY weaned off insulin completely.

Conclusion: The insulin requirements for pregnancy complicated with T1DM and MODY were higher than those for T2DM and GDM. In the subjects with PGDM, the insulin requirement and percentage of preprandial insulin increased gradually from early to mid- and late pregnancy.

KEYWORDS

pre-gestational diabetes, gestation diabetes mellitus, preprandial insulin, total daily dose, pregnancy, postpartum

Introduction

Hyperglycemia in pregnancy (HIP) could be classified as pre-gestational diabetes (PGDM), gestation diabetes mellitus (GDM), and diabetes in pregnancy (DIP) (1). Insulin is commonly prescribed as the first-line medication when lifestyle modification alone failed to achieve glycemic targets. Women with HIP who had to treat with insulin showed worse adverse maternal and infant outcomes, including a smaller gestational age at delivery and a higher neonatal ICU admission rate (2, 3). However, few studies had evaluated daily insulin dosage, composition, and change in insulin dosage during pregnancy. We aimed to compare the differences of maternal and neonatal outcomes among women with different types of HIP. In addition, we explored total daily dosage and percentage change in preprandial and basal insulin during pregnancy and postpartum period.

Materials and methods

Subjects

The study population comprised women with HIP attending Peking Union Medical College Hospital (PUMCH), Beijing, China, from April 2019 to October 2021. Subjects with renal or liver disease, tumor, and polycystic ovarian syndrome (PCOS) and those taking medication known to affect glycemic metabolism were excluded. Comorbidity data included the presence of hypertension or hypothyroidism.

In total, 17 women with type 1 diabetes mellitus (T1DM), 43 women with type 2 diabetes mellitus (T2DM), 54 women with GDM, and seven women with a genetic diagnosis of maturity onset diabetes of the young (MODY, 3 GCK-MODY, 1 HNF1A-MODY, 1 PDX1-MODY, 1 HNF1B-MODY, and 1 KLF11-MODY) who had given birth to single live babies at term were recruited. The screening of MODY was based on a predictive model previously published by our center (4). Only 2.5% of subjects with HIP (two cases of T1DM and one case of T2DM) were managed on the insulin pumps therapy. The oral glucose tolerance test was undertaken in 24–28 weeks of gestation to screen for GDM. Among pregnant women with T2DM, 51.2% (22 cases) of the subjects were treated with lifestyle interventions, 41.9% (18 cases) need oral hypoglycemic agents, and only 9.3% of subjects (four cases) were treated with insulin before pregnancy. The clinical characteristics between T1DM and MODY individuals were similar, including maternal age, duration of diabetes, body mass index (BMI) levels, lipid profile, and insulin dependency during pregnancy (Supplementary Table 1). In addition, the sample size of MODY subjects in this study was relatively small. To make the conclusion more persuasive and scientific, these two types of diabetes were combined as Group 1. Pregnant women with T2DM and GDM were classified as Group 2 and Group 3. The flow chart of cohort selection is shown in Figure 1.

Clinical and laboratory measurements

The prospective interventions were composed of lifestyle management, self-monitoring of blood glucose (SMBG), and insulin therapy. All women with HIP were treated with medical nutritional counseling based on the Dietary Reference Intakes (DRI) (5) and Chinese Recommendation for Pregnancy with Diabetes Mellitus (6). Calorie intake should be calculated on the basis of pre-pregnancy BMI and desirable weight gain as follows: 30–35 kcal/kg desirable body weight for women with normal weight and 25–30 kcal/kg desirable body weight for women with overweight/obesity. The energy supply percentages of carbohydrate, protein, and fat were 40%–60%, 15%–20%, and 20%–30% of total calories, respectively, including a minimum of 175 g of carbohydrate, a minimum of 71 g of protein, and 28 g of fiber. SMBG was performed five to eight times per day including fasting, 1 hour postprandial, 2 hours postprandial, and bedtime. Targets for glycemic control were as follows: fasting plasma glucose (FPG), 3.9–5.3 mmol/L; 1-hour value (1h-PG), 6.1-7.8 mmol/L; and 2-hour value (2h-PG), 5.6-6.7 mmol/L. All the women with diabetes were encouraged to breastfeed. The criteria for pharmacological interventions postpartum were based on the Chinese Diabetes Society guidelines for nonpregnant adults [FPG ≥ 7 mmol/L, 2h-PG ≥ 10 mmol/L, and glycosylated hemoglobin (HbA1c) ≥ 7%] (7). In view of this, insulin therapy was the only option for postpartum hyperglycemia. This study was approved by the Ethics Committee of PUMCH and conducted in accordance with the Declaration of Helsinki (Ethics Approval Number: JS-3000D).

Diabetes history and treatment details were obtained from medical records. Both preprandial and basal insulin requirements were recorded for the first trimester, second/third trimester, or postpartum period. Data including maternal demographics, comorbidity, family history of diabetes (first-degree relatives), obstetric history, gestational age at delivery, gestational weight gain (GWG), neonatal weight, and neonatal complications were collated. High-performance liquid chromatography was used for the measurement of HbA1c.

Statistical analysis

All analyses were performed in IBM SPSS software (version 26.0, Chicago, IL, USA). Normal distribution of the data was evaluated with Kolmogorov–Smirnov test. Continuous variables were described as mean ± standard deviation if normally distributed and as median (interquartile range) if not. Independent sample T-Test, Mann–Whitney U-test, one-way ANOVA test, Kruskal–Wallis H-test, and Wilcoxon rank sum

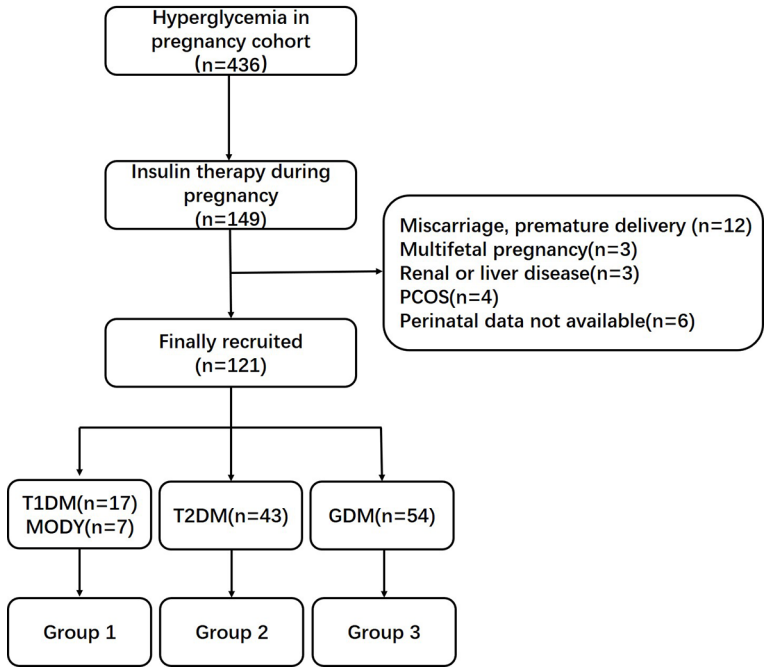

FIGURE 1
Flow chart of cohort selection. PCOS, polycystic ovarian syndrome; T1DM, type 1 diabetes mellitus; MODY, maturity onset diabetes of the young; T2DM, type 1 diabetes mellitus; GDM, gestational diabetes mellitus.

test were used as appropriate. The Chi-square statistic was utilized for categorical variables, and the results are described as frequencies (number of cases) and percentages. Levels of statistical significance were considered as $P < 0.05$.

Results

Anthropometric parameters and perinatal outcomes

Comparison of anthropometric features and perinatal outcomes among the three groups is presented in Table 1. Compared with Group 2 and Group 3, Group 1 had a younger maternal age and a lower pre-pregnancy BMI. There was no difference in mode of conception, comorbidity prevalence, family history of diabetes, and obstetric history among the three groups.

There was no significant difference in gestational age at delivery, GWG, cesarean section, neonatal weight, macrosomia, 1-min Apgar score, and other complications or congenital malformation. In first the trimester, glycated albumin (GA) and HbA1c in Group 1 were higher than those in Group 2 and Group 3. In the second/third trimester of pregnancy, there was no difference in level of HbA1c; however, GA of Group 1 was still higher than that of the other two groups.

Changes in insulin requirements throughout pregnancy

The comparisons of daily insulin requirements (U/kg/day) and dosage allocation of each group in the first trimester and the second/third trimester are shown in Figure 2. The preprandial, basal, and total insulin requirements of Group 1 [0.34 (0.21, 0.40) U/kg/day, 0.28 (0.16, 0.37) U/kg/day, and 0.65 (0.32, 0.78) U/kg/day] were higher than those of Group 2 [0.16 (0.00, 0.30) U/kg/day, 0.10 (0.00, 0.23) U/kg/day, and 0.29 (0.06, 0.53) U/kg/day] in the first trimester of pregnancy. The insulin requirements gradually increased in the second/third trimester and the dosages of Group 1 [0.51 (0.38, 0.67) U/kg/day, 0.31 (0.15, 0.43) U/kg/day, and 0.84 (0.56, 1.06) U/kg/day] remained higher than those of Group 2 [0.35 (0.20, 0.55) U/kg/day, 0.22 (0.09, 0.31) U/kg/day, and 0.53 (0.29, 0.81) U/kg/day] (Supplementary Table 2).

Because GDM screening was usually not performed until 24th gestational week, insulin therapy in Group 3 was usually initiated during the second/third trimester. In addition, the mealtime and basal insulin requirements [0.07 (0.00, 0.15) U/kg/day, 0.07 (0.05, 0.14) U/kg/day, and 0.14 (0.08, 0.24) U/kg/day] were significantly lower than those of Group 1 or Group 2.

All subjects with T1DM relied on insulin therapy postpartum, with postpartum total daily dose (TDD) reduced to 0.69 (0.48, 0.78) U/kg/day and an average reduction of 26.9%

TABLE 1 Anthropometric data and perinatal outcomes of participants by subgroups.

	Group 1	Group 2	Group 3
Cases	24	43	54
Maternal age (years)	32.48 ± 3.25	36.02 ± 4.95*	35.41 ± 3.49*
Course (years)	6.5 (3.0, 10.0)	2.0 (1.0, 3.8)*	NA
Natural conception [n (%)]	22 (91.7)	35 (81.4)	42 (77.8)
Pre-pregnancy BMI (kg/m^2)	22.00 ± 3.34	26.71 ± 3.85*	23.78 ± 4.65$^{\#}$
Delivery BMI (kg/m^2)	26.49 ± 3.48	30.28 ± 3.88*	28.34 ± 4.86$^{\#}$
Obstetric history [n (%)]	9 (37.5)	22 (51.2)	28 (51.9)
Comorbidity prevalence [n (%)]	10 (41.7)	11 (25.6)	18 (33.3)
Family history of diabetes [n (%)]	8 (33.3)	23 (53.5)	25 (46.3)
Gestational age at delivery (weeks)	38.0 (38.0, 39.0)	38.0 (37.0, 39.0)	38.0 (38.0, 39.0)
GWG (kg)	11.84 ± 4.81	9.43 ± 4.50	11.94 ± 5.54
HbA1c in the first trimester (%)	6.93 ± 1.82	6.46 ± 1.11	5.37 ± 0.37*#
GA in the first trimester (%)	18.02 ± 3.98	14.01 ± 2.32*	13.56 ± 2.42*
HbA1c in the second/third trimester (%)	5.30 ± 0.76	5.53 ± 0.45	5.34 ± 0.56
GA in the second/third trimester (%)	14.78 ± 2.34	13.46 ± 1.29*	13.73 ± 1.53
Cesarean section [n (%)]	11 (45.8)	25 (58.1)	27 (50.0)
Neonatal weight (g)	3,427.19 ± 687.83	3,337.76 ± 486.96	3,420.21 ± 506.99
Macrosomia (%)	3 (12.5)	4 (9.3)	7 (13.0)
Other complications or congenital malformation [n (%)]	5 (20.8)	6 (14.0)	4 (7.4)
Insulin therapy after gestation [n (%)]	17 (70.8)	8 (18.6)*	0 (0)*#

BMI, body mass index; GWG, gestational weigh gain; HbA1c, Glycosylated hemoglobin; GA, Glycated albumin.
*p < 0.05 compared to Group 1, #p < 0.05 compared to Group 2.

(19.0, 46.0). In contrast, only 18.6% of subjects with T2DM were insulin dependent after delivery, with TDD of 0.38 (0.27, 0.54) U/kg/day and an average reduction of 36.7% (26.9, 52.6) compared to prenatal requirements (Supplementary Table 3). All of the subjects with GDM and MODY weaned off insulin completely.

The proportion of daily insulin dosage was further compared among the three groups. It was found that preprandial insulin requirements of TDD were significantly higher in Group 2 [67.2% (51.8, 73.7)] and Group 1 [63.6% (54.9, 75.0)] when compared with that in Group 3 [50.0% (0.0, 66.7)] (P = 0.006) in the second/third trimester.

There was no difference in preprandial insulin percentage between Group 1 and Group 2 in the first trimester of pregnancy. The percentage of preprandial insulin requirements increased from 53.8% (46.7, 60.0) and 54.5% (49.3, 62.9) of TDD in the first trimester to 63.6% (54.9, 75.0) and 67.2% (51.8, 73.7) of TDD in the second/third trimester, respectively (Figure 3).

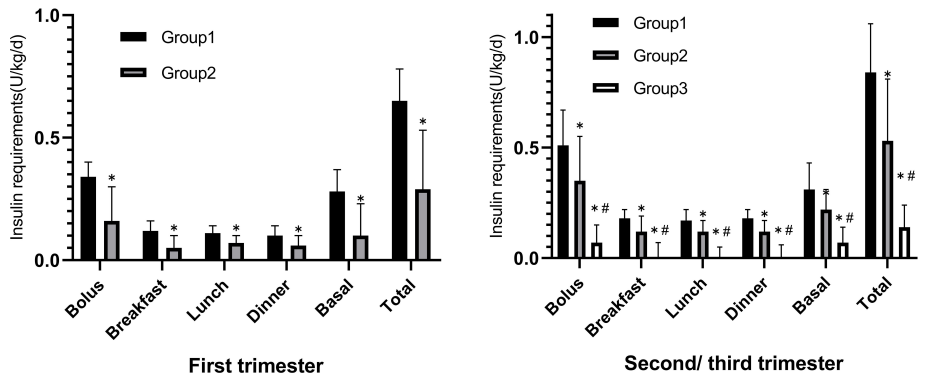

FIGURE 2
The comparison of daily Insulin dosage (U/kg/day) distribution of the three groups in the first trimester versus the second/third trimester.
*p < 0.05 compared to Group 1, #p < 0.05 compared to Group 2.

Discussion

HIP occurred worldwide and is closely related to the health problems in women and their offspring, such as maternal-infant complications and metabolic and cardiovascular diseases. Favorable glycemic control plays a major role in satisfactory perinatal outcomes.

Several risk factors for HIP have been identified, such as previous GDM, family history of diabetes, ethnicity, higher maternal age, pre-pregnancy obesity, or overweight. Excess neonatal and maternal short- and long- term complications are associated with HIP (8, 9). Furthermore, hyperglycemia is also correlated with inflammation including kisspeptin. Meanwhile, reduced kisspeptin level production may be a risk factor of GDM (10). Other studies have shown that kisspeptin may be used as a potential biomarker including GDM in maternal complications (11, 12). Concurrently assessing these risk factors may facilitate the identification of women at risk for HIP and the implementation of HIP prevention measures. In the future study, we will explore the effect of gestational biomarkers levels including kisspeptin on perinatal complications.

Lifestyle interventions such as dietary changes and physical activity were cornerstones in treating HIP. There were issues regarding efficacy and safety of oral pharmacotherapy during pregnancy; thus, insulin is commonly prescribed as the first-line treatment following lifestyle intervention. There are two insulin delivery systems for HIP: multiple daily injections (MDI) and continuous subcutaneous insulin infusion (CSII). According to recent studies, pregnant women with T1DM on CSII therapy have shown lower insulin requirements and better glycemic control compared with those on MDI (13, 14). Some studies have found no significant difference in perinatal outcomes between MDI and CSII groups (13, 15). Of note, at least one study suggested that MDI were superior to CSII in achieving lower HbA1c levels (16). A meta-analysis also reported that CSII therapy was associated with an increasing risk of higher GWG and large for gestational age (14).

Well-controlled HIP was usually defined as GA < 15.7% (17) or HbA1c < 6.0% (18). The present study showed that 73.8% of subjects achieved GA targets in the first trimester, whereas in the second/third trimester, the control rate increased to 88.2%. Likewise, the proportion of subjects with HbA1c lower than 6.0% increased from 54.4% to 90.2% (Supplementary Table 2).

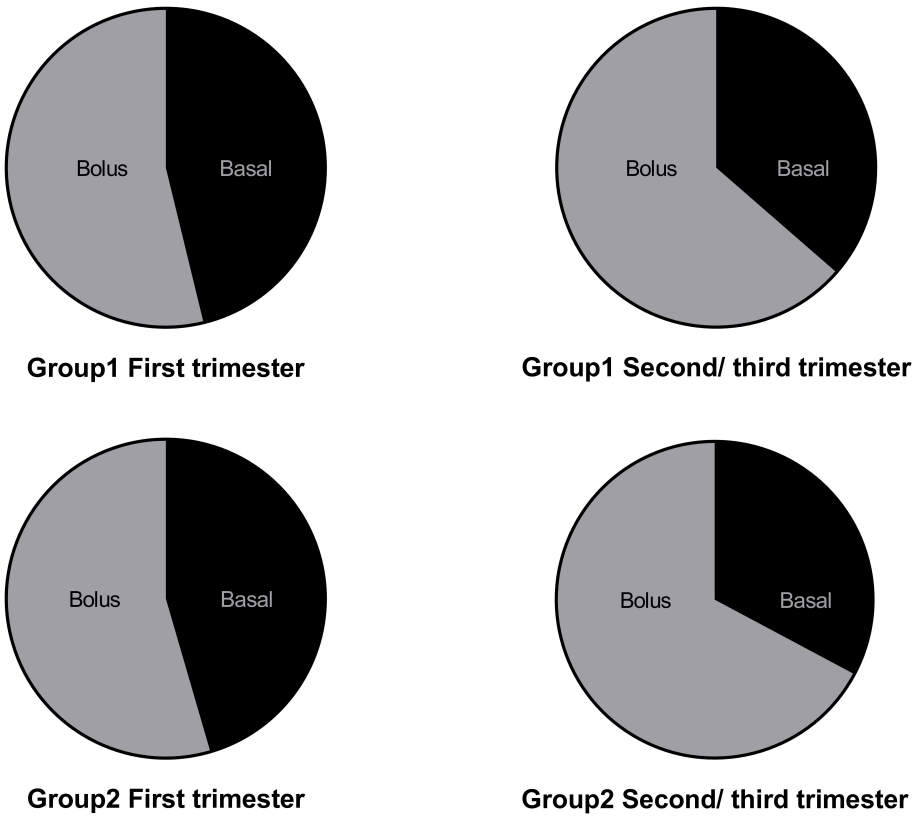

FIGURE 3
Comparison of percentage of preprandial insulin between Group 1 and Group 2.

Overall, from the first trimester to the second/third trimester, the averaged decrease of GA and HbA1c was 0.40% and 0.50%, respectively. In this study, the incidence of macrosomia was 12.5%, 9.3%, and 13.0% in the three groups, respectively (P = 0.928), which were not higher than the incidence of macrosomia in well-controlled GDM from the same center previously reported (19). These results also showed that subjects with HIP may benefit from strict glycemic control.

Although there were no significant statistical differences, pregnant women with insulin-treated T2DM seemed to have a relatively lower incidence of macrosomia and less GWG. It has reported that maternal dysglycemia in early pregnancy associated with poorer pregnancy outcomes (9, 20, 21). Therefore, we speculated that early intervention on glycemic control and GWG had a greater effect on neonatal birth weight.

Insulin requirements during pregnancy and postpartum vary greatly for the alteration of insulin sensitivity, bodyweight, and appetite, so it is important to find out the change rules of dosage and composition. Our study found that, from the first trimester to the second/third trimester, the increasing daily dosage in Group 1 and Group 2 was 0.25 (0.15, 0.82) U/kg/day and 0.24 (0.06, 0.42) U/kg/day, respectively, with an increase in the total insulin dosage of 30.0% (14.6, 82.2) and 56.4% (5.1, 130.0); in addition, the increasing preprandial dosage in Group 1 and Group 2 was 0.21 (0.15, 0.29) U/kg/day and 0.18 (0.00, 0.29) U/kg/day, respectively, with an increase in the preprandial dosage of 64.3% (30.0, 107.9) and 50.9% (0, 122.1) (Supplementary Table 3).

Moreover, the percentage of preprandial insulin increased about 10% in both groups. In early stage of pregnancy, improved insulin sensitivity might be related to the decrease of food intake and depletion of glucose and glycogen reserves (22). In the second and third trimesters of pregnancy, decreased insulin sensitivities were promoted by the progressive increase in placental hormones, such as estrogen, progesterone, prolactin, and growth hormone (23). Serum lipid levels were elevated during the second/third trimester of pregnancy (24, 25). Both of them results in higher maternal post-prandial blood glucose levels (9). A relatively high amount of carbohydrates recommended by DRI may also play a role in high percentage of preprandial insulin requirements.

Insulin requirements were drastically reduced after delivery of the placenta. Our study found that insulin was completely withdrawn in all the subjects with MODY in Group 1, 81.4% with T2DM, and 100% with GDM. The daily dosage reduction in T1DM and T2DM was 0.21 (0.13, 0.44) U/kg/day and 0.31 (0.19, 0.44) U/kg/day, respectively, with a decline by 26.9% (19.0, 46.0) and 36.7% (26.9, 52.6). In addition, the decrease in preprandial dosage was 30.5% (19.4, 38.9) and 34.7% (14.5, 50.2) in T1DM and T2DM, respectively (Supplementary Table 4). Therefore, postpartum insulin dosage should be reduced substantially to avoid the risk of hypoglycemia.

This study does have some limitations. The number of enrolled subjects was relatively limited, and data about changes in insulin requirements in each gestational week were not recorded. A small number of pregnant women with miscarriage, multiple pregnancies, and incomplete records were excluded for this study, which may lead to bias in the results. In addition, the specific mechanism of increase in the percentage of preprandial insulin in the second/third trimester of pregnancy still required further research and remained to be explored.

Conclusion

In summary, pregnancies complicated by T1DM and MODY had higher insulin requirements than that by T2DM and GDM. Percentage of preprandial insulin was roughly equivalent to basal insulin in the first trimester for PGDM but markedly increased in the second/third trimester. All women complicated by T1DM and approximately one in five women with T2DM continued to be treated with insulin administration after delivery, whereas women with GDM weaned off insulin completely. Therefore, postpartum insulin dosage should reduce timely to avoid hypoglycemia. Clarifying the changes in insulin dosing throughout pregnancy in women with HIP may contribute to achieve optimized glycemic control for physicians and patients.

Data availability statement

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

Ethics statement

This study was reviewed and approved by Ethics Committee of PUMCH. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FP carried on the study design, the data collection, the interpretation of the data, and the preparation of the manuscript. CR carried on the data collection and the analysis and interpretation of the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1013663/full#supplementary-material

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Effect of maternal body mass index on the steroid profile in women with gestational diabetes mellitus

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Objective: To explore the effect of maternal body mass index (BMI) on steroid hormone profiles in women with gestational diabetes mellitus (GDM) and those with normal glucose tolerance (NGT).

Methods: We enrolled 79 women with NGT and 80 women with GDM who had a gestational age of 24–28 weeks. The participants were grouped according to their BMI. We quantified 11 steroid hormones profiles by liquid chromatography-tandem mass spectrometry and calculated the product-to-precursor ratios in the steroidogenic pathway.

Results: Women with GDM and BMI<25kg/m^2 showed higher concentrations of dehydroepiandrosterone (DHEA) (p<0.001), testosterone (T) (p=0.020), estrone (E1) (p=0.010) and estradiol (E2) (p=0.040) and lower Matsuda index and HOMA-β than women with NGT and BMI<25kg/m^2. In women with GDM, concentrations of E1 (p=0.006) and E2 (p=0.009) declined, accompanied by reduced E2/T (p=0.008) and E1/androstenedione (A4) (p=0.010) in the BMI>25 kg/m^2 group, when compared to that in the BMI<25 kg/m^2 group. The values of E2/T and E1/A4 were used to evaluate the cytochrome P450 aromatase enzyme activity in the steroidogenic pathway. Both aromatase activities negatively correlated with the maternal BMI and positively correlated with the Matsuda index in women with GDM.

Conclusions: NGT women and GDM women with normal weight presented with different steroid hormone profiles. Steroidogenic pathway profiling of sex hormones synthesis showed a significant increase in the production of DHEA,

T, E1, and E2 in GDM women with normal weight. Additionally, the alteration of steroid hormone metabolism was related to maternal BMI in women with GDM, and GDM women with overweight showed reduced estrogen production and decreased insulin sensitivity compared with GDM women with normal weight.

