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Endocrine and metabolic effects on maternal-fetal and neonatal outcomes

Edited by
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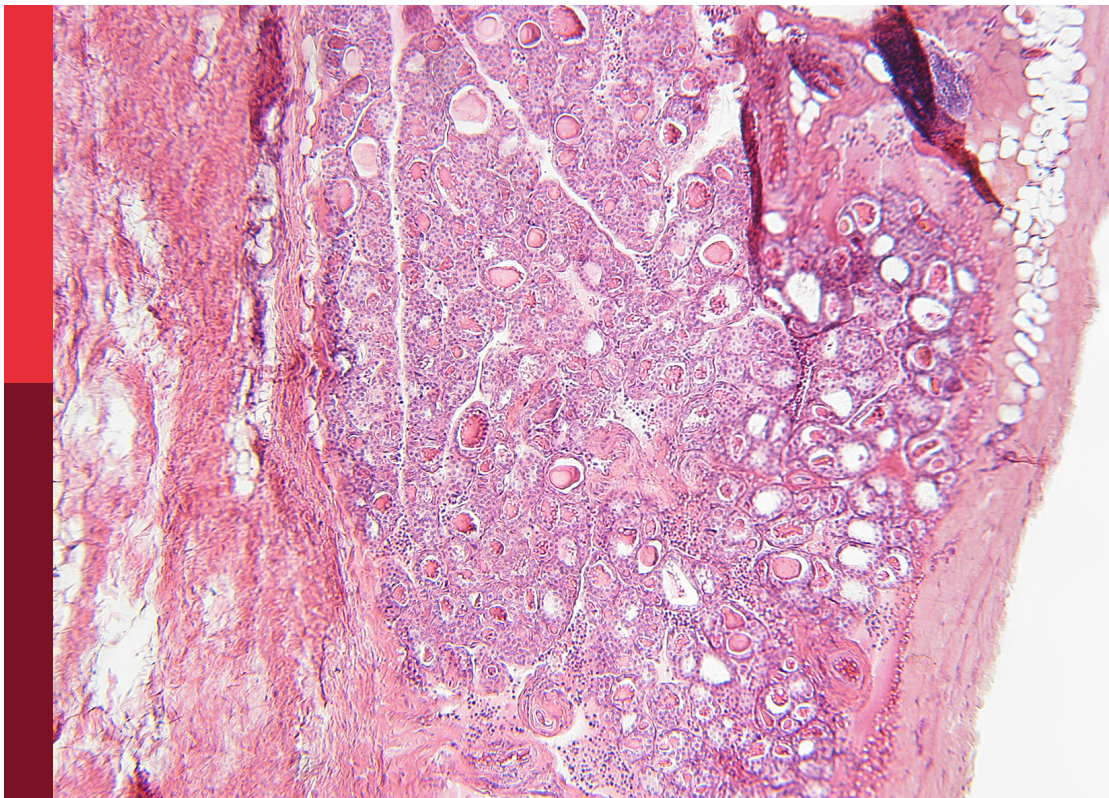

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Endocrine and metabolic effects on maternal-fetal and neonatal outcomes

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Progression to type 2 diabetes mellitus after gestational diabetes mellitus diagnosed by IADPSG criteria: Systematic review and meta-analysis

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Background: To estimate the progression rates to type 2 diabetes mellitus (T2DM) in women with gestational diabetes mellitus (GDM) diagnosed by the International Association of Diabetes and Pregnancy Study Group (IADPSG) criteria.

Methods: Systematic review and meta-analysis were conducted by searching Medline, Embase, and Cochrane between January 1, 2010 and December 31, 2021 for observational studies investigating progression to T2DM after GDM. Inclusion criteria were IADPSG-diagnosed GDM, studies with both GDM and controls, postpartum follow-up duration at least one year. Data were pooled by random effects meta-analysis models. Heterogeneity was assessed by I_2 statistic. The pooled relative risk for incidence of T2DM and pre-diabetes between GDM participants and controls were estimated. Reasons for heterogeneity among studies were investigated by prespecified subgroup and meta-regression analysis. Publication bias was assessed by the Begg's and Egger's tests.

Results: This meta-analysis of six studies assessed a total of 61932 individuals (21978 women with GDM and 39954 controls). Women with IADPSG-diagnosed GDM were 6.43 times (RR=6.43, 95% CI:3.45-11.96) more likely to develop T2DM in the future compared with controls. For GDM women, the cumulative incidence of T2DM was 12.1% (95% CI: 6.9%-17.3%), while the pooled cumulative incidence of T2DM was estimated to be 8% (95% CI: 5-11%) in studies with 1 to 5 years of follow-up and increased to 19% (95% CI: 3-34%) for studies with more than 5 years of follow-up. Women with IADPSG-diagnosed GDM had 3.69 times (RR=3.69, 95% CI:2.70-5.06) higher risk of developing pre-diabetes (including impaired fasting glucose and/or impaired glucose tolerance) than controls. Meta-regression analysis showed that the study effect size was not significantly associated with study design, race, length

of follow-up, and maternal age (P>0.05). Overall, the studies had a relatively low risk of bias.

Conclusions: Women with IADPSG-diagnosed GDM have higher risk of developing T2DM and pre-diabetes. The risk of T2DM in GDM women are higher with longer follow-up duration. Our results highlight the importance of promoting postpartum screening and keeping health lifestyle as well as pharmacological interventions to delay/prevent the onset of T2DM/pre-diabetes in GDM women.

Systematic review registration: https://www.crd.york.ac.uk/prospero, identifier (CRD42022314776)

KEYWORDS

gestational diabetes mellitus, type 2 diabetes mellitus, IADPSG criteria, systematic review, meta-analysis

Introduction

Gestational diabetes mellitus (GDM) is an established risk factor for developing type 2 diabetes mellitus (T2DM) in later life. Previous meta-analysis has highlighted a nearly 10-fold higher risk of T2DM in women with GDM compared with controls (1). In 2010, the International Association of Diabetes and Pregnancy Study Group (IADPSG) proposed new diagnostic criteria for GDM using a one-step strategy with 75g oral glucose tolerance test (OGTT) during 24–28 weeks of gestation, and the diagnostic thresholds for GDM were identified as one or more of plasma glucose values equaling or exceeding 5.1 mmol/L (92 mg/dl), 10.0 mmol/L (180 mg/dl), and 8.5 mmol/L (153 mg/dl) at fasting, 1-hour, and 2-hour after 75g OGTT (2). This was the first evidence-based, large-scale diagnostic criteria based on glucose levels associated with adverse pregnancy outcomes in the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) Study (3). The IADPSG diagnostic criteria marked a milestone in the history of GDM diagnostic criteria, and many international organizations, including American Diabetes Association (ADA) (4), World Health Organization (WHO) (5), the International Federation of Gynecology and Obstetrics (6) et al. advocated the use of 75g OGTT during 24–28 weeks of gestation as the diagnostic test and the new cutoff values recommended by IADPSG as GDM diagnostic criteria.

The application of this landmark IADPSG diagnostic criteria led to a rise in the prevalence of GDM globally, which contribute to a rise in the healthcare and economic burden worldwide (7–10). Due to changes in GDM diagnosis criteria, although the IADPSG criteria had been implemented for almost 12 year, previous systematic review and meta-analysis evaluating progression to T2DM after GDM did not distinguish different kinds of diagnosis criteria, and no available meta-analysis reported the risk of T2DM and pre-diabetes after GDM diagnosed by IADPSG criteria. As is well-known, lifestyle and pharmacological intervention could prevent or delay progression to T2DM in women with previous GDM (11). Therefore, there is an urgent need to evaluate more recent evidence on the risk of progression to T2DM in women with GDM diagnosed by IADPSG criteria. This systematic review and meta-analysis aimed to investigate progression to T2DM in women with GDM diagnosed by IADPSG criteria compared with controls.

Methods

This systematic review and meta-analysis were conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) and Meta-Analyses Of Observational Studies in Epidemiology (MOOSE) guidelines. The protocol of the study had been registered on the International prospective register of systematic reviews (PROSPERO) (registration number: CRD42022314776).

Search strategy

We conducted a comprehensive literature search using Medline, Embase, the Cochrane Library, Scopus, AJOL, and Hinari for observational studies investigating progression to T2DM after GDM diagnosed by IADPSG criteria published between January 1, 2010 and December 31, 2021. We chose 2010 as the start date because the IADPSG GDM diagnostic criteria was released in 2010. The search strategy included keywords, medical subject headings (MeSH), and free text words covering "gestational diabetes mellitus" and "type 2 diabetes mellitus",

and was restricted to studies published in English and conducted on humans. The search strategy was shown in the Supplementary Material.

Study selection

Two authors (J.J. and YY.S.) independently reviewed the titles and abstracts to identify all potentially relevant studies. All duplicate records were removed. Full text of the relevant studies were obtained and screened in details according to the following predefined eligibility criteria: GDM diagnosed by IADPSG criteria, duration of postpartum follow-up at least one year, studies with both GDM women and controls (pregnant women without GDM). All reference lists from relevant reviews were hand searched for additional eligible studies. All conference proceedings, guidelines, consensus, opinions, protocols, dissertations, case series, qualitative studies, commentaries, editorials, perspectives, and letters were excluded. Disagreements between the two authors were resolved by third party consultation. The literature review and study selection process were summarized in a PRISMA flowchart (Figure 1).

Data extraction

Two authors (J.J. and YY.S.) independently extracted information from the included studies according to the Cochrane Handbook guidelines, and findings were reported according to the PRISMA and MOOSE guidelines. The extracted data was exported to an excel sheet detailing the authors' name, country of origin of the study, year of publication, study design, sample size (including the number of GDM and control), length of follow-up, participants' age, race, diagnostic criteria for T2DM, incidence of T2DM and pre-diabetes (including impaired fasting glucose[IFG] and/or impaired glucose tolerance[IGT]) among GDM and control, etc. Disagreements were settled by consensus among all authors.

Study quality assessment and risk of bias

The Newcastle-Ottawa scale (NOS) was used to evaluate the quality of non-randomized studies (12). The scale for cohort studies consisted of three categories: Selection, Comparability, and Outcome. Based on the guideline of the scale, a cohort study could be awarded a maximum of one star for each item of the Selection and Outcome categories and a maximum of two stars for the category of comparability. Totally, a study could be awarded from zero up to nine stars. Publication bias was assessed by the Begg's and Egger's tests and funnel plots diagram for asymmetry.

Statistical analysis

The pooled relative risk for the incidence of T2DM and pre-diabetes between participants with GDM and controls was

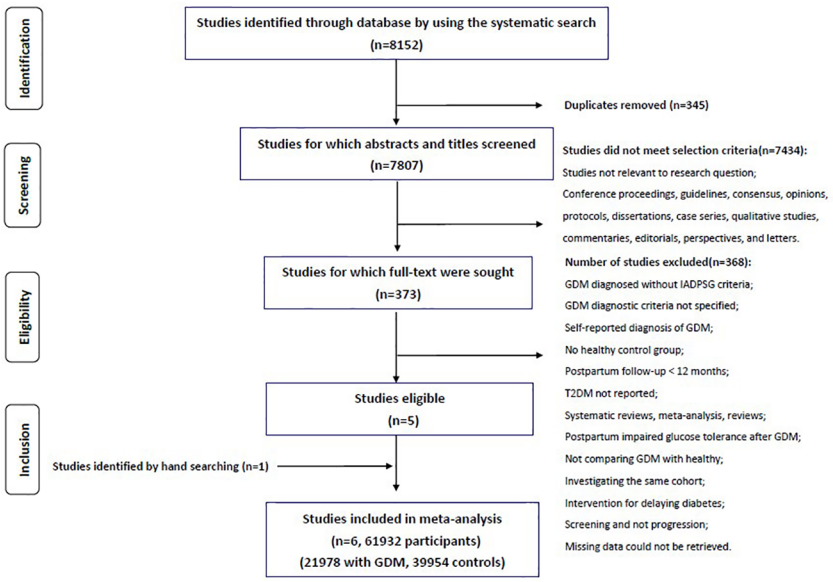

FIGURE 1
PRISMA flowchart showing literature search and study inclusion of the meta-analysis.

estimated. Data were pooled by random effects meta-analysis models with the Review Manager (version 5.4; Nordic Cochrane Centre, Cochrane Collaboration, Copenhagen, Denmark) using the DerSimonian-Laird method. In studies which no cases of T2DM and pre-diabetes was reported in any of the groups, a continuity correction was applied using the default value of 0.5 to avoid division by zero. Heterogeneity among the included studies was assessed by I^2 statistic, and graphically represented using forest plot diagram. Pre-specified subgroup analyses by race and length of study follow-up was performed to explore potential sources of heterogeneity among studies. Meta-regression models were fitted to estimate the effects of study heterogeneity and investigated the cumulative risk of developing T2DM by study length of follow-up. Sensitivity analysis were conducted by recalculating the pooled estimate with a named study removed at a time to estimate the effect of each individual study on the overall pooled estimate.

Results

Study selection

The initial comprehensive literature search identified 8152 records. After exclusion of duplicates, 7807 articles were screened, of which 373 studies were selected for further detailed review using the full text. One study retrieved from the reference lists of previous relevant reviews were also included. Finally, six studies, including a total of 61932 individuals (21978 women with GDM and 39954 controls) fulfilled all the eligibility criteria and were included in the meta-analysis. The study selection process was summarized in Figure 1.

Study quality assessment

All six included studies underwent quality assessment with NOS scale received a total of six to eight stars, suggesting that the risk of bias is relatively low. A summary of the study quality assessment was shown in the Supplementary Material-STable 1.

Study characteristics

All studies included in this systematic review and meta-analysis were observational studies, of which three were prospective cohorts and the other three were retrospective cohorts. One study was a multi-centered study including ten field centers (13), the rest five studies were conducted in Canada, Australia, United Arab Emirates, Pakistan, and Japan, respectively (14–18). The six included studies had different lengths of follow-up, ranged from 1.3 to 11.4 years. A summary of study characteristics of the included studies was presented in Table 1.

Meta-analysis

The meta-analysis of the six studies evaluated a total of 61932 participants (21978 women with GDM and 39954 controls). Among the participants, 1342 women from the GDM group subsequently developed T2DM during postpartum follow-up, while 329 women progressed to T2DM in the control group. The pooled relative risk of developing T2DM in the GDM group was 6.43 (RR=6.43, 95% CI:3.45-11.96) compared with controls (Figure 2). Significant heterogeneity was seen in the overall effect estimate (I2 = 88.2%, P<0.001) (Figure 2). In addition, women who had GDM were 3.69 times (RR=3.69, 95% CI:2.70-5.06) more likely to develop pre-diabetes (including IFG and/or IGT) than controls (Figure 3).

The risk of T2DM were assessed by pre-specified subgroup analysis based on study length of follow-up. Studies were classified by their length of follow-up into two groups: 1-5 years and more than 5 years. The estimated pooled relative risk of developing T2DM was 4.38 (95% CI:1.18-16.34) for studies with postpartum follow-up of 1-5 years, while for those with follow-up of more than five years, the pooled relative risk was 12.47 (95% CI:3.10-50.08). The pooled relative risks were not statistically significantly different between subgroups (P=0.28) (Figure 4). The relative risk of T2DM was also assessed by subgroup analysis based on race and the difference in pooled relative risks by subgroup was not statistically significant (P=0.67).

Further meta-regression analyses showed that the study effect size was not significantly associated with study design, race, length of follow-up, and maternal age (P> ˃0.05) (Table 2). Overall, in women with GDM, the cumulative incidence of T2DM and pre-diabetes were 12.1% (95% CI: 6.9%-17.3%) and 25.5% (95% CI: 4.1%-46.8%), respectively. The pooled cumulative incidence of T2DM was estimated to be 8% (95% CI: 5%-11%) in women with GDM in studies with 1 to 5 years of follow-up, increasing to 19% (95% CI: 3%-34%) for studies with more than 5 years of follow-up (Table 3).

Publication bias and sensitivity analysis

No indication of publication bias was detected among the included studies, with both Begg's and Egger's tests being statistically non-significant (P=0.71 and P=0.76, respectively). No apparent asymmetry was observed in the funnel plots diagram of the six studies included in the meta-analysis (Supplementary Material-SFigure 1).

TABLE 1 Study characteristics.

Author	Year	Country	Study design	Paticipants' age	Race	GDM sample size	Control Sample Size	Total Sample Size	Follow-up (years)	T2DM diagnostic criteria	T2DM/ GDM	T2DM/ Control	Pre-diabetes/ GDM	Pre-diabetes/ Control
Hiersch et al. (12)	2021	Canada	retrospective cohort	30.55	Mixed	20513	34848	55361	4.4	Diabetes Canada 2018 clinical practice guidelines	1132/ 20513	244/34848	–	–
Wood et al. (13)	2021	Australia	prospective cohort	GDM 29; control 25	White	172	122	294	2.5	WHO, ADA	11/172	2/122	16/172	1/122
Bayoumi et al. (14)	2021	United Arab Emirates	retrospective cohort	38.7	Non-white	362	833	1195	9	ADA	96/362	9/833	171/362	145/833
Aziz et al. (15)	2018	Pakistan	prospective cohort	GDM 28.94; control 25.68	Non-white	78	89	167	2	HbA1C: ≥6.5%; or FBG: ≥126 mg/dL (7.0 mmol/L); or 2-h blood glucose: ≥200 mg/dL (11.1 mmol/L) during an OGTT; or A random plasma glucose: ≥200 mg/dL (11.1 mmol/L)	11/78	0/89	3/78	–
Lowe et al. (11)	2018	Multi-country including 10 field centers	prospective cohort	GDM 31.9; control 29.8	Mixed	663	3946	4609	11.4	ADA	71/663	63/3946	275/663	728/3946
Kugishima et al. (16)	2018	Japan	retrospective cohort	GDM 32.9; control 33.2	Non-white	190	116	306	1.3	WHO	21/190	11/116	–	–

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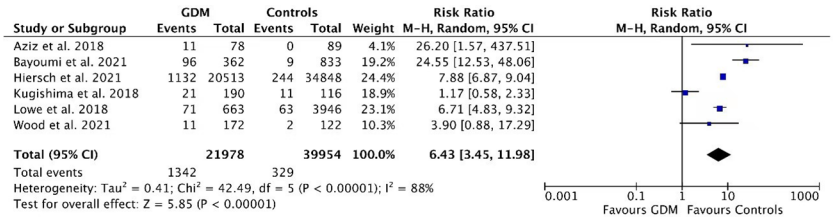

FIGURE 2
Relative risk of T2DM in women with GDM compared with controls.

Sensitivity analysis was conducted, in which a named study was removed, and the others were analyzed, the pooled estimate remained close to the overall estimate and without apparent fluctuation, indicating that no individual study had a large influence on pooled estimate.

Discussion

Main findings

The results of the systematic review and meta-analysis demonstrated that women who had GDM diagnosed by IADPSG criteria were 6.43 times (RR=6.43, 95% CI:3.45-11.96) more likely to develop T2DM in the future compared with controls. In women with previous GDM, the cumulative incidence of T2DM was 12.1% (95% CI: 6.9%-17.3%), while the pooled cumulative incidence of T2DM was estimated to be 8% (95% CI: 5-11%) in studies with 1 to 5 years of follow-up and increased to 19% (95% CI: 3-34%) for studies with more than 5 years of follow-up. Women with IADPSG-diagnosed GDM had 3.69 times (RR=3.69, 95% CI:2.70-5.06) higher risk of developing pre-diabetes than controls. Meta-regression analysis showed that the study effect size was not significantly associated with study design, race, length of follow-up, and maternal age (P>0.05). Overall, the studies had a relatively low risk of bias.

Comparison with previous studies

In this systematic review and meta-analysis, we included evidence from the most recent studies and specified GDM diagnostic criteria to IADPSG to estimate the risk of developing T2DM after GDM. The findings of our study suggested a higher risk of T2DM and pre-diabetes in women with GDM than controls, similar trends reported by previous systematic reviews and meta-analysis (1, 19, 20). However, previous studies conducted by Kim et al. published in Diabetes Care in 2002 (20), Bellamy et al. published in the Lancet in 2009 (19), and Vounzoulaki et al. published in the BMJ in 2020 (1) evaluated studies published between 1965-2001, 1960-2009 and 2000-2020, respectively, which involved GDM women diagnosed by different diagnostic criteria, thus could have led to different overall risk of subsequent T2DM in women with GDM. The systematic review and meta-analysis conducted by Bellamy et al. (19) and Vounzoulaki et al. (1) reported a 7.43 times (RR=7.43, 95% CI:4.79-11.51) and 9.51 times (RR=9.51, 95% CI:7.14-12.67) higher risk of developing T2DM in women with previous GDM than controls, respectively, which was higher than our findings. The systematic review conducted by Kim et al. (20) demonstrated that the cumulative incidence of T2DM increased markedly in the first 5 years after delivery and appeared to plateau after 10 years, which was inconsistent with our results that the risk of T2DM was higher in studies with more than 5 years of follow-up than 1 to 5 years in women with previous GDM.

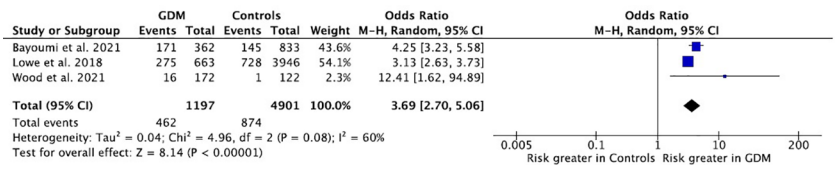

FIGURE 3
Relative risk of pre-diabetes in women with GDM compared with controls.

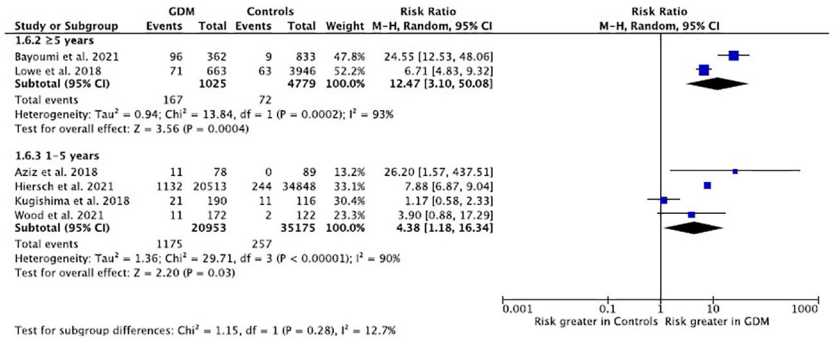

FIGURE 4
Relative risk of T2DM in women with GDM compared with controls based on study length of follow-up.

As the definition of GDM in studies conducted by previous systematic reviews and meta-analysis was all hyperglycemia first detected at any time during pregnancy, which also included women with pre-existing diabetes mellitus who were not identified prior to pregnancy and did not distinguish between diabetes in pregnancy and GDM. Women with pre-gestational diabetes mellitus indicated the underlying T2DM short-term after delivery. Therefore, the previous study found that the incidence of T2DM increased markedly in the first 5 years after delivery. Unlike previous systematic reviews, the definition of GDM we used in this study were diabetes first diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes prior to gestation (4).

Furthermore, the GDM diagnostic criteria had been constantly evolving during the past few decades, with the diagnostic strategy evolved from "two-step strategy" (GDM was diagnosed by stepwise approach: Step 1: 50g fasting plasma glucose test [GCT]; Step 2: For pregnant women whose 1-hour glucose value of 50g GCT is equal to or greater than 7.8 mmol/L [140 mg/dl], 75g/100g OGTT was conducted) (21, 22) to "one-step strategy" (GDM was diagnosed by 75g OGTT during 24-28 gestational week, and the diagnostic thresholds for GDM were identified as one or more of plasma glucose values equaling or exceeding 5.1 mmol/L [92 mg/dl], 10.0 mmol/L [180 mg/dl], and 8.5 mmol/L [153 mg/dl] at fasting, 1-hour, and 2-hour after 75g OGTT) (2); the cut-off value of fasting plasma glucose (FPG) for GDM evolved from 5.8 mmol/L (National Diabetes Data Group [NDDG], 1979) (21) to 5.3 mmol/L (Carpenter and Coustan, 1982) (22), and then to 5.1 mmol/L (IADPSG, 2010) (2); the diagnostic thresholds evolved from two or more abnormalities (21, 22) to one or more abnormalities (2). The newly released IADPSG criteria is the first scientific based diagnostic criteria predominantly based on HAPO study (3) and marked a milestone in the history of GDM diagnostic criteria. The IADPSG criteria had been advocated by many international organizations, including ADA (4), WHO (5), and the international Federation of Gynecology and Obstetrics (6). Studies had shown that the prevalence of GDM had increased significantly by implementation of IADPSG diagnostic criteria and more pregnant women with milder degrees of gestational hyperglycemia were diagnosed as GDM, which provided an opportunity for earlier detection and effective lifestyle intervention of milder GDM to prevent adverse pregnancy outcomes (7–9). We specified GDM diagnostic criteria to IADPSG in this meta-analysis. Therefore, the previous

TABLE 2 Results of meta-regression models.

Study level variables	Coefficient (95%CI)	P values	I^2 (%)
Study design			
Retrospective cohort study	0.84 (0.04, 16.90)	0.884	90.37
Prospective cohort study	Ref.		
Race			
White	1.01 (0.02, 66.58)	0.992	92.55
Non-white	0.54 (0.00,185.31)	0.757	92.55
Mixed	Ref.		
Length of follow-up (mean)	1.51 (0.84, 1.57)	0.277	89.95
Age (mean)	1.08 (0.75, 1.55)	0.589	89.58

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TABLE 3 Cumulative incidence of T2DM by length of follow-up.

	No of contributing studies	GDM (%; 95% CI)	Controls (%; 95% CI)
Length of follow-up (years):			
1-5	4	0.08 (0.05, 0.11)	0.02 (0.00, 0.03)
≥5	2	0.19 (0.03, 0.34)	0.01 (0.01, 0.02)
Overall	6	0.12 (0.07, 0.17)	0.01 (0.005,0.02)

systematic reviews might have caused a higher risk of developing T2DM after GDM than ours.

Our results indicated that even the milder degrees of gestational hyperglycemia diagnosed by the more stringent IADPSG criteria still had a higher risk of subsequent progression to T2DM and pre-diabetes. Genetic predisposition might be one possible contributor to the progression to T2DM after GDM as increasing evidence have indicated that GDM may share similar genetic susceptibilities with T2DM (23–26). Genome wide association studies (GWAS) and other studies have reported that several genetic variants (such as *MTNR1B, TCF7L2, IGF2BP2, CDKAL1, GCK*) are associated with increased risk of both GDM and T2DM, suggesting that these conditions might have a shared genetic background (27, 28). The genetic predisposition for GDM influences the health outcomes in the perinatal stage and poses a risk for T2DM in later life. Women with genetic variants for GDM and/or T2DM are expected to have a higher risk of postpartum diabetes, but further studies are needed to discover the specific genetic variants associated with postpartum diabetes (29). Pre-diabetes was considered to be a precursor for the development of T2DM and women with pre-diabetes might also indicate the underlying frequency of T2DM. So, we should pay more attention to postpartum follow-up of GDM women and introduce structured postpartum preventive care (30). Almost all guidelines recommended T2DM screening with 75g OGTT at 4-12 weeks postpartum and tested every 1-3 years thereafter for women with GDM. Women with a history of GDM found to have pre-diabetes should receive intensive lifestyle interventions and/or metformin to prevent T2DM (4). Despite the emphasis of these guidelines and the magnitude of T2DM risk after GDM, the postpartum screening rates were relatively low and the importance of postpartum follow-up and intervention had not been adequately addressed (31–35).

Both lifestyle and pharmacological intervention prevent or delay progression to T2DM in women with previous GDM. Results of the prospective Nurses' Health Study (NHS) showed that subsequent T2DM risk after GDM was significantly lower in women who following healthy eating patterns (36). According to a randomized controlled clinical trial conducted at 27 clinical centers, intensive lifestyle intervention and metformin could reduce progression to diabetes by 35% and 40%, respectively, in women with previous GDM over 10 years of follow-up compared with placebo (37). Therefore, we should highlight the importance of increased awareness in women with GDM the need to attend postpartum screening and motivated them to keep healthy lifestyle, including physical exercise and balanced diet, as well as adopt pharmacological interventions under the guidance of doctors if needed in order to delay or prevent the progression from GDM to T2DM (36–38). In the meanwhile, it is important to cooperate obstetricians, internists, pediatricians, and other healthcare providers to provide support and emphasize the importance of postpartum follow-up of GDM to reduce the future risk of T2DM.

More up-to-date large randomized controlled trials with longer follow-up are needed to investigate the lifestyle and pharmacological intervention strategies to delay or prevent the progression from GDM to T2DM. In addition, further cost-effectiveness studies of these interventions should be conducted to promote the implementation of these interventions.

Strengths and limitations

This systematic review and meta-analysis assessed the most recently published studies on the risk of progression to T2DM in women with IADPSG-diagnosed GDM, with a relatively large total number of individuals, and postpartum follow-up duration ranging from 1.3 to 11.4 years. As the diagnostic criteria of GDM had changed over the past years, this meta-analysis provided up-to-date results using the specific IADPSG criteria. Nevertheless, several limitations should also be considered. Owing to limited availability of studies evaluating the risk of progression to T2DM in women with GDM diagnosed by IADPSG criteria, only a few studies fulfilled all the eligibility criteria and were included in this meta-analysis, resulting in inclusion of a relatively small sample size. Therefore, we were unable to investigate the progression to T2DM after GDM in certain subgroups, which could have been a cause of heterogeneity among studies. Furthermore, we were unable to identify the main sources of heterogeneity in our analysis, and a more in-depth analysis could have been performed if individual patient level data were available. In addition, the IADPSG criteria was released in 2010 and many of the women might have not been developed T2DM at the time of assessment. Therefore, more high-quality studies with longer and complete follow-up are needed to accurately evaluate the progression to T2DM in women with GDM.

Conclusions

In conclusion, our systematic review and meta-analysis showed that women with GDM diagnosed by IADPSG criteria had higher risk of developing T2DM and pre-diabetes than controls. The risk of T2DM in women with previous GDM was higher in studies with more than 5 years of follow-up than 1 to 5

years. Our results highlight the importance of promoting postpartum screening and keeping health lifestyle, including physical exercise and balanced diet, as well as pharmacological interventions to delay or prevent the onset of T2DM/pre-diabetes in women with GDM.

Data availability statement

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

Author contributions

HY and JJ conceived and designed the study. JJ and YS conducted the study selection and data extraction. JJ and PZ conducted the study quality assessment and statistical analysis. JJ wrote the manuscript. HY, YW, SW, GS, and JY 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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Supplementary material

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Development and validation of a new predictive model for macrosomia at late-term pregnancy: A prospective study

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Objective: Fetal macrosomia is defined as a birth weight more than 4,000 g and is associated with maternal and fetal complications. This early metabolic disease may influence the entire life of the infant. Currently, macrosomia is predicted by using the estimated fetal weight (EFW). However, the EFW is inaccurate when the gestational week is gradually increasing. To assess precisely the risk of macrosomia, we developed a new predictive model to estimate the risk of macrosomia.

Methods: We continuously collected data on 655 subjects who attended regular antenatal visits and delivered at the Second Hospital of Hebei Medical University (Shijiazhuang, China) from November 2020 to September 2021. A total of 17 maternal features and 2 fetal ultrasonographic features were included at late-term pregnancy. The 655 subjects were divided into a model training set and an internal validation set. Then, 450 pregnant women were recruited from Handan Central Hospital (Handan, China) from November 2021 to March 2022 as the external validation set. The least absolute shrinkage and selection operator method was used to select the most appropriate predictive features and optimize them *via* 10-fold cross-validation. The multivariate logistical regressions were used to build the predictive model. Receiver operating characteristic (ROC) curves, C-indices, and calibration plots were obtained to assess model discrimination and accuracy. The model's clinical utility was evaluated *via* decision curve analysis (DCA).

Results: Four predictors were finally included to develop this new model: prepregnancy obesity (prepregnancy body mass index ≥ 30 kg/m^2), hypertriglyceridemia, gestational diabetes mellitus, and fetal abdominal circumference. This model afforded moderate predictive power [area under the ROC curve 0.788 (95% confidence interval [CI] 0.736, 0.840) for the training

set, 0.819 (95% CI 0.744,0.894) for the internal validation set, and 0.773 (95% CI 0.713,0.833) for the external validation set]. On DCA, the model evidenced a good fit with, and positive net benefits for, both the internal and external validation sets.

Conclusions: We developed a predictive model for macrosomia and performed external validation in other regions to further prove the discrimination and accuracy of this predictive model. This novel model will aid clinicians in easily identifying those at high risk of macrosomia and assist obstetricians to plan accordingly.

KEYWORDS

macrosomia, fetal growth, obesity, gestational diabetes mellitus, predictive model

Background

Macrosomia is defined as a birth weight more than 4,000 g and is one of the most common adverse neonatal outcomes worldwide. Macrosomia is strongly associated with severe adverse perinatal outcomes, including shoulder dystocia, maternal birth canal trauma, and fetal brachial plexus injury or fracture (1, 2). If the risk could be estimated more accurately, this would help reduce such outcomes (3). Several methods that were earlier developed to predict fetal birth weight remain in use in clinical practice. For example, the Hadlock formula for the estimation of fetal weight (EFW) uses fetal morphological ultrasonic or other parameters (4, 5). However, the American College of Obstetricians and Gynecologists recently reported that the accuracy of both the Hadlock formula and the formulae using clinical parameters to predict macrosomia were limited; this is because the EFW accuracy falls constantly as the gestational weeks increase, especially at late-term pregnancy (6). The use of EFW methods to predict macrosomia is associated with a high risk of incorrect delivery decisions (7, 8). A more accurate method is required. Some scholars have built predictive models to predict the newborn weight in recent years. However, these have certain limits. For example, some models are difficult to use in the clinic because they require seldom-measured fetal parameters, or some are applicable only to specific races (9–11). Moreover, the accuracy of these models has not been completely assessed and external validation evidence is lacking. In this study, we developed a novel predictive model and performed validations to identify patients at risk of delivering macrosomia easily, allowing rational intervention and appropriate prenatal decision-making.

Methods

Populations

This is a prospective study. From November 2020 to September 2021, we prospectively recruited 700 pregnant women attending the Second Hospital of Hebei Medical University (Shijiazhuang, China) to conduct model development and internal validation. From November 2021 to March 2022, in another region, 500 pregnant women attending Handan Central Hospital (Handan, China) were prospectively recruited as the model's external validation. All the data in two regions were continuously recorded in the primary healthcare systems. After excluding 95 patients (45 subjects from Shijiazhuang and 50 subjects from Handan) who did not meet the inclusion criteria, a total of 1,105 subjects were finally included in analysis. Based on the work of the two medical centers, we ultimately identified 19 relevant features, of which 17 were maternal features and 2 were fetal features. The flow chart of study design is shown in Figure 1 (see Figure 1).

Inclusion and exclusion criteria

The subject's inclusion criteria were (1) maternal age ≥ 20 years; (2) a singleton pregnancy; (3) the completion of an oral glucose tolerance test (OGTT) at 24–28 weeks of gestation; and (4) a fetal ultrasound examination at 37–41 weeks of gestation. According to the World Health Organization (WHO), a body mass index (BMI) ≥ 30 kg/m^2 reflects obesity. The diagnostic criteria for gestational diabetes mellitus (GDM) were those of the International Association of Diabetes and Pregnancy Study

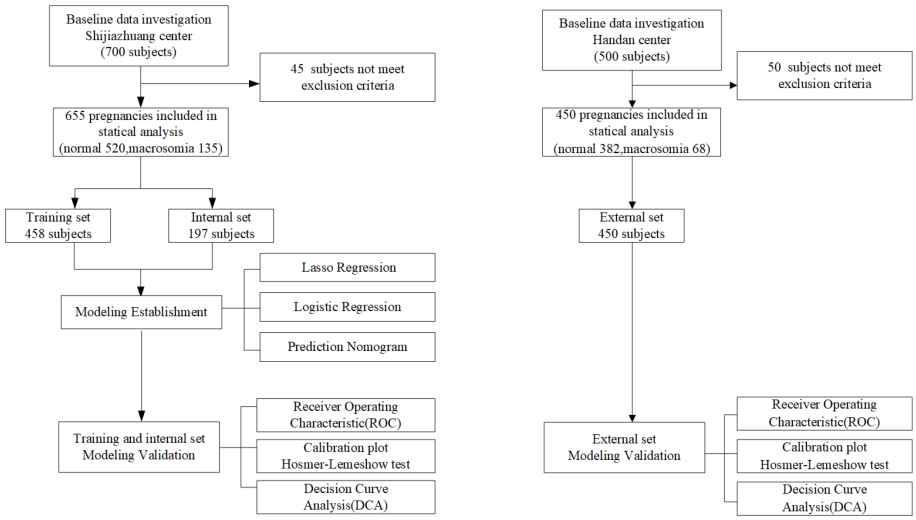

FIGURE 1
Study flow chart.

Groups (IADPSD): fasting plasma glucose (FPG) ≥ 5.1 mmol/L, oral glucose tolerance 1-h plasma glucose (OGTT 1hPG) ≥ 10.0 mmol/L, and oral glucose tolerance 2-h plasma glucose (OGTT 2hPG) ≥ 8.5 mmol/L on a 75-g OGTT test performed at 24–28 weeks of gestation; GDM was diagnosed when any of the three criteria were met (12). Hypercholesterolemia and hypertriglyceridemia were diagnosed using the criteria of the Guidelines for the American College of Cardiology/American Heart Association (13). The exclusion criteria were (1) multiple pregnancies; (2) gestational hypertension; (3) congenital heart disease; (4) a severe liver or kidney disease; (5) an autoimmune disease; (6) a psychiatric disorder; (7) the use of hormonal drugs during pregnancy; and (8) a fetal chromosomal abnormality or a congenital malformation. This study was approved by both Hebei Medical University and Handan Central Hospital.

Predictive factors choose and measurements

Several candidate predictors were referred to previous studies. Other candidate predictors were obtained based on advice from experienced obstetricians, endocrinologists, and ultrasound physicians. Finally, 17 maternal and 2 fetal characteristics were included, which were proven to be potentially related with macrosomia: 1). maternal demographic characteristics: age, gestational weeks before delivery, maternal abdominal circumference, added weight during pregnancy, prepregnancy BMI, and uterine height at late-term pregnancy; 2). metabolic-related factors: the patient's history of prepregnancy obesity, GDM, hypercholesterolemia, and hypertriglyceridemia during pregnancy; 3). biochemical features: the OGTT test results including the FPG, OGTT-1hPG, and OGTT-2hPG at gestational 24–28 weeks and the levels of low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), serum creatinine, and serum uric acid at gestational 16 weeks; and 4). fetal growth parameters: the biparietal diameter and abdominal circumference at gestational 37–41 weeks. The blood biochemical tests concluded at gestational 16 weeks and OGTT tests concluded at 24–28 weeks of gestation. The prepregnancy BMI was calculated as the self-reported prepregnancy weight (kg)/height (m^2) that was regularly registered in the patient's primary healthcare systems. The added weight during pregnancy was calculated as the weight of an inpatient before delivery minus the self-reported prepregnancy weight (14). The uterine height was measured by an obstetrician *via* abdominal palpation at late-term pregnancy. A same measurement of fetal ultrasonographic parameters was performed according to the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG) Practice Guidelines in two medical centers at a subject's gestational 37–41 weeks (4): the subject lays supine or in the lateral position during examinations. A senior physician examined the fetus *via* three-dimensional abdominal ultrasonography and recorded the fetal ultrasonographic parameters. The ultrasound examinations were performed by an experienced ultrasound physician who was blinded to the study groups at the Second Hospital of Hebei Medical University and Handan Central Hospital.

Outcome assessment

The weight of newborns was measured by nurses during the admission for delivery. All the newborns were weighed immediately after delivery by using the baby scale. Macrosomia was defined as a newborn weighing more than 4,000 g. The outcome measurement was completed by experienced obstetricians in two medical centers.

Statistical analysis

A total of 655 patients from Shijiazhuang were randomly divided into a training set with 458 participants and an internal validation set with 197 participants with a 3:1 ratio. A total of 450 patients in Handan were analyzed for an external validation set. The t-test was used for analyzing numerical variables, and the Mantel–Haenszel chi-square test was utilized for analyzing categorical variables between groups. The method to achieve model selection is the last absolute shrinkage and selection operator (LASSO) regression method. The optimal penalty (lambda, λ) was estimated by using 10-fold cross-validation. According to the lambda-choosing path, the optimal penalty lambda could be present by the lambda with a minimum mean squared error (lambda.min) or the lambda.min with one standard error (lambda.1se) (15, 16). The univariable logistic regression was first used to evaluate the relationship between all the predictive features and the outcome. Then, to screen the potential optimal features, two multivariate logistic regression models with penalty was lambda.min (model 1) and lambda.1se (model 2) were built and compared to choose the most appropriate predictive features. The features were considered as odds ratio (OR) having 95% confidence interval (CI) and as a *P*-value. The statistical significance levels were all two sided. All of the selected features had statistical significance and were applied to develop the nomogram prediction models. The discriminatory ability of the model was evaluated by using receiver operating characteristic (ROC) curve analysis and C-indices. The accuracy of the model was evaluated by drawing the calibration curves, accompanied by using the Hosmer–Lemeshow test. The calibration curves were measured by the bootstrap method for 500 repetitions. Decision curve analysis (DCA) was used to determine the clinical practicability of nomograms based on the net benefit under different threshold probabilities. For sample size simulation, we used the formula to calculate the sample size required for developing the prediction model of a binary outcome recommended by Riley et al. (17). Missing values in the data sets were handled by using the multiple interpolation method. Statistical analyses were performed using R software (version 3.6.1; R Foundation for Statistical Computing, Vienna, Austria).

Results

Population characteristics

A total of 458 subjects were used to develop the model, 197 subjects were analyzed for internal validation, and 458 subjects were finally analyzed for external validation. The prevalence of macrosomia in the model training set (development set), internal validation set, and external validation set was 23%, 15%, and 15%, respectively. There was no significant difference between the training set and the internal validation set for all the 19 features. All the alternative features characteristics of three sets are listed in Table 1 (see Table 1).

Features selection and model development

As shown in Figure 2A, we used LASSO regression to identify useful predictors from the 19 potential factors and then employed multivariate logistic regressions to build the model. In Figure 2B, nine features were saved under the optimal penalty that was lambda.min, and four features were finally saved under the penalty that was lambda.1se. Table 2 shows the regression analysis of all the features. Model 1 (Table 2) shows that the multivariate logistic regression result with penalty was lambda. min. Model 2 (Table 2) shows that the multivariate logistic regression result with the penalty being lambda.1se. After comparing the results of two multivariate regression models, four features in Model 1 (Table 2) including the added weight, 2hPG, age, and gestational weeks were excluded as they were not significantly contributing to the outcome. Four features using lambda.1se were finally included to build the predictive model: the prepregnancy obesity (BMI ≥ 30 kg/m^2), GDM, hypertriglyceridemia, and fetal abdominal circumference (Table 2). Then, we created a nomogram of macrosomia risk (See Figure 3). An example interpretation of this nomogram is as follows: a woman is not obese prepregnancy but develops GDM and hypertriglyceridemia during pregnancy, and the fetal abdominal circumference is 39 cm at 37–41 weeks of gestation. The latter three features attract the scores of 45, 52.5, and 77.5, respectively (total 175). The nomogram indicates that the risk of a macrosomia birth is almost 60%.

Macrosomia risk factors

Four features were finally included to build the predictive model: prepregnancy obesity (95% CI 1.51,4.27 $p < 0.001$), GDM (95% CI 1.49,6.03 $P = 0.002$), hypertriglyceridemia (95% CI

TABLE 1 Characteristics of the population in training set and validation sets.

Features	Training set (Shijiazhuang, n=458)	Internal Validation set (Shijiazhuang, n=197)	External Validation set (Handan, n=450)	*P*-value
Age(years)	31.21 ± 4.60	30.98 ± 4.58	28.29 ± 4.36	0.18
Gestational weeks at delivery (weeks)	38.29 ± 1.13	38.18 ± 1.05	38.46 ± 1.28	0.15
Prepregnancy BMI (kg/m^2)	24.65 ± 4.57	24.05 ± 4.60	24.35 ± 4.70	0.13
Added weight during pregnancy (kg)	13.44 ± 2.37	13.21 ± 2.32	13.52 ± 2.39	0.24
Uterine height (cm)	33.29 ± 2.26	33.29 ± 2.18	32.08 ± 2.73	0.98
Maternal abdominal circumference (cm)	96.15 ± 7.31	95.33 ± 6.90	94.68 ± 7.01	0.07
GDM (%)				
Yes	210 (46)	76 (39)	116 (35)	0.09
No	248 (54)	121 (61)	334 (64)	
Prepregnancy obesity (%)				
Yes	131 (29)	51 (26)	116 (26)	0.48
No	327 (71)	146 (74)	334 (74)	
Hypertriglyceridemia (%)				
Yes	132 (29)	49 (25)	228 (38)	0.30
No	326 (71)	148 (75)	222 (62)	
Hypercholesteremia (%)				
Yes	141 (31)	67 (34)	103 (23)	0.42
No	317 (69)	130 (66)	347 (77)	
HDL-C (mmol/L)	1.52 ± 0.41	1.58 ± 0.40	1.83 ± 0.54	0.42
LDL-C (mmol/L)	3.11 ± 0.64	3.17 ± 0.63	3.05 ± 0.55	0.27
Serum creatinine (mmol/L)	62.26 ± 15.15	64.76 ± 15.35	67.81 ± 14.26	0.12
Serum uric acid (mmol/L)	294.06 ± 63.10	297.29 ± 64.87	292.44 ± 64.27	0.73
OGTT (mmol/L)				
FPG	4.88 ± 0.68	4.78 ± 0.62	4.93 ± 0.85	0.10
OGTT 1hPG	9.04 ± 1.38	9.02 ± 1.41	9.19 ± 1.71	0.93
OGTT 2hPG	6.89 ± 1.06	6.77 ± 1.11	6.95 ± 1.08	0.22
Fetal biparietal diameter (cm)	9.37 ± 0.36	9.42 ± 0.35	9.10 ± 0.61	0.09
Fetal abdominal circumference (cm)	35.03 ± 2.12	34.70 ± 2.20	35.09 ± 2.18	0.07

Values are expressed as means ± SD (standard variation) or frequency (%). GDM, gestational diabetes mellitus; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein. OGTT, oral glucose tolerance test; FBG, fasting blood glucose; OGTT 1hPG, oral glucose tolerance 1-h plasma glucose; OGTT 2hPG, oral glucose tolerance test 2-h plasma glucose. P-values: Comparison between model development set and internal validation set by using the t-test or the Mantel–Haenszel chi-square test.

2.14,6.16 $P < 0.001$), and fetal abdominal circumference (95% CI 1.02,1.43 $P = 0.03$) were independent risk factors for macrosomia (See Table 2, Model 2).

Validation of the predictive model

The predictive power was assessed by using the area under the ROC curves (AUC). The AUCs were 0.788 (training set), 0.819 (internal validation set), and 0.778 (external validation set) separately. The optimal cutoffs were 0.367 (training set), 0.576 (internal validation set), and 0.353 (external validation set) (see Figure 4). The C-indices were 0.788 (95% CI 0.736, 0.840), 0.819 (95% CI 0.744, 0.894), and 0.773 (95% CI 0.713, 0.833), respectively. The calibration plots of all three sets fit well with the ideal curves (see Figure 5). The Hosmer–Lemeshow test revealed that the predicted and actual probabilities were consistent ($P_{\text{training set}} = 0.083$, $P_{\text{internal validation set}} = 0.762$, $P_{\text{external validation set}} = 0.074$). We then used DCA to assess clinical utility (See Figure 6). The threshold probabilities of the model for the three sets were 3%–78%, 1%–57%, and 2%–66% respectively. As the incidence rate of macrosomia is reported to be 5.47%–31.3% in China (18, 19) and 8.07%–8.84% in other countries in literatures (20), DCA exhibited positive net benefits and potential clinical utility within these thresholds' ranges (see Figure 6).

Discussion

Macrosomia is strongly associated with multiple adverse perinatal outcomes in a previous study (21). Obstetricians and gynecologists have sought to improve screening; however, the predictive accuracy remains poor. In this study, we developed a predictive model applicable at late-term pregnancy to help guide the perinatal delivery strategy. Four simple predictors were finally selected as the most appropriate features to build this model:

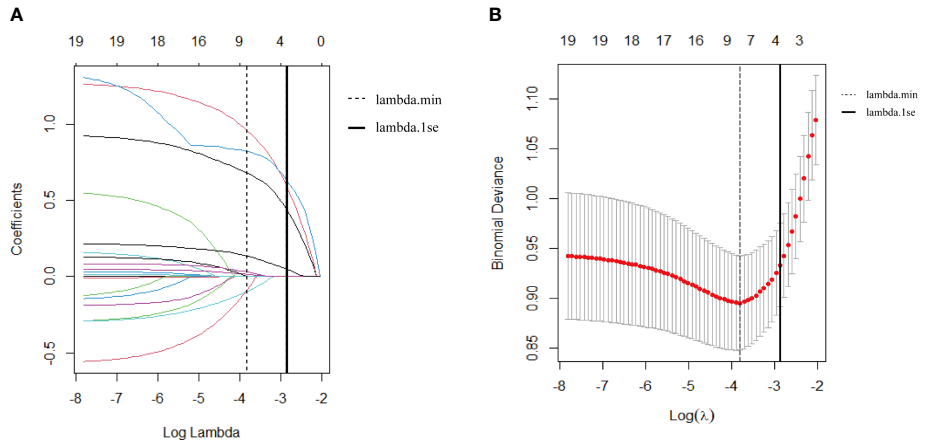

FIGURE 2
(A) Feature selection via the least absolute shrinkage and selection operator (LASSO) regression model. **(A)** The LASSO coefficient profiles of 19 features. The coefficient profile plot was conducted against the log (lambda, λ) sequence. The dotted vertical line was drawn at the lambda with a minimum mean squared error (lambda.min); nine features were selected by the LASSO regression. The solid vertical line was drawn at the lambda.min with one standard error (lambda.1se); four features were selected by the LASSO regression model. **(B)** Feature selection via 10-fold cross-validation. **(B)** The optimal parameter (lambda, λ) selection in the Lasso regression model used 10-fold cross-validation via the minimum criteria. The partial likelihood deviance (binomial deviance) curve was plotted versus logλ. The dotted vertical line was drawn at the lambda with a minimum mean squared error (lambda.min); nine features were selected. The solid vertical line was drawn at the lambda.min with one standard error (lambda.1se); four features were selected.

TABLE 2 Logistic regression analysis of the candidate predictors for macrosomia.

Candidate predictors	Univariate analysis			Multivariate analysis (Model 1)			Multivariate analysis (Model 2)		
	OR	95%CI	*P*-value	OR	95%CI	*P*-value	OR	95%CI	*P*-value
Prepregnancy obesity	4.09	2.75-6.08	<0.01	2.73	1.71-4.33	<0.01	2.54	1.51-4.27	<0.01
GDM	4.90	3.21-7.46	<0.01	2.40	1.30-4.42	0.01	2.95	1.49-6.03	<0.01
Hypertriglyceridemia	3.51	2.36-5.22	<0.01	3.04	1.89-4.89	<0.01	3.61	2.14-6.16	<0.01
Fetal abdominal circumference	1.46	1.31-1.62	<0.01	1.30	1.13-1.51	<0.01	1.21	1.02-1.43	0.03
Added weight	1.15	1.06-1.24	<0.01	1.04	0.94-1.14	0.50			
OGTT 2hPG	0.80	0.66-0.96	0.02	0.82	0.67-1.00	0.06			
Age	1.04	0.99-1.08	0.08	1.03	0.98-1.00	0.20			
Gestational weeks	1.20	1.02-1.40	0.02	1.12	0.93-1.36	0.24			
Fetal biparietal diameter	0.77	0.45-1.30	0.32	0.82	0.45-1.50	0.52			
OGTT 1hPG	1.49	1.49-1.72	<0.01						
FBG	2.29	1.71-3.08	<0.01						
HDL-C	1.06	0.78-1.45	0.71						
LDL-C	0.89	0.66-1.19	0.42						
Serum uric acid	1.00	0.99-1.00	0.32						
Serum creatinine	1.00	0.98-1.01	0.99						
Uterine height	1.03	0.95-1.12	0.47						
Prepregnancy BMI	0.97	0.93-1.01	0.11						
Hypercholesteremia	0.96	0.64-1.45	0.86						
Maternal abdominal circumference	1.03	1.00-1.06	0.04						

OR, odds radio; CI, confidence interval. GDM, gestational diabetes mellitus; FBG, fasting blood glucose; OGTT 1hPG, oral glucose tolerance test 1-h postprandial blood glucose. OGTT 2hPG, oral glucose tolerance test 2-h postprandial blood glucose. HDL-C, high-density lipoprotein cholesterol. LDL-C, low-density lipoprotein cholesterol.
Model 1: Multivariate logistic regression with penalty was lambda. min based on LASSO and ten-fold validation test.
Model 2: Multivariate logistic regression with penalty was lambda.1se based on LASSO and ten-fold validation test.

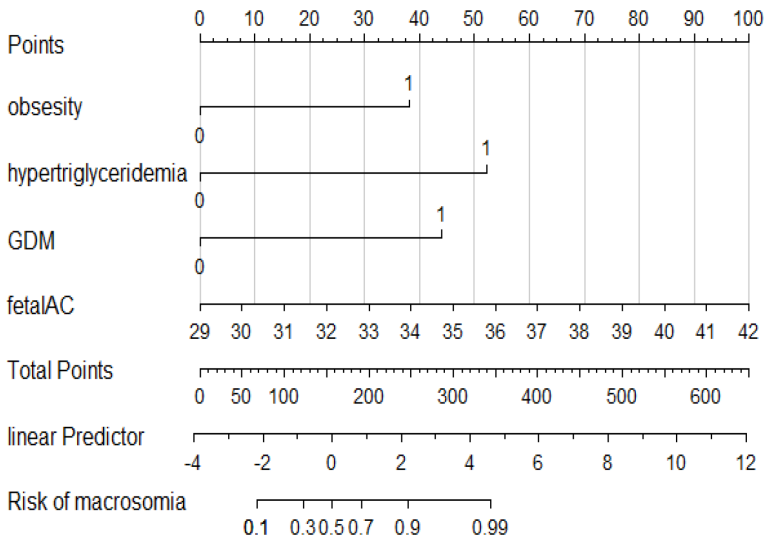

FIGURE 3
A nomogram prediction model of macrosomia. Four predictors were included: the prepregnancy obesity, hypertriglyceridemia, GDM, and fetal abdominal circumference. The score of each predictor were determined from each feature axis to the total points axis by following the vertical line. GDM, gestational diabetes mellitus; fetal AC, fetal abnormal circumference.

prepregnancy obesity (prepregnancy BMI ≥ 30 kg/m^2), GDM, hypertriglyceridemia, and fetal abdominal circumference.

Metabolic features are strongly associated with fetal macrosomia. Prepregnancy obesity is one of the most common manifestations of metabolic dysfunction in different populations. For example, a prospective study on 912 Caucasians indicated that prepregnancy obesity increased the risk of macrosomia threefold (22). Another Asian study came to the same conclusion that pregnancies with prepregnancy obesity have a higher risk to give birth to macrosomia (23). In fact, obesity is accompanied by the manifestations of abnormal metabolism such as chronic inflammation, oxidative stress, and epigenetic changes; these may affect fetal growth *in utero* by compromising the placental function (24–26). Moreover, the constant high levels of circulating adipokines (leptin, adiponectin, and tumor necrosis factor-α) may impair insulin signaling, thus reducing maternal (and even fetal) insulin sensitivity, which may, in turn, affect fetal growth (27–31). Furthermore, the adipokine secretion levels in obese women differ from those in non-obese women, perhaps explaining the relationship between obesity and fetal macrosomia (32).

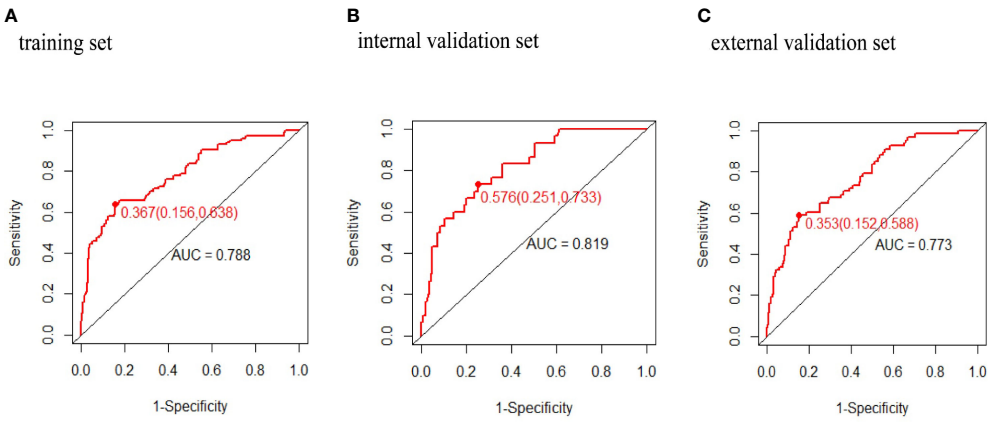

FIGURE 4
Receiver operating characteristic curves of macrosomia risk nomogram prediction. Receiver operating characteristic curve (ROC) of the **(A)** training set, **(B)** internal validation set, **(C)** external validation set. AUC, area under the receiver operating characteristic curve.

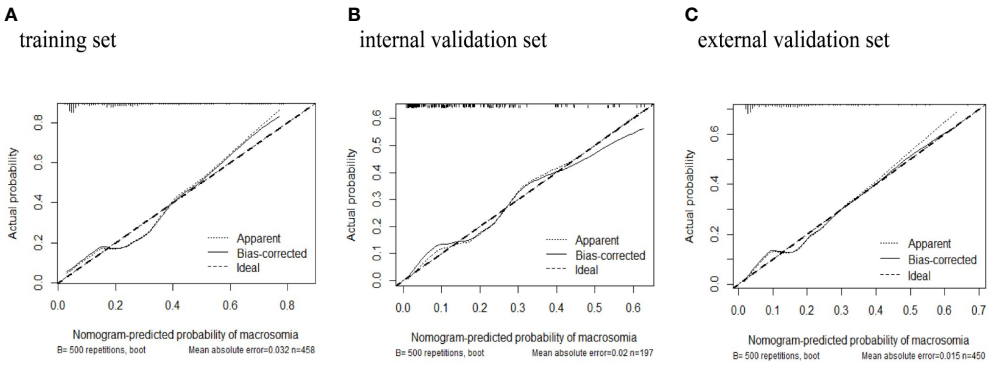

FIGURE 5
Calibration plots of macrosomia risk nomogram prediction. The x-axis represents the predicted risk of macrosomia. The y-axis represents the actual diagnosed case of macrosomia. The diagonal dotted line represents a perfect prediction by an ideal model. The solid line represents the performance of the **(A)** training set, **(B)** internal validation set, **(C)** external validation set. The closer fit of solid line to the diagonal dotted line represents a better prediction.