KEYWORDS

gestational diabetes mellitus, steroid hormone, body mass index, estrogen, androgen

1 Introduction

Gestational diabetes mellitus (GDM) is defined as glucose intolerance and hyperglycemia that occur during pregnancy. It is one of the most common complications during pregnancy, which seriously threatens maternal and fetal health (1, 2). According to clinical statistics, at least 30% of women with a history of GDM are likely to develop type 2 diabetes mellitus (T2DM) after delivery (3). The risk of T2DM in pregnant women with GDM is approximately seven times higher than that in pregnant women without GDM (4). The incidence of macrosomia in the offspring of pregnant women with GDM is approximately 15-45%, which is three times higher than that in healthy pregnant women (5). It has been reported that increasing maternal body mass index (BMI) is an independent risk factor for the development of GDM (6). During pregnancy, excessive weight gain and higher maternal BMI may result in increased insulin resistance and further exacerbate maternal hyperglycemia (7). Greater fat deposition may reduce the ability to compensate for the physiological increase in insulin resistance that occurs during gestation (8, 9). A number of interrelated factors including overweight/obesity and steroids affecting both insulin secretion and insulin resistance are involved in the pathophysiology of GDM (10).

Abnormal metabolism of steroid hormones may induce physiological disorders that lead to complications in obstetrics and gynecology, such as infertility, miscarriage, polycystic ovary syndrome (PCOS), preeclampsia, and GDM (11–13). It has been reported that the pancreas is a target of gonadal steroids, and steroids metabolites have been shown to regulate pancreatic function and insulin resistance in T2DM (14, 15). Progression of pregnancy is accompanied by significant changes in steroid hormones (16). At the beginning of pregnancy, ovarian corpus luteum cells play an essential role in progesterone production. As the placenta develops during pregnancy, the levels of various maternal hormones, including lactogen, placental prolactin, glucocorticoids, estrogen, and androgen, begin to rise rapidly at 24-28 weeks of gestation, while insulin sensitivity starts to decline simultaneously, which promote a state of insulin resistance (17, 18).

Hyperglycemia during pregnancy is the result of impaired glucose tolerance caused by pancreatic β-cell dysfunction on a background of chronic insulin resistance (10). Previous studies have shown that serum dehydroepiandrosterone sulfate (DHEAS) levels may directly affect beta cell function by enhancing glucose-stimulated insulin secretion and specific mRNA expression of beta cell mitochondria and peroxisomal lipid metabolic enzymes (19). Dokras et al. found that testosterone (T) levels in pregnant women were positively correlated with insulin responses during a glucose tolerance test (20). In addition, low levels of sex hormone-binding globulin (SHBG) in the first trimester are associated with an increased risk of developing GDM diagnosed in the second trimester (21). A clinical study has shown that T2DM and GDM are associated with specific changes in sexual steroids and insulin resistance levels during pregnancy. Hyperandrogenemia and higher insulin resistance is observed in women with pregestational T2DM, but not in women with GDM during pregnancy. Decreased estrogen and aromatase activity were found in women with pregestational T2DM and GDM during gestation (22). These studies showed that steroid hormones are related with insulin resistance and GDM development. Another study demonstrated a different metabolic profile of steroid hormones in lean and obese PCOS patients; in that, excessive androgen accumulation was observed in obese PCOS patients with higher insulin resistance than in lean ones (23). Additionally, maternal BMI is a known risk factor for GDM. However, whether the change in maternal weight or BMI has any effect on the steroid hormone profiles in women with GDM and normal pregnant women has not been reported.

Abbreviations: GDM, Gestational diabetes; T2DM, type 2 diabetes mellitus; BMI,body mass index; PCOS, polycystic ovary syndrome; DHEAS, dehydroepiandrosterone sulfate; NGT, normal glucose tolerant; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; HOMA, Homeostasis model assessment; TG, triacylglycerol; P5, Pregnenolone; P4, Progesterone; 17OHP5, 17α-hydroxypregnenolone; 17OHP4, 17α-hydroxyprogesterone; DHEA, Dehydroepiandrosteron; A4, Androstenedion; T, Testosterone; DHT, Dihydrotestosterone; E3, Estriol; E2, Estradiol; E1, Estrone; 17βHSD, 17β-hydroxysteroid dehydrogenase; CYP19A1, cytochrome P450 aromatase.

The aim of the present study was to explore the difference in steroid profiles in GDM patients and normal pregnant women at 24–28 gestational weeks and to investigate the effect of maternal BMI on steroid hormone profiles and steroid metabolic pathway in women with GDM.

2 Materials and methods

2.1 Study population and sample collection

Eighty GDM patients and 79 pregnant women with normal glucose tolerance (NGT) with a gestational age of 24–28 weeks were enrolled between April 7, 2020, and May 22, 2020, at the Women's Hospital of Zhejiang University School of Medicine, China. The pregnant women were between 23 and 35 years old, with a single fetus and normal fetal development at gestational age of 24–28 weeks. The exclusion criteria were as follows (1): *in vitro* fertilization embryo transfer (IVF-ET) (2); personal history of chronic diseases, type 1 Diabetes Mellitus and T2DM, PCOS, autoimmune or chromosomal diseases and liver, kidney, adrenal or thyroid dysfunction (3); diseases that require hormone therapy. First, we compared the differences between women with GDM and those with NGT. The participants were subdivided according to BMI: BMI>25kg/m^2 GDM group (n=24), BMI<25kg/m^2 GDM group (n=56), BMI>25kg/m^2 NGT group (n=12), and BMI<25kg/m^2 NGT group (n=67) (Figure 1). Fasting venous blood sample were collected after 8–14 hours of fasting at the date with oral glucose tolerance test (OGTT). Blood samples were left at room temperature for 30 min, and the upper serum was separated after centrifugation. Finally, serum was stored at -80°C for the detection of steroid hormones. This study was approved by the Ethics Committee of Women's Hospital School of Medicine, Zhejiang University (IRB-20200305-R).

2.2 GDM diagnostic criteria

We used 75-g OGTT at 24–28 gestational weeks for the diagnostic criteria of GDM, which is recommended by the International Association of Diabetes and Pregnancy Study Group (IADPSG) (24). A pregnant woman who meets one of the following conditions may be diagnosed with GDM (1): fasting glucose ≥5.1 mmol/L (2); 1 h glucose ≥10.0 mmol/L (3); 2 h glucose ≥8.5 mmol/L.

2.3 Methods of steroid hormones detection

2.3.1 Sample preparation

In our experiment, calibrators (Bepure, CHN) were dissolved in 10% methanol and 90% water. Internal standard (Bepure, CHN) was dissolved in methanol. Firstly, 200 µL methanol was added to an HLB SPE plate (Waters, Oasis PRiME HLB 96-well µElution Plate, USA), and was lowly flowed through the plate under low vacuum. Thereafter, 200 µL water was flowed through the SPE plate to balance the plate, after which the 200 µL calibrators, quality controls (QCs), and serum samples were placed into a 1.5 mL tube, wherein 200 µL of an internal standard mixture was added and mixed for 3 min. Finally, we added 400µL water, mixed it for 1min, and centrifuged it at 4°C for 15000 g for 10 min. The supernatant (700 µL) was then added to the SPE plates. A low vacuum causes the supernatant to flow slowly through the SPE plate. The HLB SPE plate was washed once with 200 µL 15% methanol. After

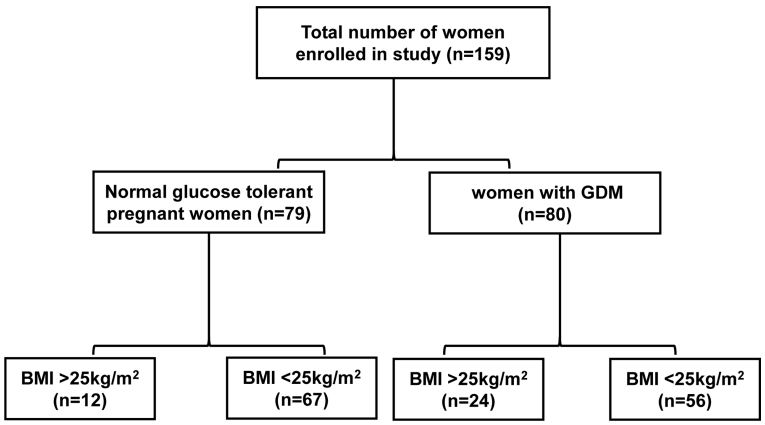

FIGURE 1
Flow chart of the study population.

60μL of methanol was eluted into a 96-well plate and mixed with 60μL of water, the extract was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The details on the methods are shown in Supplementary Materials Tables 1–5. The recovery experiment and matrix effect are referred to literatures (25, 26).

2.3.2 LC-MS/MS

LC column: Waters HSS T3 (2.1×50 mm, 1.8 μm), pre column: HSS T3 (2.1×5.0 mm, 1.8 μm).

LC method A: The mobile phase consisted of 2 mM ammonium acetate, 0.1% formic acid, and water (solvent A) or methanol (solvent B). The liquid chromatographic gradient was as follows: at 0~4 min, 40%~60% solvent B; 4~6.5 min, 60%~75% solvent B; 6.5~7.5 min, 75%~90% solvent B; 7.5~7.6 min, 90%~45% solvent B; and 7.6~8.0 min, 40% solvent B. Column temperature was 45°C, injection volume was 10 μL and flow rate was 0.45 mL/min.

LC method B: The mobile phase consisted of a 0.1% ammonia solution and water (solvent A) or methanol (solvent B). The liquid chromatographic gradient was as follows: at 0~0.6 min, 30% solvent B; 0.6~0.7 min, 30%~65% solvent B; 0.7~1.5 min, 65~85% solvent B; 1.5~2.5 min, 85%~98% solvent B; 2.5~2.6 min, 98%~30% solvent B; and 2.6~3.0 min, 30% solvent B. Column temperature was 45°C, injection volume was 10 μL and flow rate was 0.4 mL/min.

Detection was performed using LC-MS/MS (Waters TQS) equipped with an electrospray ionization probe and operated by switching between positive and negative ionization modes. The capillary potential was set at 3.2 kV. The ion-source temperature was 150°C and the desolvation gas was heated to 400°C at a flow rate of 600 L/h. The cone gas flow rate was set to 150 L/h. Multiple reaction monitoring was used for quantification, as listed in Supplementary Materials Table 6. Data acquisition was achieved using Masslynx software.

2.4 Statistical analysis

SPSS software version 22 (SPSS Inc., Chicago, IL, USA) was used for the data analysis. The student's t-test was performed using the clinical characteristics between the two groups. The data are presented as mean ± standard error (SEM). The comparison of steroid hormone profiling and product/precursor ratios was made using the non-parametric test of Mann–Whitney U. The data are presented as median (25,75 percentile). $p<0.05$ was considered statistically significant. Spearman correlation analysis was also performed. * $p<0.05$, ** $p<0.01$.

Matsuda index=$10000/(G_0\times I_0)^{\frac{1}{2}}(G_{mean}\times I_{mean})^{\frac{1}{2}}$ and HOMA-β=$20\times I_0/(G_0-3.5)$, where G_0 is fasting glucose (mmol/L) and I_0 is fasting insulin (μU/mL). G_{mean} (mmol/L) is the mean of fasting glucose, 1-h glucose OGTT, and 2-h glucose OGTT. I_{mean} (μU/mL) is the mean of fasting insulin, 1-h insulin OGTT, and 2-h insulin OGTT. Matsuda index and HOMA-β data were log transformed to meet normality.

3 Results

3.1 Baseline characteristics and steroid profiles of women with GDM and women with NGT

Women with GDM (n=80) and women with NGT (n=79) had similar maternal age, gestational age, blood pressure, total cholesterol, HDL-cholesterol and LDL-cholesterol. Women with GDM had a significantly higher level of fasting glucose ($p<0.001$), 1-h glucose ($p<0.001$), 2-h glucose ($p<0.001$), 2-h insulin ($p<0.001$), hemoglobin A1c (HbA1c) ($p=0.040$) and triacylglycerol (TG) ($p=0.03$) than women with NGT. The indexes related with insulin resistance including Matsuda index ($p<0.001$) and Homeostasis model assessment (HOMA)-β ($p=0.045$) were decreased in women with GDM (shown in Table 1). Additionally, using LC-MS/MS, we compared the serum levels of 11 steroid hormones between the two groups, including pregnenolone(P5), progesterone (P4), 17α-hydroxypregnenolone (17OHP5), 17α-hydroxyprogesterone (17OHP4), dehydroepiandrosterone (DHEA), androstenedione (A4), T, dihydrotestosterone (DHT), estriol (E3), estradiol (E2), and estrone (E1). Compared with women with NGT, women with GDM showed significantly higher concentrations of DHEA ($p=0.001$), A4 ($p=0.023$), and T ($p<0.001$) (shown in Table 2). Further analysis indicated an elevated risk of GDM in women with high levels (greater than median) of DHEA (OR=2.582, $p=0.003$) and T (OR=2.725, $p=0.002$) compared with those with low levels (less than or equal to median) (Table 3). Surprisingly, we found no significant differences in maternal BMI between the two groups. For a better understanding of the alterations in steroid hormone metabolism in women with GDM and NGT, we subdivided the participants by BMI.

3.2 Analysis of clinical characteristics and steroid profiles of GDM women and NGT women with normal weight

We observed that the GDM group (BMI<25 kg/m^2) had higher maternal age ($p=0.040$) and higher serum levels of fasting glucose (p<0.001), 1-h glucose ($p<0.001$), 2-h glucose ($p<0.001$), 2-h insulin ($p<0.001$), and TG ($p=0.007$) when compared to the NGT group (BMI<25 kg/m^2). Matsuda index and HOMA-β were decreased in the GDM group (Table 4). The levels of DHEA ($p<0.001$), T ($p=0.020$), E1

TABLE 1 Baseline characteristics in women with GDM and women with NGT.

	NGT group (n=79)	GDM group (n=80)	*p* Value
Clinical measures			
Maternal age (year)	28.94 ± 0.32	30.26 ± 0.30	ns
Maternal BMI (kg/m^2)	23.16 ± 0.23	23.49 ± 0.31	ns
Gestational age (weeks)	24.87 ± 0.13	25.15 ± 0.13	ns
Systolic blood pressure (mm Hg)	112.35 ± 1.85	113.72 ± 1.21	ns
Diastolic blood pressure (mm Hg)	65.82 ± 1.14	66.35 ± 0.95	ns
Fasting glucose (mmol/L)	4.38 ± 0.03	4.74 ± 0.06	<0.001
1-h glucose OGTT (mmol/L)	7.54 ± 0.11	10.85 ± 0.15	<0.001
2-h glucose OGTT (mmol/L)	6.48 ± 0.85	9.59 ± 0.14	<0.001
Fasting insulin (μU/mL))	7.60 ± 0.81	8.61 ± 0.47	ns
1-h insulin OGTT (μU/mL))	57.34 ± 3.14	59.24 ± 3.29	ns
2-h insulin OGTT (μU/mL))	45.79 ± 2.35	71.17 ± 4.18	<0.001
HbA1c (%)	4.94 ± 0.03	5.12 ± 0.05	0.040
TG (mmol/l)	2.03 ± 0.08	2.51 ± 0.11	0.030
Total cholesterol (mmol/L)	5.89 ± 0.10	6.12 ± 0.12	ns
HDL-cholesterol (mmol/L)	1.87 ± 0.04	1.82 ± 0.04	ns
LDL-cholesterol (mmol/L)	3.26 ± 0.08	3.34 ± 0.10	ns
Matsuda index	138.02 ± 5.78	97.79 ± 5.25	<0.001
HOMA-β	180.36 ± 16.94	133.55 ± 25.40	0.045

The student's t-test was performed between the two groups. All data are presented as mean ± standard error (SEM), p<0.05 was considered statistically significant. ns means that there is no significant difference between the two groups. Matsuda index and HOMA-β data were log transformed to meet normality. Matsuda index=$10000/(G_0\times I_0)^{1/2}$ $(G_{mean}\times I_{mean})^{1/2}$.HOMA-β=$20\times I_0/(G_0-3.5)$. G_0 is fasting glucose (mmol/L), I_0 is fasting insulin (μU/mL). G_{mean} (mmol/L) is the mean of fasting glucose, 1-h glucose OGTT and 2-h glucose OGTT. I_{mean}(μU/mL) is the mean of fasting insulin, 1-h insulin OGTT and 2-h insulin OGTT.

(*p*=0.010), and E2 (*p*=0.040) were increased in the GDM group (BMI<25 kg/m^2) compared to those in the NGT group (BMI<25 kg/m^2). Next, we used the ratio of product-to-precursor in the steroid hormone metabolic pathway to demonstrate the activity of the enzymes involved in the reaction. The results indicated that DHEA/17OHP5 (*p*=0.010) and T/A4 (*p*=0.003) were increased and DHT/T was decreased in the GDM group (*p*<0.001) (Table 5).

3.3 Analysis of clinical characteristics and steroid profiles of GDM women with normal weight and overweight

In the GDM group, women with BMI>25 kg/m^2 had significantly higher levels of diastolic blood pressure (*p*=0.023), fasting glucose (*p*=0.001), fasting insulin (*p*<0.001), 1-h insulin (*p*=0.001), 2-h insulin (*p*=0.003) and HbA1c (*p*<0.001) than

TABLE 2 The results of steroid hormones in women with GDM and women with NGT.

	NGT group (n=79)	GDM group (n=80)	*p* Value
Steroid hormones profiling (ng/mL)			
Pregnenolone (P5)	1.11 (0.86,1.42)	1.14 (0.94,1.48)	ns
Progesterone (P4)	48.61 (40.89,59.18)	52.92 (45.13,60.76)	ns
17α-Hydroxypregnenolone (17OHP5)	1.09 (0.88,1.42)	1.17 (0.87,1.53)	ns
17α-Hydroxyprogesterone (17OHP4)	2.81 (2.27,3.48)	3.12 (2.53,3.62)	ns
Dehydroepiandrosterone (DHEA)	1.84 (1.50,2.19)	2.39 (1.77,3.29)	0.001
Androstenedione (A4)	3.38 (2.57,4.29)	3.57 (2.75,6.01)	0.023
Testosterone (T)	0.69 (0.54,0.89)	0.87 (0.65,1.52)	<0.001
Dihydrotestosterone (DHT)	0.014 (0.011,0.018)	0.015 (0.01,0.02)	ns
Estrone (E1)	0.07 (0.05,0.10)	0.08 (0.06,0.11)	ns
Estradiol (E2)	0.39 (0.32,0.45)	0.39 (0.32,0.52)	ns
Estriol (E3)	0.09 (0.08,0.11)	0.10 (0.08,0.11)	ns

Mann-Whitney U test was performed between the two groups. All data are presented as median (25,75 percentile). p<0.05 was considered statistically significant. ns means that there is no significant difference between the two groups.

TABLE 3 Calculated odds ratio (OR) for GDM.

Steroid hormones	Odds ratio	95% confidence limits	*p* Value
DHEA (high versus low)	2.582	(1.362, 4.893)	0.003
A4 (high versus low)	1.388	(0.744, 2.590)	0.302
T (high versus low)	2.725	(1.435, 5.176)	0.002

"High" is the level of steroid greater than median; 'low' is the level of steroid less than or equal to median). OR and p value were obtained by Chi-square test. p<0.05 was considered statistically significant.

those with BMI<25 kg/m^2 (Table 4). The Matsuda index was lower in the BMI>25 kg/m^2 group than in the BMI<25 kg/m^2 group in women with GDM. The between-group differences in HOMA-β did not reach significance.

Steroid hormone analysis showed that E1 (p=0.006) and E2 (p=0.009) levels were significantly decreased in GDM women with BMI>25 kg/m^2. Regarding enzymatic activity, the BMI>25 kg/m^2 group showed an increased ratio of E3/E2 (p=0.003) and a decreased ratio of E1/A4 (p=0.010) and E2/T (p=0.008) compared to the BMI<25 kg/m^2 group (Table 5). No difference was observed in NGT women with normal weight and overweight (Supplementary Materials Figure 1).

3.4 Association between steroid hormones ratio with BMI in women with GDM

Correlation analysis in the GDM cohort indicated that BMI was negatively correlated with E1/A4 (r=-0.249, p=0.026) and E2/T (r=-0.267, p=0.016). The Matsuda index was positively correlated with E1/A4 (r=0.402, p<0.001) and E2/T (r=0.297, p=0.007). In addition, BMI was positively correlated with E3/E2 (r=0.272, p=0.015), and Matsuda index was negatively correlated with E3/E2 (r=-0.317, p=0.004) (Figure 2).

TABLE 4 Clinical characteristics of GDM group and NGT groups with different maternal BMI.

	NGT		GDM			
	BMI<25 (n=67)[a]	BMI>25 (n=12)	BMI<25 (n=56)[b]	BMI>25 (n=24)[c]	P VALUE (a VS. b)	P VALUE (b VS. c)
Clinical measures						
Maternal age (year)	29.06 ± 0.36	28.25 ± 0.54	30.07 ± 0.33	30.71 ± 0.63	0.04	ns
Maternal BMI (kg/m^2)	22.60 ± 0.21	26.25 ± 0.26	22.02 ± 0.24	26.91 ± 0.27	ns	<0.001
Gestational age (weeks)	24.71 ± 0.12	25.78 ± 0.41	24.98 ± 0.15	25.53 ± 0.24	ns	ns
Systolic blood pressure (mm Hg)	112.70 ± 1.59	110.17 ± 8.62	111.90 ± 1.41	117.80 ± 2.07	ns	ns
Diastolic blood pressure (mm Hg)	65.30 ± 1.27	68.75 ± 2.42	64.52 ± 1.12	70.25 ± 1.52	ns	0.023
Fasting glucose(mmol/L)	4.35 ± 0.03	4.53 ± 0.09	4.61 ± 0.06	5.03 ± 0.12	<0.001	0.001
1-h glucose OGTT (mmol/L)	7.50 ± 0.12	7.75 ± 0.26	10.78 ± 0.18	11.02 ± 0.31	<0.001	ns
2-h glucose OGTT (mmol/L)	6.47 ± 0.08	6.52 ± 0.30	9.67 ± 0.15	9.41 ± 0.32	<0.001	ns
Fasting insulin (μU/mL))	7.19 ± 0.94	9.81 ± 1.02	7.27 ± 0.36	11.74 ± 1.12	ns	<0.001
1-h insulin OGTT (μU/mL)	56.03 ± 3.29	64.7 ± 9.67	52.50 ± 3.09	74.96 ± 7.42	ns	0.001
2-h insulin OGTT (μU/mL)	44.64 ± 2.45	52.24 ± 7.28	63.20 ± 3.92	89.79 ± 9.64	<0.001	0.003
HbA1C (%)	4.92 ± 0.03	5.04 ± 0.06	5.01 ± 0.05	5.36 ± 0.07	ns	<0.001
Triacylglycerides (mmol/L)	1.96 ± 0.08	2.39 ± 0.32	2.39 ± 0.14	2.78 ± 0.16	0.007	ns
Total cholesterol (mmol/L)	5.87 ± 0.10	6.00 ± 0.39	6.20 ± 0.12	5.90 ± 0.28	ns	ns
HDL-cholesterol (mmol/L)	1.85 ± 0.04	1.99 ± 0.14	1.86 ± 0.05	1.71 ± 0.06	ns	ns
LDL-cholesterol (mmol/L)	3.27 ± 0.09	3.20 ± 0.29	3.41 ± 0.11	3.19 ± 0.22	ns	ns
Matsuda index	143.65 ± 6.25	106.61 ± 11.99	106.27 ± 5.20	78.01 ± 12.19	<0.001	<0.001
HOMA-β	173.90 ± 18.87	216.70 ± 36.37	120.50 ± 35.59	163.90 ± 16.18	0.045	ns

p value (a VS. b) is the p value of women with NGT (BMI<25kg/m^2) compared with women with GDM (BMI<25kg/m^2). p value (b VS. c) is the p value of women with GDM (BMI<25kg/m^2) compared with women with GDM (BMI>25kg/m^2). The student's t-test was performed. All data are presented as mean ± standard error (SEM), p<0.05 was considered statistically significant. ns means that there is no significant difference between the two groups.

TABLE 5 Steroid hormones profiling and product/precursor ratios of GDM group and NGT group with different maternal BMI.