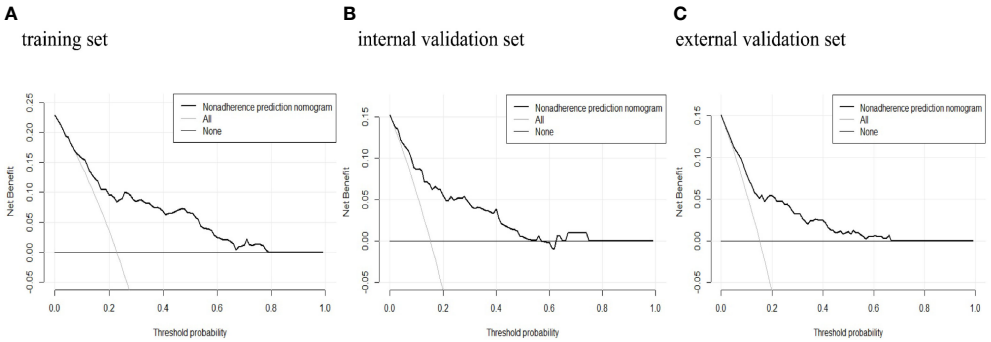

FIGURE 6
Decision curve analysis of macrosomia risk nomogram prediction. DCA of the **(A)** training set, **(B)** internal validation set, and **(C)** external validation set. The x-axis measures the threshold probability. The y-axis measures the net benefit. The thick, black solid line represents the macrosomia risk nomogram. The thin, black horizontal line (none line) represents the assumption that no patients are non-adherent to medication, which means that the net benefit is zero. The thin, gray bias (all line) represents the assumption that all patients are non-adherent to medication. DCA, decision curve analysis.

GDM was also associated with macrosomia. GDM is one of the most common metabolic diseases during pregnancy; the prevalence of GDM has gradually increased over recent decades (33). In a previous study, pregnant Asian women with GDM were at a higher risk of macrosomia than non-GDM women (34). The pathophysiological mechanism in play may be explained by the Pedersen hypothesis: GDM impairs maternal glycemic control; the serum glucose levels remain high, and then, more glucose crosses the placenta. Maternal or exogenously administered insulin does not cross the placenta. Thus, as glucose continuously crosses the placenta, compensatory hyperinsulinemia develops in the fetus (35). The risk imposed by GDM is thus twofold: not only does maternal metabolism become abnormal but also the fetus increases its adipose tissue and proprotein stores during growth, increasing the risk of macrosomia (36).

Abnormal lipid metabolism is another risk factor that may be associated with an offspring's growth. Lipid levels do not change greatly during early pregnancy; however, from gestational week 12, intestinal fat absorption increases markedly, inducing physiological hyperlipidemia (37). In this study, we found that hypertriglyceridemia was a strong predictor of macrosomia, suggesting that abnormal lipid metabolism during pregnancy is closely linked to macrosomia. Hypertriglyceridemia during pregnancy raises the levels of plasma triglycerides and free fatty acids that enter the fetal circulation *via* the placenta, increasing fetal plasma protein synthesis and decreasing lipolysis; fetal lipids accumulate (38–40). Therefore, the control of maternal lipid levels (especially the triglyceride level) should be paid high attention to reduce the risk of macrosomia.

It is well known that antenatal ultrasonography valuably assesses the fetal intrauterine growth and detects fetal structural abnormalities that predict adverse pregnancy outcomes. The three-dimensional measurements of the biparietal diameter, the

abdominal circumference, and the femoral length in late-term pregnancy can be used to derive the estimated fetal weight (EFW) (41). The question remains, which parameter is most closely related to macrosomia? Higgins et al. evaluated four common fetal ultrasonographic parameters commonly used to predict macrosomia in 416 pregnant women; their study suggested that the fetal abdominal circumference showed the highest predictive ability (42). In our model, we similarly found that the fetal abdominal circumference was the optimal predictor, especially during late-term pregnancy.

Several predictive models for macrosomia have been reported in previous studies (9, 11, 43, 44). For example, Mazouni et al. used a nomogram to predict macrosomia in 194 women (11). Their model included predictors as follows: the ultrasound-derived EFW at 37–42 weeks of gestation, parity, ethnicity, and the BMI. The AUCs of this model were 0.860 and 0.850 in the development set and internal validation set. The discrimination of their model was also better than that afforded by the Hadlock formula. However, this model was difficult to validate in Asians because one predictor, the race of subject, was limited to European, African, and Black in their study. Recently, Zou et al. developed a model to predict macrosomia for Asian GDM patients (9). This model includes the prepregnancy BMI, the gestational weight, the fasting plasma glucose and triglyceride levels, the fetal biparietal diameter, and the amniotic fluid index as predictors with the AUC of 0.813. However, the discrimination of Zou's model is limited as the external validation is lacking. Their model was also confined to GDM subjects so that may not be fit to general pregnancies. Compared with the two previous models, the ROC curves of our model in the internal set and external set were 0.819 and 0.773, which suggested that the generalization ability of this novel model is certain. As the three maternal predictors in our model were both accessible at an earlier stage of pregnancy, the early prevention of metabolic-related factors may reduce the risk of macrosomia. During late-term pregnancy, this model could screen patients with a high risk of macrosomia and help clinicians to make correct delivery decisions for each patient.

Study limitations

Although all the four predictors were easy to obtain in different populations, it should be noted that the model's generalization ability needs more validation in different populations. We have referred to the international guidelines or recommendations to formulate the inclusion and exclusion criteria in this study. Thus, theoretically, the model is applicable to different races. Second, the timeframe of all the included features was formulated to be measured during pregnancy; however, the biochemical or ultrasound examinations may remain with several days' (usually within 1 week) difference among the subjects due to patients' personal reasons. This is common in the clinical practice but may still influence the accuracy of the nomogram. Despite its limitations, our study has the strength to prove the stable discrimination ability of this new model, such as the validation at different levels, well-organized sets, and the representative samples.

Conclusion

We developed a nomogram that predicted macrosomia and confirmed both discrimination and accuracy *via* external validation. The key predictors were prepregnancy obesity, hypertriglyceridemia, gestational diabetes, and the fetal abdominal circumference. The model is easy to use and will assist obstetricians in terms of clinical decision-making.

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 Institutional Ethics in Research Committee at the second hospital of the Hebei Medical University approved the study (2020-R-125). All participants provided written informed consent.

Author contributions

YuHW wrote the manuscript. HL and JW contributed to data analyses. AW, HJX, and ZN collected the data used to develop this model. JL and XH collected the data used in validations. HZ and JZ performed the ultrasound examinations. YueHW and HX reviewed and revised the manuscript. ZL designed this study. All authors approved the final manuscript.

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Conflict of interest

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

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Urinary cortisol is lower in pregnant women with higher pre-pregnancy BMI

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Background/objectives: Although cortisol levels increase during normal pregnancy, particularly high levels of cortisol or stress have been associated with adverse maternal/child outcomes. Obesity is associated with altered cortisol metabolism, but there is limited information on pregnancy-related changes in cortisol in pregnant women with overweight/obesity. The objective of this study was to examine weekly measures of urinary cortisol and perceived stress throughout ~10-36 weeks gestation, if levels differ by pre-pregnancy BMI categories, and whether concurrent measures of urinary cortisol and perceived stress are associated.

Methods: Longitudinal observational data from Healthy Mom Zone, a gestational weight management intervention, and an ancillary fetal growth study were combined. Pregnant women with normal (n=7), overweight (n=11), or obese (n=14) pre-pregnancy BMI were recruited at >8 weeks gestation. Overnight urinary cortisol and Perceived Stress Scale were measured weekly from ~10-36 weeks gestation.

Results: Higher pre-pregnancy BMI was associated with overall lower urinary cortisol throughout gestation, but rate of increase in urinary cortisol across pregnancy was similar across weight status groups. Women with obesity reported higher levels of overall perceived stress than normal weight women. Regardless of weight status, perceived stress was not associated with gestational age or cortisol.

Conclusions: Although women with obesity reported higher perceived stress, they had lower urinary cortisol than women with normal BMI, and gestation-related increases in cortisol were similar across weight groups and unrelated to

perceived stress, suggesting that physiological factors that drive increases in cortisol as pregnancy may outweigh effects of stress and adiposity.

Clinical trial registration: https://clinicaltrials.gov/ct2/show/NCT03945266, identifier (NCT03945266)

KEYWORDS

pregnancy, cortisol, stress, obesity, intensive longitudinal data

1 Introduction

Perceived psychosocial stress during pregnancy has been associated with a number of adverse pregnancy outcomes, including preterm birth and low birth weight (1). There is also evidence that prenatal maternal perceived stress may influence longer term outcomes for offspring health (2). Maternal level of cortisol, a biomarker of stress exposure, has separately been associated with a number of child outcomes, including fetal growth and infant cognitive ability, temperament, and stress regulation (3). Although it is known that cortisol increases during normal pregnancy (4), there is little information available on the trajectory of this change, as most studies examining changes in cortisol across pregnancy have measured cortisol at three (e.g. once per trimester) or fewer times throughout gestation (5–10). More frequent assessment and detailed examination of how cortisol level changes throughout pregnancy is needed to better understand the physiology of cortisol during pregnancy.

Over half of US women enter pregnancy with overweight or obesity (11), and pregnant women with obesity report greater levels of psychological distress (12). Maternal obesity is a well-established risk factor for adverse pregnancy outcomes, including gestational diabetes, caesarean birth, and large-for-gestational age birth (13), as well as long term obesity risk for children (14). In a rodent model, animals with a genetic predisposition to obesity were more susceptible to the effects of prenatal stress (15). In non-pregnant humans, perceived psychosocial stress has been associated with weight gain, particularly among those with an already elevated BMI (16). Furthermore, adults with greater abdominal adiposity have been shown to have greater cortisol reactivity to acute physical and psychosocial stressors (17). Obesity is also associated with alterations in cortisol metabolism (18), including placental metabolism (19). However, there has been little research on how the effects of prenatal perceived stress and cortisol may differ depending on maternal weight status.

The literature examining associations between cortisol levels in pregnancy and maternal weight status is scant; studies are either cross-sectional (20, 21) or longitudinal with a limited number of time points (8, 9, 22). These studies have consistently found lower levels of cortisol in pregnant women with obesity than those with normal weight, but whether patterns of change throughout pregnancy vary by weight status remains unclear. In addition, other factors such as maternal age (21–23), parity (21, 22, 24), and fetal sex (21, 25) have been inconsistently associated with maternal cortisol in the literature. Finally, although obesity-related differences in cortisol reactivity to stress have been reported in non-pregnant populations (17), studies examining concordance between cortisol and self-reported stress during pregnancy have not considered obesity as a moderator.

The objectives of this study were to a) describe how cortisol levels change throughout pregnancy using weekly urinary cortisol assessments, b) determine whether overall urinary cortisol level and it's rate of change across gestation differ by pre-pregnancy BMI and demographic factors (i.e., maternal age, parity, fetal sex), and c) examine the association between urinary cortisol and self-reported perceived stress, and whether this relationship differs by pre-pregnancy BMI. Based on previous research (8, 10, 17, 20, 21), we hypothesized that a) urinary cortisol would increase across gestation; b) overall cortisol levels would be lower in women with pre-pregnancy overweight or obesity, older women, parous women, and women carrying male fetuses, and the increase in cortisol across gestation would be slower among women with pre-pregnancy overweight/obesity; and c) urinary cortisol would be associated with concurrent self-reported perceived stress, and pre-pregnancy BMI would moderate this association such that the magnitude of the association between cortisol and perceived stress would be greater among women with higher BMI.

2 Materials and methods

2.1 Participants

Data for this analysis were from two samples that were combined. Most participants (n=27) were from the Healthy Mom Zone (HMZ) study, a randomized-controlled trial designed to manage gestational weight gain in pregnant

women with overweight or obesity (26). Women were eligible if they were >8 weeks pregnant with a single fetus, English-speaking, non-smoking, free of significant pregnancy complications or medical conditions, and had a BMI ranging from 24.5 to 45 kg/m^2 (>40 kg/m^2 with physician consultation). Exclusion criteria included diabetes at study entry, severe allergies or dietary restrictions, and contraindications to prenatal physical activity. Participants were randomized to a standard of care or the HMZ adaptive intervention, and all participants completed an intensive longitudinal data collection protocol, including an ancillary fetal growth study. Further details of the intervention and data collection procedures have been previously published (26). The remaining participants (n=5) included pregnant women with a BMI ≥ 18.5 enrolled into an observation only group to increase sample size for the fetal growth study and incorporate a greater BMI range. These women were not randomized to an intervention condition but completed the same measurement protocols as the participants enrolled in the HMZ study. With the exception of BMI, the same eligibility criteria were used as the HMZ study. This analysis includes a final sample of 32 women who were studied through approximately 36 weeks gestation. Demographics were reported at study enrollment, and fetal sex was abstracted from medical records. Height and pre-pregnancy weight were self-reported and used to calculate pre-pregnancy BMI. Gestational age was determined using last menstrual period. This project was completed in accordance with the Declaration of Helsinki. Written informed consent was obtained for each participant prior to randomization or completion of any study measures. Participants also provided consent for the study team to access their medical records. All procedures were approved by the Pennsylvania State University Institutional Review Board.

2.2 Urinary cortisol

Overnight urinary cortisol was measured once per week throughout the study. Urinary cortisol was used to reflect the systemic production of cortisol over a standardized period of time (versus, for example, salivary sampling that only reflects a point/momentary estimate) without the requirement of a blood draw. Women were asked to collect their urine from the time they went to bed at night through the first morning void. Because the prevalence and frequency of nighttime urination increases across gestation (27), this strategy helped ensure that samples reflected cortisol excretion over a consistent time window throughout gestation. Participants were instructed to refrigerate samples after collection, and following collection study staff retrieved and aliquoted the samples. Samples were frozen at -4°C until the end of the study when all samples were analyzed. Urinary cortisol was analyzed in duplicate using a competitive enzyme immunoassay (R&D Systems #KGE008B, Minneapolis, MN, assay range 0.2-10 ng/mL, sensitivity 0.111 ng/mL), and normalized to urinary creatinine (R&D Systems #KGE005, Minneapolis, MN, assay range 0.3-20 mg/dL, sensitivity 0.07 mg/dL). The inter-assay coefficient of variation was 14.0% for cortisol and 4.1% for creatinine, and the intra-assay coefficient of variation was 5.6% for cortisol and 2.5% for creatinine.

2.3 Perceived stress

Participants completed the Perceived Stress Scale (28) weekly as part of a paper survey that was turned in with each urine sample. This 10-item scale is a measure of the degree to which a person assesses situations in their life as stressful, and is a commonly used measure of global subjective psychosocial stress. Given the weekly sampling design, the questionnaire was modified to ask about the previous week rather than previous month. Cronbach's alpha in this sample was 0.89, indicating very good internal consistency.

2.4 Statistical analysis

Descriptive statistics (mean ± SD or percentages) were generated for demographic variables. One single cortisol measurement was >15 SDs from the mean, and was thus excluded from the data set. Intraclass correlation coefficients (ICC) were calculated for urinary cortisol and perceived stress to determine the proportion of variance due to between-subjects variance. A series of simulations were used to estimate power to detect a BMI group x gestational age interaction. A set of 1000 simulated data sets were generated using variances and covariances from the current data set. Level 2 sample size was set to 32 individuals, and level 1 sample size set to 24 measurements per individual. Simulations indicated that there was 80% power to detect a BMI group by gestational age interaction with a difference in slopes of 0.08. The association between cortisol and predictor variables was evaluated using a generalized mixed-effects modeling approach (PROC GLIMMIX in SAS). All models utilized the Laplace estimation method and an unstructured covariance structure. Cortisol level across gestation was modeled using the loglinear distribution and identity link, with a random intercept and slope for gestational age in weeks. Linear, quadratic, and cubic fixed effects of gestational week were considered. Statistical significance of the fixed effects and Akaike information criterion (AIC) were used to determine that the linear model was the best fit, and a linear gestational week term was included in all subsequent models. Next, predictors including maternal pre-pregnancy BMI, age, parity, and fetal sex were examined in separate models. Pre-pregnancy BMI was analyzed both as a continuous variable to include maximal variability, and as a categorical variable, with participants classified as normal weight (BMI 18.5-24.9), overweight (BMI 25.0-29.9), or obese (BMI ≥30.0), for greater clinical interpretability. Main effects

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and interactions with gestational week were tested for each predictor. These analyses were repeated with perceived stress as the dependent variable. Finally, concurrent weekly perceived stress score was examined as a predictor of urinary cortisol. All models controlled for study group assignment (i.e. randomized to intervention, randomized to control, or non-randomized observation only). Due to variability in enrollment date and final study date, sample sizes at the earliest and latest gestational ages were small, so analyses were repeated including only data from 11-36 weeks gestation. Results were similar, so analyses with the full data set are included herein.

3 Results

Sample characteristics are described in Table 1. Participants (n=32) were predominantly non-Hispanic white (93.8%), married (90.6%), and pregnant with their first child (65.6%). The mean age of women at study entry was 30.5 ± 3.0 years and the mean pre-pregnancy BMI was 31.3 ± 7.1 kg/m^2. Fourteen women (43.8%) were classified as having obesity (BMI ≥ 30), 11 (34.4%) were classified as having overweight (BMI 25-29.9), and 7 (21.9%) were classified as having a normal BMI (18.5-24.9). All women gave birth to live infants (56.3% male) with a mean birth weight of 3386 ± 619 grams and mean gestational age of 39.5 ± 1.4 weeks.

TABLE 1 Participant characteristics (n=32).

Variable	Mean (SD) or n (%)
Maternal age (years)	30.5 (3.0)
Pre-pregnancy BMI (kg/m^2)	31.3 (7.1)
Gestational week at study entry	10.6 (1.7)
Infant birth weight (g)	3386 (619)
Gestational age at birth (weeks)	39.5 (1.4)
Race-ethnicity, n (%)	
Non-Hispanic white	30 (93.8)
Hispanic white	1 (3.1)
Asian	1 (3.1)
Marital status, n (%)	
Married	29 (90.6)
Single	2 (6.3)
Divorced	1 (3.1)
Parity, n (%)	
0	21 (65.6)
1	11 (34.3)
Pre-pregnancy BMI classification, n (%)	
Normal weight (BMI 18.5-24.9)	7 (21.9)
Overweight (BMI 25.0-29.9)	11 (34.4)
Obese (BMI ≥ 30)	14 (43.8)
Fetal sex, n (%)	
Male	18 (56.3)
Female	14 (43.8)

Adherence to the urine collection protocol was very high. On average, women collected 24.4 (SD 2.6, range 16-29) urine samples, reflecting a mean of 94.4% compliance with weekly sample collection. The ICC for urinary cortisol was 0.41, indicating both within- and between-person variability. As expected, urinary cortisol increased significantly across gestation (Table 2). Across the whole sample, mean urinary cortisol approximately doubled from the start of the second trimester at 14 weeks (66.4 ± 30.5 ng/mg creatinine) through 36 weeks (131.5 ± 84.8 ng/mg creatinine), with an average rate of increase of 3.1% per week.

As a continuous variable, pre-pregnancy BMI was negatively associated with weekly urinary cortisol (Table 2), with a 1 unit increase in BMI being associated with -1.5% lower urinary cortisol. When considered as a categorical variable, women with overweight or obesity tended to have lower urinary cortisol than women with normal weight, though this difference did not meet the threshold for statistical significance (p=0.054). Mean values by BMI category across gestational week are plotted in Figure 1. On average, women with overweight/obesity had 28.2% lower urinary cortisol compared to women with normal weight. There was no significant interaction between pre-pregnancy BMI and gestational week, indicating that the rate of increase in urinary cortisol across pregnancy was similar across the range of BMI. There were no significant associations between urinary cortisol and maternal age, fetal sex, or parity, nor were there any interactions between gestational age and these factors.

Participants completed an average of 23.7 perceived stress scale questionnaires (SD 4.4, range 3-29), reflecting a mean compliance of 91.2% with the weekly questionnaires. One participant completed only 19% of their questionnaires; all others completed ≥ 80% of questionnaires. The ICC for perceived stress was 0.63. Self-reported perceived stress did not systematically change across gestation (Table 2). Mean values by BMI category across gestational week are plotted in Figure 2. Pre-pregnancy BMI was positively associated with perceived stress, with each 1 unit increase in BMI being associated with 1.9% greater perceived stress. When categorized into pre-pregnancy BMI classes, women with obesity had significantly higher perceived stress than women with normal BMI (p=0.005, Figure 3), with the average score among women with obesity being 42% higher than women with

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TABLE 2 Predictors of maternal urinary cortisol (ng/mg creatinine) and perceived stress.

	Urinary cortisol (ng/mg creatinine)			Perceived stress		
Predictor	Model estimate	SE	p-value	Model estimate	SE	p-value
Gestational age (weeks)	0.0302	0.0036	<0.0001	0.0021	0.0023	0.36
Pre-pregnancy BMI (kg/m^2)	-0.0156	0.0072	0.03	0.0185	0.0062	0.003
Maternal age (years)	0.0079	0.0019	0.67	-0.0209	0.0159	0.19
Nulliparous	0.0220	0.1189	0.85	0.2585	0.0947	0.007
Fetal sex – female	-0.0651	0.1074	0.54	0.1100	0.0961	0.25
Perceived stress	-0.0004	0.0028	0.90	–	–	–

Estimates are from generalized linear mixed models using a loglinear distribution and including random intercept and random gestational age slope. All models controlled for study group, and gestational week was included in models testing demographic characteristics and perceived stress as predictors. Est, fixed effect estimate; SE, standard error; BMI, body mass index.

normal BMI. Women with pre-pregnancy overweight did not differ from either women with normal weight or obesity. Nulliparous women had 29.5% higher perceived stress scores than women with previous births (p=0.03, Figure 3). There was no association between perceived stress and either maternal age or fetal sex, and rate of change in perceived stress across pregnancy did not differ by these characteristics. There was also no significant association between cortisol and concurrent perceived stress in the sample as a whole (Table 2) or in any BMI group (pre-pregnancy BMI by perceived stress interaction, p=0.61).

4 Discussion

In this small but intensively characterized sample of pregnant women, we observed that urinary cortisol increased across gestation, and that levels were lower among women with higher pre-pregnancy BMI. Perceived stress was positively related to higher pre-pregnancy BMI. Unlike cortisol, perceived stress did not systematically change across gestation, and there was no association between urinary cortisol and perceived stress, regardless of BMI. These findings contribute

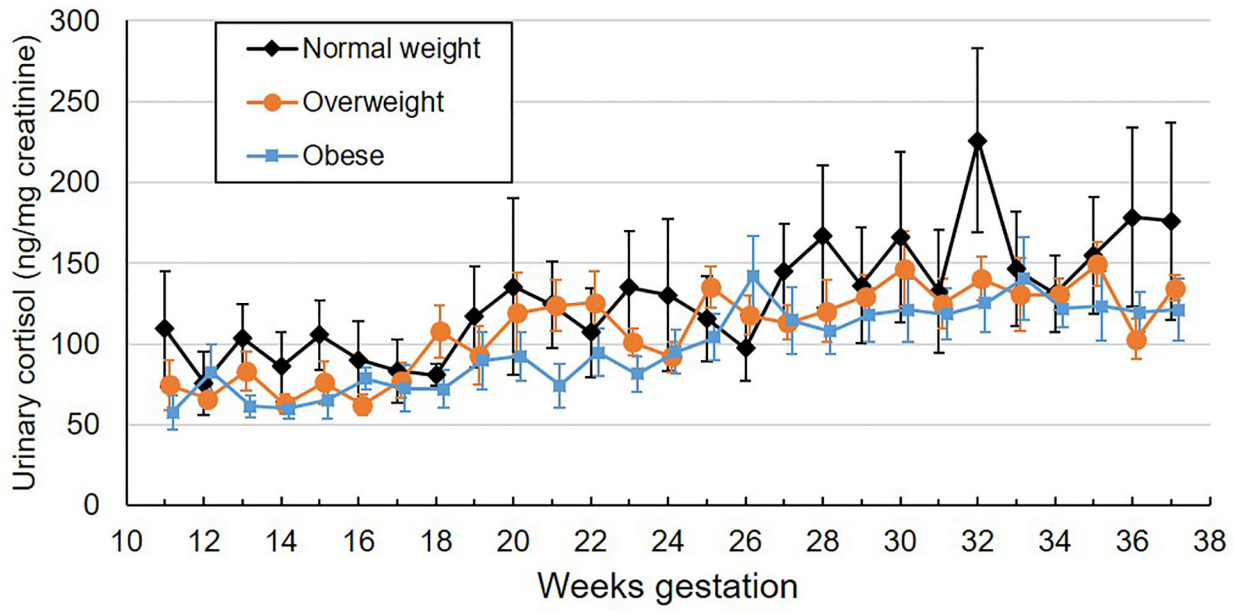

FIGURE 1
Mean weekly overnight urinary cortisol levels (ng/mg creatinine) across gestation by maternal pre-pregnancy BMI category. Values are mean ± standard error.

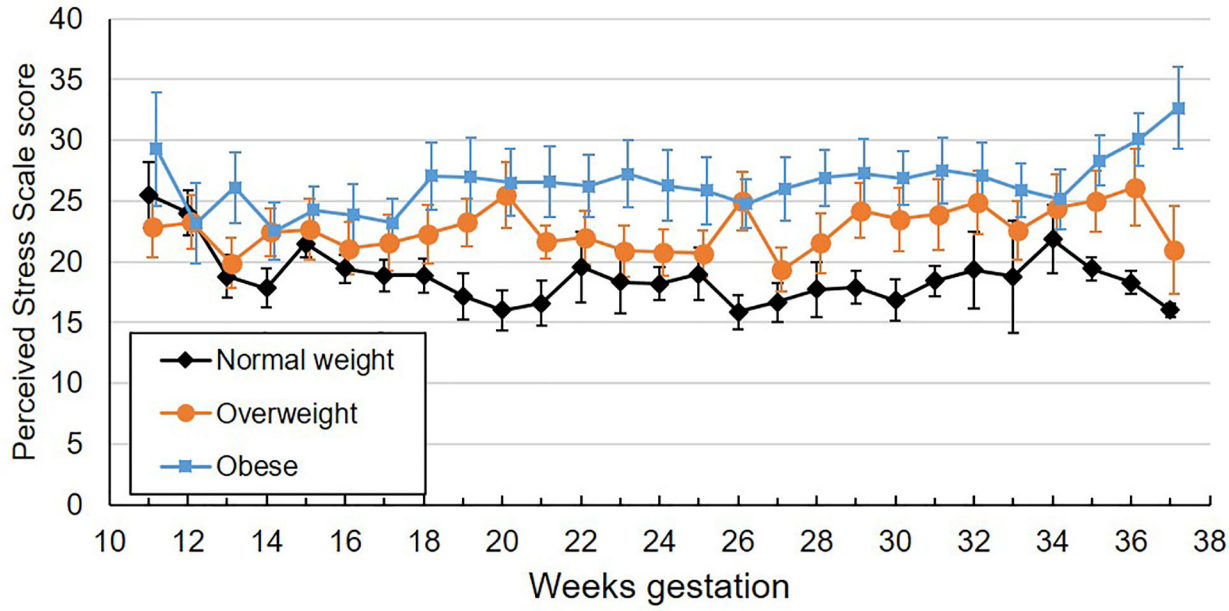

FIGURE 2
Mean weekly perceived stress scale scores across gestation by maternal pre-pregnancy BMI category. Values are mean ± standard error.

to our understanding of perceived stress and cortisol physiology in pregnancies among women with obesity.

As expected, urinary cortisol increased across gestation, approximately doubling from late first trimester through ~36 weeks gestation. This is in line with previous studies which have reported a 1.5-3 fold increase in urinary cortisol during pregnancy (7, 10). Although the physiological role of the upregulation of cortisol during pregnancy is not fully understood, evidence suggests contributes to regulation of growth and the timing of birth (4). Although cortisol increased across gestation, there was no statistically significant, systematic change in perceived stress over time in this sample.

Also as hypothesized, we observed an inverse association between pre-pregnancy BMI and urinary cortisol, a finding consistent with previous research indicating that pregnant women with obesity have lower urinary (20), serum (8, 21), or salivary (8, 22) cortisol levels than those with lower BMIs. These findings in pregnancy are in contrast to studies in non-pregnant adults, which have mostly found either a positive or null relationship between BMI and cortisol (17). However, in contrast to our hypothesis, the rate of increase in cortisol across gestation did not differ by BMI. This finding differs from a previous report suggesting that women with obesity may not experience the same pregnancy-related increase in urinary cortisol excretion that is seen in women with normal BMI (8). Compared to this previous study, our sampling began earlier in pregnancy and was more frequent, which may have allowed us to better characterize gestational changes in urinary cortisol among women with obesity. However, our finding should be interpreted with caution as our study was not adequately powered to detect small-medium sized interaction effects. Despite lower cortisol levels, women with pre-pregnancy obesity reported greater levels of perceived stress than women with normal pre-pregnancy BMI. Compared to women without obesity, rates of depression and anxiety have been reported to be higher in pregnant women with obesity, who may experience unique sources of stress, such as weight-related stigma (12).