	NGT		GDM			
	BMI<25 (n=67)[a]	BMI>25 (n=12)	BMI<25s (n=56)[b]	BMI>25 (n=24)[c]	P VALUE (a VS. b)	P VALUE (b VS. c)
Steroid hormones profiling (ng/mL)						
Pregnenolone (P5)	1.13 (0.88,1.42)	1.09 (0.74,1.37)	1.22 (0.98,1.62)	0.97 (0.75,1.20)	0.03	0.001
Progesterone (P4)	48.61 (40.74,58.95)	48.63 (41.89,64.94)	54.41 (48.50,62.61)	46.76 (42.48,55.13)	0.02	0.009
17α-Hydroxypregnenolone (17OHP5)	1.09 (0.88,1.33)	1.19 (0.85,1.50)	1.22 (0.93,1.56)	1.02 (0.72,1.43)	ns	ns
17α-Hydroxyprogesterone (17OHP4)	2.79 (2.22,3.47)	2.93 (2.61,4.17)	3.13 (2.55,3.74)	3.06 (2.53,3.47)	ns	ns
Dehydroepiandrosterone (DHEA)	1.75 (1.47,2.15)	2.05 (1.78,3.12)	2.49 (1.78,3.41)	2.06 (1.61,3.24)	<0.001	ns
Androstenedione (A4)	3.38 (2.57,4.27)	3.41 (2.52,4.83)	3.57 (2.75,5.59)	3.87 (2.73,8.43)	ns	ns
Testosterone (T)	0.69 (0.53,0.89)	0.70 (0.55,0.88)	0.84 (0.63,1.43)	0.98 (0.72,1.72)	0.020	ns
Dihydrotestosterone (DHT)	0.014 (0.011,0.019)	0.01 (0.013,0.015)	0.015 (0.01,0.02)	0.014 (0.01,0.02)	ns	ns
Estrone (E1)	0.07 (0.05,0.10)	0.07 (0.06,0.11)	0.08 (0.06,0.12)	0.06 (0.05,0.08)	0.010	0.006
Estradiol (E2)	0.39 (0.31,0.46)	0.36 (0.33,0.43)	0.40 (0.35,0.59)	0.35 (0.27,0.42)	0.040	0.009
Estriol (E3)	0.09 (0.07,0.11)	0.09 (0.08,0.10)	0.10 (0.08,0.12)	0.10 (0.08,0.11)	ns	ns
Product/precursor ratio						
P4/P5	43.70 (35.63,63.23)	56.98 (37.58,65.52)	43.27 (32.78,56.61)	51.67 (37.13,64.68)	ns	ns
17OHP4/17OHP5	2.48 (1.95,3.05)	2.81 (1.49,3.80)	2.48 (1.95,3.53)	3.02 (2.03,4.69)	ns	ns
17OHP4/P4	0.05 (0.04,0.07)	0.06 (0.05,0.07)	0.06 (0.05,0.07)	0.07 (0.06,0.08)	ns	ns
A4/17OHP4	1.12 (0.90,1.44)	1.17 (0.98,1.22)	1.25 (0.98,1.60)	1.41 (1.11,2.28)	ns	ns
17OHP5/P5	1.01 (0.66,1.40)	1.18 (0.83,1.39)	0.94 (0.74,1.18)	1.18 (0.72,1.43)	ns	ns
DHEA/17OHP5	1.79 (1.27,2.15)	1.83 (1.28,2.72)	2.04 (1.70,2.47)	2.30 (1.73,2.72)	0.010	ns
A4/DHEA	1.62 (1.21,2.44)	1.36 (1.10,2.46)	1.44 (1.09,2.39)	2.18 (1.32,3.34)	ns	ns
T/A4	0.21 (0.19,0.23)	0.20 (0.18,0.23)	0.24 (0.20,0.28)	0.23 (0.21,0.27)	0.003	ns
DHT/T	0.020 (0.017,0.029)	0.018 (0.016,0.019)	0.016 (0.013,0.020)	0.013 (0.011,0.017)	<0.001	ns
E1/A4	0.022 (0.014,0.029)	0.024 (0.016,0.027)	0.022 (0.015,0.033)	0.013 (0.001,0.029)	ns	0.010
E2/E1	5.46 (4.46,6.69)	5.55 (3.44,6.06)	4.99 (3.96,6.14)	5.17 (4.55,6.70)	ns	ns
E3/E2	0.24 (0.20,0.29)	0.26 (0.19,0.30)	0.23 (0.18,0.30)	0.27 (0.24,0.37)	ns	0.003
E2/T	0.55 (0.41,0.76)	0.59 (0.42,0.71)	0.50 (0.31,0.71)	0.33 (0.24,0.49)	ns	0.008

Mann-Whitney U test was performed. All data are presented as median (25,75 percentile). $p<0.05$ was considered statistically significant. ns means that there is no significant difference between the two groups.

4 Discussion

In this study, elevated serum glucose, insulin, HbA1c, and TG levels were expected in GDM patients, which is consistent with previous studies (27). However, there was no significant difference in maternal BMI between GDM patients and healthy pregnant women. One explanation might be that pregnant women pay greater attention to weight management, particularly to a healthy diet and maintenance/increase in physical activity (28). Steroid hormone metabolism was distinctly profiled in GDM women and NGT women, and it has been validated that BMI correlates with steroid hormone metabolism (29). We hypothesized that there would be differences based on BMI groups between women with GDM and women with NGT. We performed a comprehensive measurement of 11 known steroid hormones in the steroidogenic pathway between women with GDM and NGT using LC-MS/MS. We observed a decreased insulin sensitivity and hyperandrogenism in women with GDM compared with women with NGT. In pregnant women who were normal weight, we found a substantial alteration in androgen and estrogen synthesis between women with GDM and women with NGT. T/A4 representing 17β-hydroxysteroid dehydrogenase (17βHSD) activity increased significantly in women with GDM than in women with NGT (Pathway 1 in Figure 3). Interestingly, our results also demonstrated that the differential profile of steroid hormone is correlated with BMI in women with GDM. Specifically, in GDM women with overweight, the concentrations of E1, E2, E1/A4 and E2/T decreased significantly, representing decreased activity of cytochrome P450 aromatase (CYP19A1) in the steroidogenic pathway (Pathway 2 in Figure 3). Thus, our results shed new light on the occurrence of GDM from the perspective of steroid hormone metabolism in pregnant women with different BMI.

Disorders of steroid hormone metabolism have been associated with insulin metabolism. A cohort studies has

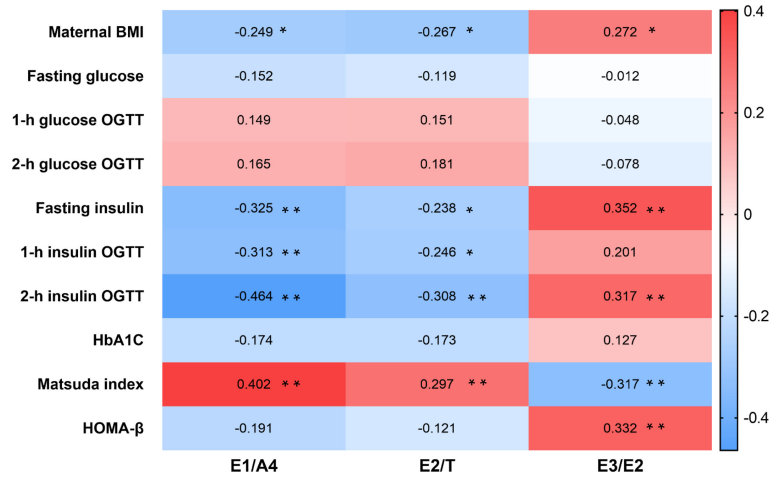

FIGURE 2
Heatmap of steroid hormones ratio and clinical characteristics in GDM patients. Correlation between steroid hormone ratio and clinical data in women with GDM using Spearman correlation analysis. *$p < 0.05$, **$p < 0.01$. GDM, gestational diabetes mellitus.

revealed increased serum T and DHEA levels in pregnant women with PCOS, who were always diagnosed with insulin resistance and higher BMI. Furthermore, a higher level of androgen has also been observed in GDM patients with insulin resistance (30, 31). Uzelac et al. found that women with GDM have higher serum androgen and lower estrogen levels than women without GDM in the third trimester of pregnancy. Their study suggests that owing to decreased conversion of T to estrogen and increased leptin production, the placenta of GDM patients has elevated levels of T and leptin. The underlying mechanism is that the androgen and leptin signaling pathways may be overactivated by the presence of excessive ligands and overexpressed receptors in the GDM placenta. Disorders of these two endocrine networks may lead

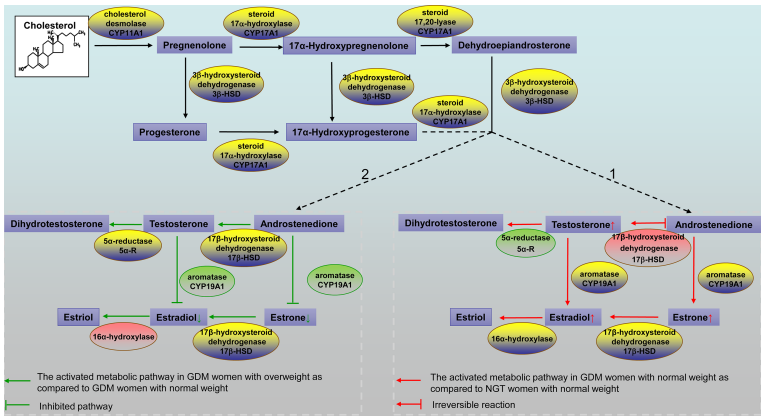

FIGURE 3
Schematic representation of the steroidogenic pathways. Pathway 1. The activated metabolic pathway in GDM women with normal weight compared to NGT women with normal weight is indicated by red arrows. Steroid hormones that showed a higher concentration in GDM women with normal weight are shown in the box with small red arrows. The red box indicates a higher product/precursor ratio in GDM women with normal weight. The green box indicates a lower product/precursor ratio in GDM women with normal weight. Pathway 2. The activated metabolic pathway in GDM women with overweight compared to GDM women with normal weight is indicated by green arrows. Steroid hormones that showed a lower concentration in women with GDM with overweight are shown in the box with small green arrows. The red box indicates a higher product/precursor ratio in GDM women with overweight. The green box indicates a lower product/precursor ratio in GDM women with overweight. GDM, gestational diabetes mellitus; NGT, normal glucose tolerance.

to placental abnormalities and maternal and fetal complications associated with GDM (32). Our study confirmed elevated serum DHEA, T, and A4 levels in women with GDM. However, a latest study reveals that serum T and E2 levels is reduced with the increasing of gestational age, while DHEA, A4 and E1 were found to be unrelated to GDM (33). This finding goes against our work. One probable reason is that our study is a one timepoint study. We can only note that steroid profile differed in women with GDM and women with NGT at 24–28 gestation weeks. Another possible reason is that the abovementioned studies had a small sample size and a larger, longitudinal cohort study is needed to validate their findings.

In pregnant women who were normal weight, women with GDM showed higher DHEA, T, E1, E2, and 17β-HSD activity than women with NGT. According to recent literature, T, E3, P5, and DHEA might be the differential metabolites for GDM. The genetic variants rs10046 of CYP19A1 and rs2257157 of 17βHSD isoform 3 could predispose to GDM in Chinese women (34). Additionally, we observed a reduced Matsuda index and HOMA-β in women with GDM, which was used to evaluate insulin sensitivity and the function of pancreatic β-cells. Studies in women with PCOS have reported that androgen excess predisposes to pancreatic β-cell dysfunction, indicating inadequate insulin release or an exaggerated insulin response to glucose. In addition, β-cell dysfunction was positively correlated with T concentration, independent of insulin resistance (35, 36). In mice, knockout of androgen receptors protects them from hyperinsulinemia and insulin resistance when exposed to chronic androgen excess (37). It is possible that androgen excess is associated with pancreatic β-cell dysfunction. Therefore, we speculated that excessive androgen synthesis may impair pancreatic β-cell function and reduce insulin sensitivity, resulting in hyperglycemia in GDM women with normal weight.

During pregnancy, the biochemical synthesis of steroid hormone including estrogens, 16α-hydroxylation, and aromatization requires interacting processing in the placenta, the fetal and maternal adrenal glands, and the fetal liver. This interdependent physiological entity is known as the feto-placental unit, which is involved in steroid hormone synthesis and metabolism (38). Within this unit, the fetal adrenal gland can synthesize steroid hormone precursors–DHEAS that can be used by the placenta to produce estrogens. DHEAS can be converted into 16αhydroxyDHEAS (16OHDHEAS) and 15,16OHDHEAS by 15αhydroxylase and 16α hydroxylase in the fetal liver. Maternal DHEAS is further catabolized by the placenta to E1 and E2, whilst the placenta converts 16OHDHEAS to E3, respectively (39). Fetal adrenal hypertrophy and DHEA production is promoted by adrenocorticotropic hormone (ACTH), which is secreted by the fetal pituitary gland. A previous study showed that pregnant women with an anencephalic fetus (in which levels of ACTH secreted from the fetal pituitary gland are markedly reduced), the levels of circulating E3 are very low as a result of impaired development of the fetal zone (40). Fetal adrenal hypoplasia is a rare condition that presents as marked low maternal serum levels of E3 during the second trimester (41). In our study, the last antenatal care recorded is normal for all subjects. In combination with stringent requirements for inclusion, differences in estrogen levels due to abnormal placental unit were excluded.

In the early second trimester of pregnancy, high concentrations of unconjugated E3 in the maternal serum have been considered to be a useful predictor of GDM development (42). However, our results revealed that different steroid hormone metabolism exist between GDM women with overweight and normal weight. GDM women with overweight showed a reduced in E1 and E2 levels, increased insulin levels, and decreased insulin sensitivity. Aromatase activity is related to estrogen generation in the placenta during pregnancy. We found that lower CYP19A1 activities was related to higher BMI and declined insulin sensitivity in GDM women with overweight. A previous finding indicates that aromatase availability in the amygdala is negatively associated with BMI. It also demonstrated that individual variations in the brain's capacity for estrogen synthesis may influence the risk of obesity and self-control (43). Previous findings have revealed a novel role for E2 in the regulation of energy metabolism and glucose homeostasis. Aromatase knockout mice have decreased E2 levels, accompanied by reduced glucose oxidation, elevated adiposity, and insulin levels (44, 45). E2 is an important antidiabetic steroid operating *via* binding to nuclear receptors as well as *via* modulation of ion channels controlling the secretion of pancreatic hormones (33, 46). Therefore, E2 deficiency in GDM women with overweight may be an important component participating in the pathophysiology of GDM.

In conclusion, NGT women and GDM women with normal weight presented with different steroid hormone profiles. Steroidogenic pathway profiling of sex hormone synthesis showed a significant increase in the production of DHEA, T, E1, and E2 in GDM women with normal weight. Additionally, the alteration of steroid hormone metabolism was related to maternal BMI in women with GDM, and GDM women with overweight showed reduced estrogen production and declined insulin sensitivity compared with GDM women with normal weight.

Our novel finding suggests that steroid hormone metabolic changes need to be considered in GDM development, especially in GDM patients with different BMI. We believe that our study makes a significant contribution to the GDM research. However, our study is limited by the small sample population and missed clinical outcomes, and a larger longitudinal cohort research is needed in the future to validate our results. In addition, more cellular protein mechanistic studies are needed for further study.

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 Women's Hospital School of Medicine, Zhejiang University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YS, BZ designed the study and drafted the manuscript. XM, BY collected the data. KW, YL performed statistical data analyses. DZo, JX performed steroid hormones measurement. XS, DZh and ZM contributed to revising the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors DZ and JX were employed by Hangzhou BIOZON Medical Laboratory Co.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.999154/full#supplementary-material

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ORIGINAL RESEARCH
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First Trimester Plasma MicroRNA Levels Predict Risk of Developing Gestational Diabetes Mellitus

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Aims: Our objective is to identify first-trimester plasmatic miRNAs associated with and predictive of GDM.

Methods: We quantified miRNA using next-generation sequencing in discovery (Gen3G: n = 443/GDM = 56) and replication (3D: n = 139/GDM = 76) cohorts. We have diagnosed GDM using a 75-g oral glucose tolerance test and the IADPSG criteria. We applied stepwise logistic regression analysis among replicated miRNAs to build prediction models.

Results: We identified 17 miRNAs associated with GDM development in both cohorts. The prediction performance of hsa-miR-517a-3p|hsa-miR-517b-3p, hsa-miR-218-5p, and hsa-let7a-3p was slightly better than GDM classic risk factors (age, BMI, familial history of type 2 diabetes, history of GDM or macrosomia, and HbA1c) (AUC 0.78 vs. 0.75). MiRNAs and GDM classic risk factors together further improved the prediction values [AUC 0.84 (95% CI 0.73–0.94)]. These results were replicated in 3D, although weaker predictive values were obtained. We suggest very low and higher risk GDM thresholds, which could be used to identify women who could do without a diagnostic test for GDM and women most likely to benefit from an early GDM prevention program.

Conclusions: In summary, three miRNAs combined with classic GDM risk factors provide excellent prediction values, potentially strong enough to improve early detection and prevention of GDM.

Keywords: biomarkers, epigenetics, next-generation sequencing, pregnancy, ribo-hormones, risk factors

INTRODUCTION

GDM is the most common pregnancy complication with a prevalence reaching up to 25% depending on ethnicity and the diagnostic criteria (1). Both the mother and her child are affected by GDM (2, 3). Mothers are at a higher risk of preeclampsia (PE), prolonged labor, and Caesarian section (2, 3). They are also at a higher risk of recurrent GDM in future pregnancies (~48%) (4) and of developing type 2 diabetes (T2D) within 5–10 years after delivery (~40%) (5). At birth, offspring are at increased risk of shoulder dystocia, prematurity, hypoglycemia, hyperinsulinemia, hyperbilirubinemia, respiratory distress syndrome, and macrosomia (2, 3, 6). They also have a higher risk of developing obesity (7), metabolic syndrome (7), T2D (8), and hypertension (9), both as children and as adults (2, 3). Treatment of GDM is effective in preventing pregnancy complications (10), but whether it prevents long-term consequences remains unclear (7, 8, 11, 12).

GDM is usually diagnosed between the 24th and 28th week of pregnancy (13). Early identification of women at a higher risk of GDM could thus help improve follow-up and prevent long-term complications as recommended by the World Health Organization (WHO) Commission on Ending Childhood Obesity (14). Lately, circulating microRNAs (miRNAs) showed promise in identifying women with GDM. However, few studies were conducted in the first trimester (15–20), and those that were had a small number of participants, or did not offer replication in independent cohort(s). Most studies also applied a targeted approach, testing selected miRNAs limiting the possibilities of novel discovery. This limitation could be overcome using a non-targeted method such as next-generation sequencing.

miRNAs are short single-stranded RNA molecules (19–24 nucleotides) involved in post-transcriptional gene expression regulation through binding to their target messenger RNAs (mRNAs) (21). MiRNAs are stable in blood and other biologic fluids (21). Several physiological mechanisms are regulated by miRNAs including glucose homeostasis, fetal growth, and development (22). Because circulating miRNAs can regulate gene translation in distant cells, they can be considered "ribo-hormones". MiRNAs from three clusters [chromosome 14 miRNA cluster (C14MC), chromosome 19 miRNA cluster (C19MC), and miR-371-3] are largely expressed by the trophoblasts in the placenta (22). C14MC miRNAs are more abundantly expressed at the beginning of the pregnancy, whereas their levels gradually decrease as the pregnancy progresses (22). Those expressed from the C19MC cluster on chromosome 19 progressively increase during pregnancy (22). Interestingly, placental miRNAs are secreted into maternal circulation suggesting they might contribute to fetal-maternal communication (23).

We hypothesized that the plasmatic microtranscriptomic profile in the first trimester of pregnancy is dysregulated in women who subsequently developed GDM. We also tested if some miRNA could help distinguish women at a higher risk of future GDM. Our objectives were thus to identify plasmatic miRNAs measured in the first trimester of pregnancy associated with and predictive of GDM development. We will also explore their implication in the pathophysiology of GDM with biological pathways analyses.

Abbreviations: BMI, Body mass index; C14MC, Chromosome 14 microRNA cluster; C19MC, Chromosome 19 microRNA cluster; CI, Confidence interval; ECM, extracellular matrix; L2FC, Log2 Fold change; FDR, False discovery rate; GCT, Glucose challenge test; GDM, Gestational diabetes mellitus; Gen3G, Genetics of Glucose regulation in Gestation and Growth; GH, Gestational hypertension; HbA1c, Glycated hemoglobin; IADPSG, International Association of the Diabetes and Pregnancy Study Groups; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, MicroRNA; mRNA, Messenger RNA; npv, negative predictive value; OGTT, Oral glucose tolerance test; PE, Preeclampsia; ppv, positive predictive value; ROC, receiver operating characteristic; rRNA, Ribosomal RNA; T2D, Type 2 diabetes; WHO, World Health Organization.

MATERIALS AND METHODS

Study Participants

Participants were selected from the Genetics of Glucose regulation in Gestation and Growth (Gen3G) prospective pregnancy and early life cohort, which aims to improve our comprehension of the mechanisms implicated in glucose regulation during pregnancy and fetal growth (24). Included women were ≥18 years old, with a singleton pregnancy and not taking medication affecting glucose tolerance, whereas women with diabetes (reported or detected by glucose or A1c screening) at their first trimester visit (between the 4th to 16th week of pregnancy) were excluded. A total of 854 women were followed until delivery and had a complete 75-g oral glucose tolerance test (OGTT) between the 24th and 29th week of pregnancy. International Association of Diabetes and Pregnancy Study Groups (IADPSG) guidelines (13) were applied in GDM diagnosis. For this study, we selected the 444 women of European descent (only a very small number of women were of non-European descent in Gen3G and were as thus too small to consider including them in this discovery step analysis) for which a plasma sample (500 µl) collected at the first trimester of pregnancy, and with follow-up visits at 3 or 5 years postpartum, was available. A total of 56 women developed GDM. The ethics review board of *CIUSSS de l'Estrie-CHUS* approved the study and all participants provided informed written consent.

Participants in the replication cohort were selected from the 3D cohort which was described previously (25). We selected women of European descent (women of non-European descent were excluded to ensure that this replication step is valid when compared to the discovery step using Gen3G) with plasma samples collected at the first trimester of pregnancy and complete OGTT data performed between the 24th and 28th week of pregnancy. Women with a diagnosis of pre-gestational diabetes (either Type 1 or 2) or chronic hypertension without further PE diagnosis at the first trimester of pregnancy were excluded. A total of 148 eligible women, 76 with GDM, were sampled and included in this analysis. In the 3D cohort, women were recruited at multiple university hospital centers where different GDM diagnosis procedures and criteria were applied.

For the purpose of this study and to improve harmonization with Gen3G, we applied the IADPSG criteria retrospectively to categorize GDM status (including only women with complete OGTT data for both GDM and non-GDM categories).

RNA Extraction and Library Preparation

RNA extraction and library preparation were described in the work of Légaré et al. (26). Briefly, we used the standard protocol of the MirVana PARIS kit (Thermo Fisher Scientific, catalog # AM1556) for total RNA extraction from 500 µl of plasma stored at −80°C until processing. Plasma samples were collected between the 4th and 16th week of pregnancy and randomly ordered before extraction. Total RNA was eluted in 75 µl of nuclease-free water and then precipitated with ammonium acetate and ethanol and resuspended in 5 µl of RNAse-free water as described by Burgos et al. (27). RNA samples were then randomized again before library preparation. We applied the standard protocol of the TruSeq Small RNA Sample Prep kit (Illumina, BC, Canada; catalog # RS-200-0012) adapted by Burgos et al. (27). Only half of the recommended reagents volumes for ligation (3′ and 5′ ends) of RNA samples (5 µl), reverse transcription, barcoding (index 1–48: one index per sample), and PCR amplification (15 cycles) were used to ensure the optimal ratio between reagents and RNA amount. The libraries were re-suspended in 25 µl of 10 mM tris-HCl (pH 8.5) buffer.

Library Quality Control and Sequencing

As reported previously (26), for Gen3G miRNAs sequencing, the McGill University and Génome Québec Innovation Centre (Québec, Canada) performed the library quality control, quantification, pooling, and sequencing of the replication samples as well. Quality control of the libraries (verification of concentration, library length, and the absence of primer dimers) was done with either the Agilent High Sensitivity DNA Kit (Agilent, Mississauga, ON, Canada; catalog #5067-4626) on the Agilent 2100 Bioanalyzer or the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems; concentration) and the LabChip GX instrument (PerkinElmer, catalog# CLS760672; library length and absence of primer dimers). Quantitative PCR (qPCR) was used for library quantification.

For Gen3G samples, the libraries were equimolarly pooled (HiSeq 2500: 7 pM final molarity; 12 libraries with different indexes per lane; HiSeq 4000: 10 pM final molarity; 20 libraries with different indexes per lane), denatured, and clustered on single-read Illumina flow cells (catalog #GD-401-3001 and catalog #GD-410-1001) following the manufacturer's protocol. Either an Illumina HiSeq 2500 or HiSeq 4000 sequencing platform with 50 cycles, and seven cycles indexing read, was used for sequencing. Twelve samples were extracted twice and sequenced on the two platforms. MiRNA levels were highly correlated (i.e., Pearson's correlation coefficient ≥0.94) (26) confirming that the sequencing results obtained on both platforms do not vary significantly. For the replication cohort, the Illumina NovaSeq platform was used. Each library pool (48 libraries per lane) was loaded at 225 pM on an Illumina NovaSeq S1 lane using the Xp protocol as recommended by the manufacturer. The run was performed for 1 × 100 cycles (single-end mode).