In contrast to our hypothesis, there was no association between urinary cortisol and perceived stress score; however, this is perhaps not surprising. The utility of cortisol as a biomarker of stress in pregnant women has been debated, as physiological changes in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis that occur during pregnancy result in increased cortisol levels (4). In line with our findings, studies comparing cortisol and survey measures of stress during pregnancy have found little evidence of correlation between these measures (22, 29). However, studies using experimental protocols to induce stress responses (30) or experience sampling methods to measure subjective stress 'in the moment' (31) have found that cortisol remains responsive to stress during pregnancy. It is possible in our sample that more subtle fluctuations in cortisol due to within-person changes in weekly perceived stress were not detectible amid larger physiological changes related to advancing gestation. Future studies of the effects of stress during pregnancy would benefit from using a combination of different types of measures to assess stress levels.

We did not observe any of the hypothesized associations between cortisol levels and demographic factors including maternal age, parity, and fetal sex, although perceived stress was higher among

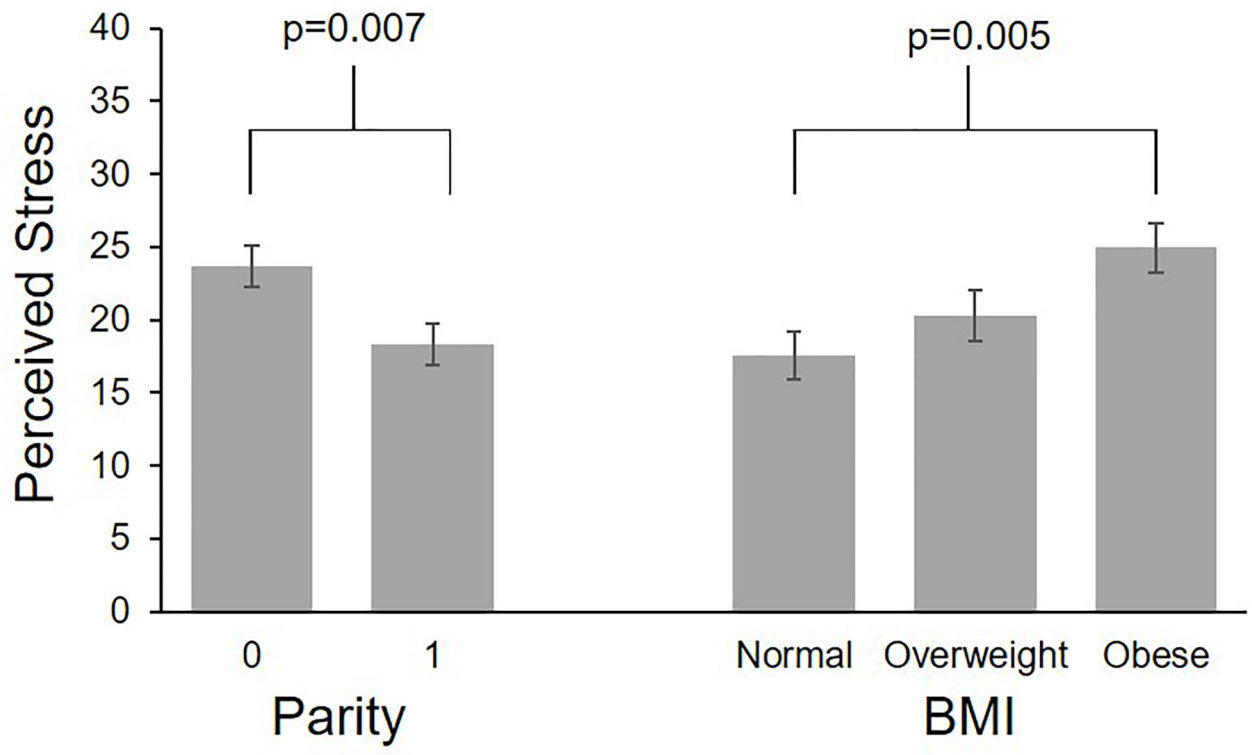

FIGURE 3
Differences in perceived stress by parity and pre-pregnancy BMI. Values are least square means ± standard error from multilevel models controlling for study group and gestational age.

nulliparous women. Previous studies have found higher levels of stress among younger pregnant women (32), older women experiencing their first pregnancy (32), and multiparous women (33), whereas nulliparous women may experience greater stress related to pregnancy-specific anxiety (34). Whether these relationships are reflected in cortisol levels is unclear. One cross-sectional study found that serum cortisol, assessed at an average of 12.9 weeks gestation, was higher among younger women (21). Studies examining cortisol later in gestation, however, have not found significant associations with maternal age (22, 23). Our sample lacked women at the extremes of child-bearing age range, which may have limited ability to detect such associations. The association between parity and cortisol level is also uncertain. Two studies have reported higher serum cortisol among nulliparous women (21, 24), while another found salivary cortisol to be higher in nulliparous women in early second trimester but not in later pregnancy (22). While fetal sex is likely unrelated to maternal exposure to stress, the effect of maternal stress on fetal programming of many outcomes is sex dependent (35), which could be related to differences in cortisol metabolism. One study found that women carrying female fetuses had higher serum cortisol levels (21). A longitudinal examination of salivary cortisol across the second half of pregnancy found that cortisol was higher in mothers of male fetuses from 24 to 30 week gestation, but mothers of female fetuses had higher levels after 30 weeks (25). Further research is needed to understand how cortisol metabolism and stress effects differ by fetal sex.

The intensive longitudinal characterization of cortisol and perceived stress across gestation is a strength of our study, but there are some limitations of note. The sample size was small and likely underpowered for analyses of interactions, and racially and socioeconomically homogenous, limiting generalizability. Maternal report of stress was collected using one instrument, the Perceived Stress Scale. Although this instrument is widely used and well-validated, it does not capture all types or sources of stress. Additional questions assessing pregnancy-specific stress would have strengthened the study.

In conclusion, this study demonstrated that pregnant women with overweight and obesity had consistently lower urinary cortisol than women with normal pre-pregnancy BMI across the study period of ~10-36 weeks gestation. The rate of increase in cortisol across gestation, however, was similar across BMI category, in turn suggesting that the physiological upregulation of cortisol that occurs as gestation advances may be common feature of pregnancy across all weight statuses. This finding, coupled with the fact that studies in non-pregnant individuals tend to observe positive or null associations between adiposity and cortisol, suggests that obesity-

related differences in maternal cortisol may arise early in gestation. Further research is needed to evaluate the mechanisms and consequences of obesity-related alterations of cortisol metabolism in early pregnancy. Regardless of BMI status, weekly reports of subjective stress throughout gestation were not predictive of concurrent weekly urinary cortisol levels in this sample. Longitudinal studies of pregnancy with intensively collected data using a combination of biomarkers (e.g. cortisol), ambulatory assessment (e.g. heart rate monitoring), and subjective measures of stress (e.g. ecological momentary assessment, pregnancy specific stress) would help to determine if stress management is a viable intervention target to optimize maternal and fetal outcomes.

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 Pennsylvania State University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

EH contributed to study design, analysed and interpreted the data, and drafted the manuscript. JS contributed to study design, data interpretation, and critical revision of the manuscript. KM and AP contributed to study design, data collection, and critical revision of the manuscript. DS and JS lead the study design, contributed to data interpretation, and critical 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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Longitudinal trends in lipid profiles during pregnancy: Association with gestational diabetes mellitus and longitudinal trends in insulin indices

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Objective: To investigate the correlation of trends in lipid profiles from first to second trimester with trends in insulin indices and gestational diabetes mellitus (GDM).

Methods: Secondary analysis of an ongoing prospective cohort study was conducted on 1234 pregnant women in a single center. Lipid profiles, glucose metabolism and insulin indices were collected in the first and second trimesters. Trends in lipid profiles were divided into four subgroups: low-to-low, high-to-high, high-to-low and low-to-high group. Insulin indices including homeostasis model assessment of insulin resistance and quantitative insulin sensitivity check index were calculated to evaluate insulin resistance (IR). Trends in insulin indices were described as: no IR, persistent IR, first-trimester IR alone and second-trimester IR alone. Pearson correlation analysis and multivariate logistic regression were performed to assess the associations of lipid profiles subgroups with insulin indices and GDM.

Results: First- and second-trimester total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol were strongly correlated to first- and second-trimester insulin indices. Only TG had a sustained correlation with glucose metabolism indices. High-to-high low-density lipoprotein cholesterol (LDL-c) was an independent risk factor for GDM. High-to-high TG and high-to-low TG groups were independent risk factors for persistent IR. High-to-high TG and low-to-high TG groups were independent risk factors for second-trimester IR alone.

Conclusion: TG has a sustained correlation with insulin indices and glucose metabolism indices. Persistently high TG is an independent risk factor for persistent IR and second-trimester IR alone. Regardless of whether pregnant women have first-trimester IR, lower TG levels help reduce the risk for persistent IR or subsequent development of IR. These results highlight the benefit of lowering TG levels in early and middle pregnancy to prevent the development of IR.

KEYWORDS

lipid profiles, insulin, gestational diabetes mellitus, insulin resistance, pregnancy, trends

Introduction

Gestational diabetes mellitus (GDM) is one of the most common complications during pregnancy in China (1–3) and is associated with short-term and long-term adverse outcomes in the mother and her offspring. GDM increases the risks of maternal and perinatal morbidities, such as cesarean delivery, preterm labor, fetal macrosomia, neonatal hypoglycemia and the need for neonatal unit admission (4, 5). Moreover, women with a previous history of GDM and the offspring of affected pregnancy are predisposed to future type 2 diabetes mellitus, obesity, and cardiovascular and metabolic diseases (6–8).

Excessive insulin resistance (IR), commonly calculated by two insulin indices: the homeostasis model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI), is a contributory factor in the pathogenesis of GDM. It is suggested that IR during pregnancy increases the risk of GDM (9–11). Furthermore, GDM with IR increases adverse pregnancy outcomes, such as preterm labor, large for gestational age (LGA) fetuses and hypertensive disorders of pregnancy, compared with GDM without IR (12, 13).

Dyslipidemia plays important roles in impaired glucose metabolism and IR (14, 15). These three conditions interact and can aggravate adverse maternal and fetal outcomes. Previous studies have shown that dyslipidemia during pregnancy is associated with the subsequent development of GDM, IR, preeclampsia and LGA (12, 13, 16, 17). However, it is unclear whether the longitudinal trends in IR status parallel trends in lipid profiles throughout pregnancy. We hypothesized that trends in lipid profiles from first to second trimester would influence the incidence of GDM and relate to the trends in insulin indices.

This study aimed to investigate the correlation of trends in lipid profiles from first to second trimester with trends in insulin indices and GDM.

Material and methods

This secondary analysis of data from a prospective cohort study included pregnant women who performed routine prenatal care at First Affiliated Hospital of Sun Yat-sen University between July 2021 and July 2022.

Inclusion criteria for the study were: age ≥18 years old; singleton pregnancy; underwent blood tests for lipid profiles and fasting insulin in both the first (<14 weeks of gestation) and second (24-28 weeks of gestation) trimesters; Exclusion criteria were: women with pregestational diabetes mellitus or impaired fasting glucose; pregnancy loss or termination of pregnancy before 28 weeks of gestation; missing data on lipid profiles, insulin or GDM diagnosis.

All the following data were extracted from the electronic medical records in our hospital: maternal demographic characteristics including maternal age, body mass index (BMI, calculated in kg/m^2) before pregnancy, method of conception, smoking, first-degree family history of diabetes mellitus and parity; blood test results in the first and second trimesters including lipid profiles: total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c); glucose metabolism indices: hemoglobin A1c (HbA1c), oral glucose tolerance test (OGTT) result between 24 and 28 weeks of gestation, first-trimester fasting plasma glucose (FPG, second-trimester FPG was included in OGTT test); and insulin indices: fasting insulin (FINS), HOMA-IR and QUICKI.

Pregestational diabetes mellitus included diabetes diagnosed before pregnancy and overt diabetes in pregnancy (18). Impaired fasting glucose was defined as FPG 5.6-6.9 mmol/L in the first trimester (19). GDM was diagnosed when one or more of the following one-step 75g OGTT criteria were first met between 24 and 28 weeks of gestation: FPG 5.1-6.9 mmol/L, 1-hour plasma glucose ≥10.0 mmol/L or 2-hour plasma glucose 8.5-11.0 mmol/L (20).

Definition and subgroups of abnormal lipid profiles and insulin indices

Each lipid parameter was stratified into two levels. For TC, TG and LDL-c, concentrations above the 75th percentile of the study population were defined as high, otherwise were specified as low. For HDL-c, concentrations below the 25th percentile of the study population were defined as low, otherwise were specified as high. Accordingly, four lipid subgroups (G1 to G4) were derived based on the lipid levels from first to second trimester. Among them G1 denoted low-to-low group, G2 denoted high-to-high group, G3 denoted high-to-low group, and G4 denoted low-to-high group, respectively. For example, for TG, women in G1 group represented those whose TG levels were below the 75th percentile of the study population across the two trimesters.

Insulin indices including HOMA-IR and QUICKI were applied to evaluate IR. HOMA-IR was calculated as FINS (μU/mL) × FPG (mmol/L)/22.5. QUICKI was calculated as 1/(Log FPG (mg/dL) + Log FINS (μU/mL). In the present study, IR was defined by a HOMA-IR index above the 75th percentile of the study population, and a QUICKI index lower than the 25th percentile of the study population (21). Four HOMA-IR subgroups were derived: IR-H1 group, defined as pregnancies without high HOMA-IR in both the first and second trimesters; IR-H2 group, defined as pregnancies with persistently high HOMA-IR in both the first and second trimesters; IR-H3 group, defined as pregnancies with first-trimester high HOMA-IR alone; and IR-H4 group, defined as pregnancies with second-trimester high HOMA-IR alone. Similarly, four QUICKI subgroups were derived: IR-Q1 group, defined as pregnancies with persistently high QUICKI in both the first and second trimesters; IR-Q2 group, defined as pregnancies with persistently low QUICKI in both the first and second trimesters; IR-Q3 group, defined as pregnancies with first-trimester low QUICKI alone; and IR-Q4 group, defined as pregnancies with second-trimester low QUICKI alone. Based on that, trends in IR were described as no IR, persistent IR, first-trimester IR alone and second-trimester IR alone.

Statistical analysis

Continuous variables were presented in mean ± standard deviation and categorical variables were represented in counts and

proportions. Continuous variables were compared across different subgroups by Mann-Whitney U test, and categorical variables were compared by $\chi 2$ test or continuity correction test. Pearson correlation analysis was performed to evaluate the associations among lipid concentrations, insulin indices and glucose metabolism indices.

Multivariate logistic regression was performed to explore whether trends in lipid profiles from first to second trimester were associated with trends in insulin indices and GDM. The adjusted odds ratios (aOR) and 95% confidence intervals (CI) were calculated by adjusting maternal age, BMI before pregnancy, conception by *in vitro* fertilization (IVF), first-degree family history of diabetes mellitus, smoking and multiparous. The relationships of lipid profiles with insulin indices and GDM were also explored within subgroups stratifying BMI before pregnancy, which were categorized as underweight (< 18.5 kg/m^2), normal weight (18.5-23.9 kg/m^2), overweight or obese (≥ 24.0 kg/m^2).

Statistical software package SPSS Statistics 26.0 (SPSS Inc., Chicago, IL, USA) and R software (version 4.1.3) were used for data analyses. In all analyses, *P* value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics, lipid profiles, glucose metabolism indices and insulin indices in the study population

The total study population consisted of 2021 women with singleton pregnancies that underwent all the required blood tests during the study period. Seven hundred and eighty-seven cases were excluded due to pregnancy loss or termination of pregnancy (n=34), preexisting diabetes mellitus (n=55) and missing data (n=698). The remaining 1234 cases comprised 233 (18.9%) GDM and 1001 (81.1%) non-GDM cases.

In the present study, the 75th percentile of HOMA-IR in the study population were 1.60 and 1.96 in the first and second trimesters, respectively; and the 25th percentile of QUICKI were 0.36 and 0.34 in the first and second trimesters, respectively. Accordingly, 778 (63.0%) women had persistently low HOMA-IR in both the first and second trimesters (IR-H1). By contrast, 159 (12.9%), 148 (12.0%) and 149 (12.1%) women had high HOMA-IR in both trimesters (IR-H2), in first trimester alone (IR-H3) and second trimester alone (IR-H4), respectively. Similarly, 783 (63.4%), 155 (12.6%), 142 (11.5%) and 154 (12.5%) women had persistently high QUICKI (IR-Q1), persistently low QUICKI (IR-Q2), first-trimester low QUICKI alone (IR-Q3) and second-trimester low QUICKI alone (IR-Q4), respectively.

In the first and second trimesters, high TC, TG and LDL-c levels were defined as above 5.50 and 7.00 mmol/L, 1.59 and 2.49 mmol/L, 3.10 and 4.03 mmol/L, respectively; low HDL-c level was defined as below 1.56 and 1.83 mmol/L, respectively in the present study. Each lipid parameter was divided into four subgroups (G1-G4) as described in the methods part. Over half of our study population had normal lipid levels in the first and second trimesters (numbers and percentages in each lipid were TC: 858, 69.5%; TG: 813, 65.9%; HDL-c: 825, 66.9% and LDL-c: 817, 66.2%, respectively). Conversely, numbers and percentages in women with persistently abnormal lipid levels were TC: 175, 14.2%; TG: 194, 15.7%; HDL-c: 193, 15.6% and LDL-c: 194, 15.7%, respectively.

Compared to non-GDM group, maternal age, BMI before pregnancy, the proportion of conception by IVF, first-degree family history of diabetes mellitus and first-trimester TC and LDL-c were higher in GDM group (Table 1). TG, HbA1c, FINS and HOMA-IR were higher, and QUICKI was lower in GDM group than those in non-GDM group in both first and second trimesters (Table 1).

Compared to IR-H1 group, women had higher maternal BMI before pregnancy, first-trimester LDL-c, and both first- and second-trimester TG, HDL-c and FINS in IR-H2, IR-H3 and IR-H4 groups, respectively (all P <0.05) (Table 2). First-trimester TC was higher in IR-H2 group than that in IR-H1 group (Table 2). Comparison of lipid profiles and glucose metabolism indices between IR-Q1 group and IR-Q2, IR-Q3 and IR-Q4 groups demonstrated similar results (Table 3). We further compared lipid profiles among women with different IR statuses separately in first and second trimesters. The results showed that first-trimester TC and LDL-c, and first- and second-trimester TG and HDL-c were significantly different in those with high HOMA-IR compared to those without (sTable 1). Comparison of lipid profiles and glucose metabolism indices between women with and without low QUICKI also demonstrated the analogous results (sTable 2).

Correlation between lipid profiles, glucose metabolism indices and insulin indices

The first-trimester levels of TC, TG, LDL-c and HDL-c showed significant correlation to first- and second-trimester FINS, HOMA-IR and QUICKI (Figure 1). Among them TG had stronger connection to insulin indices (First trimester: FINS: r=0.32, HOMA-IR: r=0.31, QUICKI: r=-0.31; second trimester: FINS: r=0.30, HOMA-IR: r=0.30; QUICKI: r=-0.30; all P < 0.05) (Figure 1). The second-trimester levels of TG and HDL-c represented positive relationship to first- and second-trimester FINS, HOMA-IR and QUICKI (Figure 2). As shown in Figure 2, TG still had relatively higher connection to insulin indices (First trimester: FINS: r=0.26, HOMA-IR: r=0.25, QUICKI: r=-0.27; second trimester: FINS: r=0.30, HOMA-IR: r=0.29; QUICKI: r=-0.30; all P < 0.05).

For glucose metabolism indices, only rises in TG paralleled increases in all the glucose metabolism indices across the two trimesters (Figures 1, 2). Overall, TG showed a more significant connection to insulin indices than to glucose metabolism indices. The relationship between first- and second-trimester lipid profiles were depicted in sFigure 1.

Trends in lipid profiles and risk of GDM and IR

After adjustment for covariates, high-to-high (G2) LDL-c was an independent risk factor for GDM (aOR 1.661, 95% CI 1.139-2.422), but not high-to-low (G3) or low-to-high LDL-c (G4) group (Figure 3). Trends in TG were not associated with the incidence of GDM (Figure 3). Stratified analysis indicated that high-to-high LDL-c

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TABLE 1 Maternal characteristics, lipid profiles and glucose metabolism and insulin indices in the first and second trimesters in women with and without gestational diabetes mellitus.

Characteristics	GDM N=233	Non-GDM N=1001	*Z* or $\chi 2$ value	*P* value
Maternal age, years	33.30 ± 4.60	31.14 ± 4.12	6.581	< 0.001
BMI, kg/m^2	21.94 ± 3.05	20.99 ± 2.71	4.339	< 0.001
Conception by IVF, n (%)	60 (25.8)	161 (16.1)	12.014	0.001
Smoking, n (%)	2 (0.9)	10 (1.0)	0.039	1.000
Family history of diabetes mellitus, n (%)	22 (9.4)	44 (4.4)	9.508	0.002
Multiparous, n (%)	89 (38.2)	323 (32.3)	2.988	0.084
Blood tests in the first trimester				
TC, mmol/L	5.11 ± 0.97	4.94 ± 0.79	2.466	0.014
TG, mmol/L	1.47 ± 0.56	1.33 ± 0.53	4.015	< 0.001
HDL-c, mmol/L	1.78 ± 0.32	1.79 ± 0.33	-0.076	0.939
LDL-c, mmol/L	2.88 ± 0.68	2.75 ± 0.55	2.876	0.004
HbAlc, %	5.25 ± 0.34	5.11 ± 0.29	5.670	< 0.001
FPG, mmol/L	4.42 ± 0.37	4.32 ± 0.33	4.168	< 0.001
FINS, μU/mL	7.53 ± 3.67	6.56 ± 3.39	4.174	< 0.001
HOMA-IR	1.50 ± 0.80	1.27 ± 0.69	4.483	< 0.001
QUICKI	0.37 ± 0.03	0.38 ± 0.03	-4.483	< 0.001
Blood tests in the second trimester				
TC, mmol/L	6.35 ± 1.20	6.33 ± 1.06	0.270	0.788
TG, mmol/L	2.22 ± 0.79	2.12 ± 0.85	2.517	0.012
HDL-c, mmol/L	2.06 ± 0.42	2.08 ± 0.36	-0.869	0.385
LDL-c, mmol/L	3.64 ± 0.95	3.59 ± 0.74	0.566	0.571
HbAlc, %	5.05 ± 0.35	4.84 ± 0.30	8.152	< 0.001
FINS, μU/mL	9.42 ± 4.70	8.11 ± 3.77	4.488	< 0.001
HOMA-IR	1.95 ± 1.10	1.55 ± 0.77	5.851	< 0.001
QUICKI	0.35 ± 0.03	0.37 ± 0.03	-5.851	< 0.001
Oral glucose test result				
0h, mmol/L	4.56 ± 0.45	4.25 ± 0.30	9.740	< 0.001
1h, mmol/L	10.02 ± 1.39	7.43 ± 1.33	19.702	< 0.001
2h, mmol/L	8.80 ± 1.14	6.40 ± 1.04	20.930	< 0.001

BMI, body mass index; IVF, in vitro fertilization; TC, total cholesterol; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; FINS, fasting insulin; HOMA-IR, homeostasis model assessment of insulin resistance; QUICKI, quantitative insulin sensitivity check index.

group contributed to risk of GDM in women with underweight and obese or overweight, not in women with normal weight (sTable 3).

High-to-high (G2) TG group was an independent risk factor for IR-H2 and IR-H4 (aOR 2.222, 95% CI 1.408-3.506; aOR 1.823, 95% CI 1.149-2.894, respectively) after the adjustment for covariates (Figure 4). High-to-low (G3) TG group was associated with IR-H2 (aOR 2.162, 95% CI 1.226-3.812), and low-to-high (G4) TG group was correlated with IR-H4 (aOR 2.744, 95% CI 1.666-4.520) (Figure 4). The associations of G2 group of TGs with IR-Q2 and IR-Q4 were also validated (Figure 5). Besides, G2 group of TGs increased the risk of IR-Q3 (aOR 1.605, 95% CI 1.015-2.539) (Figure 5). Low-to-low (G1) HDL-c demonstrated comparable risk of IR-H4 (aOR 1.732, 95% CI 1.105-2.715) and IR-Q4 (aOR 1.740, 95% CI 1.117-2.712). High-to-high (G2) LDL-c revealed comparable risk of IR-H1 (aOR 1.690, 95% CI 1.076-2.654) and IR-Q1 (aOR 1.773, 95% CI 1.126-2.791) (Figures 4, 5). Stratified analysis showed significant association of high-to-high TG with persistent IR and second-trimester IR alone, respectively for women with normal weight, but not for women with underweight and overweight or obese (sTables 4, 5).

TABLE 2 Maternal characteristics, lipid profiles and glucose metabolism indices in different HOMA-IR subgroups.

Characteristics	IR-H1 N=778	IR-H2[a] N=159	IR-H3[a] N=148	IR-H4[a] N=149
Maternal age, years	31.41 ± 4.24	32.34 ± 4.72*	31.29 ± 3.91	31.72 ± 4.44
BMI, kg/m^2	20.42 ± 2.32	23.76 ± 3.30**	21.95 ± 3.02**	21.54 ± 2.30**
Conception by IVF, n (%)	133 (17.1)	25 (15.7)	30 (20.3)	33 (22.1)
Smoking, n (%)	5 (1.9)	3 (0.6)	3 (2.0)	1 (0.7)
Family history of diabetes mellitus, n (%)	33 (4.2)	12 (7.5)	10 (6.8)	11 (7.4)
Multiparous, n (%)	243 (31.2)	63 (39.6)*	52 (35.1)	54 (36.2)
Blood tests in the first trimester				
TC, mmol/L	4.91 ± 0.77	5.18 ± 0.83**	5.01 ± 0.82	5.03 ± 1.07
TG, mmol/L	1.26 ± 0.49	1.72 ± 0.67**	1.47 ± 0.53**	1.44 ± 0.45**
HDL-c, mmol/L	1.83 ± 0.32	1.71 ± 0.34**	1.73 ± 0.33**	1.72 ± 0.29**
LDL-c, mmol/L	2.71 ± 0.52	2.95 ± 0.58**	2.85 ± 0.56**	2.88 ± 0.77**
HbAlc, %	5.12 ± 0.29	5.21 ± 0.33**	5.12 ± 0.35	5.19 ± 0.31**
FPG, mmol/L	4.27 ± 0.32	4.49 ± 0.36**	4.57 ± 0.33**	4.31 ± 0.33
FINS, μU/mL	5.03 ± 1.63	12.21 ± 4.05**	10.16 ± 2.60**	6.48 ± 1.50**
Blood tests in the second trimester				
TC, mmol/L	6.33 ± 1.05	6.27 ± 1.11	6.34 ± 1.11	6.41 ± 1.25
TG, mmol/L	1.96 ± 0.72	2.54 ± 1.00**	2.34 ± 0.86**	2.42 ± 0.91**
HDL-c, mmol/L	2.11 ± 0.37	1.99 ± 0.35**	2.03 ± 0.36*	2.02 ± 0.35**
LDL-c, mmol/L	3.58 ± 0.73	3.56 ± 0.80	3.64 ± 0.76	3.70 ± 1.03
HbAlc, %	4.83 ± 0.30	5.04 ± 0.34**	4.86 ± 0.28	4.96 ± 0.34**
FINS, μU/mL	6.41 ± 1.87	14.78 ± 5.01**	7.76 ± 1.58**	12.24 ± 2.30**
Oral glucose test result				
0h, mmol/L	4.21 ± 0.30	4.60 ± 0.40**	4.33 ± 0.30**	4.52 ± 0.37**
1h, mmol/L	7.70 ± 1.58	8.80 ± 1.70**	7.80 ± 1.64	8.26 ± 1.84**
2h, mmol/L	6.66 ± 1.37	7.51 ± 1.43**	6.86 ± 1.33*	7.13 ± 1.43**

[a] In comparison with IR-H1 group; *P < 0.05; **P < 0.01.

Discussion

This study has demonstrated that first, the concentration of first- and second-trimester lipid profiles are significantly different between women with and without GDM, and between women with and without IR; second, both first- and second-trimester TC, TG, and HDL-c are strongly correlated to first- and second-trimester insulin indices, while only TG has sustained correlation with glucose metabolism indices; third, persistently high TG is an independent risk factor for persistent IR and second-trimester IR alone.