Bioinformatics Analysis of the Sequencing Data

The extracellular RNA processing toolkit (exceRpt) pipeline version 4.6 from Rozowsky et al. was used in the analysis of our sequencing data (28). Briefly, exceRpt removes the sequences of the adapters and bad quality reads (Phred score <20 for 80% or more of the read) with FASTX-Toolkit (28). Remaining sequences were mapped to the human genome (GRCh37) and miRbase version 21 using STAR (28). Although exceRpt was optimized for low concentration small RNA analysis, we decided not to exclude the reads mapping to the ribosomal RNAs (rRNAs) to reduce computation time because there was very little contamination by rRNA sequences in our samples (average of 1.04 ± 0.90% of total reads mapped) (26). Visualization of raw read counts was used to identify and exclude eight outlier samples in the Gen3G (seven with <500,000 and one with >25 million miRNAs reads), and nine samples with <1 million miRNAs reads in the 3D.

Statistical Analysis

Participants' characteristics were compared with the Kruskal–Wallis test and a Dunn's *post-hoc* test with Bonferroni correction or Mann–Whitney U-test if only two groups were compared. The DESeq2 R package (29) was used to identify miRNAs associated with GDM. Default parameters were applied, including Wald test to assess differential expression as well as the collapseReplicates function to combine read counts from samples that were sequenced twice (n = 12). The associations between miRNAs and GDM were adjusted for the sequencing run and lane as well as gestational age at the time of plasma collection and a nominal p-value <0.05 was considered significant. Results were considered replicated in the 3D when the fold changes were in the same direction with nominal one-sided p-values <0.05. The EnhancedVolcano package was used to produce volcano plot (30).

Prediction Modeling Analyses

Gen3G samples were randomly assigned to training (70%) and test (30%) sets. We used data from the 3D as an external replication cohort. Since about twice more read counts were obtained in the 3D as compared to the Gen3G cohort, normalized DESeq2 counts were z-score–transformed to allow applying the same GDM prediction equation to both cohorts. This strategy improves the robustness of the replication. Stepwise logistic regression analyses using only replicated miRNAs were applied on the training set to select the miRNAs independently associated with GDM. To assess whether miRNAs improve GDM prediction value over that of the GDM classic risk factors, logistic regression analyses were also conducted with classical risk factors for GDM (maternal age, BMI, familial history of T2D, history of GDM, or macrosomia) and biomarkers [HbA1c, 1-h post–50-g glucose challenge test (GCT) glucose levels] with or without miRNA levels. The

pROC package (31) was applied to build receiver operating characteristic (ROC) curves on either the remaining of the Gen3G cohort (30% test set) or the 3D cohort, to assess specificity and sensitivity of the test and to compare ROC curves using the DeLong test. All statistical analyses were performed in R version 4.0.2 in RStudio-server version 1.2.1335.

KEGG Pathway Analysis

The mirPath v.3 software (32) was used for a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the miRNAs associated with GDM in both cohorts. Analyses were restricted to experimentally validated miRNA:mRNA interactions (Tarbase v7.0 database) (33). The default settings of mirPath v.3 were applied including a p-value threshold of 0.05, application of a false discovery rate (FDR) correction and the Fisher's exact test (hypergeometric distribution) for enrichment analysis. The pathway union parameters were employed to merge results.

RESULTS

Participants' Characteristics

Characteristics of study participants from both the discovery cohort (Gen3G) and the replication cohort (3D) are shown in **Table 1**. Women from the 3D were ~2 years older and had their first visit about 2 weeks later on average in their pregnancy compared to women from the Gen3G [mean: 11.9 (controls) and 11.9 (GDM) for 3D vs 9.7 (controls) and 9.5 (GDM) for Gen3G]. Women with and without GDM from both cohorts had similar BMI.

Identification of the miRNAs Associated With GDM

A total of 2,170 plasmatic miRNAs were identified in Gen3G (discovery cohort; mean = 2,484 reads ± 71,800). These results were previously reported in detail (26). In Gen3G, we found 73 miRNAs nominally associated with GDM ($p < 0.05$). Of these, 20 were upregulated [$\log_2$ fold changes (L2FC) between 0.187 and 0.788] in women with GDM compared to normoglycemic women, whereas 53 were downregulated (L2FC between −1.01 and −0.121) (**Figure 1**). **Supplementary Table 1** shows the complete list of miRNAs, their mean of normalized read counts, and the percentage of women in which they were detected both in the GDM and normoglycemic groups as well as their fold change, nominal p-value and FDR q-value. One miRNA called hsa-miR-517a-3p|hsa-miR-517b-3p passed our FDR threshold (q-value < 0.1). This miRNA was 1.8 times (L2FC= −0.862) less abundant in women with GDM as compared to normoglycemic women. The findings for hsa-miR-517a-3p|hsa-miR-517b-3p were replicated in the 3D cohort (L2FC = −0.409 p = 0.023). A total of 16 additional miRNAs (23% of the total miRNAs were associated with GDM in the Gen3G) had a fold change in the same direction (lower values in GDM groups) and nominal p-value <0.05 in our replication cohort 3D (**Table 2**). The concordance in direction and nominal p-value threshold was the criteria for inclusion in the list of miRNAs considered for GDM prediction modeling (next step).

Plasma miRNAs Improve GDM Prediction

We first performed a stepwise analysis on the Gen3G training set using the 17 miRNAs associated with GDM in both cohorts. Three miRNAs were retained in the model: hsa-miR-517a-3p|hsa.miR-517b-3p, hsa-miR-218-5p, and hsa-let-7a-3p (**Table 3**). When tested in the Gen3G test set, these three miRNAs combined provided good discrimination with an area under the curve (AUC) of 0.78 [95% confidence interval (CI) 0.62–0.94; **Figure 2A**]. This discrimination level was similar to any combination of classic GDM risk factors without miRNAs in the Gen3G (**Figure 2B**). By sequentially adding the classic GDM risk factors and available biomarkers to the miRNA model, the overall prediction model reached up to an AUC of 0.90 (95% CI 0.83–0.97; **Figure 2A**). In addition to the three miRNAs, this model included maternal age, BMI, family history of T2D, history of GDM or macrosomia, HbA1c, and glucose levels 1-h post–50-g GCT at the first trimester. A model excluding glucose levels from the GCT (rarely used in clinical practice at the first trimester) provided an AUC of 0.84 (95% CI 0.73–0.94).

Models considering miRNAs alone or their addition to GDM classical risk factors that provide higher specificity than models with classical risk factors as shown by their ROC curves are shifted to the left. The model including miRNAs, maternal age, BMI, family history of T2D, history of GDM or macrosomia, and HbA1c is the one that would be easier to implement in clinical settings. Based on this model, we suggest thresholds which identify women at a very low or higher risk of GDM. For the low-risk threshold, the goal was to exclude, with a margin, all women that developed GDM, whereas the higher risk group had to include as much as possible GDM cases while retaining an acceptable sensitivity. For the low risk, we have set the threshold at 0.036 for a specificity of 0.393 and a sensitivity of 1 (35% of women had lower values, none developed GDM): the first woman who developed GDM was ranked 49 (or 40.5%) of the 121 participants. The high-risk threshold was set at 0.269 for a specificity of 0.907 and a sensitivity of 0.571 (15% of women had higher values and 44% of them had developed GDM). Combining the high- and low-risk thresholds will leave 50% of women that will need to undergo GDM screening between the 24th and 28th week of pregnancy. These thresholds and the number of women in each group are presented in **Figure 3**.

We also applied the same equation (built on the training set of the Gen3G cohort) to 3D samples as part of our planned replication step. In the 3D, the only GDM classic risk factors available were age, BMI, and family history of T2D; together, they offer a model with a modest predicting value (AUC = 0.588; CI= 0.490–0.685). Predicting models using the three selected miRNAs combined (alone or in addition to classic risk factors) in the 3D offer predicting ability greater than chance alone (AUC ~0.67; **Figure 2C**). However, the performance of the prediction models in the 3D was overall less convincing when compared to the Gen3G results (by the AUCs).

TABLE 1 | Characteristics of study participants from the Gen3G and 3D (replication) cohorts.

Characteristics	Controls Gen3G (380) Mean ± SD (Range)	GDM Gen3G (56) Mean ± SD (Range)	Controls 3D (63) Mean ± SD (Range)	GDM 3D (76) Mean ± SD (Range)	Difference between groups[a]
First trimester variables					
Age (years)	28.32 ± 4.0 (18–41)	29.71 ± 5.6 (21–47)	31.37 ± 4.5 (23–45)	31.76 ± 4.6 (20–42)	b, c
Body mass index (kg/m^2)	25.63 ± 5.7 (16.10–54.10)	28.16 ± 7.3 (17.80–47.10)	25.03 ± 5.3 (18.14–45.49)	27.40 ± 7.1 (16.77–47.22)	NS
Gestational age (weeks)	9.65 ± 2.2 (4.10-16.30)	9.51 ± 2.8 (5.10–15.10)	11.90 ± 1.8 (5.57–14.86)	11.94 ± 1.3 (8.43–15.57)	b, c
HbA1c (%)[d]	5.20 ± 0.3 (2.9–6.1)	5.37 ± 0.3 (4.7–5.9)	NA	NA	f
HbA1c (mmol/mol)	33.33 ± 3.1 (8–43)	35.22 ± 3.1 (28–41)	NA	NA	f
1-h post-GCT glycemia (mmol/L) [d]	5.51 ± 1.4 (2.6–10.0)	6.70 ± 1.3 (4.5–10.2)	NA	NA	f
Second trimester variables					
Gestational age (weeks)	26.41 ± 1.0 (24.10–29.40)	26.32 ± 1.0 (24.30–28.20)	27.35 ± 1.3 (24.86–30.00)	27.12 ± 1.5 (24.43–29.71)	b, c
Fasting OGTT glycemia (mmol/L)	4.17 ± 0.3 (3.4–5.0)	4.66 ± 0.6 (3.7–7.3)	4.47 ± 0.3 (3.7–5.0)	5.02 ± 0.6 (3.8–6.6)	b, c, e, f,
1-h post-OGTT glycemia (mmol/L)	6.90 ± 1.4 (3.6–9.9)	9.85 ± 1.4 (6.3–13.0)	8.05 ± 1.3 (4.7–9.9)	10.04 ± 1.2 (7.6–12.3)	b, e, f
2-h post-OGTT glycemia (mmol/L)	5.61 ± 1.1 (3.0–8.3)	8.24 ± 1.3 (4.9–11.4)	6.43 ± 1.1 (3.8–8.4)	8.27 ± 1.3 (5.0–12.0)	b, e, f

[a]*Kruskal–Wallis test and a Dunn's post-hoc test with Bonferroni correction (results were considered significant at Bonferroni adjusted p-value < 0.025 or Mann–Whitney U-test if only two groups were compared).*
[b]*Controls from 3D vs. controls from Gen3G.*
[c]*GDM from 3D vs. GDM from Gen3G.*
[d]*Data available only for 51 GDM and 352 controls.*
[e]*Controls from 3D vs. GDM from 3D.*
[f]*Controls from Gen3G vs. GDM from Gen3G.*
GCT, glucose challenge test; GDM, Gestational diabetes mellitus; NA, Not applicable; NS, not significant; OGTT, 75-g oral glucose tolerance test; SD, standard deviation.

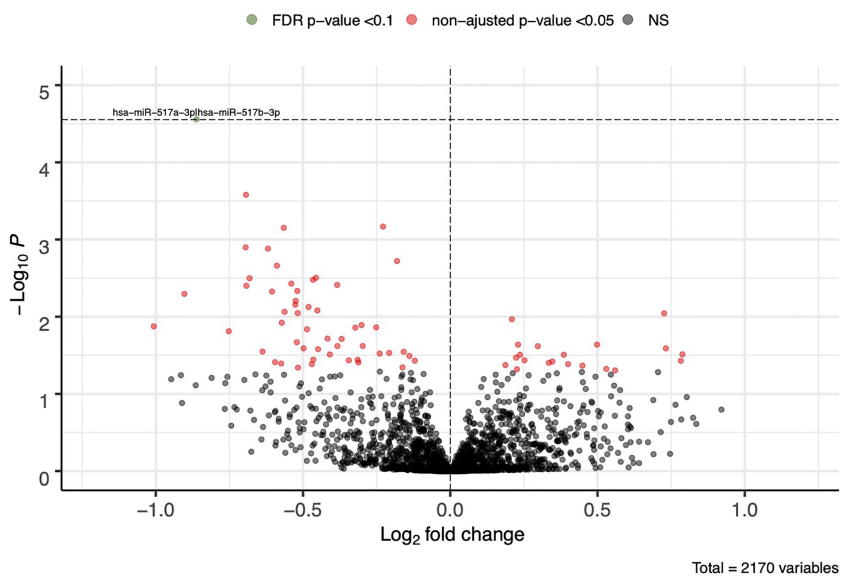

FIGURE 1 | First trimester plasmatic miRNAs associated with GDM. Fold change represents the change in plasmatic miRNA abundance in GDM compared to normoglycemic women. Model adjusted for sequencing runs and lanes as well as gestational age. FDR cutoff of 0.1 is represented by a horizontal dotted line. FDR, false discovery rate; NS, non-significant.

Biological Pathways Potentially Regulated by the miRNAs Associated with GDM

First, we have identified 16 KEGG pathways targeted by the 17 miRNAs associated with GDM in both cohorts. This list of identified pathways is presented in **Figure 4** with the top three pathways related to lipid metabolism and extracellular matrix (ECM) receptor interaction pathways.

Moreover, in another study in the Gen3G (same data set), we have recently identified miRNAs associated with insulin sensitivity estimated with the Matsuda index between the 24th and 29th week of pregnancy (34). Of the 17 miRNAs that we found associated with GDM, 10 were positively associated with insulin sensitivity: they are all decreased in GDM women in the current study (**Figure 5**).

DISCUSSION

In this study, we have used comprehensive and replicated microtranscriptomic data from large cohorts of plasma

TABLE 2 | First trimester plasmatic miRNAs associated with GDM in both Gen3G and 3D cohorts.

miRNAs	Gen3G controls		Gen3G GDM		Gen3G		3D controls		3D GDM		3D	
	% women	Mean ± SD	% women	Mean ± SD	L2FC	P-value	% women	Mean ± SD	% women	Mean ± SD	L2FC	P-value
hsa-miR-517a-3p| hsa-miR-517b-3p[a]	96.58	21.33 ± 26.07	87.50	12.41 ± 12.51	−0.862	2.76E-05	96.83	60.39 ± 46.20	98.68	41.40 ± 38.45	−0.409	0.023
hsa-miR-141-3p	100.00	154.42 ± 255.27	100.00	98.18 ± 62.21	−0.566	0.0007	100.00	477.57 ± 326.66	100.00	379.41 ± 416.74	−0.346	0.029
hsa-miR-519c-3p[a]	92.37	11.82 ± 13.41	71.43	6.04 ± 6.10	−0.695	0.0013	95.24	33.27 ± 29.49	86.84	24.75 ± 29.52	−0.474	0.036
hsa-miR-520a-3p[a]	99.47	91.18 ± 107.60	98.21	57.66 ± 55.98	−0.619	0.0013	98.41	180.89 ± 158.19	100.00	144.28 ± 146.03	−0.395	0.027
hsa-miR-1323[a]	100.00	149.59 ± 167.60	98.21	124.97 ± 104.80	−0.589	0.0022	100.00	610.56 ± 445.66	100.00	435.49 ± 390.55	−0.478	0.005
hsa-miR-524-5p[a]	88.16	9.12 ± 10.32	73.21	5.64 ± 5.92	−0.682	0.0032	96.83	47.58 ± 46.95	94.74	31.65 ± 40.53	−0.555	0.005
hsa-miR-516b-5p[a]	99.74	105.22 ± 101.37	96.43	77.36 ± 60.26	−0.540	0.0037	100.00	293.70 ± 207.06	100.00	228.52 ± 185.46	−0.343	0.029
hsa-miR-218-5p	76.32	6.34 ± 14.87	51.79	2.07 ± 3.84	−0.903	0.0051	90.48	11.60 ± 16.16	69.74	8.75 ± 15.29	−0.719	0.011
hsa-miR-429	97.11	14.09 ± 18.05	89.29	9.47 ± 7.21	−0.481	0.0075	95.24	29.28 ± 31.61	94.74	22.25 ± 23.97	−0.380	0.043
hsa-miR-516a-5p[a]	97.37	32.78 ± 35.83	91.07	21.24 ± 17.90	−0.518	0.0090	98.41	126.45 ± 108.98	98.68	92.73 ± 93.42	−0.348	0.047
hsa-miR-196a-5p	95.26	8.40 ± 7.07	91.07	7.16 ± 4.65	−0.369	0.0194	84.13	7.94 ± 7.29	76.32	5.99 ± 6.36	−0.415	0.047
hsa-miR-215-5p	100.00	578.70 ± 914.43	100.00	413.62 ± 236.44	−0.383	0.0240	100.00	1120.92 ± 880.97	100.00	857.98 ± 769.45	−0.357	0.023
hsa-miR-515-3p[a]	65.53	3.32 ± 4.81	50.00	1.82 ± 2.26	−0.638	0.0283	85.71	13.68 ± 15.29	71.05	10.35 ± 17.08	−0.632	0.020
hsa-miR-424-5p	99.74	49.62 ± 29.91	100.00	33.79 ± 20.65	−0.240	0.0300	98.41	117.75 ± 83.98	100.00	87.02 ± 63.01	−0.275	0.036
hsa-let-7a-3p	100.00	93.18 ± 27.58	100.00	71.68 ± 32.80	−0.139	0.0323	100.00	199.54 ± 93.99	100.00	166.08 ± 73.42	−0.181	0.037
hsa-miR-525-5p[a]	90.26	9.41 ± 10.98	80.36	6.98 ± 7.16	−0.465	0.0360	98.41	73.74 ± 64.36	93.42	50.03 ± 67.23	−0.445	0.022
hsa-miR-518f-5p[a]	66.58	2.78 ± 3.42	51.79	1.63 ± 2.12	−0.574	0.0405	90.48	20.68 ± 20.59	86.84	14.37 ± 15.43	−0.500	0.018

[a]*C19MC miRNAs. Fold change represents difference in miRNA abundance in GDM compared to controls. % women, percentage of women for which the miRNA was detected (at least one normalized read count); C19MC, Chromosome 19 miRNA cluster; GDM, Gestational diabetes mellitus; L2FC, log2 fold changes; p-value, nominal p-value; Mean ± SD, mean and standard deviation of DESeq2 normalized reads counts.*

TABLE 3 | Stepwise logistic regression model of miRNAs predicting GDM in the Gen3G training set.

Coefficients	Estimate	Std.Error	z value	Pr(>|z|)
Intercept	-2.8602	0.3609	-7.926	2.27e-15
hsa-miR-517a-3p-has-miR-517b-3p[a]	-0.7861	0.4387	-1.792	0.0732
hsa-miR-218-5p[a]	-2.4198	1.1005	-2.199	0.0279
hsa-let-7a-3p[a]	-0.5261	0.2067	-2.546	0.0109

[a]*z-score of DESeq2 normalized reads counts.*

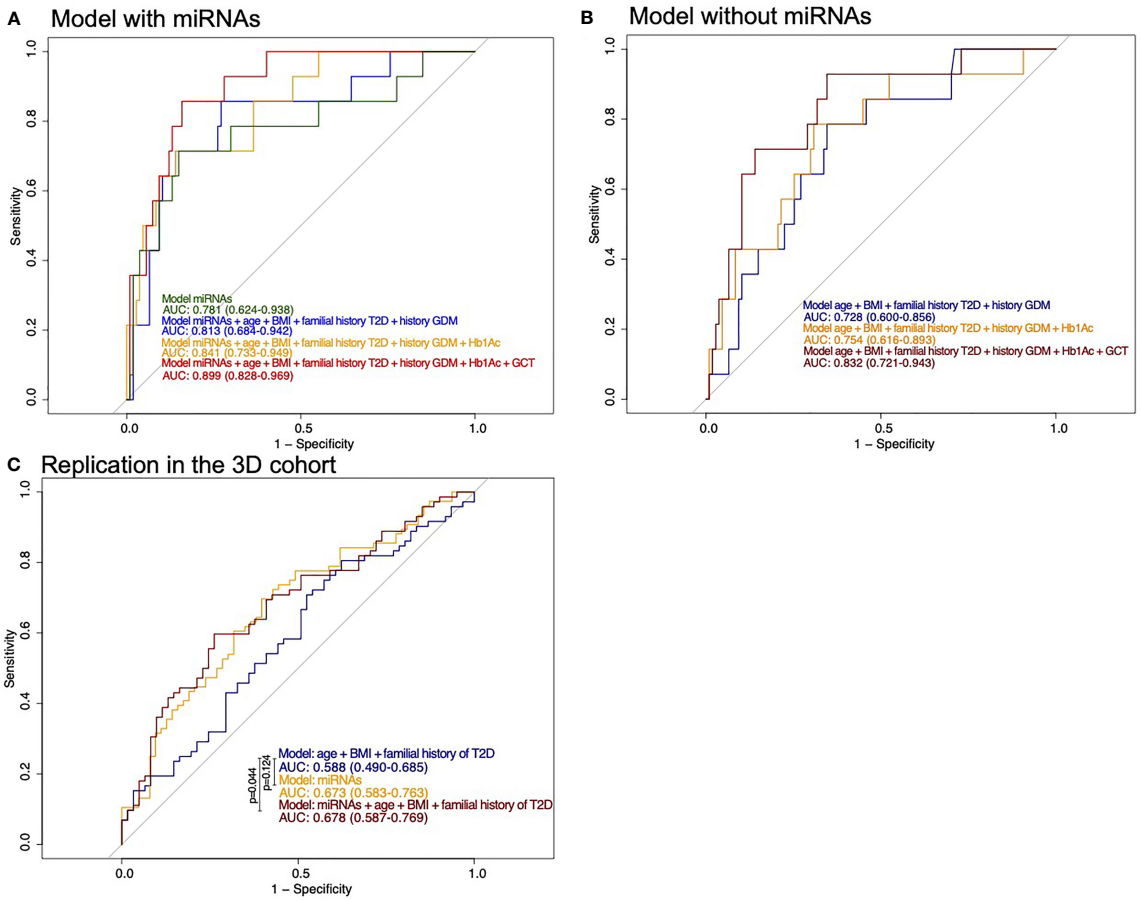

FIGURE 2 | ROC curves for prediction of the risk of developing GDM. **(A)** Models including miRNAs as well as classical risk factors and biomarkers of GDM in the Gen3G cohort test set. History of GDM also includes history of macrosomia. **(B)** Models with classical risk factors and biomarkers of GDM in the Gen3G cohort test set. History of GDM also includes history of macrosomia **(C)** Models including miRNAs as well as classical risk factors of GDM in the replication cohort (3D). They are compared with the DeLong test. BMI, body mass index; GCT, 1-h post–50-g glucose challenge test value; GDM, Gestational diabetes mellitus; HbA1c, Glycated hemoglobin; T2D, Type 2 diabetes.

samples collected before the 16th week of pregnancy to identify miRNAs associated with and predictive of GDM. In brief, 17 miRNAs were associated with GDM in both cohorts, and hsa-miR-517a-3p|hsa-miR-517b-3p passed pre-specified significance threshold in the Gen3G (FDR $q < 0.1$) and was replicated in the 3D cohort. A prediction model with three miRNAs showed good discrimination of women at higher risk to develop GDM 3 months later, which was improved by the addition of GDM classic risk factors and two classic glycemic biomarkers.

The model based only on miRNAs performed generally well—similar to the GDM classic risk factors alone with or without HbA1c and glucose levels 1-h post-GCT. The best model (AUC ~0.90) combined miRNAs, age, BMI, familial history of T2D, history of GDM or macrosomia, HbA1c, and 1-h post–50-g GCT values. If we exclude the GCT which is rarely performed in the first trimester, the model that only included miRNAs, age, BMI, family history of T2D, history of GDM or macrosomia, and HbA1c values provides also a very strong predictive value (AUC ~0.84) and would be easier to translate into the clinical setting.

Interestingly, the comparison of the various models supports that miRNAs have more impact on the specificity of the models when compared to GDM classical risk factors alone or combination with its biomarkers. Another study used clinical measures as well as candidate biomarkers (HbA1c, random glucose, fructosamine, sex hormone binding globulin, adiponectin, and triglycerides) to predict women at risk of GDM and made several models including different variables to obtain the best prediction that could also be translated in a clinical setting (35). Contrary to our study, they only included obese women in their analyses, which is itself a risk factor for GDM. Nevertheless, their models had lower AUC (35) than ours, with their best model having an AUC of 0.77.