The association between hypertriglyceridemia and GDM has been well elaborated. Recent meta-analysis has reported that among studies exploring the relationship between lipid profiles and GDM, TG is the most crucial with most included studies reporting higher TG levels in women with GDM (22). Further analysis has supported that higher TG levels in women with GDM occur in the first trimester and persist across pregnancy (22). Our study also has shown elevated TG levels in women with GDM in both first (1.47 ± 0.56 mmol/L vs 1.33 ± 0.53 mmol/L, $P < 0.001$) and second trimester (2.22 ± 0.79 mmol/L vs 2.12 ± 0.85 mmol/L, $P < 0.001$), compared to women without GDM. However, results are less consistent for the other lipids. Some evidence has shown elevated TC and LDL-c levels in women with GDM compared to those without. While a nested case-control study that has measured first- and second-trimester lipid profiles levels among 318 pregnant women conclude that there is no statistical difference in TC and LDL-c between women with GDM and those without (23). This conclusion is also supported by another meta-analysis (24). Our study has observed higher TC and LDL-c levels in women with GDM than those without in the first trimester but not in the second trimester. However, no significant difference in HDL-c has been found between women with and without GDM across two trimesters.

Consistent with previous research, TG levels strongly correlate with OGTT test results in both the first and second trimesters (16, 22). Nevertheless, trends in TG are not an independent risk for GDM in

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TABLE 3 Maternal characteristics, lipid profiles and glucose metabolism indices in different QUICKI subgroups.

Characteristics	IR-Q1 N=783	IR-Q2[a] N=155	IR-Q3[a] N=142	IR-Q4[a] N=154
Maternal age, years	31.42 ± 4.24	32.30 ± 4.76*	31.25 ± 3.91	31.76 ± 4.41
BMI, kg/m^2	20.43 ± 2.33	23.83 ± 3.31**	21.98 ± 3.04**	21.53 ± 2.27**
Conception by IVF, n (%)	134 (17.1)	25 (16.1)	29 (20.4)	33 (21.4)
Smoking, n (%)	5 (0.6)	3 (1.9)	3 (2.1)	1 (0.6)
Family history of diabetes mellitus, n (%)	34 (4.3)	11 (7.1)	9 (6.3)	12 (7.8)
Multiparous, n (%)	245 (31.3)	62 (40.0)*	50 (35.2)	55 (35.7)
Blood tests in the first trimester				
TC, mmol/L	4.91 ± 0.77	5.19 ± 0.84**	5.01 ± 0.83	5.02 ± 1.05
TG, mmol/L	1.26 ± 0.49	1.73 ± 0.68**	1.48 ± 0.54**	1.44 ± 0.45**
HDL-c, mmol/L	1.83 ± 0.32	1.71 ± 0.34**	1.73 ± 0.33**	1.72 ± 0.29**
LDL-c, mmol/L	2.71 ± 0.52	2.97 ± 0.58**	2.86 ± 0.56**	2.87 ± 0.76**
HbAlc, %	5.12 ± 0.29	5.20 ± 0.33**	5.11 ± 0.35	5.19 ± 0.31**
FPG, mmol/L	4.27 ± 0.32	4.49 ± 0.36**	4.58 ± 0.34**	4.31 ± 0.33
FINS, μU/mL	5.05 ± 1.65	12.32 ± 4.05**	10.24 ± 2.62**	6.51 ± 1.51**
Blood tests in the second trimester				
TC, mmol/L	6.32 ± 1.05	6.28 ± 1.12	6.35 ± 1.12	6.40 ± 1.24
TG, mmol/L	1.96 ± 0.72	2.55 ± 1.01**	2.35 ± 0.87**	2.41 ± 0.90**
HDL-c, mmol/L	2.11 ± 0.37	1.99 ± 0.35**	2.03 ± 0.37*	2.02 ± 0.35**
LDL-c, mmol/L	3.58 ± 0.73	3.56 ± 0.81	3.65 ± 0.76	3.69 ± 1.02
HbAlc, %	4.83 ± 0.30	5.05 ± 0.34**	4.85 ± 0.28	4.96 ± 0.33**
FINS, μU/mL	6.41 ± 1.86	14.88 ± 5.03**	7.78 ± 1.59**	12.19 ± 2.29**
Oral glucose test result				
FPG, mmol/L	4.21 ± 0.30	4.60 ± 0.40**	4.33 ± 0.30**	4.51 ± 0.37**
1-h PG, mmol/L	7.71 ± 1.58	8.82 ± 1.71**	7.77 ± 1.63	8.27 ± 1.82**
2-h PG, mmol/L	6.66 ± 1.37	7.53 ± 1.43**	6.84 ± 1.35	7.14 ± 1.43**

[a] In comparison with IR-Q1 group; *P < 0.05; **P < 0.01.

our study after adjusting for covariates. In stratified analysis, persistently high TG is associated with GDM in women with underweight (aOR 7.626, 95% CI 1.268-45.857), but the wide CI indicates that this result is not sufficiently powered. Several risk factors have been proposed to contribute to GDM, such as being overweight or obese before pregnancy, genetic factors, inflammatory factors and dyslipidemia (especially hypertriglyceridemia irrespective of the period in pregnancy) (8, 16, 25–27). A large-scale retrospective study in China has reported that persistently high TG levels (defined as above the 90th percentile of the population) in the first and third trimesters increase the risk of GDM (aOR 1.97, 95% CI 1.57-2.47), compared to those with persistently low TG levels throughout pregnancy (16). It seems that our results with respect to TG and risk of GDM are rather incompatible with existing conclusions. This inconsistency may be due to the one-step approach for screening GDM in our cohort, which is still under debate whether it will identify more women that are considered as low risk for GDM compared to the two-step approach (28). The lower definition thresholds may lead to overdiagnosis and thus reduce the efficacy of TG to detect the risk for GDM.

Hypertriglyceridemia is an outstanding reflection of insulin resistance. It is an essential criterion for diagnosing metabolic syndrome (29) and both of which are known to be associated with adverse pregnancy outcomes, such as hypertensive disorders of pregnancy (12, 16, 30), preterm labor (13) and LGA (12, 13, 16, 31); and with long-term risk for cardiovascular disease (32, 33). The relationship between elevated TG levels and increased risk of IR has been clearly stated in non-pregnant individuals (29, 34, 35). Nevertheless, only a few studies have reported such a correlation in pregnant population in the first (9) or second trimester (36, 37). The current study has directly indicated the sustained correlation between TG and insulin indices in the first and second trimesters. Also, it has been denoted that high-to-high TG is an independent risk factor for persistent IR and second-trimester IR alone. In stratified analysis, the associations of persistently high TG with persistent IR and second-trimester IR alone exist in women with normal weight (IR-H2: aOR

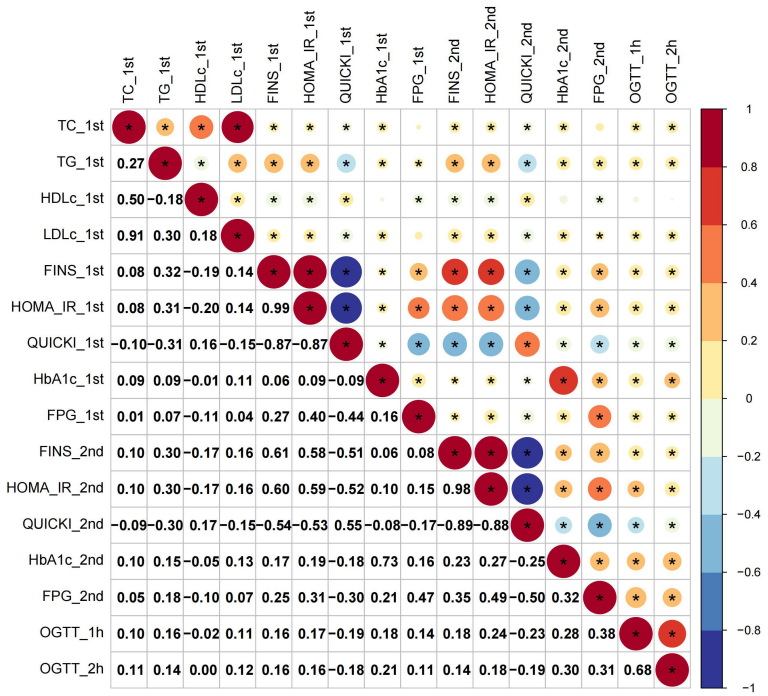

FIGURE 1
Correlations of first-trimester lipid profiles with glucose metabolism and insulin indices. Asterisk (*) in the circles denoted $P < 0.05$; _1st and _2nd denoted first and second trimester, respectively.

2.470, 95% CI 1.391-4.383; IR-H4: aOR 2.389, 95% CI 1.437-3.969; IR-Q2: aOR 2.487, 95% CI 1.398-4.422; IR-Q4: aOR 2.382, 95% CI 1.436-3.950), but not in women with underweight and overweight or obesity. Another retrospective study including 2647 GDM women in China has demonstrated the connections between HOMA-IR and adverse pregnancy outcomes (13). However, stratified analysis of their study also fails to support such results in women with underweight, overweight, or obesity. The authors have claimed that they could not

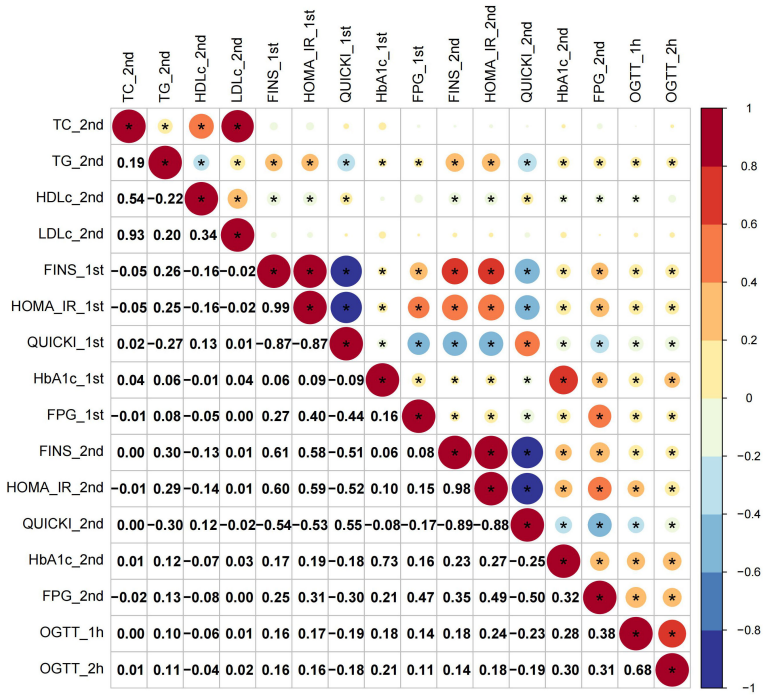

FIGURE 2
Correlations of second-trimester lipid profiles with glucose metabolism and insulin indices.

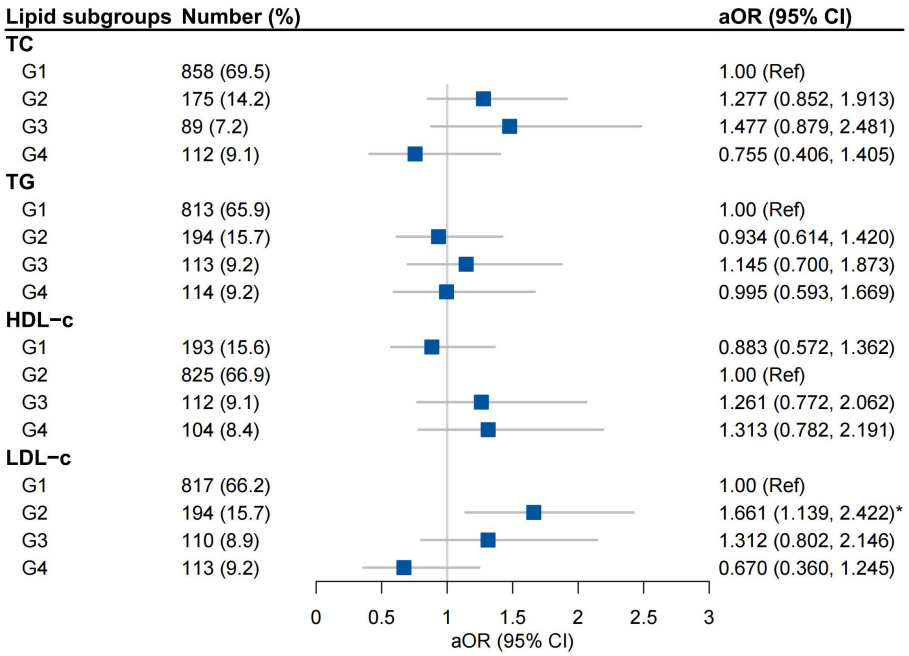

FIGURE 3
Associations between trends in lipid profiles and GDM Adjusted for Maternal age, BMI, Conception by IVF, Family history of diabetes mellitus, Smoking and Multiparous; G1, low-to-low group; G2, high-to-high group; G3, high-to-low group; G4 low-to-high group.

differentiate whether nonsignificant association is contributed by early lifestyle interventions in obese women. In our cohort, no interventions have been applied to overweight or obese women attending for first antenatal visit, but we have not recorded their lifestyle such as physical activities and dietary patterns before and during pregnancy. Therefore, further research is needed to investigate whether the underlying pathogenesis of IR during pregnancy is discordant between women with overweight or obese and with normal weight.

Both high-to-high TG group and high-to-low TG group are associated with the risk of persistent IR, implying that no matter the TG levels in the second trimester, pregnant women would suffer an increased risk of persistent IR so long as the TG levels are high in their first trimester. This result emphasizes the importance of lowering lipid levels in early pregnancy or even before conception to prevent the development of persistent IR in women with first-trimester IR. On the other hand, high-to-high TG group and low-to-high TG group are associated with the risk of women who have no IR in the first trimester but develop IR afterward, whereas high-to-low TG group is not. Consequently, for women without first-trimester IR, it is still reasonable to lower their TG levels in the first and second trimesters to reduce the risk of the later development of IR. Since

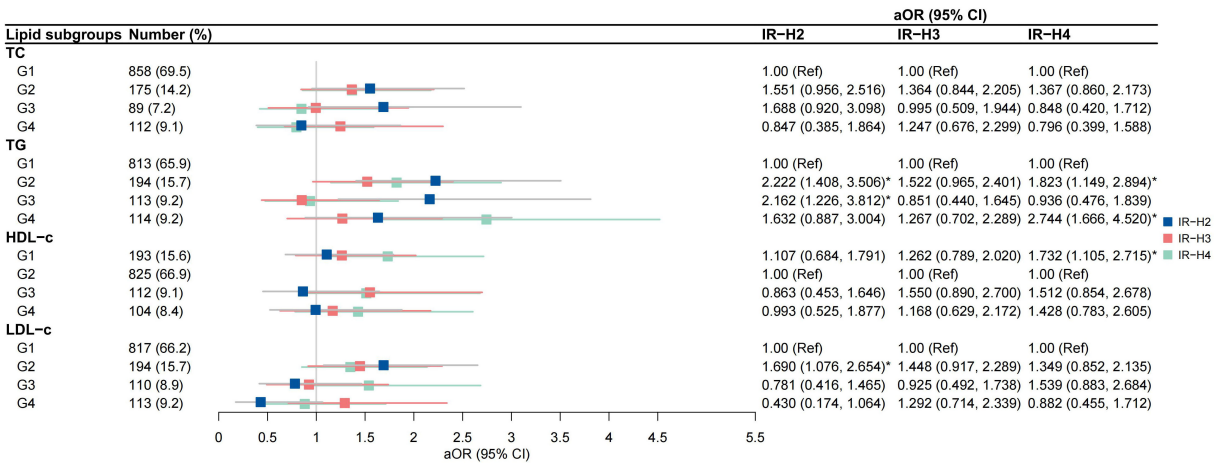

FIGURE 4
Associations between trends in lipid profiles and trends in insulin resistance calculated by HOMA-IR IR-H2, pregnancies with persistently high HOMA-IR in both the first and second trimesters; IR-H3 group, pregnancies with first-trimester high HOMA-IR alone; IR-H4 group, pregnancies with second-trimester high HOMA-IR alone.

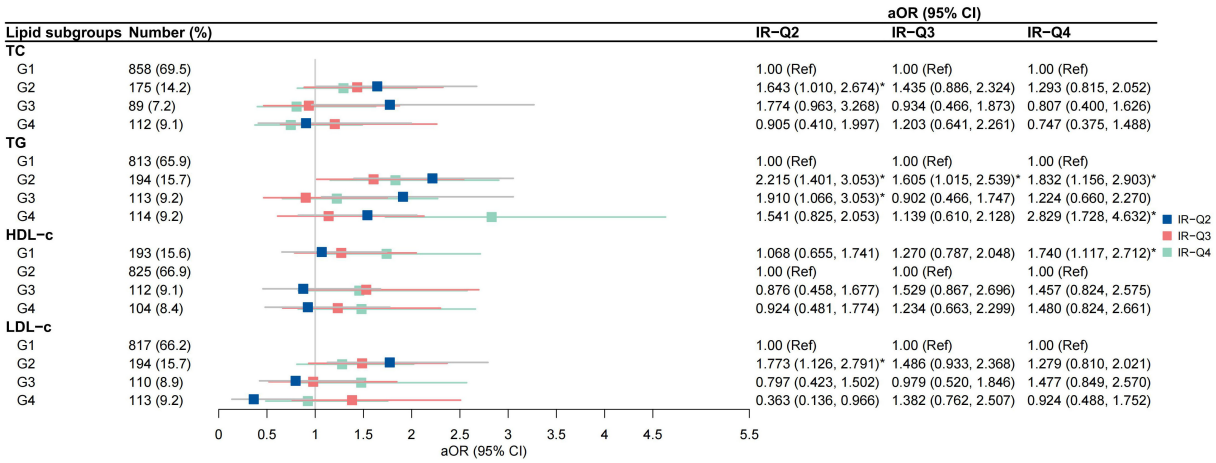

FIGURE 5
Associations between trends in lipid profiles and trends in insulin resistance calculated by QUICKI IR-Q2, pregnancies with persistently low QUICKI in both the first and second trimesters; IR-Q3 group, pregnancies with first-trimester low QUICKI alone; IR-Q4 group, pregnancies with second-trimester high low QUICKI alone.

plasma insulin concentration is not a routine blood test during antenatal visits in most care centers, TG may be additionally used as a surrogate estimate of insulin resistance during pregnancy. The associations between longitudinal trends in TG and IR in our study highlight the benefit of lowering lipid levels in early and middle pregnancy to prevent IR.

The main strength of our study included the synchronous screening of first- and second-trimester lipid profiles, glucose metabolism and insulin indices in the same population. Outcome variables were analyzed in continuous and categorical forms to strengthen the robustness of the results. The main baseline characteristics that may contribute to the outcomes of our cohort were almost completely extracted. There are also some limitations of our study. We only collected first- and second-trimester blood tests, making it difficult to depict the trends in lipid profiles and insulin indices throughout the pregnancy. In line with previous studies, we have confirmed the relationships between TG and IR in the first and second trimesters, therefore more effort is needed to identify the association between lipid profiles and insulin indices in the third trimester. The setting of single center and exclusion of multiple pregnancies limit the generalizability of our results. The third limitation was that the comparison of maternal and fetal outcomes between lipid subgroups and IR subgroups was lacking, albeit the effect of dyslipidemia and IR on adverse outcomes has been widely discussed in previous research. Finally, due to the study design, we did not collect other factors which may influence maternal lipid levels, including thyroid hormone, weight gain during pregnancy and factors related to hypercoagulability such as antenatal hospital admission and family history of venous thromboembolism.

In conclusion, our results suggest that TG has a sustained correlation with insulin indices and glucose metabolism indices in both the first and second trimesters. In addition, persistently high TG is an independent risk factor for persistent IR and second-trimester IR alone. For women with first-trimester IR, it is still important to lower their lipid levels in early pregnancy or even before conception to prevent the development of persistent IR. For women without first-trimester IR, it is still reasonable to lower TG levels in the first and second trimesters to reduce the risk of the later development of IR. These results together highlight the benefit of lowering TG levels in early and middle pregnancy to prevent the development of IR.

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

Approval for the study was obtained from the Independent Ethics Committee for Clinical Research and Animal Trials of First Affiliated Hospital of Sun Yat-sen University in Guangzhou. All eligible women were given written information about the study and those who agreed to participate provided written informed consent.

Author contributions

LS, DW and YH performed the statistical analysis and wrote the manuscript. LY and CZ contributed to the planning of the study, collected study data and reviewed the manuscript. SZ and SC collected the study data. ZW and HC designed the study and revised the manuscript critically. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.1080633/full#supplementary-material

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Effects of galactooligosaccharides on maternal gut microbiota, glucose metabolism, lipid metabolism and inflammation in pregnancy: A randomized controlled pilot study

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Background: Gut microbiota of pregnant women change with the gestational week. On the one hand, they participate in the metabolic adaptation of pregnant women. On the other hand, the abnormal composition of gut microbiota of pregnant women is more likely to suffer from gestational diabetes mellitus (GDM). Therefore, gut microbiota targeted treatment through dietary supplements is particularly important for prevention or treatment. Prebiotic supplements containing galactooligosaccharides (GOS) may be an intervention method, but the effect is still unclear.

Objective: This study aims to evaluate the feasibility and acceptability of prebiotic intervention in healthy pregnant women during pregnancy, and to explore the possible effects of intervention on pregnant women and the influence on gut microbiota as preliminaries.

Methods: After recruitment in first trimester, 52 pregnant women were randomly assigned to receive GOS intervention or placebo containing fructooligosaccharides. 16S rRNA sequencing technology was used to detect the composition, diversity and differential flora of gut microbiota. Lipid metabolism, glucose metabolism and inflammatory factors during pregnancy were also analyzed.

Results: The adverse symptoms of GOS intervention are mild and relatively safe. For pregnant women, there was no significant difference in the GDM incidence rates and gestational weight gain (GWG) in the GOS group compared with placebo ($P > 0.05$). Compared with the placebo group, the levels of FPG, TG, TC, HDL-C LDL-C, and IL-6 had no significant difference in GOS group ($P > 0.05$). For newborns, there was no significant difference between GOS group and placebo group in the following variables including gestational week, birth weight, birth length, head circumference, chest circumference, sex, and delivery mode (P >

0.05). And compared with the placebo group, the GOS group had a higher abundance of *Paraprevotella* and *Dorea*, but lower abundance of *Lachnospiraceae*UCG_001.

Conclusions: GOS prebiotics appear to be safe and acceptable for the enrolled pregnancies. Although GOS intervention did not show the robust benefits on glucose and lipid metabolism. However, the intervention had a certain impact on the compostion of gut microbiota. GOS can be considered as a dietary supplement during pregnancy, and further clinical studies are needed to explore this in the future.

KEYWORDS

gut microbiota, pregnancy, galactooligosaccharides, prebiotic, gestational diabetes mellitus, metabolism

1 Introduction

With the change of gestational age, gut microbiota is participated in the physiological adaptation of maternal metabolism (1). Meanwhile, the abnormal composition of gut microbiota in pregnant women is related to the high possibility of complications during pregnancy, such as gestational diabetes mellitus (GDM) (2). The higher bacterial richness detected in GDM patients is also correlated with metabolic and inflammatory indicators (3). Some clinical trials suggested that intervention with dietary supplements during pregnancy may have different benefits for pregnant women (4). Some probiotics containing Lactobacillus or Bifidobacterium reduced the incidence of GDM to a certain extent (5). However, considering different intervention durations, strains, and doses, some studies did not support this view (6, 7). And other studies have shown that some dietary fiber can help alleviate type 2 diabetes (T2D) in non-pregnant people by regulating gut microbiota (8). Therefore, developing strategies to regulate gut microbiota is a potential direction to improve maternal metabolic health.

Different from probiotics, galactooligosaccharides (GOS) is a kind of prebiotics that aren't digested and absorbed by the host, but can selectively promote the metabolism and proliferation of beneficial bacteria in the body, particularly by Lactobacillus and Bifidobacterium (9). GOS is a functional oligosaccharide with natural properties, and are composed of 3-10 molecules of galactose and glucose (10). GOS has the potential to protect against lipopolysaccharide (LPS) induced intestinal barrier injury (11). GOS can promote the increase of intestinal butyrate producing bacteria and promote the production of short-chain fatty acids (SCFAs) (12). SCFAs and G protein–coupled receptors 41/43 (GPR41/43) promote acute inflammatory responses in the intestine for tissue inflammation and protective immunity (13). GOS was also found to improve lipid metabolism in mice experiments (14). And for humans, GOS prebiotic supplements have certain effects on immune response. After prebiotics supplementation, pro-inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) decreased, while anti-inflammatory cytokines interleukin-10 (IL-10) raised (15). GOS intervention can also increase *Bifidobacterium* that beneficial to human health (16). The use of prebiotics and other dietary supplements during pregnancy or lactation can produce beneficial gut microbiota in cesarean-delivered newborns, especially *Bifidobacterium* colonization (17).

Therefore, prebiotics have the potential to promote health and regulate gut microbiota. However, the beneficial effects of prebiotics during pregnancy remain unclear, the study of GOS prebiotics intervention on pregnant women is still in the preliminary exploration stage. This pilot randomized controlled pilot study aims to evaluate the feasibility, acceptability, and safety of prebiotic intervention for healthy pregnant women, and preliminarily explore the possible benefits for pregnant women.

2 Materials and methods

2.1 Study population

We conducted a prospective double-blinded randomized clinical trial involving singleton pregnancy women. Inclusion criteria were: 18-40 years of age; living in Beijing; understanding and willing to sign informed consent; singleton pregnancy; first prenatal care visit between 5-8 weeks of gestation. Exclusion criteria were: smoking, excessive alcohol or drug abuse; pregnancy complicated with chronic diseases (pre-existing diabetes, impaired glucose tolerance, impaired fasting glucose, chronic hypertension and so on); taken any prescribed chronic medications; steroids use.

The trial was recruited at Peking University First Hospital (PUFH), which is a public hospital located in Beijing, China. This study protocol has been approved by PUFH Clinical Trial Ethics Committee (reference number: 164). All patients provided written informed consent. The clinical trial was registered on www.chictr.org.cn (trial registration number: ChiCTR1800017192). The protocol of this study has been published online, which shows the whole recruitment process in detail (18). Our pilot RCT is conducted and reported in accordance with the Consolidated Standards of Reporting Trials guidelines for randomized pilot and feasibility trials (19). Recruitment commenced in August 2020 and finished in December 2021.

2.2 Study design and intervention

During this double-blinded, parallel-group clinical study, participants were randomly assigned to the control group and the intervention group at a 1:1 ratio. Women participants who meet the eligibility criteria were recruited and stratified according to their body mass index (BMI). All participants were divided into four groups underweight (BMI<18.5 kg/m^2), normal weight (BMI 18.5–23.9 kg/m^2) overweight (BMI 24–27.9 kg/m^2) and obesity (BMI>28 kg/m^2) (20). Computer-generated random numbers are used on the 'H6WORLD' platform (www.h6world.cn) to produce the randomized sequences. Based on BMI stratification, participants were automatically assigned to the control group or intervention group according to random sequences.

Subsequently, participants took GOS supplements in the intervention group or placebo containing fructooligosaccharides (FOS) in the control group from the first trimester (T1). In intervention group, GOS (6 g/100 g) and sialic acid (3 g/100 g) were the primary ingredients. The control group mainly contained FOS (3 g/100 g). The purities of GOS and FOS were 90% and 93% (w/w) on dry matter respectively. The dietary supplements were provided by the Beijing Sanyuan Foods Co. Ltd, Beijing, China. The dosage of the supplements was 60g per day. In order to improve pregnancy health care and strengthen adherence, both the two groups were provided with supplements containing nutrients, minerals and vitamins at each visit timepoint. The trial process followed the double-blind principle of researchers and participants.

2.3 Data and sample collection

Participants were enrolled at 5-8 weeks of gestation. Blood and stool samples were collected and followed up at 11-13 weeks of gestation and 24-28 weeks of gestation. During the follow-up period, filled in the questionnaire during the corresponding pregnancy, and left the participants' blood samples and stool samples at two time points. All the 52 participants who were finally included in the study took blood samples and stool samples in both periods. All samples were collected in sterile tubes and stored at -80 °C until testing. The data of biochemical indexes such as glucose and lipid metabolism of pregnant women were obtained through the medical record system. After blood samples were collected, the immunological parameters IL-6 level was detected in the laboratory department. Fecal samples were collected for gut microbiota analysis.

2.4 Study outcomes

For the primary study outcomes, the effect of GOS on maternal gut microbiota were reported. At the same time, for those who have been followed up to the second trimester of pregnancy, based on the results of 75g oral glucose tolerance test (OGTT) at 24-28 gestational weeks (21), GDM incidence rates in these populations were reported.

For pregnant women, baseline data such as age, gravidity, parity, BMI, history of GDM, and family history of diabetes were described. For secondary outcomes, the biochemical parameters of glucose and lipid metabolism (fasting plasma glucose (FPG), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C)), IL-6, and gestational weight gain (GWG) in the intervention group and the control group were included respectively. As for Newborns, included gestational age, the mode of delivery, sex, birth length, birth weight, head circumference and chest circumference. We evaluated the safety and adverse reactions of prebiotics intervention.