We have also identified thresholds that could be useful to identify women at a very low and high risk of GDM. We fully understand that the algorithm and the suggested thresholds must

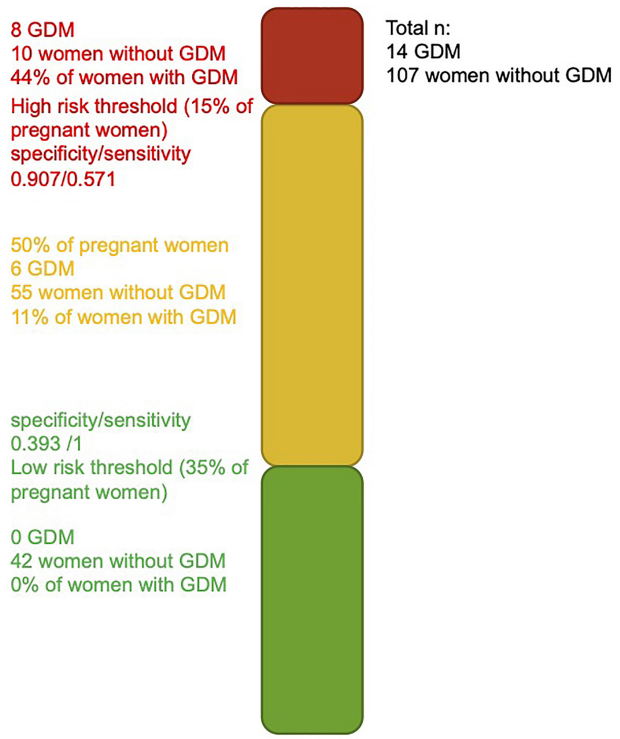

FIGURE 3 | Threshold for identification of women at higher and lower risk of developing GDM. Green and red bars represent the women at a very low and higher risk of developing GDM.

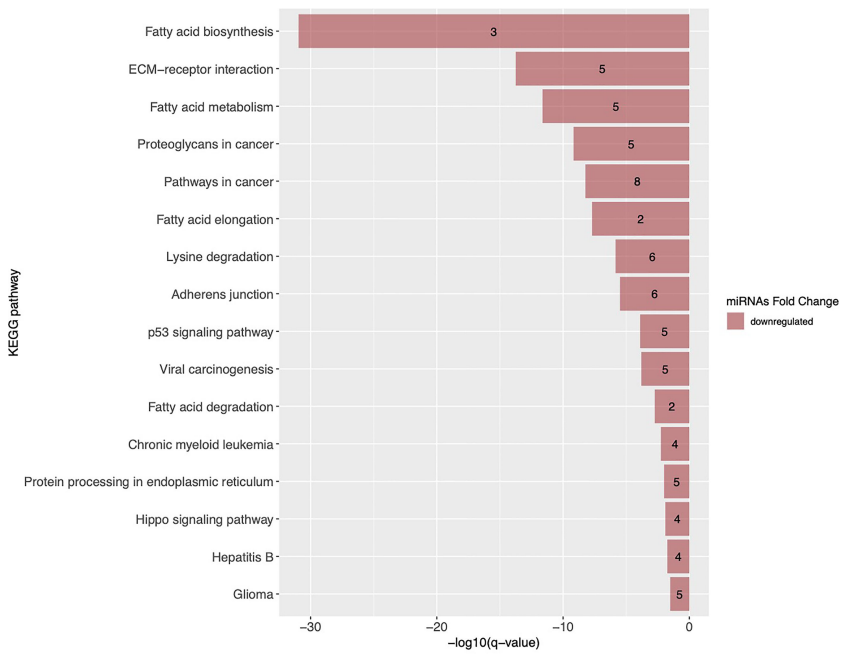

FIGURE 4 | KEGG pathways targeted by miRNAs associated with GDM in both Gen3G and 3D cohorts. KEGG pathways are ranked by their FDR adjusted q-value. Pathways enriched with miRNAs negatively associated with GDM are shown as red bars and the number inside each bar represents the number of miRNAs regulating the pathway. ECM, extracellular matrix; KEGG, Kyoto Encyclopedia of Genes and Genomes.

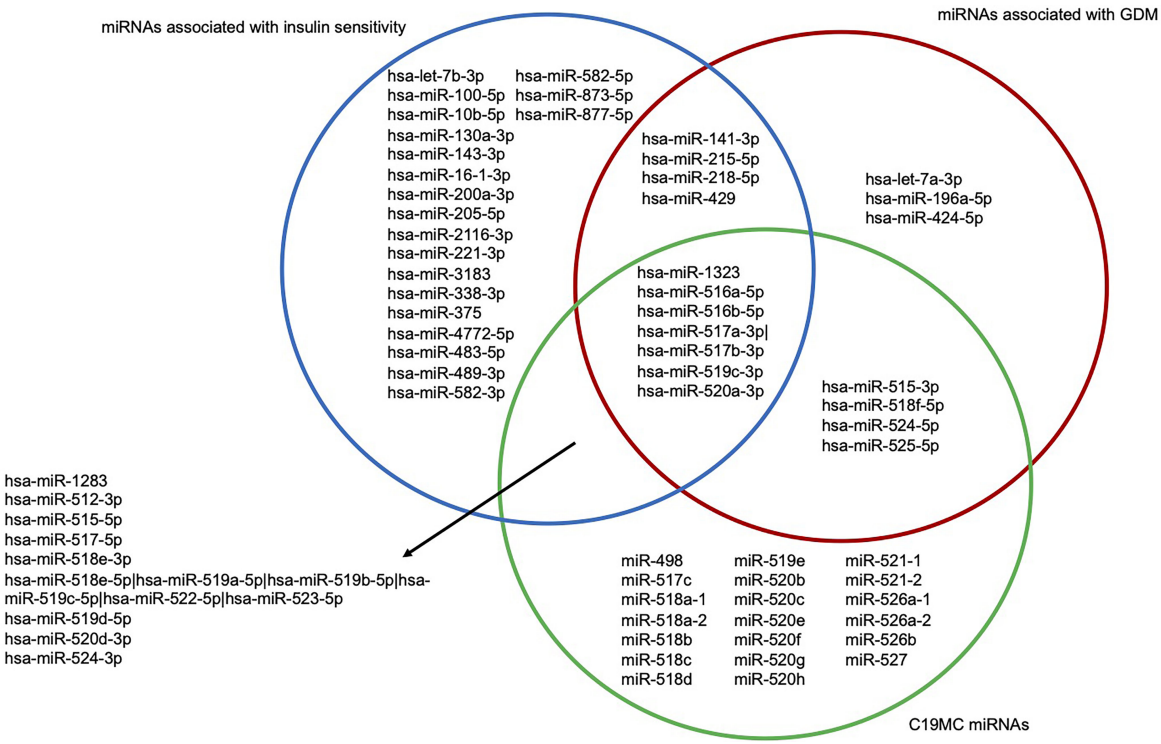

FIGURE 5 | Venn diagram of first trimester miRNAs associated with GDM and insulin sensitivity as well as being expressed from the C19MC. Blue circle represents miRNAs associated with insulin sensitivity assessed with the Matsuda index between the 24th and 29th week of pregnancy. Red circle represents miRNAs associated with GDM. Green circle represents C19MC miRNAs. C19MC, Chromosome 19 miRNA cluster; GDM, Gestational diabetes mellitus.

be validated in similar populations and replicated in populations of different ethnic origins, but, in principle, they could be used to identify women who could do without a diagnostic test for GDM and women most likely to benefit from an early GDM prevention program. The costs for the early prevention program would be partially borne by the savings made by not carrying out GDM diagnostic test in 35% of pregnant women, increasing feasibility of such program. Combining the two thresholds, only 50% of the women remain in the "gray" zone for which standard GDM screening between the 24th and 28th week of pregnancy will be needed.

Indeed, recently, there is a growing interest in using miRNAs to identify women with GDM earlier than the diagnosis that is currently used. So far, we did find nine studies (15–20, 36–38), of which six (15–20) were conducted during the first trimester of pregnancy. Three studies (15, 18, 20) also used the miRNAs they identified to predict women with GDM and two of them (15, 20) included a replication step. One study found urine hsa-miR-517a-3p|hsa-miR-517b-3p predictive of GDM but only in the second trimester of pregnancy and in the opposite direction than in the current study (19). This discrepancy could be explained by the analysis of urine instead of plasma and the timing of sampling (second vs. first trimester) as hsa-miR-517a-3p|hsa-miR-517b-3p is part of a cluster showing increasing expression from the first trimester to the end of pregnancy (19, 22). In a previous study from our group in the Gen3G, hsa-miR-517a-3p|hsa-miR-517b-3p (and hsa-miR-218-3p) measured in the first trimester was positively associated with Matsuda-insulin sensitivity index assessed between the 24th and 29th week of pregnancy (34). Hsa-miR-517a-3p|hsa-miR-517b-3p was found downregulated in women with GDM, suggesting that its expression is insufficient to exert its potential effects on insulin sensitivity and counterbalance the insulin resistance state in pregnancy. Hsa-miR-517a-3p|hsa-miR-517b-3p has been found to be downregulated in the placentas of diabetic women with fetal macrosomia (39), which is consistent with our results. Our results are in line with Santovito et al. (40) who found downregulation of let-7a in plasma of non-treated T2D patients and that its levels were increased 12 months after treatment initiation.

The exact mechanisms involved in gestational insulin resistance and GDM development in pregnancy remain poorly understood. Lipid metabolism–related pathways are of particular interest because early pregnancy is characterized by a lipogenic profile allowing to store nutrients that will be later used to meet the metabolic demand of both the mother and her growing fetus during pregnancy (41). By targeting fatty acid biosynthesis and metabolism, some pregnancy-associated miRNAs (5 miRNAs out of 17) could play a role in the regulation of the lipid metabolism pathways which might have be related to decreasing insulin sensitivity later in pregnancy.

Moreover, 10 of the 17 miRNAs that we found to be less abundant in women with GDM were also positively associated with insulin sensitivity assessed between the 24th and 29th week of pregnancy (34). In brief, our miRNAs could be implicated in the pathophysiology of GDM through their roles in lipid metabolism and insulin sensitivity regulation.

Remarkably, 10 of the 17 miRNAs associated with GDM were from the C19MC, a miRNA cluster mainly expressed in the placenta (trophoblasts) (22) that could contribute to feto-maternal communication and consequently to metabolic adaptation in pregnancy when secreted into maternal circulation (**Figure 5**) (23). All 10 miRNAs were less abundant in GDM compared to normoglycemic women.

STRENGTHS AND LIMITATIONS

To the best of our knowledge, this is the first study including the largest number of pregnant women and using miRNA sequencing as an agnostic approach. We replicated our results and prediction algorithm in a completely independent cohort to demonstrate external validity and help assess generalizability (3D first-trimester samples were collected at random, non-fasting at ~11 weeks). Gen3G samples were collected mostly following a 50-g GCT in the first trimester. The effect of a 50-g GCT on miRNA expression is not currently known, but because the results were replicated in the 3D cohort, we believe that our models are robust. Also, we did not have access to as many clinical characteristics in the 3D cohort, which could also explain why the model's AUC values were not as high as in Gen3G. Our study has also other limitations. We did not validate our results using another method such as qPCR or digital PCR, which will be needed if clinical applications are intended. Also, replication in other ethnicities will be needed to confirm our miRNA selection and also their weight in the algorithm in other population. Our prediction algorithm performed better when other clinical values (HbA1c and 1-h post–50-g GCT) were considered, but we were not able to confirm these results in the 3D as these were not available.

CONCLUSION

We have identified plasmatic miRNAs measured between the 4th and 16th week of pregnancy that are associated with subsequent development of GDM. Three of these miRNAs could be used in combination with GDM classical risk factors to identify women at a low or higher risk of GDM 12 weeks on average prior to its current diagnostic. This would allow to decrease the need for GDM screening between 24th and 28th week for women at low risk and opens the window for early GDM prevention.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: GEO repository, accession numbers GSE216275 and GSE216997. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Centre intégré universitaire de santé et de services sociaux de l'Estrie-CHUS, Sherbrooke, Canada. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CL was responsible for data collection, statistical analysis, writing of the manuscript and contributes to bioinformatics analysis. VD, CP, and KT contributed to data collection and manuscript revision. FW and A-AC contributed to bioinformatics analyses and the revision of the manuscript. MS, ZL, and P-EJ contributed to data analyses and manuscript revision. PP, M-FH, RG, and LB elaborated the study design, supervised all steps of the study, and participated in manuscript writing and revision. LB is the guarantor of this work. 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.2022.928508/full#supplementary-material

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Maternal serum NGAL in the first trimester of pregnancy is a potential biomarker for the prediction of gestational diabetes mellitus

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Objective: Gestational diabetes mellitus (GDM) has adverse effects on the health of mothers and their offspring. Currently, no known biomarker has been proven to have sufficient validity for the prediction of GDM in the first trimester of pregnancy. The aim of this study was to investigate the potential relationship between serum neutrophil gelatinase-associated lipocalin (NGAL) levels in the first trimester of pregnancy and later GDM risk and to evaluate the performance of serum NGAL as a biomarker for the prediction of GDM.

Methods: The study was conducted by recruiting participants at 8–13 weeks of gestation from The First Affiliated Hospital of Chengdu Medical College between January and June 2021; participants were followed up for oral glucose tolerance test (OGTT) screening at 24–28 gestational weeks. We examined the serum NGAL levels of all subjects in the first trimester who met the inclusion and exclusion criteria. Anthropometric, clinical, and laboratory parameters of the study subjects were obtained during the same study period. A logistic regression model was carried out to investigate the potential relationship between serum NGAL levels in the first trimester of pregnancy and later GDM risk. The receiver operating characteristic (ROC) curve and area under the curve (AUC) were used to assess the discrimination and calibration of serum NGAL as a biomarker for the prediction of GDM in the first trimester of pregnancy.

Results: Serum NGAL levels in the first trimester of pregnancy were significantly higher in women who later developed GDM than in those who did not develop GDM. Serum NGAL levels in the first trimester of pregnancy were positively associated with an increased risk of GDM after adjustment for potential confounding factors. The risk prediction model for GDM constructed by using serum NGAL levels in the first trimester of pregnancy achieved excellent performance.

Conclusions: Maternal serum NGAL in the first trimester of pregnancy is a potential biomarker for the prediction of GDM, which could help guide the clinical practice of antenatal care.

KEYWORDS

biomarker, first trimester, gestational diabetes mellitus, neutrophil gelatinase-associated lipocalin (NGAL), insulin resistance

Introduction

Gestational diabetes mellitus (GDM) is a common gestational disorder characterized by glucose intolerance during the second or third trimester (1, 2). Depending on ethnicity and the screening methods and diagnostic tests used, it is estimated that GDM occurs in 6-18% of all pregnancies worldwide (3–5). GDM affects approximately 15% of all Chinese pregnant women according to the International Association of Diabetes diagnostic criteria for GDM (6).

Currently, GDM is diagnosed with an oral glucose tolerance test (OGTT), which is generally performed in the second trimester of pregnancy. However, according to the recent guidelines of the American Congress of Obstetricians and Gynecologists (ACOG), early screening in the first trimester is recommended in women with risk factors for GDM (e.g., BMI above 25, hypertension, family history of diabetes, and known impaired glucose metabolism) (7). Moreover, the effective early detection of women at risk in the first trimester could be beneficial for reducing disease onset and associated maternal and perinatal complications by providing timely interventions such as physical exercise and dietary changes (8). Therefore, it is necessary to identify some appropriate biomarkers that would allow early prediction of GDM risk.

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2 (LCN-2), is a 25-kDa secretory glycoprotein that was originally identified in mouse kidney cells and human neutrophil granules (9–11). It plays an important role in the regulation of the immune response, inflammation, and tumour metastasis (9, 12–14). In addition, several studies have also reported that NGAL is associated with obesity through the induction of IFNγ expression, resulting in subsequent adipogenesis in adipose tissues (15–17). In addition to the close associations of NGAL with obesity observed in previous studies, it has also been reported that NGAL plays an important role in the pathophysiology of other metabolic diseases, such as dyslipidaemia, dysglycaemia, and bone metabolic disease (18–20). However, the role of NGAL in regulating blood glucose levels is controversial, as different studies have reported discrepant results. Although some studies suggest that NGAL has a role in promoting glucose intolerance, insulin resistance, and obesity, there is also evidence related to its beneficial anti-diabetic role (16, 19, 21). Several studies reported that circulatory NGAL levels were elevated and positively correlated with obesity, hypertriglyceridaemia, hyperglycaemia, and insulin resistance in type 2 diabetes mellitus patients (16, 22). In contrast, other studies reported that NGAL has an important role in improving insulin sensitivity and glucose metabolism, either by stimulating insulin secretion or by controlling the food intake behaviours of mice (19, 23). Therefore, the above findings suggest that NGAL plays an important role in glucose homeostasis, making it a potential new biomarker of abnormal glucose metabolism during pregnancy.

To the best of our knowledge, maternal serum NGAL levels in the first trimester of pregnancy in women with GDM have been little studied and are poorly understood. Two previous studies reported that maternal serum NGAL levels were significantly higher in women with GDM than in those without GDM and correlated positively with fasting plasma glucose in the third trimester (24, 25). However, the studies were conducted in the third trimester when OGTT screening had already been performed. Thus, it may not be helpful for the early detection of GDM in the first trimester of pregnancy, as the levels of NGAL may be different between the first trimester and third trimester. The aim of this study was to investigate the potential relationship between serum NGAL levels in the first trimester of pregnancy and later GDM risk and to evaluate the performance of serum NGAL as a biomarker for the prediction of GDM.

Materials and methods

Study participants and design

The prospective cohort study was conducted by recruiting participants at 8–13 weeks of gestation from The First Affiliated Hospital of Chengdu Medical College between January and June 2021; participants were followed up for OGTT screening at 24–28 gestational weeks. A total of 824 pregnant women visited the antenatal clinic at the hospital for their first prenatal examination during the study period. The inclusion criteria

were as follows: (1) women with a singleton pregnancy; (2) women between 18 and 40 years of age; (3) women at 8–13 weeks of gestation; (4) women who planned to receive antenatal care and deliver in the study hospital; and (5) women who signed the consent form. The exclusion criteria were as follows: (1) women with a previous history of chronic diseases such as chronic kidney disease, diabetes, hypertension, polycystic ovary syndrome, malignant tumours, autoimmune diseases, blood system diseases, or infectious diseases; (2) women with foetal malformation or those who experienced miscarriage; and (3) women with incomplete or unavailable data. A total of 516 participants met the inclusion and exclusion criteria and had ELISA performed for serum NGAL detection at 8-13 gestational weeks. However, 28 participants were lost to follow-up because of miscarriage or opting to receive prenatal care at other hospitals. Ultimately, 488 women were recruited for the study, and 74 were diagnosed with GDM by OGTT screening at 24–28 gestational weeks. The overall sample size was calculated using the following formula as described in previous studies: $n= Z^2 \times P \times (1-P)/e^2$, where n= the required sample size, Z= 1.96 at a 95% confidence interval (CI), P= the prevalence of GDM (14.8%) and e= the margin of error (5%) (6, 26, 27). The total sample size was calculated to be 194. However, 488 eligible participants were recruited for this study. PASS (Power Analysis and Sample Size) software was used to calculate the statistical power, effect size, and smallest sample size for different statistical tests.

The study flowchart is presented in Figure 1. The study was approved by the Ethics Committee of The First Affiliated Hospital of Chengdu Medical College (No. CYFY17032024). Informed consent was obtained from all participants, and all procedures were conducted according to the Declaration of Helsinki.

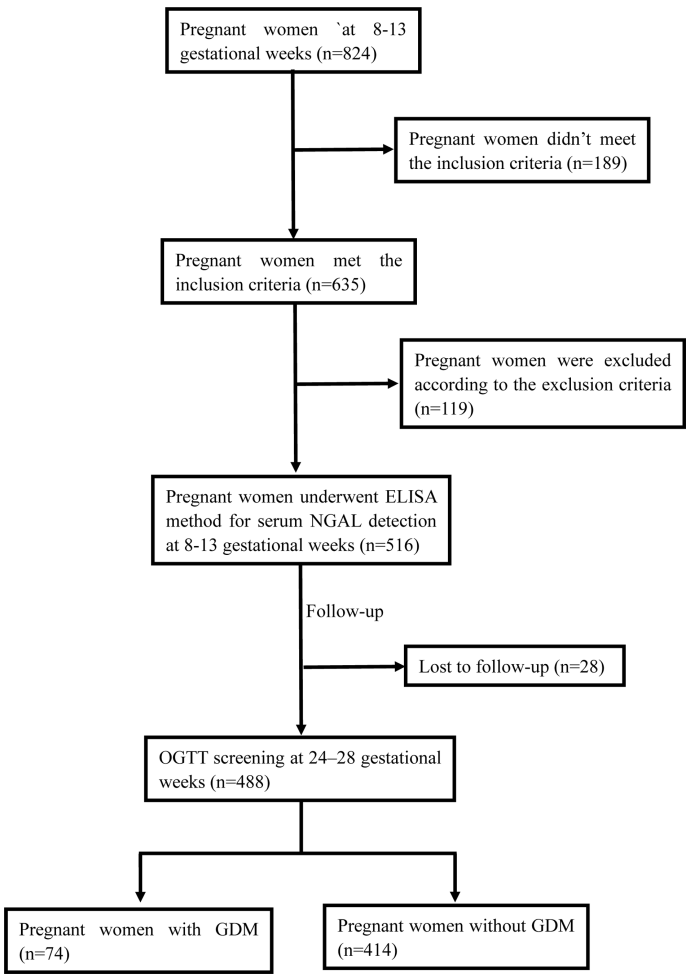

FIGURE 1
Flow graph of the study design.

Assessment of GDM and glucose homeostasis

A 75-g OGTT was performed to screen for GDM at 24–28 gestational weeks. According to the criteria of the American Diabetes Association, GDM was diagnosed when any of the following plasma glucose values were met or exceeded: a fasting glucose level ≥5.1 mmol/L, a 1-h glucose level ≥10.0 mmol/L, or a 2-h glucose level ≥8.5 mmol/L (1). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to published formulas (28).

Clinical characteristics and laboratory tests

Basic information, including name, age, prepregnancy weight, height, gravidity and parity, diastolic blood pressure (DBP), systolic blood pressure (SBP), and history of diseases, was collected by trained nurses when the study subjects visited the obstetrics clinic for their first prenatal examination. For the laboratory tests, fasting plasma samples taken at 8–13 weeks of gestation were used. Blood glycosylated haemoglobin (HbA1c), serum insulin, uric acid, serum creatinine (Cr), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT), and aspartate transaminase (AST) levels were measured on the same day by standard methods in the clinical laboratory of our hospital.

The participants' serum NGAL levels were detected by using a commercially available enzyme-linked immunosorbent assay kit (Abcam, Shanghai, China). All samples were analysed in three duplicates according to the manufacturer's instructions.

Statistical analysis

Data were analysed by using SPSS software (version 16.0, Chicago, IL, USA). Data with normal distributions are presented as the mean ± SD, and nonnormally distributed data are presented as frequencies. To compare differences between the GDM group and the non-GDM group, the independent-samples *t* test or Wilcoxon test was used for continuous variables, and the chi-square test or Fisher's exact test was used for categorical variables. The relationship between serum NGAL levels and clinical and laboratory parameters was determined by Pearson correlation analysis. Logistic regression was used to analyse the association between GDM risk and serum NGAL levels with adjustment for potential confounding factors. The results are presented as the odds ratio (OR) and 95% CI. The receiver operating characteristic curve (ROC) and area under the curve (AUC) were used to evaluate the specificity and sensitivity of serum NGAL as a potential biomarker for the prediction of GDM. A p <0.05 was considered statistically significant.

Results

Clinical and laboratory characteristics of the study participants

The clinical and laboratory characteristics of the GDM group and non-GDM group are shown in Table 1. There were no statistically significant differences between the two groups in gestational age, parity, SBP, or DBP at sampling. However, maternal age and pre-pregnancy BMI were significantly higher in the GDM group than in the non-GDM group. Regarding laboratory characteristics, serum HbA1c, fasting insulin, TC, TG, LDL, OGTT glucose and HOMA-IR levels were significantly higher in the GDM group than in the non-GDM group (P<0.05). However, no differences in HDL-C, AST, ALT, serum creatinine, or uric acid levels were observed between the two groups (P>0.05). We also observed that the serum NGAL levels were significantly higher in the GDM group than in the non-GDM group (P <0.001, Table 1).

Correlations between serum NGAL levels and clinical and laboratory parameters

Correlations between the maternal serum NGAL levels and clinical and laboratory parameters in the GDM group and non-GDM group are presented in Table 2. Pearson correlation analysis showed that serum NGAL levels were positively correlated with pre-pregnancy BMI and HbA1c, fasting insulin, TG, TC, HOMA-IR, OGTT glucose and LDL-C levels in the GDM group (P < 0.05). However, there were no significant correlations between serum NGAL levels and other clinical and laboratory parameters in the GDM group (P > 0.05). In addition, no correlations were observed between maternal serum NGAL levels and clinical and laboratory parameters in the non-GDM group.

Clinical and laboratory risk factors for GDM explored by univariable logistic regression

Univariable logistic regression was carried out to identify the risk factors for GDM. The results showed that maternal age and pre-pregnancy BMI were significantly and positively correlated with the risk of GDM, with ORs of 3.142 (95% CI = 0.812-7.143) and 4.318 (95% CI = 0.914-8.317), respectively. Regarding laboratory parameters, HbA1c, HOMA-IR, TG, and LDL-C

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TABLE 1 Clinical and laboratory characteristics of the study participants.

Parameters	GDM group (n=74)	non-GDM group (n=414)	*p-value*
Maternal age (years)	28 ± 3.45	26 ± 3.23	**0.013**
Gestational age (weeks)	11.23 ± 2.43	11.75 ± 2.71	0.214
Pre-pregnancy BMI (kg/m^2)	25.47 ± 4.67	23.46 ± 4.22	**0.023**
SBP (mm/Hg)	116.17 ± 12.32	114.78 ± 15.72	0.128
DBP (mm/Hg)	75.52 ± 9.34	72.48 ± 8.67	0.273
Parity			0.899
Primiparous	39	212	
Multiparous	35	202	
HbA1c (%)	5.51 ± 0.82	4.22 ± 0.78	**0.024**
Fasting insulin (mIU/ml)	9.73 ± 0.21	7.2 ± 0.13	**<0.001**
Fasting glucose (mmol/L)	5.45 ± 1.29	4.28 ± 0.63	**0.031**
1 h-glucose (mmol/L)	10.09 ± 1.91	7.35 ± 1.82	**0.027**
2 h-glucose (mmol/L)	8.31 ± 1.21	6.72 ± 1.29	**0.012**
HOMA-IR	2.18 ± 0.89	1.05 ± 0.22	**<0.001**
TC (mmol/L)	6.62 ± 0.61	5.12 ± 0.53	**0.021**
TG (mmol/L)	2.73 ± 0.21	1.62 ± 0.14	**0.022**
LDL-C (mmol/L)	4.65 ± 0.54	3.64 ± 0.47	**0.017**
HDL-C (mmol/L)	2.51 ± 0.13	2.09 ± 0.23	0.318
AST (U/L)	21 ± 3.27	22 ± 3.95	0.223
ALT (U/L)	19 ± 3.58	20 ± 3.14	0.274
Creatinine (μmol/L)	69 ± 6.73	71 ± 7.45	0.121
Uric acid (μmol/L)	305 ± 16.56	312 ± 18.27	0.217
Serum NGAL levels (ng/ml)	72.37 ± 8.24	47.25 ± 7.38	**<0.001**

Data are presented as the means ± standard deviations for continuous variables or frequencies for categorical variables. P values were calculated by using independent-samples t tests for normally distributed continuous variables and chi-square tests for categorical variables. Statistically significant values at $P < 0.05$ are shown in bold.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

levels were significantly and positively correlated with the risk of GDM (P<0.05) (Table 3). These findings suggested that pre-pregnancy BMI, maternal age, and HbA1c, HOMA-IR, TG, and LDL-C levels are risk factors for GDM.

According to univariable logistic regression, we used these identified risk factors to build a risk prediction model for GDM. An ROC curve was constructed using the model with an AUC of 0.705 (sensitivity of 55.3% and specificity of 91.3%, 95% CI=0.598–0.812, P<0.001, Figure 2A).

Association of serum NGAL levels with the risk of GDM

The association of serum NGAL levels with the risk of GDM was assessed by a logistic regression model. Serum NGAL levels were divided into tertiles according to the cut-off points of the distribution of serum NGAL levels. The lowest tertile was considered as a reference.

The results showed that the prevalence of GDM increased stepwise from 7.33% to 23.60% across serum NGAL tertiles, with a threefold increase in the highest tertile versus the lowest tertile (Figure 3). Women with serum NGAL levels in the highest tertile as well as those with levels in the middle tertile had a higher risk of GDM than those with levels in the lowest tertile, with adjusted ORs of 4.513 (95% CI = 1.209-10.224) and 2.231 (95% CI = 0.814-6.217), respectively (Table 4). When regarded as a continuous variable, serum NGAL levels were significantly associated with an increased risk of GDM (adjusted OR = 1.512, 95% CI = 0.491–3.013, $P = 0.012$).

Association of serum NGAL levels with the risk of adverse pregnancy outcomes

Previous studies have reported that GDM is associated with a series of adverse pregnancy outcomes, such as foetal growth restriction, large for gestational age, caesarean section, preterm delivery, postpartum haemorrhage, polyhydramnios, preeclampsia, and preterm premature rupture of membranes. Thus, we also investigated the associations between serum NGAL levels and these adverse pregnancy outcomes. The results showed that compared to the lowest tertile, the middle and highest tertiles of serum NGAL levels were associated with a

TABLE 2 Correlations between serum NGAL levels and clinical and laboratory parameters in the two groups.

Variable	GDM group (n=74)		non-GDM group (n=414)	
	r	*p-value*	*r*	*p-value*
Pre-pregnancy BMI (kg/m^2)	0.411	**<0.001**	0.083	0.532
SBP (mm/Hg)	-0.131	0.418	-0.247	0.342
DBP (mm/Hg)	0.081	0.336	0.135	0.415
HbA1c (%)	0.341	**0.012**	0.179	0.132
Fasting insulin (mIU/ml)	0.672	**<0.001**	0.154	0.253
Fasting glucose (mmol/L)	0.713	**0.021**	0.214	0.314
1 h-glucose (mmol/L)	0.801	**0.032**	0.143	0.146
2 h-glucose (mmol/L)	0.632	**0.019**	0.227	0.273
HOMA-IR	0.631	**<0.001**	-0.337	0.371
TC (mmol/L)	0.268	**0.032**	0.153	0.214
TG (mmol/L)	0.391	**<0.001**	0.132	0.115
LDL-C (mmol/L)	0.513	**0.017**	0.112	0.421
HDL-C (mmol/L)	-0.132	0.552	-0.274	0.218
AST (U/L)	0.222	0.118	0.172	0.098
ALT (U/L)	0.371	0.243	0.762	0.327
Creatinine (μmol/L)	0.432	0.347	0.271	0.514
Uric acid (μmol/L)	0.586	0.175	0.192	0.205

P values were calculated by Pearson correlation analysis. Statistically significant values at $P < 0.05$ are shown in bold.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

TABLE 3 Clinical and laboratory risk factors for GDM explored by univariable logistic regression.

Parameters	OR	95% CI	*p-value*
Maternal age (years)	3.142	0.812-7.143	**0.013**
Gestational age (weeks)	3.271	0.337-10.165	0.428
Pre-pregnancy BMI (kg/m^2)	4.318	0.914-8.317	**<0.001**
SBP (mm/Hg)	1.318	0.227-7.025	0.332
DBP (mm/Hg)	2.102	0.153-9.067	0.298
Parity			
Primiparous	1.000	Reference	–
Multiparous	1.287	0.318-8.132	0.465
HbA1c (%)	2.292	0.104-5.145	**0.002**
Fasting insulin (mIU/ml)	3.321	0.227-9.172	0.312
HOMA-IR	4.732	0.106-10.287	**<0.001**
TC (mmol/L)	7.293	1.007-18.023	0.127
TG (mmol/L)	3.784	0.803-10.174	**0.002**
LDL-C (mmol/L)	4.313	0.514-12.637	**0.007**
HDL-C (mmol/L)	1.145	0.125-7.472	0.226
AST (U/L)	2.773	0.104-9.381	0.114
ALT (U/L)	1.467	0.187-8.451	0.179
Creatinine (μmol/L)	7.578	1.241-15.752	0.225
Uric acid (μmol/L)	4.331	0.453-10.713	0.341

P values were calculated by univariable logistic regression analysis after adjustment for potential confounding factors, including a family history of diabetes, dietary habits, physical activity during pregnancy, and economic status. Statistically significant values at $P < 0.05$ are shown in bold.
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, glycated haemoglobin; HOMA-IR, homeostasis model assessment-insulin resistance; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

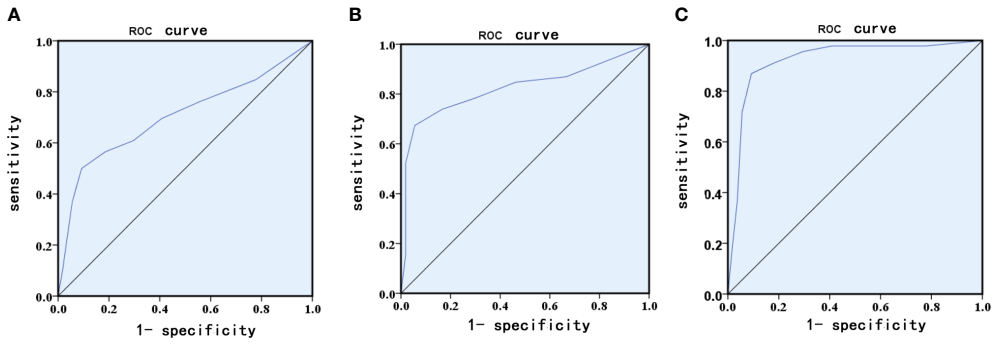

FIGURE 2
Receiver operating characteristic (ROC) curves for logistic regression models utilizing clinical and laboratory risk factors **(A)**, serum NGAL **(B)** serum NGAL levels and clinical and laboratory risk factors **(C)**.

higher risk of these adverse pregnancy outcomes, except foetal growth restriction and preeclampsia (Table 5).

Predictive model construction and evaluation of the prediction efficacy of serum NGAL levels for GDM

To evaluate the prediction efficacy of serum NGAL levels for GDM, ROC curves were constructed using the serum NGAL level model with an AUC of 0.823 (sensitivity of 96.3% and specificity of 82.2%, 95% CI=0.733–0.914, P<0.001, Figure 2B). Therefore, the performance of the model constructed by using serum NGAL levels was greater than that of the model constructed by using the identified risk factors, including prepregnancy BMI, maternal age, and HbA1c, HOMA-IR, TG, and LDL-C levels (Figure 2A). When the serum NGAL level was combined with the identified risk factors, the combined risk prediction model achieved an AUC of 0.923 (sensitivity of 91.2% and specificity of 90.8%, 95% CI=0.863-0.982, P<0.001, Figure 2C).

Discussion

Our study found that serum NGAL levels in the first trimester were significantly higher in women who later developed GDM than in those who did not develop GDM. Moreover, a positive association was found between serum NGAL levels in the first trimester and the risk of GDM after adjustment for potential confounding factors. We also used serum NGAL levels to construct a risk prediction model for

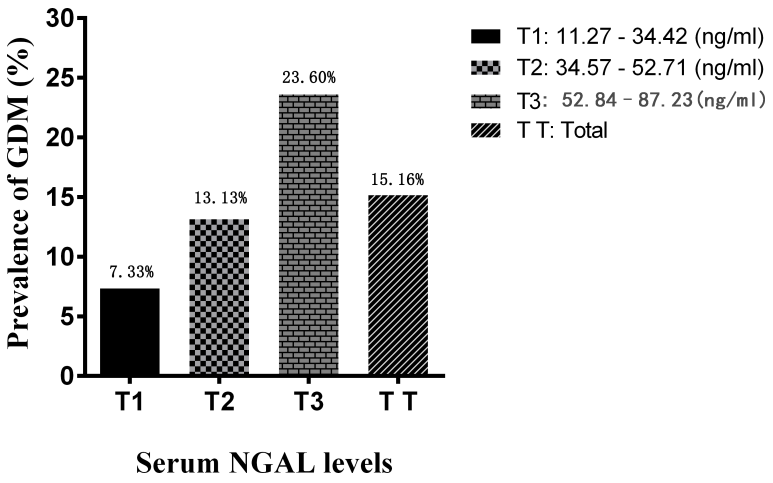

FIGURE 3
Prevalence of GDM for each tertile of serum NGAL levels.

TABLE 4 Association of serum NGAL levels with the risk of GDM.

Serum NGAL levels (ng/ml)	GDM, n (%)	adjusted OR	95% CI	*p-value*
T1: 11.27 - 34.42	11 (7.33)	1	Reference	-
T2: 34.57 - 52.71	21 (13.13)	2.231	0.814-6.217	**0.017**
T3: 52.84 – 87.23	42 (23.60)	4.513	1.209-10.224	**<0.001**
As a continuous variable	–	1.512	0.491–3.013	**0.012**

P values were calculated by logistic regression analysis after adjustment for potential confounding factors, including pre-pregnancy BMI, maternal age, and HbA1c, HOMA-IR, TG, and LDL-C levels. Statistically significant values at $P < 0.05$ are shown in bold.

GDM, and the model achieved excellent performance, with an AUC of 0.823. These findings indicate that serum NGAL in the first trimester of pregnancy is a potential new biomarker for the prediction of GDM.

NGAL is an adipocytokine that is highly expressed in adipose tissues and implicated in various metabolic and inflammatory diseases (9, 29). Previous studies reported that serum NGAL levels were significantly elevated in type 2 diabetes mellitus patients and that NGAL levels exhibited a positive correlation with obesity, hypertriglyceridaemia, hyperglycaemia, and insulin resistance (16, 30). Xiaoqian Yin et al. reported that maternal serum NGAL levels were significantly higher in women with GDM than in those without GDM and correlated positively with fasting plasma glucose levels in the third trimester (24). Lou et al. demonstrated that plasma NGAL levels were significantly increased in women with GDM, particularly among those with a pre-pregnancy BMI over 25 kg/m^2 (25). Our results were consistent with these studies since we found that serum NGAL levels and insulin resistance index scores in the first trimester were significantly higher in women who later developed GDM than in those who did not develop GDM. Moreover, we also found that serum NGAL levels were positively correlated with pre-pregnancy BMI and HbA1c, fasting insulin, TG, TC, HOMA-IR, OGTT glucose and LDL-C levels in the first trimester in women who later developed GDM. These findings suggest that NGAL may be an indicator of disorders of glucose metabolism, lipid metabolism, and insulin resistance, which are closely associated with GDM (31–33).

Although the study has revealed that serum NGAL levels are positively correlated with glucose metabolism disorders in women with GDM, the main mechanism for mediating NGAL expression is largely unknown. Zhang et al. studied the metabolic regulation of NGAL production in adipocytes and found that insulin can promote the expression and secretion of NGAL in a dose-dependent manner (34). Similarly, another study by Tan et al. showed that insulin treatment in a conditioned medium could significantly increase the secretion of NGAL protein in omental adipose tissue *in vitro* in a dose-dependent manner (35). Furthermore, they found that circulating NGAL levels were also increased in human subjects after insulin treatment, and the mechanisms involved in insulin-induced NGAL expression were the activation of phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling pathways (35). However, other studies demonstrated that insulin induces NGAL expression by promoting glucose metabolism (oxidation) and the production of reactive oxygen species (ROS) and then activating the NF-κB signalling pathway (34). This has also been confirmed by other studies. Studies by Zhao et al. provided

TABLE 5 Association of serum NGAL levels with the risk of adverse pregnancy outcomes.

Adverse pregnancy outcomes	Serum NGAL levels (ng/ml)			
	T1 (11.27 - 34.42)OR (95% CI)	T2 (34.57 - 52.71)OR (95% CI)	T3 (52.84 -87.23)OR (95% CI)	*p-value*
FGR	1.00 (reference)	0.94 (0.23–6.27)	1.58 (0.12–6.35)	0.341
LGA	1.00 (reference)	2.51 (0.21–4.36)	4.71 (1.313–8.47)	**0.001**
CS	1.00 (reference)	2.73 (1.05–7.42)	3.21 (1.14–9.04)	**0.028**
PD	1.00 (reference)	1.78 (0.18–5.16)	2.81 (0.26–6.13)	**0.012**
PH	1.00 (reference)	2.13 (0.31–6.13)	3.17 (1.04–6.52)	**0.021**
PHD	1.00 (reference)	2.34(0.37–5.21)	4.51 (1.31–7.04)	**0.001**
PE	1.00 (reference)	0.67 (0.15–5.12)	1.04 (0.21–4.15)	0.122
PPROM	1.00 (reference)	2.71 (0.31–6.15)	4.05 (1.14–8.19)	**0.003**

P values were calculated by logistic regression analysis after adjustment for potential confounding factors, including gravidity, parity, gestational age, family history of diabetes, pre-pregnancy BMI, and TC, TG, HbA1c, HDL-C, and LDL-C levels.
OR, odds ratio; CI, confidence interval; FGR, foetal growth restriction; LGA, large for gestational age; CS, caesarean section; PD, preterm delivery; PH, postpartum haemorrhage; PHD, polyhydramnios; PE, preeclampsia; PPROM, preterm premature rupture of membranes.

direct evidence that NF-κB bonded to the NGAL promoter of human adipocytes and that the NF-κB signalling pathway was activated when insulin induced NGAL expression (15, 36).

Insulin resistance occurs when normal concentrations of insulin fail to achieve an appropriate biological response downstream of the insulin receptor. As a result, β-cells must release more insulin than usual to modulate glucose homeostasis, ultimately leading to hyperinsulinaemia (37). In fact, the presence of insulin resistance is observed long before the clinical manifestations of GDM (38). Therefore, in pregnant women who later develop GDM, insulin resistance and hyperinsulinaemia may already be present in the first trimester. Our study confirmed that serum insulin levels and insulin resistance assessed by HOMA-IR in the first trimester were significantly increased in women who later developed GDM compared with those who did not develop GDM. These results suggest that insulin resistance and hyperinsulinaemia may be responsible for the elevated levels of NGAL in the first trimester of women who later develop GDM. This finding indicates that serum NGAL in the first trimester of pregnancy is a potential new biomarker for the prediction of GDM.

Over the years, numerous biomarkers, such as metabolic biomarkers, inflammatory biomarkers, and placental biomarkers, have been identified for the prediction of GDM (39–41). However, none of these biomarkers have sufficient validity for clinical practice (7). In this study, we found that serum NGAL in the first trimester of pregnancy is a potential new biomarker for the prediction of GDM, and the prediction model achieved great performance. We hope that this can be further verified in clinical trials in future studies and ultimately help guide the clinical practice of antenatal care.

Our study has many strengths. First, our study was conducted using a prospective design, which could avoid the recall bias that is usually present in a retrospective study. Second, we focused on the early prediction of GDM in the first trimester before OGTT screening. Thus, this study has clinical practical value and can provide a reliable basis for early clinical decision-making. Third, all laboratory and clinical measurements were carried out according to standardized procedures with high reliability. Fourth, our study constructed a risk prediction model for predicting GDM before OGTT screening by evaluating serum NGAL levels in the first trimester; the model achieved excellent performance. Nevertheless, our study also has limitations. First, although we adjusted for many potential confounding factors, we cannot rule out the possible influence of other unmeasured factors on the results. Second, we did not investigate the underlying mechanisms that mediate the association between serum NGAL levels and the risk of GDM. Finally, since our participants were mainly of Han ethnicity, our findings may not be generalizable to other racial groups when considering different dietary habits among racial groups.

Conclusions

Our findings demonstrated a positive association between serum NGAL levels in the first trimester of pregnancy and later GDM risk. We further used the observed association to construct a risk prediction model for GDM, which achieved excellent performance. This study suggests that maternal serum NGAL in the first trimester of pregnancy can serve as an early predictive biomarker for GDM, which could help guide the clinical practice of antenatal care.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of The First Affiliated Hospital of Chengdu Medical College (No. CYFY17032024). The patients/participants provided their written informed consent to participate in this study.

Author contributions

LL contributed to the study design and interpretation of the data. CL and JD contributed to the collection of data. JL and CH contributed to the drafting and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Trends in hospitalizations and emergency department visits among women with hyperglycemia in pregnancy between 2008 and 2017 in Taiwan

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Introduction: We investigated health service utilization, including hospitalizations and emergency department visits, for women with hyperglycemia in pregnancy between 2008 and 2017 in Taiwan.

Methods: Data from the Health and Welfare Data Science Center were used to conduct this nationwide population-based study. We identified pregnant women and the date of childbirth according to Birth Certificate Applications from 2007 to 2018. The study population was divided into four groups: known DM, newly diagnosed DM, GDM, and no DM/GDM. To assess quality of healthcare during the gestation period, trends in 30-day readmission rate, number of emergency department visits/hospitalizations per 100 childbirths, and length of hospital stay from 2008 to 2017 were examined.

Results: A total of 1830511 childbirths and 990569 hospitalizations were identified for analyses. Between 2008 and 2017, women with hyperglycemia in pregnancy (known DM, newly diagnosed DM, and GDM) had a higher rate of hospitalization, a longer length of hospital stay, and higher rates of various maternal and fetal outcomes, compared with women with no DM/GDM. Nevertheless, the differences between women with GDM and those with no DM/GDM in the aforementioned outcome measures were modest. Women with GDM had a modest decrease in the 30-day readmission rate (p for trend 0.046) with no significant difference in the number of emergency department visits during the study period.

Discussion: Our findings provide evidence of the quality of healthcare for women with GDM between 2008 and 2017 in Taiwan.

KEYWORDS

diabetes mellitus, gestational diabetes mellitus, hospitalization, hyperglycemia in pregnancy, emergency department

Introduction

Diabetes mellitus (DM) has been associated with adverse pregnancy outcomes (1, 2). Even in women with no history of DM, hyperglycemia may be noted during the gestation period, especially in those with risk factors (3). The diagnosis of gestational diabetes mellitus (GDM) is usually confirmed using an oral glucose tolerance test between 24 weeks and 28 weeks of gestation (3, 4). Similar to pregnant women with preexisting DM, women with GDM have a higher risk of adverse maternal and neonatal outcomes (5–7). Furthermore, glycemic control for GDM may improve outcomes (8, 9). Hence, screening for GDM in women at risk is recommended (3, 10).

Given the various screening tests used in clinical practice (4, 11–14), the prevalence of GDM varies widely in previous studies (15, 16). According to a recent report (17) from the International Diabetes Federation, around 21.1 million (16.7%) of live births in 2021 were to women who had hyperglycemia during the pregnancy. Among these, most (80.3%) were due to GDM. Moreover, more than half of the affected live births were in the South-East Asia and Western Pacific regions (17). In a recent study (18), the prevalence of GDM in Taiwan has increased from 7.6% in 2004 to 13.4% in 2015. Therefore, improving healthcare for women with GDM has emerged as an important issue (19).

Despite the rapid increase in the prevalence of GDM, data on healthcare resource utilization by women with GDM are scarce. Most of the healthcare costs for women with diabetes during pregnancy were attributed to hospital inpatient care, according to a recent study (20). In this study, we aimed to investigate health service use, including hospitalizations and emergency department visits, for women with hyperglycemia during pregnancy in Taiwan.

Materials and methods

Study ethics and database

Data from the Health and Welfare Data Science Center were used to conduct this population-based study. We identified pregnant women and the date of childbirth according to Birth Certificate Applications from 2007 to 2018. Relevant data (including diagnosis, treatment, outpatient clinic visits, emergency department visits, and hospitalizations) were retrieved by linking to the Registry for Beneficiaries, Ambulatory Care Expenditures by Visits, and Inpatient Expenditures by Admissions. This study was approved by the Research Ethics Committee of the National Health Research Institutes (EC1110505-E), and the requirement for patient consent was waived as the retrospective data were de-identified prior to analyses.

Diagnostic categories of hyperglycemia in pregnancy

The date of childbirth was defined as the end of the gestation period. The study population was divided into four groups according to the diagnosis of diabetes at an outpatient clinic visit or in a hospitalization:

1. Known DM: a diagnosis of DM (ICD-9-CM Codes 250 or ICD-10-CM Codes E08-E13) was noted within one year prior to the start of the gestation period.
2. Newly diagnosed DM: a diagnosis of DM (ICD-9-CM Codes 250 or ICD-10-CM Codes E08-E13, O24.0, O24.1, O24.3, O24.8) was noted between the start and the 24th week of the gestation period.
3. GDM: a diagnosis of DM (ICD-9-CM Codes 250, 648.00-648.04, or ICD-10-CM Codes E08-E13, O24.4, O24.9, O99.810, O99.814, O99.815) was noted between the 24th week and the end of the gestation period.
4. No DM/GDM: no DM (any of the above diagnosis codes) was noted between one year prior to the start of the gestation period and the date of childbirth.

The Diabetes Association of the Republic of China (DAROC) (http://www.endo-dm.org.tw/dia/) recommends universal screening for all pregnant women with no history of DM. Screening for newly diagnosed DM is suggested using fasting plasma glucose (≥ 126 mg/dl) or glycated hemoglobin (HbA1c) (≥ 6.5%) before gestational week 24. Screening for GDM is suggested using either one-step or two-step approach at gestational week 24-28. For one-step approach, a 75-g oral glucose tolerance test is administered and plasma glucose is measured at three time points (fasting, 1 hour, and 2 hour). The thresholds of plasma glucose for each time point are 92, 180, and 153 mg/dl, respectively (21). GDM is diagnosed if any one of the plasma glucose is higher than the threshold. For two-step approach, a 50-g glucose challenge test is conducted. Plasma glucose is measured at 1 hour, and the threshold is 130 (90% sensitivity) or 140 (80% sensitivity) mg/dl. A 100-g oral glucose tolerance test is conducted for women who have a positive 50-g glucose challenge test. Plasma glucose is measured at four time points (fasting, 1 hour, 2 hour, and 3 hour). The thresholds of plasma glucose for each time point are 95, 180, 155, and 140 mg/dl, respectively (22). GDM is diagnosed if any two of the plasma glucose is higher than the threshold. Medical care for women with hyperglycemia in pregnancy is usually provided by an obstetrician and an endocrinologist. The therapeutic targets for these women are HbA1c < 6.0-7.0% (if this can be achieved without significant hypoglycemia), fasting glucose ≤ 95 mg/dl, 1-hour postprandial glucose ≤ 140 mg/dl, and 2-hour postprandial glucose ≤ 120 mg/dl (23).