2.5 DNA extraction and V3–V4 region of 16SrRNA gene sequencing

A commercial kit (Qiagen, Hilden, Germany) were used to extract faecal DNA. Faecal DNA was amplified by PCR using 16S amplicon PCR forward primer and 16S amplicon PCR reverse primer. After PCR amplification, the amplicons in each library were purified by Qiagen for library preparation. Subsequently, the qualified library was sequenced by Illumina Hiseq 2500 high-throughput sequencing platform. Sequences were clustered into operational taxonomic units (OTUs) based on Silva database v128, at a similarity level of 97%. Alpha and Beta diversity were generated in Quantitative Insights Into Microbial Ecology (QIIME). And the abundance of bacterial OTUs were divided into several levels (phyla, class, order, family and genus). The laboratory technicians were blinded to the clinical status (intervention or control group) of study participants.

2.6 Sample size

The purpose of this pilot study was to eavluate the feasibility and acceptability of prebiotics for pregnant women. A total of 52 pregnant women were considered enough to provide practical recruitment, feedback and compliance information. The findings will provide basis and support for future a large sample trial to evaluate the effects of prebiotics supplementation in early pregnancy on gut microbiota, glucose metabolism and immunity of pregnant women and newborns.

2.7 Statistical analysis

Data were represented as mean ± standard deviation (SD) or count (%). All data were input into SPSS (version 25.0) to analyze. GraphPad prism (version 8.0) was used to draw diagrams. $\chi 2$ and Fisher's exact test was used for categorical variables, and t-test or non-parametric Wilcoxon test was used for continuous variables where appropriate. $P < 0.05$ was considered to be statistically significant. And bioinformatics analysis for microbiome used R software (Bell Laboratories). Alpha and beta diversities were generated in the Quantitative Insights Into Microbial Ecology (QIIME) and calculated based on weighted or unweighted Unifrac distance matrices. We used the linear discriminant analysis (LDA) effect size (LEfSe) method to identify species that show statistically significant differential abundances between groups.

3 Results

3.1 Participants enrollment and clinical baseline

Flow of participants through the study is shown in Figure 1. In total 216 women were assessed for eligibility. Of these, 124 did not meet the inclusion criteria, 38 declined the invitation to participate and 2 were excluded for other reasons. Fifty-two women were randomized, 26 to GOS and 26 to the placebo group. One woman in the GOS group withdrew early. Baseline characteristics were similar in GOS and placebo group, including age, height, pre-pregnancy weight, pre-pregnancy BMI, gravidity, parity, family history of diabetes, and history of GDM (Table 1).

3.2 Effects of prebiotics on gut microbiota in pregnant women

3.2.1 Overall microbial structures of gut microbiota

We studied gut microbiota of women in placebo and GOS groups. Figure 2 shows the overall microbiota structure at the phylum level in each group. The main phyla of placebo and GOS groups were *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*, with *Firmicutes* the most abundant.

3.2.2 Changes of gut microbiota diversity

To assess the gut microbiota community structure, richness (Chao 1 index) and diversity (Simpson index, Shannon index) were calculated (Figures 3A–C). There was no significant difference in Chao 1 index between GOS group and placebo group ($P > 0.05$). For Simpson index and Shannon index, compared with placebo group, the data of the GOS group were similarly ($P > 0.05$).

To compare overall gut microbiota structure in pregnant women, PCoA according to OTUs of each sample were implemented to provide a glimpse of gut microbial dynamics between placebo and GOS groups. The results of PCoA were PC1 = 54.49% and PC2 = 11.26% of total variations (Figure 3D).

3.2.3 Changes in specific bacterial taxa

For identify the changes in specific bacterial taxa after prebiotics supplemented intervention. We utilized the linear discriminant analysis (LDA) effect size (LEfSe) to compare the gut microbiota composition between placebo and GOS groups. The LDA score was selected to discriminate specific taxa in two groups. Compared with the placebo group, the GOS group had a higher abundance of *Paraprevotella* and *Dorea*, but lower abundance of *Lachnospiraceae*UCG_001 (Figures 4A–D).

3.3 Participants clinical outcomes

3.3.1 GDM diagnosis and OGTT values

Serum levels of FBG, 1-hour and, 2-hour OGTT plasma glucose measured at 24–28 weeks of pregnancy in women who received either GOS or placebo are illustrated in Table 2. As can be seen, there was no significant difference between the intervention and the control group regarding FBG (4.75 ± 0.30 mmol/L vs 4.73 ± 0.41

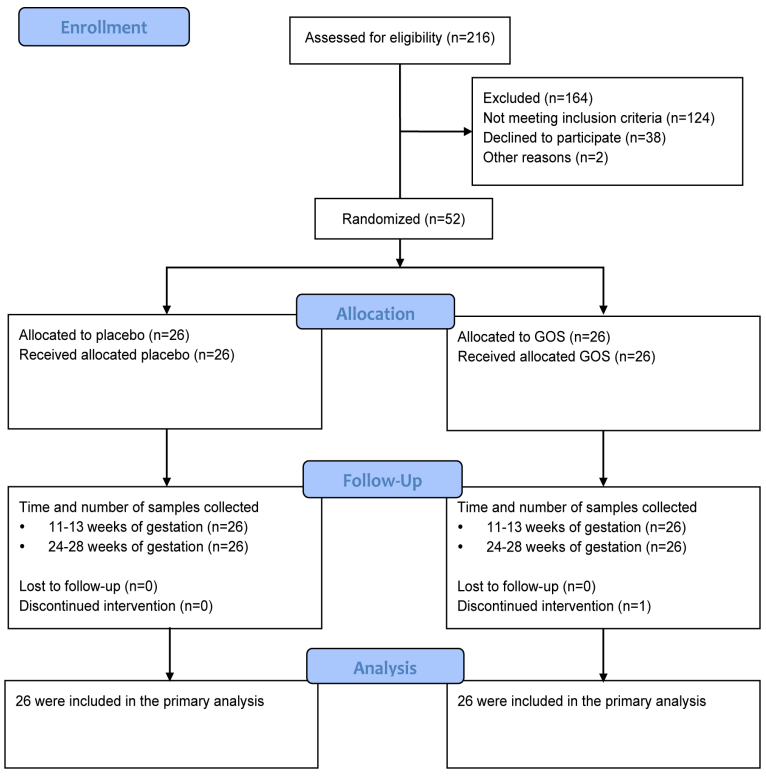

FIGURE 1
Flow chart of participants through the study.

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TABLE 1 Baseline characteristics of study participants.

	GOS (n=26)	Placebo (n=26)	P-value
Age (years)	33.42 ± 3.71	32.35 ± 3.62	0.295
Height (cm)	164.04 ± 6.20	163.36 ± 5.64	0.682
Pre-pregnancy weight (kg)	59.23 ± 8.18	61.42 ± 11.90	0.444
Pre-pregnancy BMI (kg/m^2)	22.05 ± 3.17	22.98 ± 4.20	0.368
BMI classification [n (%)]			0.914
Underweight (BMI<18.5 kg/m^2)	1 (3.8)	2 (7.7)	
Normal weight (BMI 18.5–23.9 kg/m^2)	20 (76.9)	18 (69.2)	
Overweight (BMI 24–27.9 kg/m^2)	3 (11.5)	3 (11.5)	
Obesity (BMI>28 kg/m^2)	2 (7.7)	3 (11.5)	
Gravidity	0.65 ± 0.94	0.92 ± 1.16	0.362
Parity	0.23 ± 0.43	0.27 ± 0.53	0.776
Family history of diabetes [n (%)]			0.191
Yes	5 (19.2)	1 (3.8)	
No	21 (80.8)	25 (96.2)	
History of GDM [n (%)]			1.000
Yes	0 (0.0)	1 (3.8)	
No	26 (100.0)	25 (96.2)	

Data presented are mean ± SD or n (%).
P-values for comparisons between the 2 groups in t-tests for continuous variables, and $\chi 2$ and Fisher's exact tests for categorical variables.
GDM, gestational diabetes mellitus; BMI, body mass index.

mmol/L; P = 0.883), OGTT-1 h (7.81 ± 1.55 mmol/L vs 8.57 ± 2.03 mmol/L; P = 0.133), and OGTT-2 h (6.57 ± 1.44 mmol/L vs 6.87 ± 1.33 mmol/L; P = 0.434) measured at 24–28 weeks of pregnancy. The incidence of GDM in the GOS and placebo group are provided in Table 2. The incidence of GDM in the GOS group was 30.8% which was not significantly different from the placebo group (30.8%) (P = 1.000).

3.3.2 Changes in weight and BMI during pregnancy

With the change of gestational weeks, we collected the weight gain during pregnancy of two groups of pregnant women, and calculated the changes of BMI (Table 3). There was no significant difference in these indicators between GOS group and placebo group regarding gestational weight gain (GWG) (12.42 ± 3.63 kg vs 13.19 ± 3.94 kg; P = 0.466), BMI gain (4.65 ± 1.51 kg/m^2 vs 4.93 ± 1.44 kg/m^2; P = 0.497).

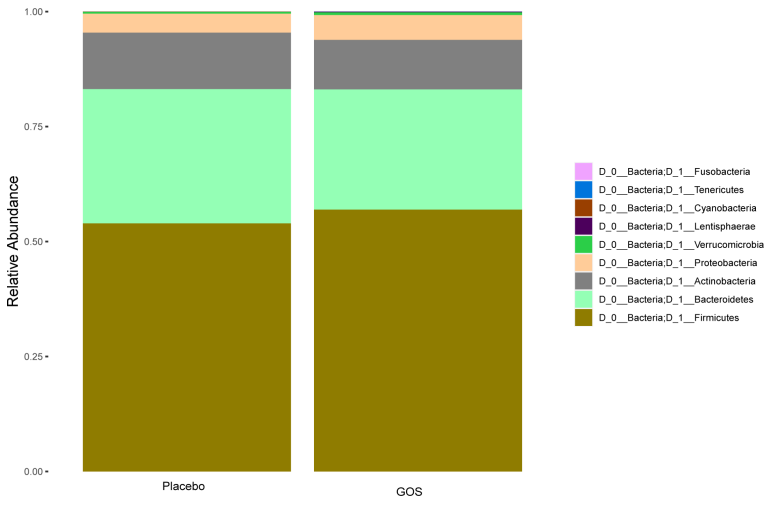

FIGURE 2
Relative abundance at the level of bacterial phylum.

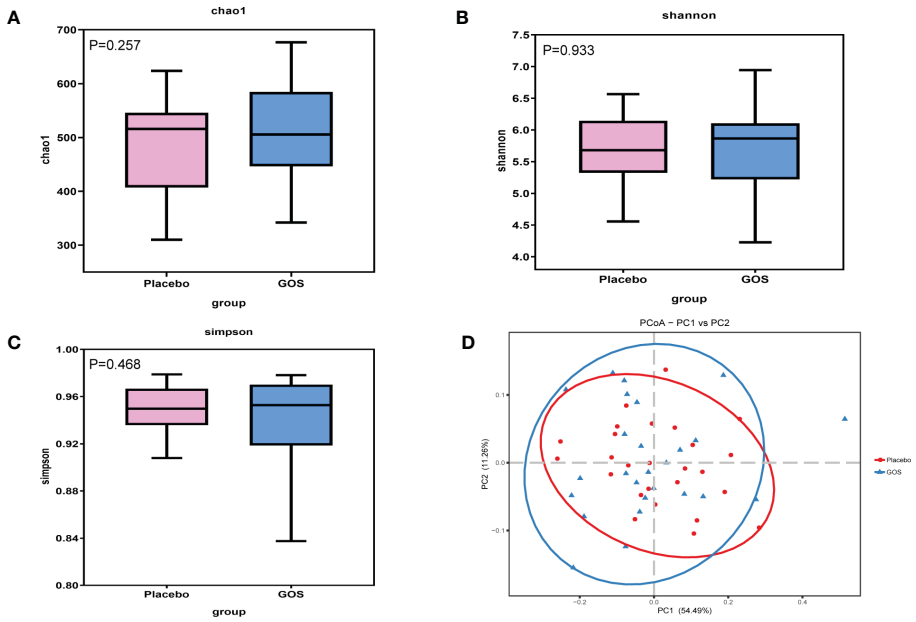

FIGURE 3
Alpha and beta diversity of gut microbiota in placebo and GOS groups. **(A)** Comparisons of Chao 1 index. **(B)** Comparisons of Shannon's index. **(C)** Comparisons of Simpson's index. **(D)** PCoA calculated based on Weighted unifrac distances.

3.3.3 Clinical characteristics of neonates

To investigate the impact of the intervention on neonatal outcomes, we measured the following variables including gestational week, birth weight, birth length, head circumference, chest circumference, sex, and delivery mode. No significant difference was found between the GOS and placebo group (all P-values were > 0.05) (Table 4).

3.3.4 Glucose metabolism, lipid metabolism and inflammatory factor levels

In order to further explore the effect of prebiotics intervention on glucose metabolism, lipid metabolism, and immunity, we analyzed the following indicators (Table 5). There was no significant difference in glucose metabolism levels between GOS group and placebo group regarding FPG (4.75 ± 0.30 mmol/L vs 4.73 ± 0.41 mmol/L; P =

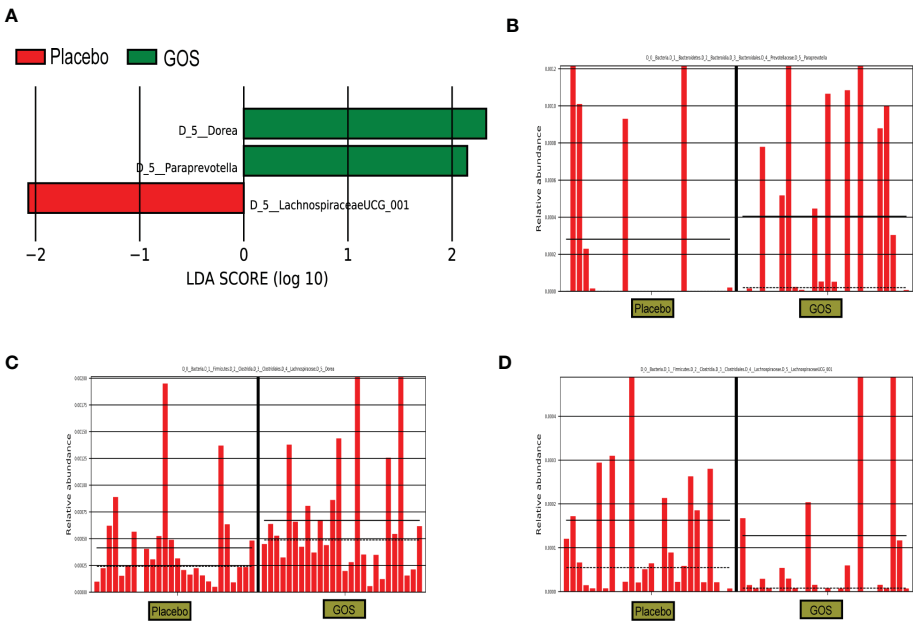

FIGURE 4
Identification of the most differentially abundant analyzed by the LEfSe method. **(A)** LDA scores of differentially abundant taxa. **(B)** Relative abundance of Paraprevotella. **(C)** Relative abundance of Dorea. **(D)** Relative abundance of LachnospiraceaeUCG_001.

TABLE 2 GDM diagnosis and OGTT values.

	GOS (n=26)	Placebo (n=26)	P-value
Plasma glucose in OGTT (mmol/L)			
Fasting	4.75 ± 0.30	4.73 ± 0.41	0.883
1h	7.81 ± 1.55	8.57 ± 2.03	0.133
2h	6.57 ± 1.44	6.87 ± 1.33	0.434
GDM diagnosis [n (%)]			1.000
Yes	8 (30.8)	8 (30.8)	
No	18 (69.2)	18 (69.2)	

GDM, gestational diabetes mellitus; OGTT, oral glucose tolerance test.

0.883). Meanwhile, there was no significant difference in lipid metabolism levels between GOS group and placebo group regarding TG (1.93 ± 0.75 mmol/L vs 2.05 ± 0.87 mmol/L; P = 0.615), TC (5.09 ± 1.27 mmol/L vs 5.20 ± 1.17 mmol/L; P = 0.737), HDL-C (1.56 ± 0.35 mmol/L vs 1.61 ± 0.37 mmol/L; P = 0.610), and LDL-C (2.65 ± 0.89 mmol/L vs 2.64 ± 0.80 mmol/L; P = 0.983). There was also no significant difference in IL-6 levels between GOS group and placebo group (1.55 ± 0.58 pg/mL vs 2.02 ± 1.20 pg/mL; P = 0.080).

3.3.5 Incidences of maternal and infant complications

Clinical data on the incidences of maternal and infant complications were also collected (Table 6). The incidence of gestational hypertension was 0.0% in the GOS group and 11.5% in the placebo group (P = 0.235). The incidence of thyroid dysfunction was 3.8% in the GOS group and 15.4% in the placebo group (P = 0.350). The incidence of fetal growth restriction was 3.8% in the GOS group and 0.0% in the placebo group (P = 1.000). The incidence of anemia was 34.6% in the GOS group and 42.3% in the placebo group (P = 0.569). And the incidence of postpartum hemorrhage was 7.7% in the GOS group and 3.8% in the placebo group (P = 1.000).

3.4 Safety of intervention

A questionnaire was used to record the possible severity of adverse symptoms in pregnant women and the relationship between symptoms and the ingestion of preparations. The results showed that one participant in GOS group had abdominal distension and one participant had nausea. These symptoms have little to do with the intake of prebiotic preparations, and may be related to appetite and hormone changes during pregnancy. Therefore, for the existing included cases, it can be considered that supplementing prebiotic preparations during pregnancy is relatively safe.

4 Discussion

During pregnancy, the disorder of gut microbiota and abnormal glucose metabolism may be the possible mechanism of pregnancy complications such as GDM (22). Moreover, patients with gestational diabetes have a higher chance of developing type 2 diabetes in the long term (23). Maternal GDM is also associated with overweight and obesity status in offspring (24). Therefore, it is necessary to seek safe and effective interventions to improve the adverse status of pregnant women. There have been studies on the use of probiotics, synbiotics and other dietary supplements during pregnancy to prevent and treat gestational diabetes (5, 25). The purpose of this study is to explore the effects of prebiotic preparations containing GOS on glucose metabolism, lipid metabolism, inflammation and gut microbiota of pregnant women during early pregnancy, and the feasibility and acceptability of using prebiotics as dietary supplements during pregnancy.

The preliminary conclusion of this study is that GOS intervention has no significant effect on reducing the incidence of GDM and improving glucose and lipid metabolism. GOS a kind of prebiotics that can be selectively and selectively utilized by host microorganisms that confer a health benefit, while probiotics are defined as live microorganisms (26). Previous clinical studies using probiotic

TABLE 3 Changes in weight and BMI during pregnancy.

	GOS (n=26)	Placebo (n=26)	P-value
BW 1st (kg)	59.23 ± 8.18	61.42 ± 11.90	0.444
BMI 1st (kg/m2)	22.05 ± 3.17	22.98 ± 4.20	0.368
GWG (kg)	12.42 ± 3.63	13.19 ± 3.94	0.466
BW 3rd (kg)	71.65 ± 8.96	74.61 ± 13.43	0.355
BMI 3rd (kg/m2)	26.70 ± 3.71	27.91 ± 4.66	0.303
BMI gain (kg/m2)	4.65 ± 1.51	4.93 ± 1.44	0.497

BW, body weight; BMI, body mass index; BW 1st, body weight at the beginning of 1st trimester; BW 3rd, body weight at the end of 3rd trimester; BMI 1st, BMI at the beginning of 1st trimester; BMI 3rd, body mass index at the end of 3rd trimester; GWG, gestational weight gain.

TABLE 4 Clinical characteristics of neonates.

	GOS (n=26)	Placebo (n=26)	P-value
Gestational week (weeks)	38.95 ± 1.58	38.86 ± 1.51	0.838
Birth weight (g)	3242.12 ± 484.20	3235.38 ± 443.53	0.959
Birth length (cm)	49.77 ± 1.53	49.65 ± 1.32	0.773
Head circumference (cm)	33.88 ± 0.44	33.94 ± 0.45	0.621
Chest circumference (cm)	32.64 ± 0.57	32.79 ± 0.57	0.356
Sex [n (%)]			0.578
Male	13 (50.0)	11 (42.3)	
Female	13 (50.0)	15 (57.7)	
Delivery mode [n (%)]			0.080
Spontaneous delivery	20 (76.9)	14 (53.8)	
Cesarean delivery	6 (23.1)	12 (46.2)	

supplements for intervention have some similarities with this study, the results showed that probiotics did not reduce the incidence of GDM in pregnant women (6, 7, 27), there is a study with different conclusion (5). There were no significant changes in FBG and insulin resistance index for some synbiotics containing fructooligosaccharide (28). An animal study in GDM mice showed that inulin-type fructose-oligosaccharide treatment alleviated glucose and lipid metabolism disorders mediated by the gut microbiota (29). Dietary supplements intervention may be beneficial in improving inflammatory status. A clinical trial supplemented with probiotics also observed that dietary supplements reduced the expression of pro-inflammatory factors TNF-α (30). However, as far as we know, there are few clinical studies on prebiotic supplements for pregnant women. The different results may be related to the type, dose, dosage form, intervention time and intervention population of dietary supplements. Our intervention seems to be safe and well tolerated in view of the minimal adverse reactions. A meta-analysis also thought that probiotics and prebiotics are safe during pregnancy and lactation, and adverse reactions related to the use of probiotics and prebiotics will not cause any serious health problems to mothers or infants (31). In this study, GOS supplementation was started in early pregnancy. GOS may have some effect as the duration of the intervention increases if taken before pregnancy or even earlier. From the follow-up, participants gave us feedback that taking such dietary supplements was convenient and easy to implement. Prebiotics can usually be added to common foods, they are mainly used for fermented dairy products (yogurt, cheese), nonfermented dairy products (milk formula for pregnant women or infant) (9). Therefore, in addition to the need to supplement essential nutrients during pregnancy, it is also possible for pregnant women to take appropriate food containing prebiotics. At the same time, the clinical conditions of the pregnant women themselves and their daily energy intake should also be considered.

Both the intervention group and the control group have similar relative abundances at the phylum level, including *Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria*. *Paraprevotella* and *Dorea* were enriched in the intervention group during the second trimester, suggesting that prebiotics could affect the composition of gut microbiota. A study has shown that the abundance of *Paraprevotella* is negatively correlated with serum TG, TC and LDL-C levels, suggesting that *Paraprevotella* may have anti-obesity effects (32). Prebiotics are not digested and absorbed by the host, but can promote the proliferation of target flora and improve intestinal microecology by increasing the abundance of beneficial bacteria in the intestine (33). In our work, after the intervention of GOS prebiotics, the relative abundance of *Paraprevotella* and *Dorea* increased specifically, and the relative abundance of *Lachnospiraceae*UCG_001 was higher in the placebo group containing FOS prebiotics. Although in our study, after the GOS intervention, some serum indicators, such as TG, LDL, etc. had no beneficial effects, but the intervention did not seem to have some adverse

TABLE 5 Glucose metabolism, lipid metabolism and inflammatory factor levels.

	GOS (n=26)	Placebo (n=26)	P-value
FPG (mmol/L)	4.75 ± 0.30	4.73 ± 0.41	0.883
TG (mmol/L)	1.93 ± 0.75	2.05 ± 0.87	0.615
TC (mmol/L)	5.09 ± 1.27	5.20 ± 1.17	0.737
HDL-C (mmol/L)	1.56 ± 0.35	1.61 ± 0.37	0.610
LDL-C (mmol/L)	2.65 ± 0.89	2.64 ± 0.80	0.983
IL-6 (pg/mL)	1.55 ± 0.58	2.02 ± 1.20	0.080

FPG, fasting plasma glucose; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; IL-6, interleukin-6.

TABLE 6 Incidences of maternal and infant complications.

	GOS (n=26)	Placebo (n=26)	P-value
Gestational hypertension [n (%)]	0 (0.0)	3 (11.5)	0.235
Thyroid dysfunction [n (%)]	1 (3.8)	4 (15.4)	0.350
Fetal growth restriction [n (%)]	1 (3.8)	0 (0.0)	1.000
Anemia [n (%)]	9 (34.6)	11 (42.3)	0.569
Postpartum hemorrhage [n (%)]	2 (7.7)	1 (3.8)	1.000

effects on these indicators. Based on the limited participants and the individual differences among pregnant women, it is necessary to explore the effect of prebiotics on serum indicators in the future.

Several strengths and limitations should be taken into consideration. First, this study is a randomized controlled pilot trial. Subsequently, 16S rRNA gene was sequenced by Illumina Hiseq 2500 sequencing platform, a widely and reliable used high-throughput sequencing platform, which can ensure gut microbiota can be successfully identified. Secondly, the quality control in the process of sample collection can be guaranteed, which makes the sequencing quality high and accurate. However, some limitations should also be considered. The sample size of our pilot study is limited, and some confounding factors such as diet and exercise have caused some interference. Although it is difficult to control these confounding factors, we recorded these situations in the form of health education and questionnaire records. Moreover, this study was recruited in the same hospital, and the potential regional differences of microbiota cannot be evaluated. In general, our study provides an important basis for the intervention of prebiotic dietary supplements targeting gut microbiota in pregnancy on metabolic diseases of pregnancy. In the future, clinical trials with higher quality and larger sample size are needed to further verify the effect of prebiotic supplements.

5 Conclusion

GOS prebiotics appear to be safe and acceptable for the enrolled pregnancies. Although GOS intervention did not show the robust benefits on glucose and lipid metabolism. However, the intervention had a certain impact on the compostion of gut microbiota. GOS can be considered as a dietary supplement during pregnancy, and further clinical studies are needed to explore this in the future.

Data availability statement

The raw sequence data of the 16S rRNA gene supporting the results of this article are available in the NCBI database, SRA data (Accession number: PRJNA925813).

Ethics statement

The studies involving human participants were reviewed and approved by Clinical Trial Ethics Committee, Peking University First Hospital, Beijing, China. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JW collected the data, prepared tables and figures, and drafted the paper. LA and ZR analyzed the data and prepared tables. SW, LA, HY, and JM conceived and designed the research. JM revised the manuscript. HY and JM provided clinical supervision. 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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Association between preconception anti-androgen therapy and pregnancy outcomes of patients with PCOS: A prospective cohort study

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Background: Polycystic ovary syndrome (PCOS) not only increases fertility challenges for women of reproductive age, but also leads to increased complications during pregnancy and even affects the birth weight of newborns. Also, hyperandrogenemia is associated with lower pregnancy rates and lower live birth rates and may even play a role in preterm delivery and pre-eclampsia in patients with PCOS. However, it is still controversial whether PCOS patients are treated with androgen-lowering therapy before pregnancy.

Objective: To assess the effect of anti-androgen therapy prior to ovulation induction on maternal and infant pregnancy outcomes in patients with PCOS.

Methods: Prospective cohort study.

Results: A total of 296 patients with PCOS were enrolled in the study. The prevalence of adverse pregnancy outcomes, and neonatal complications was lower in DRSP(with drospirenone ethinyl estradiol tablets (II) pretreatment) group than in NO-DRSP (without drospirenone ethinyl estradiol tablets (II) pretreatment) groups (DRSP *vs*. NO-DRSP: adverse pregnancy outcomes, 12.16% *vs*. 27.03%, P=0.001; neonatal complications, 17.16% *vs*. 36.67%, P<0.001). No significant difference was found in maternal complications. Further subgroup analysis revealed that PCOS with pretreatment decreased the risk of preterm delivery (2.99% *vs*. 10.00%; Adjusted RR, 3.80; 95% CI, 1.19-12.13), pregnancy loss (9.46% *vs*. 18.92%; Adjusted RR, 2.07; 95% CI, 1.08-3.96), low birth weight (0.75% *vs* 7.50%; Adjusted RR, 12.08; 95% CI, 1.50-97.31), fetal malformations(1.49% *vs*. 8.33%; Adjusted RR, 5.63; 95% CI, 1.20-26.33).There were no significant differences in the incidence of DM and PIH as pregnancy complications between the two groups (P>0.05).

Conclusion: Our findings suggest that preconception androgen-lowering therapy in patients with PCOS improves pregnancy outcomes and reduces neonatal complications.

KEYWORDS

anti-androgen therapy, polycystic ovary syndrome, adverse pregnancy outcomes, preconception intervention, newborn complications

Introduction

Polycystic ovary syndrome (PCOS), the most common complex and heterogeneous endocrine disorder in premenopausal women, has a prevalence of approximately 8%-13% based on foreign studies (1). A survey conducted in 2020, which included 28,739 participants, reported an updated prevalence estimate of 7.8% for PCOS in China (2).

Most women with PCOS experience corresponding problems, such as anovulation, irregular menstruation, and infertility, all of which can lead to a reduced quality of life (3), and an increased incidence of depression and anxiety (4). Therefore, a growing number of studies has focused on PCOS treatment (5) in recent years. Lifestyle changes, including diet, exercise, and behavioral changes, are the first-line treatment recommended for women with PCOS. However, these measures alone have been reported to be ineffective in reducing weight or treating symptoms associated with PCOS (6). Medications are the second-line treatment, and can help to improve pregnancy rates by adjusting hormone levels and improving insulin resistance. In addition to pharmacological interventions, assisted reproductive technologies can also improve pregnancy rates in PCOS patients (7).

PCOS has not only been shown to increase the reproductive burden in women of reproductive age, but it has also been reported to be a risk factor for increased complication rates during pregnancy (8). Complications PCOS patients may experience in early pregnancy include emesis, miscarriage (9). Further risks include gestational diabetes, pre-eclampsia, gestational hypertension, preterm delivery, perinatal fetal death, and increased risk of neonatal intensive care hospitalization (10, 11). With regard to neonatal birth weight, the offspring of PCOS patients have an increased risk of low birthweight babies and oversized babies (11). Despite emerging evidence that PCOS is an unfavorable risk factor for some pregnancies and perinatal outcomes, no guidelines or pharmacological strategies exist for the treatment of PCOS in pregnancy.