Outcomes of interest

Health service utilization, including hospitalizations and emergency department visits, within the gestation period was analyzed. We defined the first discharge diagnosis code as the primary diagnosis, and the causes of hospitalization were grouped as obstetric-related (ICD-9-CM Codes 630-639, 640-646, 651-676, 760-779, or ICD-10-CM Codes O00-O99, P00-P96), diabetes-related (ICD-9-CM Codes 250.1-250.3, 250.8, 251.0-251.2, or ICD-10-CM Codes E08.0, E09.0, E10.1, E10.62-E10.65, E10.69, E11.0, E11.6, E13.0, E13.11, E08.641, E09.641, E10.610, E10.618, E13.641), and others. Causes of emergency department visits were also grouped as obstetric-related (ICD-9-CM Codes 630-639, 640-647, 650-669, or ICD-10-CM Codes N96, O00-O04, O07-O09, O10-O16, O20, O21, O23, O26, O30-O36, O40-O48, O60-O66, O69, O70-O76, O80, O89, O98, O99.21, Z33), diabetes-related (ICD-9-CM Codes 250 or ICD-10-CM Codes E08, E11, E13), and others. Among diabetes-related causes, hyperosmolar hyperglycemic state (HHS)/diabetic ketoacidosis (DKA) (ICD-9-CM Codes 250.10, 250.12, 250.20, 250.22 or ICD-10-CM Codes E11.00, E11.01, E11.65, E11.69) and hypoglycemia (ICD-9-CM Codes 250.30, 250.32, 251.0, 251.1, 251.2 or ICD-10-CM Codes E16.0-E16.2, E11.641) were identified and reported.

To assess quality of healthcare during the gestation period, we examined the 30-day readmission rate, number of emergency department visits/hospitalizations per 100 childbirths, and length of hospital stay. The 30-day readmission rate (%) was calculated as the number of 30-day readmissions*100 divided by the number of hospitalizations. The number of emergency department visits (or hospitalizations) per 100 childbirths was calculated as the number of emergency department visits (or hospitalizations)*100 divided by the number of childbirths. Maternal (pregnancy induced hypertension, pre-eclampsia, and prolonged labor) and fetal (preterm birth [before 37 weeks of pregnancy], birth weight <10th percentile, birth weight >90th percentile, and hypoglycemia) outcomes were determined and compared among the four groups.

Statistical analysis

Data manipulation and statistical analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA). Data were examined according to the diagnostic categories (known DM, newly diagnosed DM, GDM, and no DM/GDM) and maternal age (<30 years, 30-<35 years, and ≥ 35 years). Statistical differences in the number of hospitalizations per 100 childbirths, length of hospital stay, and causes of hospitalizations across the diagnostic categories were examined using one-way ANOVA. Trends of 30-day readmission rate and number of emergency department visits per 100 childbirths from 2008 to 2017 were examined for statistical significance (p for trend). A p value of less than 0.05 was considered statistical significance.

Results

A total of 1830511 childbirths and 990569 hospitalizations were identified for analyses. Table 1 shows the number of

TABLE 1 Number of childbirths, hospitalizations, and length of hospital stay during gestation period from 2008 to 2017.

	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Number of childbirths	179881	175302	151306	179607	211768	176776	192206	195612	189580	178473
Maternal age <30 years	47.07%	43.44%	39.51%	36.78%	34.90%	32.71%	31.45%	30.90%	30.89%	30.97%
Maternal age 30 to <35 years	37.15%	39.63%	40.37%	42.05%	43.19%	41.86%	42.17%	40.96%	38.68%	36.90%
Maternal age ≥ 35 years	15.78%	16.92%	20.12%	21.17%	21.91%	25.43%	26.38%	28.15%	30.42%	32.13%
Number of hospitalizations	96108	94302	84084	98293	113872	96034	103869	106543	99754	97710
Maternal age <30 years	46.0%	42.6%	38.8%	35.9%	34.1%	32.0%	30.7%	30.4%	30.4%	30.7%
Maternal age 30 to <35 years	37.6%	39.8%	40.3%	42.1%	43.1%	41.6%	42.2%	40.6%	38.2%	36.1%
Maternal age ≥ 35 years	16.4%	17.6%	20.9%	22.0%	22.7%	26.4%	27.1%	29.0%	31.4%	33.2%
Number of hospitalizations per 100 childbirths	53.4	53.8	55.6	54.7	53.8	54.3	54.0	54.5	52.6	54.7
Known DM	66.3	70.4	72.2	69.4	65.3	64.4	67.2	67.2	62.9	68.6
Newly diagnosed DM	70.3	85.8	80.1	87.4	74.4	85.7	76.8	69.4	67.1	74.0
GDM	56.6	56.6	59.2	57.6	56.2	56.8	56.1	56.9	55.2	57.0
No DM/GDM	52.9	53.2	54.8	54.1	53.2	53.7	53.5	53.8	52.0	54.1
Length of hospital stay, days	4.69	4.77	4.91	4.92	4.79	4.89	4.90	4.84	4.18	4.87
Known DM	6.41	6.56	5.84	6.82	6.87	6.19	7.03	6.89	4.97	6.89
Newly diagnosed DM	5.56	6.85	7.26	6.67	6.21	6.78	7.05	5.54	4.56	5.82
GDM	4.78	4.90	4.99	4.96	4.87	4.91	4.88	4.87	4.23	4.92
No DM/GDM	4.63	4.69	4.83	4.87	4.70	4.84	4.85	4.78	4.14	4.83

Data are presented as number or %. DM, diabetes mellitus; GDM, gestational diabetes mellitus.

childbirths, hospitalizations during gestation period, and length of hospital stay from 2008 to 2017. The total number of childbirths in each year was relatively stable during the study period. However, the proportion of maternal age <30 years declined from 47.07% in 2008 to 30.97% in 2017. Conversely, the rate of maternal age ≥ 35 years increased from 15.78% to 32.13%. Similar findings were noted regarding the total number and maternal age distribution of hospitalizations during the gestation period. The number of hospitalizations per 100 childbirths was around 53-55, and the mean length of hospital stay was 4-5 days.

Table 2 shows the number of hospitalizations per 100 childbirths, length of hospital stay, and the most frequent causes of hospitalizations between 2008 and 2017, according to the diagnostic categories. Overall, the number of hospitalizations per 100 childbirths was higher in women with known DM

TABLE 2 Number of hospitalizations, length of hospital stay, and causes of hospitalization during the gestation period, 2008-2017.

	Known DM	Newly diagnosed DM	GDM	No DM/GDM
Maternal age, years	32.97 ± 4.95*	32.81 ± 5.04*	32.29 ± 4.69*	30.94 ± 4.89
Number of hospitalizations per 100 childbirths	67.16*	75.90*	56.74*	53.49
Maternal age <30 years	61.57*	64.22	51.60*	48.79
Maternal age 30 to <35 years	59.70*	65.12*	51.78*	49.43
Maternal age ≥ 35 years	61.08*	66.41*	52.14*	48.98
Length of hospital stay, days	6.47 ± 8.92*	6.03 ± 8.66*	4.84 ± 5.42*	4.72 ± 6.32
Maternal age <30 years	5.79 ± 6.62*	5.55 ± 7.30*	4.40 ± 4.49*	4.52 ± 5.50
Maternal age 30 to <35 years	6.35 ± 8.59*	5.89 ± 7.88*	4.77 ± 5.35*	5.03 ± 6.94
Maternal age ≥ 35 years	7.00 ± 10.28*	6.49 ± 10.12*	5.36 ± 7.74*	5.68 ± 8.91
Causes of hospitalization, %				
Obstetric related	71.90%	71.76%	69.81%	67.55%
Diabetes related	0.66%	0.52%	0.01%	—
Others				
Renal disease	0.54%	0.64%	0.19%*	0.22%
Respiratory disease	0.32%*	0.67%	0.18%*	0.20%
Pneumonia	0.28%	0.26%*	0.06%*	0.07%

Data are presented as mean ± SD, number, or %. DM, diabetes mellitus; GDM, gestational diabetes mellitus. *$p < 0.05$ vs. No DM/GDM.

(67.16), newly diagnosed DM (75.90), and GDM (56.74), compared with no DM/GDM (53.49). The findings were similar across the maternal age subgroups. The length of hospital stay was longer in women with known DM (6.47 ± 8.92), newly diagnosed DM (6.03 ± 8.66), and GDM (4.84 ± 5.42), compared with no DM/GDM (4.71 ± 6.32). The main causes of hospitalizations were obstetric-related (~70%), while few hospitalizations were diabetes-related (<1%).

Figure 1 shows the 30-day readmission rates according to the diagnostic categories. Women with known DM and newly diagnosed DM had a higher 30-day readmission rate than those with GDM and no DM/GDM. The trends were similar and the changes were modest during the study period (there was a modest decrease in women with GDM, p for trend 0.046). Figure 2 shows the number of emergency department visits per 100 childbirths according to the diagnostic categories. Women with known DM and newly diagnosed DM were more likely to have an emergency department visit than those with GDM and no DM/GDM. The number significantly increased from 2008 to 2017 in women with known DM and no DM/GDM (both p for trend <0.001), but not in those with newly diagnosed DM and GDM.

Table 3 shows the most frequent causes of emergency department visits according to the diagnostic categories. Causes other than obstetric-related and diabetes-related constituted 60-65% of the emergency department visits. Among these, the most frequent cause was unspecified abdominal pain. Around 35% were obstetric-related, among which the most frequent cause was hemorrhage in early pregnancy, followed by early or threatened labor. Among the diabetes-related emergency department visits, the rate of hypoglycemia was higher than that of acute hyperglycemia (HHS or DKA).

Maternal and fetal outcomes among the four groups are shown in Table 4. There were significant differences between women with hyperglycemia in pregnancy (known DM, newly diagnosed DM, and GDM) and those with no DM/GDM in these outcomes. Nevertheless, the differences between women with GDM and those with no DM/GDM were modest. For example, the rates of preterm birth (before 37 weeks of pregnancy) were 23.18%, 20.01%, 9.92%, and 9.76%, respectively.

Discussion

In this study, we examined health service utilization in women with various glucose regulation states (known DM, newly diagnosed DM, GDM, and no DM/GDM) during the gestation period from 2008 to 2017. We found that the rate of maternal age ≥ 35 years progressively increased during the study period (from 15.78% in 2008 to 32.13% in 2017, Table 1). Women with hyperglycemia in pregnancy (known DM, newly diagnosed DM, and GDM) had a higher rate of hospitalization, a longer length of hospital stay, and higher rates of various maternal and fetal outcomes, compared with women with no DM/GDM (Tables 2, 4). Nevertheless, the differences between women with GDM and those with no DM/GDM in the aforementioned outcome measures were modest. Furthermore, women with GDM had a modest decrease in the 30-day readmission rate (p for trend 0.046, Figure 1), with no significant difference in the number of emergency department visits (Figure 2) between 2008 and 2017. Our findings provide evidence of the quality of healthcare for women with GDM in Taiwan.

Preexisting DM and GDM in women have been associated with adverse maternal and neonatal outcomes (1, 24–26). However, there are few data on health service utilization with respect to hospitalizations and emergency department visits in women with known DM and GDM during the gestation period. In a recent report (20), women with diabetes had a higher

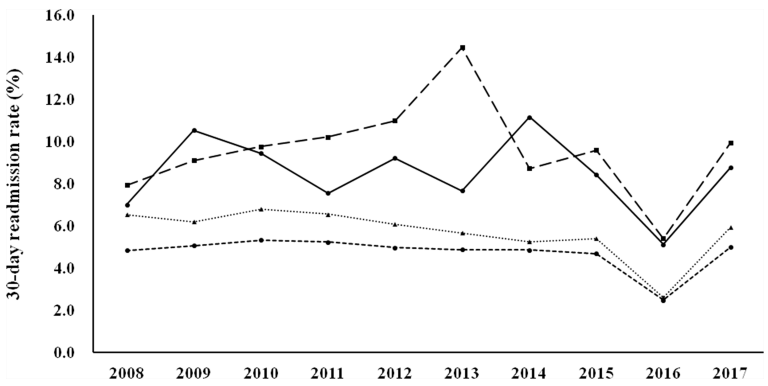

FIGURE 1
30-day readmission rates from 2008 to 2017 according to the diagnostic categories. Circle spots and solid line, known DM. Square spots and dashed line, newly diagnosed DM. Triangle spots and dashed line, GDM. Circle spots and dashed line, no DM/GDM. P for trend 0.046 for GDM.

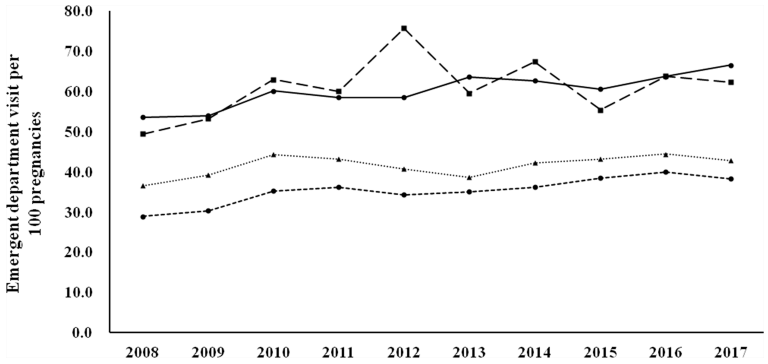

FIGURE 2
Number of emergency department visits per 100 childbirths from 2008 to 2017 according to the diagnostic categories. Circle spots and solid line, known DM. Square spots and dashed line, newly diagnosed DM. Triangle spots and dashed line, GDM. Circle spots and dashed line, no DM/GDM. P for trend <0.001 for known DM and no DM/GDM.

healthcare expenditure during pregnancy than those without diabetes, and most of the expenditure was attributed to hospital inpatient cost. Our findings showing that women with known DM and newly diagnosed DM had a higher number of hospitalizations and a longer length of hospital stay than women with no DM/GDM during the gestation period (Table 2) are in line with the aforementioned results (20). The differences were modest (number of hospitalizations per 100 childbirths 56.74 vs. 53.49, length of hospital stay 4.84 ± 5.42 vs. 4.72 ± 6.32) between women with GDM and those with no DM/GDM. Similar findings were noted regarding the maternal and fetal outcomes among the four groups (Table 4). Since the prevalence of GDM significantly increased from 2004 (7.6%) to 2015 (13.4%) in Taiwan (18), our findings provide an important reference for healthcare quality in women with GDM.

It is interesting to note that the number of emergency department visits per 100 childbirths increased from 2008 to 2017 (p for trend <0.001 for known DM and no DM/GDM), while there was no significant increase in 30-day readmission rates (a modest decrease in GDM group, p for trend 0.046) during the same period. The increase in emergency department utilization in pregnant women merits further investigation. We found that less than 40% of the emergency department visits were due to obstetric or diabetes-related causes (Table 3). In contrast, most of the hospitalizations (~70%) during the same period were due to obstetric-related causes (Table 2). It seems

TABLE 3 Most frequent causes for emergency department visits during gestation period, 2008-2017.

	Known DM	Newly diagnosed DM	GDM	No DM/GDM
Obstetric related, %	33.67	35.23	39.16	33.89
Diabetes related, %	5.16	1.29	0.14	—
Others, %	61.17	63.48	60.70	66.10
Obstetric related, %				
Hemorrhage in early pregnancy	37.03	35.07	35.07	32.62
Early or threatened labor	28.29	26.98	26.98	28.85
False labor	2.41	1.44	1.44	2.10
Other complications of pregnancy	4.08	1.35	1.35	4.41
Diabetes related, %				
HHS or DKA	4.37	3.03	2.04	—
Hypoglycemia	23.91	3.03	6.80	—
Others	71.72	93.94	91.16	—
Others, %				
Unspecified abdominal pain	15.01	17.13	17.27	16.16
Noninfective gastroenteritis and colitis	4.99	5.03	5.94	5.85
Fever	4.56	4.78	4.73	4.74

Data are presented as %. DKA, diabetic ketoacidosis; DM, diabetes mellitus; GDM, gestational diabetes mellitus; HHS, hyperosmolar hyperglycemic state.

TABLE 4 Maternal and fetal outcomes by diagnosis of diabetes, 2008-2017.

	Known DM	Newly diagnosed DM	GDM	No DM/GDM
Maternal outcomes, per 100 childbirths				
Pregnancy induced hypertension	28.06*	27.98*	5.31*	2.54
Pre-eclampsia	8.58*	8.86*	3.62*	1.85
Prolonged labor	5.08*	5.87*	4.52*	4.22
Fetal outcomes, %				
Preterm birth (before 37 weeks of pregnancy)	23.18*	20.01*	9.92*	9.76
Birth weight <10th percentile	9.17*	9.85	9.31*	10.59
Birth weight >90th percentile	23.53*	19.62*	10.02*	6.82
Hypoglycemia	3.47*	2.02*	1.29*	1.09

Data are presented as number or %. DM, diabetes mellitus; GDM, gestational diabetes mellitus. *$p < 0.05$ vs. No DM/GDM.

that hospitalization was usually not required for emergency department visits that were unrelated to obstetric-causes. With regard to diabetes-related emergency department visits, the rate of hypoglycemia was higher than acute hyperglycemia (HHS or DKA) (Table 3). As insulin therapy is recommended as the first-line pharmacologic treatment for preexisting diabetes (27) and GDM (4), our findings suggest that interventions to decrease emergency department utilization due to hypoglycemia should be considered to improve healthcare quality for women with hyperglycemia during pregnancy.

The main strengths of this study were the use of a nationwide, population-based dataset and a large study population. Nevertheless, there were several limitations in this study. First, we did not have data on maternal body mass index. Second, laboratory data related to glycemic control (such as fasting plasma glucose or HbA1c) were not available. Third, some confounding factors, such as nulliparous, urbanization of residence city (which may affect healthcare accessibility), primary or tertiary care, and socioeconomic status were not addressed. All of the aforementioned factors (28–30) may influence healthcare resource utilization during the gestation period. Final, we cannot be sure if universal screening for GDM was carried out in all medical institution since our analyses were based on retrospectively collected data. The prevalence of GDM in Taiwan increased from 7.6% in 2004 to 13.4% in 2015 (18). In a recent pragmatic, randomized clinical trial of GDM screening (31), the prevalence of GDM was 16.5% based on one-step approach (maternal age 29.4 ± 5.5 years) and 8.5% based on two-step approach (maternal age 29.3 ± 5.5 years). Thus we suggest that the screening for GDM in Taiwan should have been conducted in an efficient manner to disclose an increase in the prevalence of GDM. Despite these limitations, our results based on 10-year nationwide data still have important implications for the medical care of women with hyperglycemia in pregnancy.

In conclusion, the rate of maternal age ≥ 35 years significantly increased from 2008 (15.78%) to 2017 (32.13%) in Taiwan. Among women with GDM, there was a modest increase in the number of hospitalizations per 100 childbirths (56.74 vs. 53.49) and length of hospital stay (4.84 ± 5.42 vs. 4.72 ± 6.32) compared with those with no DM/GDM. From 2008 to 2017, women with GDM had a modest decrease in the 30-day readmission rate (p for trend 0.046) with no significant difference in the number of emergency department visits. Our findings provide evidence of healthcare quality for women with GDM in Taiwan.

Data availability statement

The datasets presented in this article are not readily available because of privacy/ethical restrictions. Requests to access the datasets should be directed to Chih-Cheng Hsu, cch@nhri.edu.tw.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee of the National Health Research Institutes. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

J-SW, J-FC, C-NH, C-MH, and C-YW contributed to conception and design of the study. M-CC, H-YO, Y-SY, and C-CH organized the database. M-CC and C-CH performed the statistical analysis. J-SW, M-CC, and C-CH wrote the first draft of the manuscript. J-FC, C-NH, C-MH, H-YO, Y-SY, and C-YW reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Trends in epidemiology of hyperglycemia in pregnancy in Taiwan, 2008-2017

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Background: Hyperglycemia in pregnancy (HIP) increases the risk of adverse pregnancy outcomes. The increasing prevalence of overweight or obesity and the increasing proportion of pregnant women with advanced maternal age (AMA) in the recent decade may affect its prevalence. We analyzed the secular trend of HIP prevalence in 2008-2017 in Taiwan and investigated the impact of AMA in this study.

Methods: This cross-sectional study used data from Health and Welfare Data Science Center. Pregnant women who registered their data in the Birth Certificate Application in 2008-2017 were recruited. Diagnosis of HIP was defined by ICD-9-CM and ICD-10-CM codes.

Results: In 2008-2017, 151,306-211,768 pregnant women were recruited in different years. The proportion of women with AMA increased from 15.8% to 32.1%. Meanwhile, the prevalence increased from 0.5% to 0.9% for preexisting diabetes, 0.2% to 0.4% for undiagnosed diabetes, and 11.4% to 14.5% for GDM. Maternal age was significantly associated with the prevalence of HIP. For women aged <30 years, 30-34 years and ≥35 years, the prevalence of preexisting diabetes were 0.51%, 0.75% and 1.24%, respectively ($p<0.05$); the prevalence of undiagnosed diabetes were 0.18%, 0.24% and 0.37%, respectively ($p<0.05$); and the prevalence of GDM were 10.57%, 14.77% and 18.13%, respectively ($p<0.05$). In all age groups, the prevalence of HIP increased over time in 2008-2017.

Conclusion: The prevalence of HIP increased in Taiwan in 2008-2017, which may result from the increasing proportion of pregnant women with AMA and the change in the diagnostic criteria for GDM.

KEYWORDS

gestational diabetes mellitus, gestational hyperglycemia, pregestational diabetes, undiagnosed, maternal age

Introduction

Hyperglycemia in pregnancy (HIP) is an important health threat to the pregnant women and the fetus (1). It includes preexisting diabetes and gestational diabetes mellitus (GDM). In women with preexisting diabetes, diabetes is diagnosed before pregnancy; whereas in women with GDM, diabetes is diagnosed during pregnancy, usually at 24 to 28 weeks of gestation. Besides, since screening for diabetes is suggested in early pregnancy by several academic associations (2), undiagnosed diabetes mellitus may also be detected in early pregnancy for the first time. Women with HIP have higher risk of receiving Cesarean section, gestational hypertension and preeclampsia (1). Newborns delivered by women with HIP are at increased risk of macrosomia, neonatal hypoglycemia and hyperbilirubinemia, preterm delivery, birth trauma and admission to neonatal intensive care unit. In women with preexisting diabetes or undiagnosed diabetes, risk of congenital abnormalities is also higher. A recent report on the TODAY study subjects has demonstrated that women with preexisting diabetes had very high rates of maternal complications (3).

In Taiwan, the percentage of women with overweight or obesity increased gradually. A report has demonstrated that women with overweight increased from 9.7% in 2011 to 11.1% in 2016, and women with obesity increased from 5.8% in 2011 to 7.4% in 2016 (4). The increasing prevalence of overweight and obesity may increase the prevalence of HIP. Indeed, Su et al. has shown that the prevalence of GDM increased gradually from 2004 to 2015 (5). However, there is no data reported on the prevalence of preexisting diabetes and undiagnosed diabetes in Taiwan during this period of time. On the other hand, with the advance in the technologies of artificial reproduction, the increase in women's employment, and the changes in social and cultural factors, the proportion of pregnant women with advanced maternal age (AMA, ≥35 years old) is increasing, especially in developed countries (6, 7). Women with AMA have a higher risk of GDM, as shown in our previous cohort study (8, 9). Besides, from the third decade of life, age-related glucose intolerance generally becomes more pronounced (10, 11). Theoretically, the risk of preexisting diabetes or undiagnosed diabetes may increase in women with AMA. However, data on the relationship of AMA, preexisting diabetes during pregnancy, and undiagnosed diabetes during pregnancy are limited in the literature. Taken together, these facts highlight the need for a detailed analysis on the prevalence of HIP and the impact of AMA on the prevalence of HIP.

In this study, we analyzed the change in prevalence of HIP over time in 2008-2017, including preexisting diabetes, undiagnosed diabetes, and GDM, using a nationwide database in Taiwan including 151,306-211,768 pregnant women in different years. The distribution of age in women with and without HIP were calculated, and the impact of maternal age on the prevalence in this period were investigated.

Materials and methods

Data source

The present study used data from Health and Welfare Data Science Center. Pregnant women whose data registered in the Birth Certificate Application in 2008-2017 were used for the analyses. The beginning of pregnancy and the 24th gestational week were calculated based on the birth date of the newborn and gestational weeks at delivery which were recorded in the Birth Certificate Application. The link between the pregnant woman and her newborn was confirmed by the Birth Certificate Application and the Maternal and Child Health Database. We also linked data from the Registry for Beneficiaries such as the Ambulatory Care Expenditures by Visits and the Inpatient Expenditures by Admissions, in order to acquire information about the presence of diseases and the treatments performed.

Research ethics approval

The study was approved by the Ethics Committee of our National Health Research Institute (NHRI IRB EC1020408-E).