Further analysis of some studies has shown that hyperandrogenemia may contribute to ovulatory drug resistance, lower pregnancy rates, and lower live birth rates in patients with PCOS (12), and even play an important role in preterm delivery and preeclampsia (13). The potential impact of hyperandrogenemia on ovulation and pregnancy in PCOS patients suggests that anti-androgen pretreatment may also help to improve fertility in PCOS patients.

Combined Oral Contraceptive (COC) is a class of oral contraceptives that combines estrogen and progestin, and has been used as a first-line treatment for improving hyperandrogenemia and regulating menstrual cycle in PCOS patients of reproductive or adolescent age. Oral COC not only regulates menstrual cycle and reduces androgens in patients with PCOS, but also suppresses hirsutism, treats acne, and prevents endometrial lesions (8). However, controversy persists among researchers as to whether COC should be used before ovulation induction, and whether preconception use of COC improves pregnancy outcomes in patients with PCOS. Palomba et al. reported that COC pretreatment before ovulation induction increased ovulation and pregnancy rates (14), Pan et al. showed that continuous preconception COC interventions not only increased pregnancy rates (14) but also reduced the incidence of small-for-gestational age (15). While a retrospective study by Li et al. found that PCOS patients treated with ethinyl estradiol cyproterone tablets to reduce androgen therapy, followed by ovulation induction, reduced the risk of gestational diabetes mellitus, gestational hypertension-related disorders, and preterm delivery during pregnancy in PCOS patients (16). A randomized controlled trial study by Lergo et al. found that pregnancy rates increased, but live birth rates did not improve in PCOS patients after COC preconception intervention (17). The lack of high-quality evidence on antiandrogen preconception therapy means the effect of COC preconception intervention on pregnancy outcomes in PCOS patients has remained unknown. Consequently, clinicians lack sufficient evidence to convince infertile PCOS patients to spend time and effort to undergo COC preconception treatment before pregnancy, and clinicians continue to disagree on whether to give COC treatment before ovulation promotion and assisted reproductive technology in actual clinical practice.

This study was conducted to settle the aforementioned debate by determining the effect of preconception androgen-lowering treatment intervention on pregnancy outcomes and pregnancy complications in patients with PCOS through a prospective cohort study, and to investigate the need for preconception COC treatment in patients with PCOS. Finally, this study investigates the factors that contribute to adverse pregnancy outcomes in PCOS patients and further help clinicians to recommend possible treatment strategies to prevent adverse pregnancy outcomes in PCOS patients.

Materials and methods

Study setting and participants

This is a prospective cohort study with a study population of patients aged 20-35 years with PCOS and fertility needs, who visited the Guangdong Provincial Maternal and Child Health Hospital from September 2019 to April 2022. The study was conducted with the approval of the Ethics Committee of Guangdong Maternal and Child Health Hospital and was successfully registered with the China Clinical Trials Registry (ChiCTR2100052703). All participants gave informed consent prior to enrolling in the study.

Inclusion criteria: (i) meeting the 2003 Rotterdam PCOS diagnostic criteria (2); (ii) PCOS patients aged 20-35 years; (iii) meeting the diagnostic criteria for hyperandrogenemia: elevated total testosterone hormone levels or elevated androstenedione levels (laboratory-defined hyperandrogenemia as total testosterone >1.97 nmol/L and/or androstenedione >10.8 nmol/L).

Exclusion criteria: (1) severe reproductive tract abnormalities; (2) other endocrine disorders, such as diabetes mellitus, thyroid dysfunction and hyperprolactinemia; (3) other systemic diseases, such as cardiovascular, hepatic and renal diseases; (4) malignant tumors; (5) mental challenges that could prevent compliance with treatment or follow-up; (6) the use of any hormones or drugs affecting endocrine and glucolipid metabolism in the 3 months before or during the study period.

Study cohort

All patients with confirmed PCOS and fertility needs had basic endocrine and glucolipid metabolism examinations on days 3-5 of their menstrual cycle. Anthropometric examinations were performed under the supervision of a professional physician at the time of enrollment. After enrollment, patients in both groups underwent lifestyle modifications under the guidance of professional physicians. Modifications included reducing sugar and fat intake, abstaining from smoking, abstaining from alcohol, and engaging in strengthening exercises. Our exposure factors were 3 consecutive oral cycles of drospirenone and ethinylestradiol tablets (II)(DRSP/EE(II)) before pregnancy in patients with PCOS. Subjects were divided into DRSP group and NO-DRSP group according to whether they voluntarily took DRSP drugs for preconception pretreatment or not. The DRSP group started on the 1st day of the menstrual cycle in the order indicated by the arrows, and 28 consecutive days of oral intake was considered to be 1 complete cycle. Basal endocrine and glucolipid metabolism were rechecked across 3 cycles of treatment. Pregnancy was induced after ovulation immediately after discontinuation of the drug. In the NO-DRSP group, ovulation is induced directly under basic lifestyle guidance. No additional treatment to lower androgen levels was received. Patients were followed up on and monitored for their maternal status, pregnancy complications, delivery and neonatal status.

Data collection and outcomes

Demographics: age, ethnicity, previous maternal history, height, weight, body mass index (BMI), hip circumference, waist circumference, waist-to-hip ratio (WHR).

Clinical characteristics: All subjects were enrolled with fasting blood sampling for basal endocrine tests, including follicle stimulating hormone (FSH), luteinizing hormone (LH), LH/FSH, estradiol (E2), progesterone (P), serum prolactin (PRL), anti-Müllerian hormone (AMH) on days 3-5 of their menstrual cycles; androgenic parameters: total testosterone (T), androstenedione (AND), dehydroepiandrosterone sulfate (DHEA-S), sex hormone binding globulin (SHBG), free androgen index (FAI); glucose and lipid metabolism indicators: fasting blood glucose (FPG), fasting insulin (FINS), triglycerides (TCH), total cholesterol (TG), low density lipoprotein (LDL), and high density lipoprotein (HDL).

Primary outcome: An adverse pregnancy outcome is a composite indicator that refers to the occurrence of one or more outcomes, such as abortion or preterm labor, during the follow-up window.

Secondary outcomes: Pregnancy complications (gestational diabetes, hypertensive disorders in pregnancy), and neonatal complications (low birthweight babies, oversized babies, neonatal malformations, neonatal referral treatment).

Statistical analysis

In our study, our main primary outcome was adverse pregnancy outcome, and the DRSP group was designed as a 1:1 cohort study with the NO-DRSP group. The incidence of adverse pregnancy outcomes in the NO-DRSP group was expected to be approximately 39.7%, while the incidence of adverse pregnancy outcomes in the DRSP group was anticipated to be approximately 24.3%. Based on statistical formulae, 143 cases each in the DRSP group and NO-DRSP group were calculated; the follow-up loss rate stayed at about 10%, and the sample size was expanded to 160 cases each in both groups.

Statistical analysis was performed using SPSS 22.0 and GraphPad Prism 8 for graphs. Normality tests were performed on both data sets. Continuous variables that conformed to a normal distribution were expressed as ± SD. Data that did not conform to a normal distribution were expressed as median and quartiles, and categorical variables were expressed as frequencies and percentages. During baseline data collection, missing values were interpolated using single values, where the mean was used for normal continuous variable data, the median for skewed continuous variables, and the plural for categorical variables. Continuous variables were statistically analyzed *via* t-test, while categorical variables were statistically analyzed using Chi-square test or Fisher's exact probability method. The significance level was determined using a two-sided $\alpha = 0.05$, when $P < 0.05$ differences were statistically significant.

Results

Patient characteristics

As shown in Figure 1, a total of 320 pregnant women with PCOS were initially included in our study. During follow-up, three cases were lost in the exposed group, including two cases who requested to withdraw from the study, and one case involving contact error. Four cases were lost in the non-exposed group, including two cases who terminated their pregnancies due to personal factors, and two others who requested to withdraw from the study. A total of 296 patients were included in the final statistical analysis, including 148 patients with PCOS who had undergone pharmacological intervention with drospirenone and ethinyl estradiol tablets (II), and 148 patients with PCOS who had not undergone pharmacological intervention. The clinical and demographic characteristics of the patients are listed in Table 1. The results of age, BMI, glucose, lipids, and sex hormone water ($P > 0.05$) at baseline examination were similar in both groups. Overall, the two groups were well matched at the baseline examination.

Adverse pregnancy outcomes

In our study, no postpartum hemorrhage, neonatal asphyxia, or neonatal death events were reported during follow-ups with either group of PCOS patients. As shown in Table 2 and Figure 2A, among 296 subjects, a total of 58 PCOS patients had adverse pregnancy outcome events, including 18 in the DRSP group, and 40 in the NO-DRSP group. The incidence of adverse pregnancy outcomes was significantly lower in PCOS patients treated with DRSP before pregnancy compared to those who were not treated with DRSP, (12.16% *vs.* 27.03%, Adjusted RR, 2.35; 95% CI, 1.34-4.14). In Table 2 and Figure 2B, further subgroup analysis of adverse pregnancy outcomes showed a significant reduction in the incidence of preterm delivery and pregnancy loss in the DRSP group compared with the NO-DRSP group, with a 7.01% reduction in

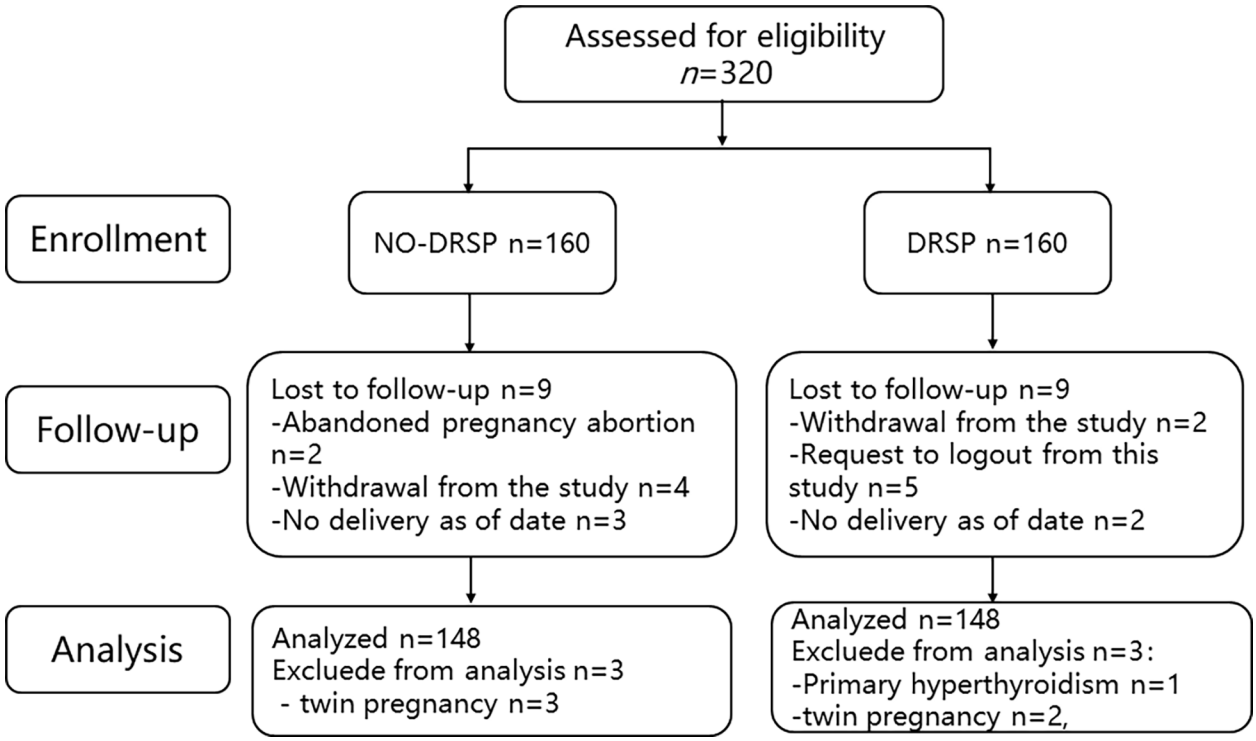

FIGURE 1
Flow chart of patient registration and follow-up.

incidence of preterm delivery (2.99% *vs.* 10.00%; Adjusted RR, 3.80; 95% CI, 1.19-12.13) and a 9.46% reduction in incidence of pregnancy loss (9.46% *vs.* 18.92%; Adjusted RR, 2.07; 95% CI, 1.08-3.96).

Neonatal complications

A total of 254 newborns were included in our study: 134 in the DRSP group and 120 in the NO-DRSP group. As shown in Table 2 and Figure 2C there was a significant difference in the incidence of neonatal complications in the offspring of PCOS patients in the DRSP group (17.16% *vs.* 36.67%, Adjusted RR, 2.46; 95% CI, 1.51-4.01), where the risk of low birthweight infants was significantly lower in PCOS patients treated with DRSP before pregnancy compared to the control group (0.75% *vs.* 7.50%; Adjusted RR, 12.08. 95% CI, 1.50-97.31). The risk of congenital malformations in the offspring of PCOS patients treated with DRSP before pregnancy was also significantly lower than in the control group (1.49% *vs.* 8.33%; Adjusted RR, 5.63; 95% CI, 1.20-26.33). The incidence of gigantism in the offspring and neonatal treatment referral was similar in both groups of PCOS patients.

Maternal complications

In Table 2 and Figure 2D, we counted the PCOS patients with severe gestational complications like gestational diabetes mellitus and gestational hypertension-related disorders among the 120 live births in the NO-DRSP group and 134 live births in the DRSP group. Overall, there was no significant difference in the incidence of pregnancy complications in PCOS patients in the DRSP group compared with the NO-DRSP group (19.40% *vs.* 25.00%; Adjusted RR, 1.06; 95% CI, 0.61-1.85), where the incidence of gestational diabetes mellitus and gestational hypertension-related disorders in the DRSP group compared with the NO-DRSP group were not significantly lower (P >0.05).

Subgroup analysis

We performed a further subgroup analysis of factors influencing total adverse pregnancy outcomes and found that the incidence of adverse pregnancy outcomes was significantly reduced by 14.79% in patients with PCOS less than 30 years of age after preconception androgen-lowering therapy. Pre-pregnancy androgen-lowering therapy significantly reduced the incidence of adverse pregnancy outcomes in both primipara and multipara women, but the effect was more pronounced in the multipara women. The findings also showed that PCOS patients with BMI <25 had a significantly lower incidence of adverse pregnancy outcomes after preconception androgen reduction therapy. When the preconception HOMA-IR of the PCOS population was ≥2.69, the risk of adverse pregnancy outcome after preconception androgen-lowering therapy was significantly lower than that of the PCOS population with HOMA-IR <2.69. These results are available in Table 3.

Discussion

Our study is a prospective cohort study that included 296 patients with PCOS tracked to pregnancy outcomes. The findings

TABLE 1 Baseline Characteristics of participants.

	NO-DRSP	DRSP	*T*	*P value*
N	148	148		
Age (year)	28.03±2.64	27.53±3.15	3.383	0.067
BMI (kg/m2)	22.24±0.16	22.35±3.22	0.581	0.446
WHR	0.84±0.03	0.85±0.05	33.277	0.000
Pre-BMI (kg/m^2)	27.93±3.72	26.83±3.19	3.463	0.064
parity				
primipara	139 (91.45%)	119 (79.33%)	8.901	0.003
multiparous	13 (8.55%)	31 (20.67%)		
AMH (ng/ml)	10.54±5.64	11.16±5.57	0.117	0.732
LH (IU/L)	8.25±4.63	9.40±5.08	1.425	0.234
FSH (IU/L)	5.60±1.49	5.38±1.44	0.192	0.661
LH/FSH	1.51±0.91	1.77±0.95	2.707	0.101
T (nmol/L)	1.39±0.53	1.59±0.55	0.160	0.690
AND (mmol/L)	10.53±3.99	12.31±3.99	0.114	0.736
DHEA-S (ug/dl)	272.78±105.81	310.13±111.04	0.016	0.899
FAI	4.57±3.56	4.77±3.39	0.173	0.665
SHBG (nmol/L)	53.34±32.57	46.41±35.36	0.028	0.868
FPG (mmol/L)	4.88±0.34	4.96±0.33	1.057	0.305
FINS (uU/mL)	10.08±1.91	11.37±1.90	11.186	0.003
HOMA-IR (uU/mL)	2.20±0.98	2.42±1.17	6.590	0.011
TC (mmol/L)	4.83±0.87	4.92±0.96	1.222	0.270
TG (mmol/L)	1.49±1.25	1.31±0.76	4.311	0.395
LDL (mmol/L)	4.46±0.87	2.80±0.89	3.194	0.075
HDL (mmol/L)	1.50±1.47	1.39±0.28	2.419	0.121

suggest that preconception standardized androgen-lowering therapy with DRSP/EE(II) may not only significantly reduce the incidence of adverse pregnancy outcomes in patients with PCOS, but also benefit the offspring of pregnant women with PCOS by reducing the risk of neonatal complications, although no significant benefit was observed in terms of complications during pregnancy in the study.

Further subgroup analysis of adverse pregnancy outcomes revealed that the incidence of pregnancy loss and preterm delivery were significantly lower in PCOS patients who had used preconceptional androgen-lowering therapy with COC, and that age, BMI, number of births, and preconceptional HOMA-IR were influential factors in the relationship between preconceptional androgen-lowering therapy and adverse pregnancy outcomes in PCOS patients. These findings may be related to the interaction between hyperandrogenemia and insulin resistance as key factors in the pathogenesis of PCOS (18–20), in line with Naver et al.'s cohort analysis, which found that pregnant PCOS patients diagnosed with hyperandrogenemia had a nearly 2-fold increased risk of preterm delivery and preeclampsia (21).

Other literature has also suggested PCOS-related risks in pregnancy. A large cohort study conducted in Sweden by Fornes et al. showed that PCOS patients were at higher risk for miscarriage, preterm birth, and low birthweight babies (22). McDonnell R et al. suggested that polycystic ovary syndrome additionally increases the risk of ectopic pregnancy in women with PCOS (23). Further studies have reported a potential association with embryonic exposure to a hyperandrogenic environment in early pregnancy, which leads to abnormal embryonic development (24), affects placental function, and interferes with normal placental implantation and other causes of early pregnancy loss (25). Our study found that preconception COC treatment significantly reduced the incidence of miscarriage and congenital malformations in the offspring of PCOS patients, probably due to the effective reduction of androgens associated with the regulated use of COC. Early pregnancy loss is associated with endometrial growth in early pregnancy as well, and studies have found high expression of androgen receptors and estrogen receptors in hyperandrogenemic patients with PCOS (25), resulting in an endometrial environment that is not conducive to embryo implantation or placenta formation. Hyperandrogenemia in

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TABLE 2 Comparison of pregnancy outcomes between two groups. (VS. *DRSP*).

Outcomes	*DRSP n (%)*	NO-DRSP *n(%)*	*Difference (95% CI, %)*	*P value*	*Crude RR (95% CI)*	*P value*	*Adjusted RR (95% CI)*	*P value*[a]
N of sample size	148	148						
Adverse pregnancy outcome								
Adverse pregnancy outcome	18(12.16)	40(27.03)	14.86(5.98~23.75)	0.001	2.22(1.27~3.88)	0.005	2.35(1.34~4.14)	0.003
Pregnancy loss	14(9.46)	28(18.92)	9.46(1.58~17.34)	0.020	2.00(1.05~3.80)	0.034	2.07(1.08~3.96)	0.030
Preterm delivery	4(2.99)	12(10.00)	7.01(0.92~13.11)	0.022	3.35(1.08~10.39)	0.036	3.80(1.19~12.13)	0.024
N of neonates	134	120						
Neonatal complications								
total	23(17.16)	44(36.67)	19.50(8.77~30.23)	<0.001	2.46(1.53~3.94)	<0.001	2.46(1.51~4.01)	<0.001
Low birth weight	1(0.75)	9(7.50)	6.75(1.82~11.69)	0.006	10.05(1.27~79.33)	0.029	12.08(1.50~97.31)	0.019
macrosomia	1(0.75)	6(5.00)	4.25(0.09~8.42)	0.039	6.70(0.81~55.65)	0.078	6.54(0.76~56.52)	0.088
Fetal malformations	2(1.49)	10(8.33)	6.84(1.49~12.20)	0.010	5.58(1.22~25.48)	0.026	5.63(1.20~26.33)	0.028
Transfer to NICU	21(15.67)	30(25.00)	9.33(-0.57~19.22)	0.064	1.59(0.91~2.79)	0.101	1.53(0.86~2.73)	0.150
Maternal complications								
Total	26(19.40)	30(25.00)	5.60(-4.64~15.84)	0.283	1.29(0.76~2.18)	0.344	1.06(0.61~1.85)	0.82
PIH	8(5.97)	9(7.50)	1.53(-4.66~7.72)	0.626	1.26(0.48~3.26)	0.639	1.23(0.45~3.33)	0.686
GDM	21(15.67)	24(20.00)	4.33(-5.11~13.77)	0.367	1.28(0.71~2.29)	0.414	1.12(0.60~2.06)	0.727

[a]Calibration for age, gestational age, BMI, HOMA-IR index.

pregnant women with PCOS can additionally cause a series of changes in the placenta by affecting the function of intravascular trophoblast cells, thereby causing vasculopathy, inflammation, and abnormal chorionic villus development, which can lead to preterm labor and preeclampsia (26). Other relevant studies have shown that the coexistence of hyperandrogenemia with insulin resistance and/or hyperinsulinemia may increase the risk of adverse pregnancy outcomes such as miscarriage, fetal growth abnormalities, GDM, gestational hypertension, preterm delivery and postpartum hemorrhage in women with PCOS (24, 27).

While Li et al. found that the incidence of gestational hypertension-related disorders incidence was significantly reduced after preconception pretreatment with COC in patients with PCOS, GDM (16), our results to-date have not indicated a significant benefit of preconception androgen reduction therapy in pregnant women with PCOS in terms of the occurrence of gestational hypertension or gestational diabetes mellitus during pregnancy. The inconsistency between our findings and those of Li et al. may be related to differences in the inclusion and exclusion criteria of the study population. For example, Li et al. did not exclude multiple pregnancies, and recruited PCOS patients aged 20-40 years, whereas the inclusion criteria for our study specified an age range of 20-35 years and excluded PCOS patients who had experienced multiple pregnancies. Our deeper analysis of factors influencing adverse pregnancy outcomes in both groups of PCOS patients suggested that the occurrence of adverse pregnancy outcomes in PCOS patients was related to age and BMI. Therefore, a randomized controlled study with a large sample is still needed at a later stage to further determine the effect of preconception COC pharmacological intervention on pregnancy complications.

Our findings not only confirm that preconception COC intervention significantly reduces the risk of low birth weight infants in the offspring of PCOS patients, but also demonstrate that preconception intervention does not increase the incidence of congenital malformations in the offspring. The main types of congenital malformations we observed were developmental abnormalities of the nervous system, musculoskeletal system, and urogenital tract system, while other types of developmental abnormalities were not observed in our study. A higher rate of neonatal transfer in PCOS patients compared to the general population has been associated with preterm birth, hypoglycemia, jaundice and respiratory distress syndrome (28). Although our study found that preconception intervention with DRSP/EE(II) though did not significantly reduce the neonatal transfer rate, there may be a diversity of reasons for neonatal referrals. Increased neonatal perinatal mortality has also been reported in pregnant women with polycystic ovary syndrome compared to those without (29, 30). Currently, there have been no reports of neonatal mortality in our study or other studies on preconception interventions for COC.

Based on various reports of the effects of androgens on pregnancy outcomes and our findings suggesting beneficial effects of preconception COC with androgen-lowering therapy, we believe that preconception administration of standardized androgen-lowering therapy should be a standard of care for patients with PCOS.

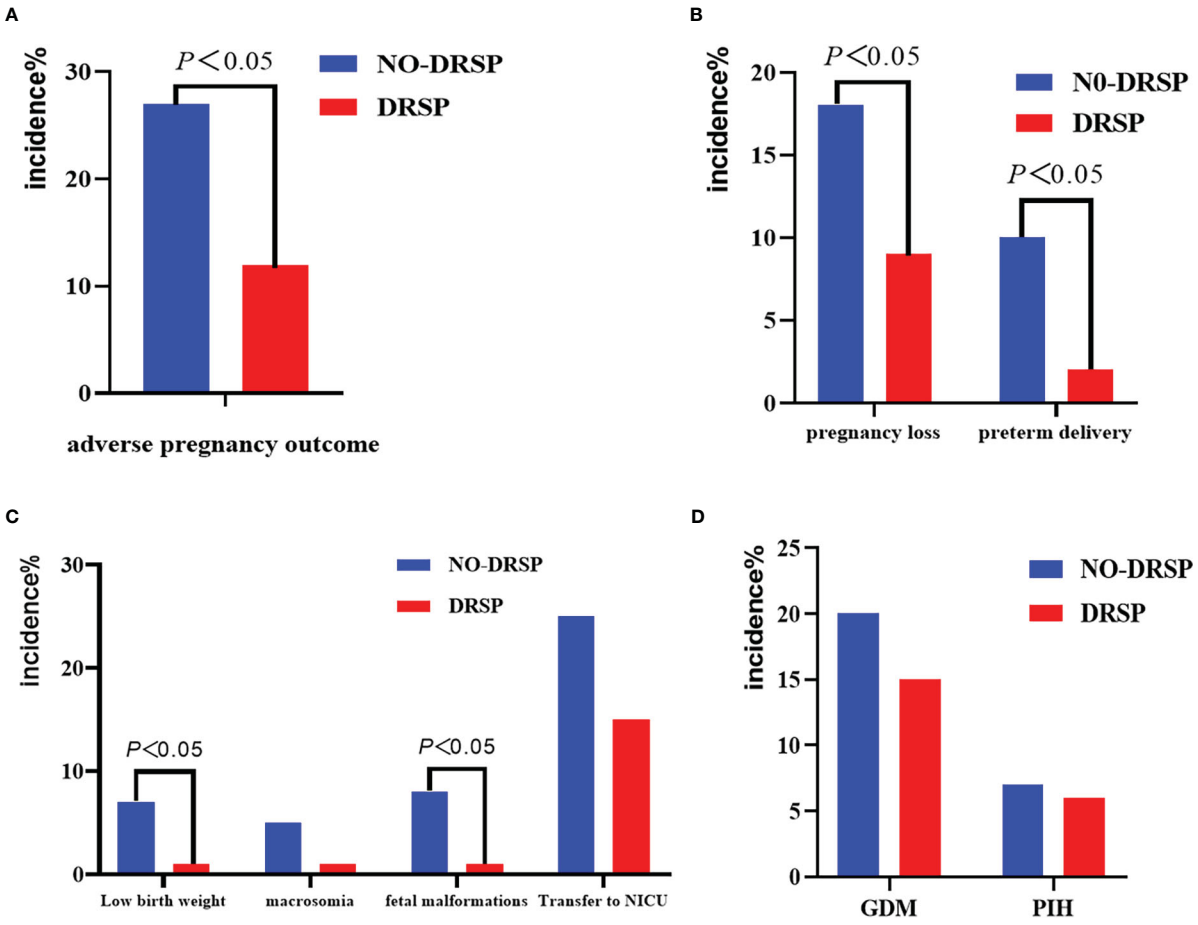

FIGURE 2
Primary and secondary outcomes in patients with polycystic ovary syndrome (PCOS).

TABLE 3 Comparison of total adverse pregnancy outcomes between the two groups in different subgroups (*vs.* DRSP).

	N	*DRSP n (%)*	NO-DRSP *n (%)*	*Difference (95% CI, %)*	*P value*	*Crude RR (95% CI)*	*P value*	*Adjusted RR (95% CI)*	*P value*
N	296	148	148						
Age									
<30 years	212	15(13.04)	27(27.84)	14.79(3.95~25.63)	0.007	2.13(1.14~4.01)	0.019	2.22(1.17~4.20)	0.015[a]
≥30 years	84	3(9.09)	13(25.49)	16.40(0.93~31.87)	0.062	2.80(0.80~9.84)	0.108	3.01(0.84~10.84)	0.091[a]
Parity									
primipara	240	14(11.86)	27(22.13)	10.27(0.87~19.66)	0.035	1.87(0.98~3.56)	0.058	1.97(1.02~3.81)	0.044[b]
multipara	56	4(13.33)	13(50.00)	36.67(13.92~59.41)	0.003	3.75(1.22~11.50)	0.021	3.70(1.16~11.78)	0.027[b]
BMI									
<25	240	16(13.68)	34(27.64)	13.97(3.91~24.03)	0.008	2.02(1.12~3.66)	0.020	2.12(1.17~3.87)	0.014[c]
≥25	56	2(6.45)	6(24.00)	17.55(-1.29~36.39)	0.062	3.72(0.75~18.43)	0.108	5.78(1.10~30.43)	0.038[c]
HOMA-IR									
<2.69	205	12(13.04)	29(25.66)	12.62(2.03~23.21)	0.025	1.97(1.00~3.86)	0.049	2.23(1.11~4.48)	0.025[d]
≥2.69	91	6(10.71)	11(31.43)	20.71(3.33~38.10)	0.014	2.93(1.08~7.93)	0.034	2.68(0.97~7.38)	0.057[d]

[a]Calibration for gestational age, BMI, HOMA-IR index;
[b]Corrected for age, BMI, HOMA-IR index;
[c]Corrected age, gestational age, HOMA-IR index;
[d]Corrected for age, gestational age, BMI.