Study population

In this cross-sectional study, pregnant women who registered their data in the Birth Certificate Application in 2008-2017 were recruited. According to the diagnosis made in outpatient clinic or during admission one year before pregnancy and during pregnancy, these women were classified into four groups according to a previous publication with some modification (12), as follows:

1. Preexisting diabetes mellitus: defined by at least one diagnosis of diabetes one year before pregnancy (by International Classification of Diseases, 9th Revision, Clinical Modification (ICD-9-CM) codes 250 or International Classification of Diseases, 10th Revision, Clinical Modification (ICD-10-CM) codes E08-E13).
2. Undiagnosed diabetes mellitus: defined by at least one diagnosis of diabetes before the week 24 of the pregnancy (by ICD-9-CM codes 250 or ICD-10-CM codes E08-E13, O24.0, O24.1, O24.3 or O24.8).
3. GDM: defined by at least one diagnosis of GDM from the 24 weeks of gestation to delivery (by ICD-9-CM codes 250, 648.00-648.04 or 648.8, or ICD-10-CM codes E08-E13, O24.4, O24.9, O99.810, O99.814, O99.815).
4. Women without diabetes or GDM: pregnant women without preexisting diabetes mellitus, undiagnosed

diabetes mellitus and gestational diabetes mellitus from one year before pregnancy to delivery.

Statistical analysis

In women who got pregnant in certain year and delivered in the next year, the year of delivery was used to calculate the data. Data of gestational weeks in different subgroups were derived from the Birth Certificate Application. Categorical variables were presented as number and percentages, and continuous variables were summarized by means and standard deviations, such as age and gestational weeks. The secular trend of prevalence in different groups were analyzed by p for trend. We categorized these women into three age groups for subgroup analysis, including <30 years old, 30-34 years old and ≥35 years old. The distribution of age at delivery in women with preexisting diabetes, undiagnosed diabetes, gestational diabetes and without diabetes or gestational diabetes was tested by the chi-square test. Prevalence of preexisting diabetes, undiagnosed diabetes and gestational diabetes in different age groups were compared by chi-square test. Two-tailed testing was used for statistical significance testing, and a value of $p < 0.05$ was considered statistically significant. All statistical analyses were conducted with SAS software version 9.4 (SAS Institute Inc., Cary, NC).

Results

In 2008-2017, the number of pregnancies ranged from 151,306 to 211,768 per year (Table 1). According to Chinese zodiac, the year 2010 was the year of Tiger, and the year 2012 was the year of Dragon. Traditionally, some women are more willing to deliver in the year of Dragon and may avoid to deliver in the year of Tiger. Excluding the highest number in 2012 and the lowest number in 2010, the number of pregnancies were between 175,302 to 195,612. During this period, the mean age at delivery increased gradually, from 29.84 years old to 31.86 years old (p<0.001). The proportion of women with AMA was also increased, from 15.8% to 32.1% (p<0.001). There was a slight change in gestational weeks at delivery during this period, and the average gestational weeks were 38.08-38.23 weeks. The comorbidity of hypertension and polycystic ovary syndrome also slightly increased from 2008 to 2017 (p for trend <0.05).

The prevalence of HIP, including preexisting diabetes, undiagnosed diabetes and GDM, increased in 2008-2017 (all p<0.05). The prevalence of preexisting diabetes increased from 0.5% in 2008 to 0.9% in 2017. The prevalence of undiagnosed

TABLE 1 Clinical characteristics and prevalence of hyperglycemia in pregnancy in pregnant women in Taiwan in 2008-2017.

	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	P for trend
Number	179,881	175,302	151,306	179,607	211,768	176,776	192,206	195,612	189,580	178,473	
Age (years)	29.84 ± 4.71	30.20 ± 4.64	30.62 ± 4.77	30.86 ± 4.63	31.03 ± 4.61	31.33 ± 4.80	31.47 ± 4.78	31.59 ± 4.88	31.75 ± 4.98	31.86 ± 5.09	<0.001
Age ≥35 years old (N)	28,382	29,666	30,442	38,021	46,399	44,950	50,703	55,057	57,671	57,343	
Age ≥35 years old (%)	15.8	16.9	20.1	21.2	21.9	25.4	26.4	28.1	30.4	32.1	<0.001
Gestational weeks (weeks)	38.20	38.23	38.17	38.18	38.19	38.17	38.16	38.13	38.08	38.08	<0.001
Comorbidity (%)											
Hypertension	3.3	3.5	3.6	3.4	3.5	3.8	3.5	3.8	4.1	4.6	<0.05
Polycystic ovary syndrome	1.1	1.2	1.4	1.0	1.1	1.1	1.2	1.5	1.6	2.0	<0.05
Prevalence (%)											
Preexisting diabetes (%)	0.5	0.7	0.8	0.7	0.7	0.8	0.9	0.9	0.9	0.9	<0.05
Undiagnosed diabetes (%)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.4	<0.05
GDM (%)	11.4	12.6	13.3	14.0	14.1	14.7	15.2	16.0	14.7	14.5	<0.05

*Preexisting diabetes mellitus, diabetes diagnosed before pregnancy; undiagnosed diabetes, diabetes mellitus first detected in early pregnancy, before 20 weeks of gestation; GDM, gestational diabetes mellitus.

diabetes was 0.2% from 2008 to 2014, and increased gradually to 0.4% in 2017. The prevalence of GDM increased gradually from 11.4% in 2008, reached a plateau of 16.0% in 2015, and then became 14.7% and 14.5% in 2016 and 2017, respectively.

In 2008-2017, the mean age at delivery increased in women with preexisting diabetes, undiagnosed diabetes and GDM and in women without HIP (Supplementary Figure 1). Table 2 shows the distribution of age in different groups. For the risk of preexisting diabetes, the risk was the highest in women aged ≥40 years (OR 4.10, 95% CI 3.83-4.38), followed by women aged 35-39 years (OR 2.48, 95% CI 2.37-2.60) and women aged 30-34 years (OR 1.57, 95% CI 1.50-1.64), compared with women aged <30 years. There was a linear trend between maternal age and the risk of preexisting diabetes (p for trend <0.0001). Similarly, for undiagnosed diabetes and GDM, the risk also increased by maternal age (both p for trend<0.0001). Women aged ≥40 years had the highest risk of undiagnosed diabetes (OR 3.56, 95% CI 3.16-4.01) and GDM (OR 2.23, 95% CI 2.19-2.28), followed by women aged 35-39 years (undiagnosed diabetes OR 2.13, GDM OR 1.84) and women aged 30-34 years (undiagnosed diabetes OR 1.40, GDM OR 1.47), compared with women aged <30 years. The proportion of women aged ≥40 years were the highest in women with preexisting diabetes (8.3%) and undiagnosed diabetes (7.9%), followed by women with GDM (5.2%), and the lowest was women without HIP (3.3%). Similarly, the proportion of women aged 35-40 years were the highest in women with preexisting diabetes (29.8%) and undiagnosed diabetes (28.2%), followed by women with GDM (25.6%), and the lowest was women without HIP (19.4%). Besides, age at delivery was significantly associated with the risk of preexisting diabetes, undiagnosed diabetes, and GDM (all p for trend <0.0001). When analyzed by year (Supplementary Figures 2–4), the proportion of AMA increased over time. In women with preexisting diabetes, the proportion of women with AMA increased from 29.4% in 2008 to 45.6% in 2017 (p for trend <0.001); while the proportion of women aged <30 years decreased from 32.6% to 20.7% in this period (p for trend <0.001). In women with undiagnosed diabetes, the proportion of women with AMA increased from 25.5% to 39.0% in 2008-2017 (p for trend, 0.011). In women with GDM, the proportion of women with AMA increased from 20.4% in 2008 to 40.5% in 2017 (p for trend <0.001), and the proportion of women aged <30 years decreased from 36.7% to 22.8% (p for trend <0.001).

In Figure 1, the prevalence of HIP increased by age. The prevalence of preexisting diabetes was the lowest in women aged < 30 years (0.51%), followed by women aged 30-34 years (0.75%, p<0.05 vs. women aged <35 years) and was the highest in women aged ≥35 years (1.24%, p<0.05 vs. women aged <30 years or women aged 30-34 years). Similar trend was observed in women with undiagnosed diabetes, the prevalence of undiagnosed diabetes in women aged <30 years, 30-34 years and ≥35 years were 0.18%, 0.24% and 0.37%, respectively (all p<0.05 comparing each other). In women with GDM, the prevalence was the lowest in women aged < 30 years (10.57%), followed by women aged 30-34 years (14.77%, p<0.05 vs. women aged <35 years) and was the highest in women aged ≥35 years (18.13%, p<0.05 vs. women aged <30 years or women aged 30-34 years). In Table 3, the prevalence of preexisting diabetes, undiagnosed diabetes and GDM in all age groups increased by time, from 2008 to 2017.

TABLE 2 The distribution of age at delivery in women with preexisting diabetes mellitus, undiagnosed diabetes mellitus, gestational diabetes mellitus (GDM) or without hyperglycemia in pregnancy (HIP) in Taiwan in 2008-2017.

	Preexisting diabetes			Undiagnosed diabetes			GDM			Without HIP		P value
Age at delivery	n	(%)	OR (95% CI)	n	(%)	OR (95% CI)	n	(%)	OR (95% CI)	n	(%)	
<30 years old	3,303	(23.1)	1.00	1,156	(25.5)*	1.00	69,006	(26.8)*	1.00	579,677	(37.3)*†‡	<0.001
30-34 years old	5,565	(38.9)	1.57(1.50- 1.64)	1,739	(38.3)	1.40 (1.30-1.51)	109,078	(42.3)¥ϕ	1.47 (1.46-1.49)	622,353	(40.0)¥£	
35-39 years old	4,258	(29.8)	2.48 (2.37-2.60)	1,281	(28.2)	2.13 (1.97-2.31)	66,065	(25.6)αβ	1.84 (1.82-1.86)	301,438	(19.4)αβγ	
≥40 years old	1,182	(8.3)	4.10 (3.83-4.38)	359	(7.9)	3.56 (3.16-4.01)	13,449	(5.2)§‖	2.23 (2.19-2.28)	50,602	(3.3)§‖¶	
Total	14,308	(100.0)	P<0.0001	4,535	(100.0)	P<0.0001	257,598	(100.0)	P<0.0001	1,554,070	(100.0)	

Preexisting diabetes mellitus, diabetes diagnosed before pregnancy; undiagnosed diabetes, diabetes mellitus first detected in early pregnancy, before 20 weeks of gestation.
*p < 0.05 vs. preexisting diabetes mellitus in women <30 years old. †p < 0.05 vs. undiagnosed diabetes mellitus in women <30 years old. ‡p < 0.05 vs. GDM in women <30 years old.
¥p < 0.05 vs. preexisting diabetes mellitus in women 30-34 years old. ϕp < 0.05 vs. undiagnosed diabetes mellitus in women 30-34 years old.£p < 0.05 vs. GDM in women 30-34 years old.
αp < 0.05 vs. preexisting diabetes mellitus in women 35-39 years old. βp < 0.05 vs. undiagnosed diabetes mellitus in women 35-39 years old.γp < 0.05 vs. GDM in women 35-39 years old.
§p < 0.05 vs. preexisting diabetes mellitus in women ≥40 years old. ‖p < 0.05 vs. undiagnosed diabetes mellitus in women ≥40 years old. ¶p < 0.05 vs. GDM in women ≥40 years old.

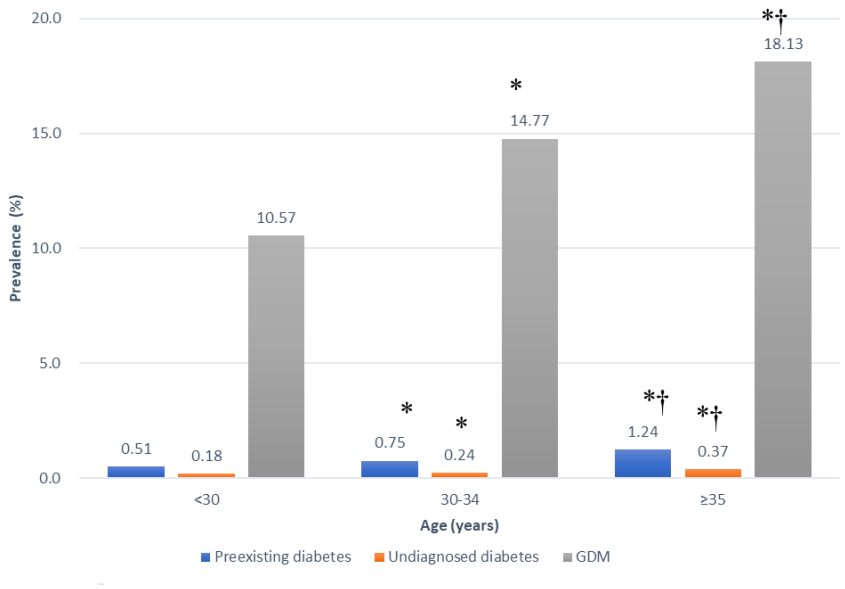

FIGURE 1
Prevalence of preexisting diabetes mellitus, undiagnosed diabetes mellitus and gestational diabetes mellitus (GDM) in different age groups in Taiwan in 2008-2017. *p < 0.05 vs. women aged <30 years. †p < 0.05 vs. women aged 30-35 years.

Discussion

In the present study, we have shown that the mean age of delivery and the proportion of pregnant women with AMA increased during 2008 to 2017. In the same period of time, the prevalence of preexisting diabetes, undiagnosed diabetes, and GDM also increased, and the trend could be observed when analyzed in all population and in different age groups. The proportions of women with AMA were significantly higher in women with preexisting diabetes, undiagnosed diabetes or GDM

TABLE 3 Prevalence of preexisting diabetes mellitus, undiagnosed diabetes mellitus and gestational diabetes mellitus (GDM) in different age groups by year in Taiwan in 2008-2017*.

	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	P for trend
Preexisting diabetes (%)											
<30 years old	0.37	0.49	0.50	0.44	0.53	0.55	0.58	0.52	0.56	0.59	0.004
30-34 years old	0.55	0.75	0.78	0.73	0.71	0.81	0.82	0.80	0.78	0.80	0.032
≥35 years old	1.00	1.19	1.22	1.24	1.17	1.17	1.31	1.28	1.39	1.25	0.015
Undiagnosed diabetes (%)											
<30 years old	0.16	0.13	0.14	0.12	0.14	0.15	0.16	0.24	0.24	0.34	0.006
30-34 years old	0.22	0.21	0.19	0.16	0.22	0.23	0.22	0.24	0.32	0.36	0.010
≥35 years old	0.34	0.34	0.35	0.34	0.37	0.34	0.36	0.35	0.41	0.47	0.015
GDM (%)											
<30 years old	8.89	9.77	10.04	10.42	10.63	11.07	11.76	12.47	10.87	10.65	0.016
30-34 years old	12.87	14.12	14.47	14.95	14.66	15.03	15.51	16.24	14.94	14.38	0.053
≥35 years old	15.49	16.25	17.24	18.19	18.30	18.90	18.78	19.39	18.19	18.24	0.009

Preexisting diabetes mellitus, diabetes diagnosed before pregnancy; undiagnosed diabetes, diabetes mellitus first detected in early pregnancy, before 20 weeks of gestation.
*Prevalence was calculated by using the number of women with the indicated disease in the age group in the year as the numerator and the number of all pregnant women in the age group in the year as the denominator.

than women without HIP. In 2008-2017, the proportions of women with AMA increase by time in women with preexisting diabetes, undiagnosed diabetes, and GDM. On the other hand, the prevalence of HIP, including preexisting diabetes, undiagnosed diabetes, and GDM, increased with advanced age.

According to the estimation by the International Diabetes Federation (IDF), the global prevalence of HIP was 16.7% in 2021, affecting 21.1 million women (13). Among them, 80.3% were women with GDM, 9.1% were women with undiagnosed diabetes and 10.6% were women with preexisting diabetes. Except for the highest prevalence of 25.9% in South-East Asia, the prevalence of HIP in other regions ranged from 13.0% in Africa to 17.2% in North America and Caribbean. In Asia, the prevalence of HIP was 14.0% by the estimation of the IDF. In the present study, the prevalence of HIP ranged from 12.2% in 2008 to 15.7% in 2017. Most pregnant women with HIP were GDM, accounting for more than 90%, and women with preexisting diabetes or undiagnosed diabetes were 4.4%-5.7% and 1.2%-2.5% of all women with HIP, respectively. For the secular trend of HIP, there are only a few reports in the literature. In a systemic review and meta-analysis for the prevalence of preexisting diabetes, the combined analysis of different studies showed that the prevalence of preexisting diabetes doubled from 0.5% to 1.0% during 1990-2020 (14), which was in concordance with the findings from the present study. For GDM, a previous report in Taiwan showed that the prevalence of GDM increased from 7.6% to 13.4% in 2004-2015 (5). Besides, two reports from Korea also demonstrated the increase in the prevalence of GDM in 2009-2011 (15) and 2012-2016 (16). Taken together, findings from the literature and the present study suggest that HIP, including preexisting diabetes, undiagnosed diabetes and GDM, is still a growing health threat for the pregnant women.

Generally, AMA is defined as age of delivery greater than 35 years (8). In the United States, the proportion of pregnant women with AMA increased from 1% in 1970s (6) to 14% in 2005 (17). In the present study, the mean age of delivery increased from 29.84 years old in 2008 to 31.86 years old in 2017, and the proportion of women with AMA increased from 14.5% to 29.6% in 2008-2017. In addition, we have shown that the prevalence of preexisting diabetes, undiagnosed diabetes, and GDM increased by age. In a meta-analysis, there is a linear relationship between maternal age and the risk GDM, and every 1-year increase in maternal age is associated with a 7.9% increase in the risk of GDM (9). Besides, age-related glucose intolerance generally becomes more pronounced in the reproductive age (10, 11), which may lead to a higher risk of preexisting diabetes during pregnancy. Taken together, these findings suggest that the increasing trend of women with AMA could be one of the reasons for the increased prevalence of HIP in 2008-2017 in the present study. Furthermore, AMA also results in increased risk of various adverse pregnancy outcomes, such as preeclampsia, intrauterine growth restriction, preterm delivery and others (8). Therefore, development of strategies to decrease the proportion of women with AMA is important to improve the health of pregnant women and their offspring. For example, education of the adverse pregnancy consequence of AMA, both to students and to young women, is a key step to lower the proportion of pregnant women with AMA. Besides, the government can make some policies to shape a friendlier environment for young women to get pregnant. In addition, researches for the treatments to lower AMA-related complications are also important and should be investigated in the future.

In the present study, there was a clinically significant increase in the prevalence of GDM, from 11.4% in 2008 to 14.5% in 2017. In the same period of time, the prevalence of preexisting diabetes and undiagnosed diabetes increased only modestly. A possible explanation is the change of the diagnosis of GDM. Currently, there are two different diagnostic criteria of GDM, the two-step method (18, 19) and the one-step method (20). Different academic associations or organizations have different suggestions, including the one-step method only, the two-step method only, or both methods (2). In the literature, the prevalence of GDM diagnosed by the one-step method is higher than that by the two-step method (21, 22). Shifting from the two-step method to the one-step method resulted in an increase in the prevalence of GDM, from 10.6% to 35.5% in a Spanish study (21), and from 2.59% to 13.44% in our previous report in Taiwan (22). Therefore, the ratio of women receiving the two-step method to the one-step method would affect the overall prevalence of GDM. In Taiwan, after the one-step method was proposed in 2010 by the IADPSG, the academic associations, including the Diabetes Association of the Republic of China (DAROC), Taiwan Association of Obstetrics and Gynecology, Taiwan Society of Perinatology, and Taiwanese Association of Diabetes Educators, have held a series of educational programs promoting the use of the one-step method, and only the one-step method was recommended in the clinical practice guideline of DAROC in 2012 (23). As a result, it is possible that more obstetricians may use the one-step method to diagnose GDM, which may be another cause for the increase in the prevalence of GDM in this study. Since 2015, the clinical practice guideline of the DAROC recommends both the one-step and the two step methods to screen GDM (24–26), which may be one potential explanation for the decline in GDM prevalence in 2016 and 2017. In addition, these educational programs may also increase the awareness of screening for undiagnosed DM and GDM for both the obstetricians/physicians and the pregnant women, which may be another reason for the increase of the prevalence of undiagnosed DM and GDM. Furthermore, according to a study in Taiwan, the percentage of women with overweight or obesity increased gradually from 2011 to 2016 (9.7%-11.1% for women with overweight, 5.8%-7.4% for women with obesity) (4). This may also contribute to the increase in the prevalence of HIP.

The strength of the study is its large sample size and the inclusion of almost all pregnant women in Taiwan in 2008-2017. Besides, we have analyzed the secular trend in the prevalence of different types of HIP, including preexisting diabetes, undiagnosed

diabetes and GDM, which is rarely reported in the literature. In contrast, this study has limitations. Pregnant women without national health insurance were unrecorded and therefore not included in our study and would be misclassified as women without HIP. However, because the coverage of the national health insurance is extremely high in Taiwan (over 99%), this may comprise only a minority of patients. In addition, we cannot distinguish between one-step and two-step methods used to screen gestational diabetes in this study. Since this may be one potential reason for the increasing incidence of GDM by time observed in this study, further researches are needed to confirm the impact of screening method on the incidence of GDM in population level.

In conclusion, the prevalence of preexisting diabetes, undiagnosed diabetes and GDM increased in Taiwan in 2008-2017, which may result from the increasing proportion of pregnant women with AMA and the change in the diagnostic criteria for GDM. In the future, we should develop strategies to decrease the proportion of women with AMA, such as education programs about the adverse pregnancy consequence of AMA or policies to shape a friendlier environment for young women to get pregnant, and conduct researches to investigate novel treatments to lower AMA-related complications, including HIP, both of which are important for the reduction of the health threat of AMA to pregnant women and their offspring.

Data availability statement

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

Author contributions

S-YL wrote the manuscript and researched data. Y-LW help in the statistical analysis. CHK and C-NL researched data and contributed to discussion. C-CH and H-YL initiated the study and edited the manuscript. C-CH was responsible for the statistics and the H-YL was the clinician. C-CH and H-YL contributed equally to this paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1041066/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Mean age at delivery in pregnant women with preexisting diabetes mellitus (dot line), undiagnosed diabetes mellitus (short dash), gestational diabetes mellitus (long dash) or without hyperglycemia in pregnancy (HIP, solid line) in Taiwan in 2008-2017. Preexisting diabetes mellitus, diabetes diagnosed before pregnancy; Undiagnosed diabetes mellitus, diabetes mellitus first detected in early pregnancy, before 20 weeks of gestation. P for trend <0.05 for women with preexisting diabetes, women with GDM and women without HIP. P for trend=0.050 for women with undiagnosed diabetes.

SUPPLEMENTARY FIGURE 2
Distribution of age at delivery in pregnant women with preexisting diabetes mellitus in Taiwan in 2008-2017. Preexisting diabetes mellitus, diabetes diagnosed before pregnancy. P for trend over years, <0.001 for women aged <30 years, 0.030 for women aged 30-34 years and <0.001 for women aged ≥35 years.

SUPPLEMENTARY FIGURE 3
Distribution of age at delivery in pregnant women with undiagnosed diabetes mellitus in Taiwan in 2008-2017. Undiagnosed diabetes mellitus, diabetes mellitus first detected in early pregnancy, before 20 weeks of gestation. P for trend over years, 0.138 for women aged <30 years, 0.175 for women aged 30-34 years and 0.011 for women aged ≥35 years.

SUPPLEMENTARY FIGURE 4
Distribution of age at delivery in pregnant women with gestational diabetes mellitus (GDM) in Taiwan in 2008-2017. P for trend over years, <0.001 for women aged <30 years, 0.023 for women aged 30-34 years and <0.001 for women aged ≥35 years.

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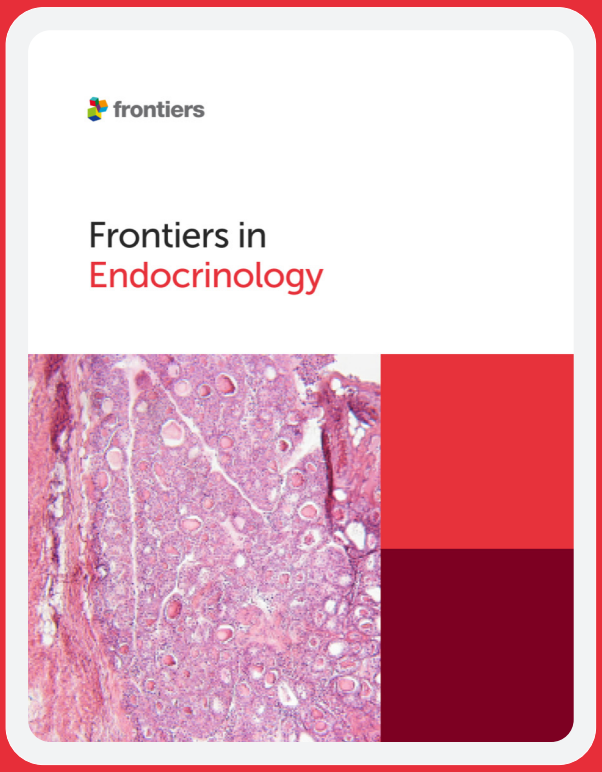

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