Strengths and limitations

A strength of our study is that it is the first prospective cohort study that examines the effect of preconceptional hypoandrogenic therapy on pregnancy outcomes in patients with PCOS. Our study not only followed subjects until the conclusion of their pregnancies and focused on the effect of preconceptional androgen lowering therapy on pregnancy outcome in PCOS patients, but also further stratified analysis and corrected for other influencing factors that may affect adverse pregnancy outcomes. Our study can thus provide evidence-based medical guidance on whether to treat PCOS patients with preconception androgen-lowering therapy with COC, and provide clinicians with possible treatment strategies to reduce adverse pregnancy outcomes in PCOS patients.

However, our study does have some limitations. First, our study was a single-center prospective study. Further multi-center randomized controlled trial validation studies are needed in the future. Furthermore, our study had a short follow-up period for PCOS offspring and only assessed the effect of preconceptional hypoandrogenic treatment with COC drugs on congenital malformations in offspring; it could not clarify the effect of drugs on offspring growth or reproductive health. Some findings were inconsistent with the results after correcting for possible interactions, and the sample size should be expanded for future validation.

Conclusions

Our study confirms that preconception anti-androgen therapy in patients with polycystic ovary syndrome is effective in reducing both the incidence of adverse pregnancy outcomes as well as the incidence of neonatal complications in the offspring of patients with PCOS. Based on the results of our study, which was a prospective cohort study with strict case-selection criteria, we conclude that preconception androgen-lowering therapy with COC medications in patients with PCOS would be beneficial for pregnancy outcomes and offspring outcomes, thus supporting the standardization of preconception androgen-lowering therapy as a treatment for patients with PCOS.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Guangdong Women and Children's Hospital (NO. 202101273). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Concept and design: All authors. Acquisition, analysis, and interpretation of data: LL, XZ, HM, JZ, YC, YO, YS, XP, YL. Drafting of the manuscript: XZ, HM. Statistical analysis: XZ, HM. Securement of funding: LL. Administrative, technical, and material support: LL. Supervision: LL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1109861/full#supplementary-material

SUPPLEMENTARY TABLE 1
The occurrence of congenital malformations in the offspring of both groups.

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Analysis of the volatile organic compounds of epidural analgesia-ameliorated metabolic disorder in pregnant women with gestational diabetes mellitus based on untargeted metabolomics

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Gestational diabetes mellitus (GDM) is a metabolic disease with an increasing annual incidence. Our previous observational study found that pregnant women with gestational diabetes had mild cognitive decline, which may be related to methylglyoxal (MGO). This study aimed to investigate whether labor pain aggravates the increase in MGO and explored the protective effect of epidural analgesia on metabolism in pregnant women with GDM based on solid-phase microextraction gas chromatography/mass spectrometry (SPME/GC-MS). Pregnant women with GDM were divided into a natural birth group (ND group, n = 30) and epidural analgesia group (PD group, n = 30). After fasting for ≥ 10 h overnight, venous blood samples were collected pre- and post-delivery to detect MGO, interleukin-6 (IL-6), and 8-epi-prostaglandin F2 alpha (8-iso-PGF2α) by ELISA. Serum samples were analyzed for volatile organic compounds (VOCs) using SPME-GC-MS. MGO, IL-6, and 8-iso-PGF2α levels in the ND group increased significantly post-delivery ($P < 0.05$) and were significantly higher in this group than the levels in the PD group ($P < 0.05$). Compared to the PD group, VOCs in the ND group increased significantly post-delivery. Further results indicated that propionic acid may be associated with metabolic disorders in pregnant women with GDM. Epidural analgesia can effectively improve the metabolism and immune function in pregnant women with GDM.

KEYWORDS

gestational diabetes mellitus, volatile organic compounds, humoral biomarkers, epidural analgesia, solid-phase microextraction gas chromatography/mass spectrometry

1 Introduction

With the implementation of the three-child policy in China, pregnancy complications have gradually increased. Gestational diabetes mellitus (GDM) is the most common complication (1). GDM can lead to maternal metabolic disorders and adversely affect the health of the mother and fetus (2, 3). In pregnant women with GDM labor pain stimulation during the perinatal period further aggravates metabolic disorders. Metabolomics is the study of biological systems, more specifically on investigating the changes in low molecular metabolites in the body after stimulation (4, 5). It mainly reflects the changes in endogenous metabolites caused by pathophysiological stimulation and disturbance. Metabolomics has great advantages in biomarker discovery, early disease diagnosis, pathogenesis research, and pharmacological and pharmacodynamic evaluations (6–12).

Childbirth is a physiological process that is accompanied by pain. labor pain is an unpleasant feeling due to the contraction of the uterus and transit of the fetus. Approximately 50% of the pregnant women regard labor pain as unbearable, which can instill fear (13). Labor pain can also be detrimental to the health of the child and the mother. Adverse effects on pregnant women and fetus include prolonged childbirth, uterine weakness, postpartum hemorrhage, fetal distress, and others (14, 15). labor pain can also lead to neuroendocrine changes in pregnant women.

Ding et al. (16) showed that the incidence of postpartum depression was as high as 34.6%, while epidural analgesia reduced the incidence of postpartum depression to 14.0%. The findings highlight the need for an analgesic method during delivery that does not affect the normal childbirth process, but which relieves pain. This innovation would be valuable for the physical and mental health of the mothers. An epidural analgesia technology is needed, especially for pregnant women with GDM. We have previously reported that GDM may cause memory loss in pregnant women (17, 18). labor pain may aggravate various GDM complications, including memory loss. This study investigated whether epidural analgesia technology in delivery can improve metabolism and serum methylglyoxal (MGO) and inflammatory, and oxidative stress factors in pregnant women with GDM.

2 Materials and methods

2.1 Subjects and protocol

Patients aged 18–35 years with American Society of Anesthesiologists physical status I-II were admitted to the study. Sixty pregnant women with GDM were selected and divided into either a natural delivery group (ND group, n = 30) or epidural analgesia group (painless delivery [PD] group, n = 30), according to whether the patient chooses labor analgesia. The study protocol was approved by the Ethics Committee of First Affiliated Hospital of Harbin Medical University and registered with the Chinese Clinical Trial Registry (registration number: ChiCTR2000038703).

Patients with pre-gestational type 1 or type 2 diabetes mellitus (T1DM and T2DM, respectively) were not included in the study. Patients with unnatural pregnancy or gestational periods <37 weeks or >41 weeks were excluded. Subjects taking medications, including corticosteroids, antidepressants, or antiepileptics, were also excluded. Additionally, subjects with chronic metabolic, endocrine, inflammatory diseases, cancer, drug or alcohol dependency, history of major brain abnormalities (e.g., tumors and hydrocephaly), epilepsy, or Parkinson's disease were excluded. The psychological status of the pregnant women was assessed using the Hamilton Depression Scale. Those who scored > 7 and those who may have depression were excluded.

After the informed consent of the pregnant women, first of all, the pregnant women and their fetuses were monitored for oxygen inhalation and routine monitoring. At the beginning of the first stage of labor, when the uterine contraction is regular and the uterine orifice is opened to 2~3cm, let the pregnant woman take a lateral lying position, try to lower her head and hold her knees with both hands, conduct epidural puncture at the waist L2-L3 space, slowly inject the needle, place a tube 3cm to the head after the puncture is successful, and connect the syringe to draw back the bloodless and brain free spinal fluid, then inject 3 ml of 1.5% lidocaine into the epidural cavity. The epidural catheter was excluded from entering the blood vessel or subarachnoid space by mistake. When the vital signs of pregnant women are stable and there is no abnormality in the anesthesia level, the loaded dose of drug is 6 ml by epidural injection, and the drug is 0.1% ropivacaine+0.5 μg/mL sufentanil. Continuously observe the vital signs of pregnant women, and measure and maintain the anesthesia level at the T10 level. The continuous dose of the epidural link self-control analgesia pump is 4-6 mL/h, and the self-control dose is 2 ml. The administration will be stopped after the uterine orifice of the parturient is completely opened. During delivery, the two groups of pregnant women can drink sugar water and honey water to provide energy supply for pregnant women.

On the survey date, all the enrolled patients underwent routine medical history inquiries, physical examinations, and provided samples for laboratory measurements. The clinical research coordinators used a standard questionnaire to collect information on demographic characteristics and medical history. All pregnant women were instructed to maintain their usual physical activity and diet for at least three days before the survey. In order to evaluate the onset of analgesia, mothers' pain was estimated using the Visual Analogue Score (VAS, 0: no pain 10: the worst pain) at analgesia request and at 20 minutes after administration of the initial bolus. After overnight fasting for ≥10 h, venous blood samples were collected to detect levels of glycosylated hemoglobin (HbA1c) and blood glucose (Glu). For each participant, blood was collected (3 mL) and centrifuged. The serum was recovered. Post-delivery, visual analog scale (VAS) scores were determined and the venous blood of each woman was collected and treated as just described. Blood samples were stored in a -80°C deep cryogenic refrigerator. MGO, interleukin-6 (IL-6), and 8-epi-prostaglandin F2 alpha (8-iso-pgf2α) were detected using ELISA. All measurements were performed within 6 months of sample collection.

2.2 Solid-phase microextraction

Post-delivery, the venous blood of the two groups was analyzed using SPME. In this study, we selected a 75 μM extraction head. The coating material was carbon molecular sieve/polydimethylsiloxane (car/PDMS). An automatic sample injector was used for heating and extraction. The liquid sample bottle was accessed *via* a puncture. An extraction method for headspace extraction was adopted. The SPME fiber was inserted into evacuated 20ml glass vials and exposed to the headspace of a blood sample (blood: 2 ml, taken from the ulnar vein) for 20 min at 40°C. After the extraction and concentration of the samples, the automatic sampling device inserted the extraction head into the gas chromatography-mass spectrometry (GC-MS) injection port for analysis. The desorption of volatiles occurred in a hot GC injector at 200°C for 2 min.

2.3 GC-MS analysis

All analyses were performed on a model QP2010 GC/MS (Shimadzu) equipped with a DB-5MS porous layer open tubular column (length: 30 m; internal diameter: 0.250 μm; film thickness, 0.25 mm: Agilent Technologies). The injections were performed in splitless mode, with a splitless time of 1 min. The injector temperature was set to 200°C and the carrier gas was helium at a flow rate of 2 mL/min. The temperature in the column was maintained at 40°C for 2 min to condense the hydrocarbons. The temperature was then increased to 200°C at 70°C/min and held for 1 min. Subsequently, the temperature was ramped to 230°C at a rate of 20°C/min and maintained for 3 min. MS analyses were performed in full-scan mode with an associated m/z range of 35–200 amu. An ionization energy of 70 eV was used for each measurement, and the ion source maintained at 200°C.

2.4 Statistical analyses

SPSS19.0 software was used for the statistical analyses. All data were tested for normality and variance. Normally distributed data are expressed as mean ± standard deviation (mean ± SD). Continuous variables with normal distribution were compared using Student's t-test. Variables with abnormal distribution were compared using the Mann-Whitney U test. The least significant difference method was used to make multiple comparisons among the groups. Categorical data are expressed as counts and percentages, and comparisons between groups were performed by bilateral χ 2 inspection. $P < 0.05$ indicated statistical significance.

SIMCA-p +11.5 software was used for multivariate data analysis and model establishment. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were used for statistical analysis. At the same time, the PLS-DA sample distribution score map was used to divide different samples into different clusters. The default seven-round cross-validation method was used, and the value of the variable importance in projection (VIP) of relevant variables in the PLS-DA model was calculated. Two hundred iterative permutation tests were conducted to verify the supervision mode and to prevent overfitting of the PLS-DA model. The nonparametric Kruskal–Wallis rank sum test was used to calculate the *P*-value. When VIP > 1.0, and $P < 0.05$, the difference variable was statistically significant (i.e., the metabolite was a significantly different metabolite).

3 Results

3.1 Demographic information

There were no significant differences in body weight, blood glucose level, or HbA1c level between the two groups ($P > 0.05$) (Table 1). The VAS score of the PD group was significantly better than that of the ND group ($P < 0.05$) (Table 1).

3.2 Level of serum indicators

Pre-delivery, the levels of MGO, IL-6, and 8-iso-pgf2α in the ND group were not significantly higher than the levels in the PD group ($P > 0.05$). Compared to pre-delivery, the levels of MGO, IL-6, and 8-iso-pgf2α in the PD group post-delivery showed an upward trend, but the difference was not statistically significant. However, post-delivery, the levels of MGO, IL-6, and 8-iso-pgf2α in the ND group significantly increased ($P < 0.05$). These levels in the ND group post-delivery were also significantly higher than those in the PD group ($P < 0.05$) (Table 2).

3.3 Metabolic volatile organic compounds

Compared to the PD group, the metabolic VOCs in the ND group increased significantly post-delivery. In this study, we found 17 different metabolites (Table 3). Of these, 11 were in the ND group

TABLE 1 Demographic characteristics.

	PD	ND	F	*P*
Sample	30	30		
Age, years	30.16 ± 3.27	30.79 ± 4.16	0.65	0.52
Height, cm	165.32 ± 2.89	164.91 ± 2.51	0.59	0.60
Weight, kg	79.36 ± 11.58	78.35 ± 13.57	0.31	0.76
Glucose, mmol/L HbA1c, % VAS	4.90 ± 1.32 5.31 ± 0.61 3.1 ± 0.7	5.39 ± 0.71 4.99 ± 0.93 7.8 ± 2.0	1.79 1.78 6.215	0.07 0.08 0.05

Vas, Visual Analogue Scale/Score; ND, Natural Delivery Group; PD, Epidural Analgesia Group; HbA1c, Glycosylated Hemoglobin.

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TABLE 2 Comparison of prenatal and postpartum serum MGO, IL-6, and 8-iso-PGF2α levels between the PD and ND groups.

Group	n	MGO		*P*
		PD	ND	
Pernatal	30	46.15 ± 8.27	45.34 ± 7.79	0.69
Postpartum	30	67.34 ± 18.81	95.35 ± 18.27	<0.001
P		<0.001	<0.001	
Group	n	IL-6		
		PD	ND	*P*
Pernatal	30	28.31 ± 4.26	26.86 ± 4.01	0.18
Postpartum	30	35.51 ± 4.21	44.17 ± 5.51	<0.001
P		<0.001	<0.001	
Group	n	8-iso-PGF2α		
		PD	ND	*P*
Pernatal	30	47.13 ± 8.26	48.19 ± 7.97	0.61
Postpartum	30	61.24 ± 7.22	81.15 ± 6.98	<0.001
P		<0.001	<0.001	

ND, Natural Delivery Group; PD, Epidural Analgesia Group; MGO, methylglyoxal; IL-6, interleukin-6; 8-iso-pgf2α, 8-epi-prostaglandin F2 alpha.

and six were in the PD group. In the two-dimensional PCA score diagram, data from the two groups showed a good separation trend (Figure 1A). When using a single prediction component and three orthogonal components, the PLS-DA score chart displayed the data of the two groups and also had a good separation effect (Figures 1B, C). Two hundred iterations were performed to test the supervision model. The R2 and Q2 values calculated from the converted data were lower than their original verification values, which proved the effectiveness of the supervision model (Figure 1D). Based on the heat map (Figure 1E), the expression of six types of VOCs increased in the PD group as indicated by the red color. Eleven types of VOCs were increased in the ND group.

TABLE 3 Differential metabolites in blood VOCs of pregnant women with GDM.

VOCs	VIP	*P*	RT	FC (PD/ND)
Alpha-L-galactopyranose, 6-deoxy-, cyclic 1,2:3,4-bis(butylboronate)	1.33661	0.0000	10.69167	-0.191668188
1-Phenyl-1-(trimethylsilyloxy)ethylene	1.26562	0.0000	7.816667	-0.861086889
Decane, 4-methyl	1.40738	0.0000	8.991667	0.634477509
Malonic acid, bis(2-trimethylsilylethyl ester	1.29241	0.0000	18.10833	-0.953999982
Propanoic acid, 2-methyl-, 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester	1.31679	0.0000	15.91488	-1.001862334
Trans-beta-ocimene	1.07303	0.0003	6.916667	-0.424311265
Trans-2-dodecen-1-ol	1.12905	0.0000	12.3649	-1.13140546
1-Diisopropylsilyloxycyclohexane	1.13159	0.0000	13.10833	-0.483747132
Cycloheptane	1.24853	0.0000	6.3	-0.59981966
Decane, 2,5,6-trimethyl-	1.28815	0.0000	8.777957	0.709318109
Decane, 3,7-dimethyl-	1.42028	0.0000	9.725	0.561697272
Heptane, 2,4-dimethyl-	1.15657	0.0000	4.45	0.521234226
Heptane, 3,4-dimethyl-	1.08788	0.0000	3.491667	0.478836917
Hexanal	1.09592	0.0000	4.375	-0.829923822
Hexane, 2,3,4-trimethyl-	1.04946	0.0000	5.484654	0.504141371
Octanal	1.30959	0.0000	4.422312	-1.054138731

VIP, Variable importance in the projection; FC, Fold change; VOCs, volatile organic compounds.

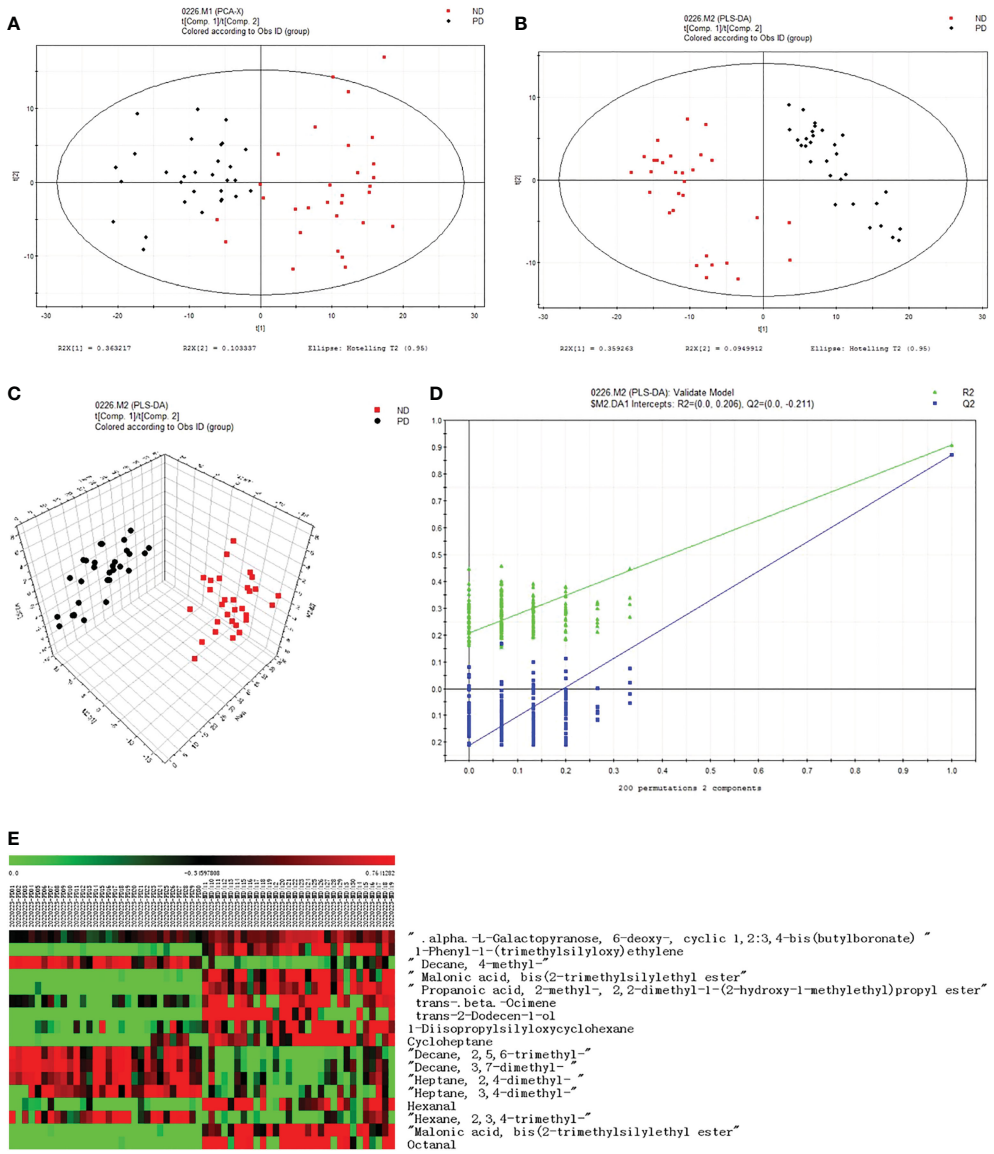

FIGURE 1
VOCs analysis chart. **(A)** PCA score plot, (R2X (1)=0.363217; R2X (2)=0.103337). The confidence level was the default of the software SIMCA-P 11.5, and its threshold was 95%. **(B)** PLSDA score plot, (R2X (1)=0.359263; R2X (2)=0.0949912). **(C)** PLS-DA score plot. **(D)** PLS-DA validation plot intercepts, R2=(0.0,0.206), Q2=(0.0,-0.211). **(E)** Heat map of two groups of different substances.

4 Discussion

Severe labor pain makes pregnant women vulnerable to fear and anxiety, and heightens their stress. These feelings can be exacerbated in pregnant women who develop GDM. These stressors have several adverse effects on the mother and fetus. During delivery, the body is in a state of stress due to stimulations that include pain and childbirth trauma (19). The immune function of the body is disrupted, which increases the risk of maternal infection and other complications. In recent years, epidural analgesia has been used in many patients. An increasing number of pregnant women and obstetricians are accepting epidural analgesia, which has an obvious effect on reducing labor pain.

Under physiological conditions, a variety of enzymes participate in the metabolism of MGO. A small amount of MGO in the body is not sufficient to cause toxic reactions. However, metabolic disorders in patients with GDM lead to an increase in MGO production. Simultaneously, the strong stress response caused by delivery leads to an increase in levels of reactive oxygen species, which stimulate the production of MGO (20). In a previous study, we found that pregnant women with GDM were in a state of metabolic disorder (21). In the process of delivery, this metabolic disorder is aggravated because the delivery process itself can be considerably traumatic. In this study, the results are consistent with this phenomenon. The level of MGO in both groups increased post-delivery. However, compared with the PD group, the level of MGO in the ND group increased significantly, indicating that epidural analgesia can ameliorate metabolic disorders of MGO caused by labor pain.

Under physiological conditions, immune changes produced by delivery have protective significance for the body (22). However, abnormal immune function disorders are closely related to perinatal

diseases. Studies have shown that labor pain is the main factor that causes changes in immune function (23–25). Epidural analgesia can effectively protect the body from excessive stress and inhibit immune function. IL-6 and 8-iso-pgf2α were also detected in this study. Compared with the PD group, the levels of IL-6 and 8-iso-pgf2α in the ND group post-delivery increased significantly, indicating that epidural analgesia can reduce the inflammatory and oxidative stress response of pregnant women with GDM.

This study mainly observed the effects of different delivery methods (ND and PD) on the metabolism of pregnant women with GDM. GC-MS was used in a novel analysis of the changes in VOCs in pregnant women with GDM post-delivery. In the two groups, we found 15 kinds of VOCs, Including 10 VOCs in the ND group and 5 VOCs in the PD group. The results revealed obvious metabolic disorders in the ND group. Ten differential substances were found in the ND group, including alpha-l-galactopyranose; 6-deoxy-, cyclic 1,2:3,4-bis(butylboronate); 1-phenyl-1-(trimethylsilyloxy)ethylene; malonic acid; bis(2-trimethylsilylethyl ester); propanoic acid; trans-beta-ocimene; trans-2-dodecen-1-ol; 1-diisopropylsilyloxycyclohexane; cycloheptane; hexanal; octanal。

Among the different substances in the ND group, alcohols (trans-2-dodecen-1-ol) and aldehydes (hexanal and octanal) were identified. Active aldehydes are mainly produced during lipid and glucose metabolism (including enzymatic and non-enzymatic pathways). The enzyme pathway is usually an aldehyde intermediate or by-product produced during glucose and lipid metabolism *in vivo* (26). This is also consistent with the disorder of active aldehyde metabolism observed in pregnant women with GDM. Under pathological conditions, aldehyde metabolism is disordered, resulting in abundant accumulation of aldehyde and formation of an aldehyde microenvironment (27). Similar to saturated aldehydes, hexanal is oxidized to the corresponding caboxylic acid by aldehyde dehydrogenase mainly in the liver, but also in other tissues and cells. The acid can serve as a substrate for the Krebs cycle or is excreted as a salt. Alternatively, it can conjugate with glutathione or the sulfhydryl group of other proteins. Free radical induced lipid peroxidation may play a role in neurodegeneration and peroxidation leads to the formation of hexanal from omega-6 fatty acids. We have previously demonstrated *in vitro* that pyruvate dehydrogenase (PDH) catalyzes the condensation of saturated aldehydes with pyruvate to form acyloins. We have further shown in perfused rat heart that hexanal, presumably *via* PDH, is converted to 3-hydroxyoctan-2-one and that it in turn can be reduced to 2,3-octanediol (28).

Aldehyde metabolism disorders are involved in the occurrence and development of various diseases. α-l-Galactopyranose is a carbohydrate metabolized by hexose (glucose) and cyclic sugar. L-Galactose, also known as α-L-galactose or L-galactopyranose, belongs to the class of organic compounds known as hexoses. These are monosaccharides in which the sugar unit is a is a six-carbon containing moeity. L-Galactose is a primary metabolite. Primary metabolites are metabolically or physiologically essential metabolites. They are directly involved in an organism's growth, development or reproduction. Based on a literature review very few articles have been published on L-Galactose. L-Galactose can be metabolized into vitamins.This may be related to the supplementary food provided to pregnant women in the ND group during delivery.

Propionic acid was identified as an important component in this study. Three different biochemical pathways for propionic acid production are succinic acid, acrylate, and propylene glycol. Propionic acid is the main end-product of succinic acid fermentation. The abundance of Bacteroides and Parabacteroides, the main producers of succinic acid, increased due to a high-fat diet and were positively correlated with body weight. Succinic acid produced by *Bacteroides thetaiotaomicron* supports the growth of *Phascolarctobacterium* and the accompanying production of propionic acid through the succinic acid pathway (29). High concentrations of *Phascolarctobacterium* have been reported in severe depression, Alzheimer's disease, autism, and other diseases, although the heterogeneity within the disease group was also high. Therefore, the increase in propionic acid was related to the tricarboxylic acid cycle, and the metabolism of propionic acid was also related to MGO (Figure 2). A previous study described a metabolic pathway in athletes after marathon competition. *Veillonella* metabolizes exercise-induced lactic acid into propionic acid, which improves running time, determines a natural and microbial-encoded enzyme process, and improves sports performance (30). The delivery process is also relatively long, lasting for several hours or even longer, and the intensity is high. In pregnant women with GDM, increased glycolysis produces more pyruvate that is metabolized to lactic acid. Lactic acid is likely to be metabolized into propionic acid in the body. According to this reasoning, pyruvate can also be converted to acetone to produce MGO and aggravate nerve injury. Therefore, propionic acid may be the product of metabolic disorders in the ND group, or it may be a potential marker of cognitive dysfunction in pregnant women with GDM aggravated by labor pain. Epidural analgesia can prevent this type of injury.Malonic acid is also a kind of propionate, which may have the same metabolic pathway as propionic acid (31).

Six substances were found in the PD group. These included decane, heptane, and other alkanes. Alkanes were also observed in the ND group. Lipid peroxidation is a pathophysiological change in various diseases, including cancer, inflammatory diseases, atherosclerosis, and aging (32). Alkane metabolism is unrelated to branched-chain hydrocarbons and is the product of lipid peroxidation. Unbound alkane hydrocarbons eventually appear in the blood, urine, and exhalate. Ethane and pentane are saturated hydrocarbons produced by lipid peroxidation chain reactions (33). Therefore, these aliphatic hydrocarbons are considered biomarkers of lipid peroxidation both *in vivo* and *in vitro* (34). This indicates that pregnant women with GDM may have an oxidative stress reaction.

This study found that both groups of pregnant women had a certain degree of stress response, but the metabolic disorder of pregnant women in the ND group was more obvious, indicating that labor pain caused an increase in oxidative stress and metabolic disorders in pregnant women with GDM. In contrast, epidural analgesia can significantly improve oxidative stress metabolites in pregnant women with GDM. This is also one of the main reasons for the changes in metabolites between the two groups.

Severe labor pain activates the hypothalamus pituitary adrenal axis and sympathetic adrenal medullary axis, causing a series of neuroendocrine disorders, which also promote the secretion of glucocorticoids and catecholamines. This stress also affects the body's immune system, thus activating inflammatory factors and oxidative stress responses, including an increase in MGO. Neuroendocrine dysfunction is one of the pathophysiological mechanisms of GDM. In the process of delivery, epidural analgesia reduces the neuroendocrine imbalance in pregnant women with GDM, adjusts the immune response, and reduces the damage caused by stress trauma to the body.

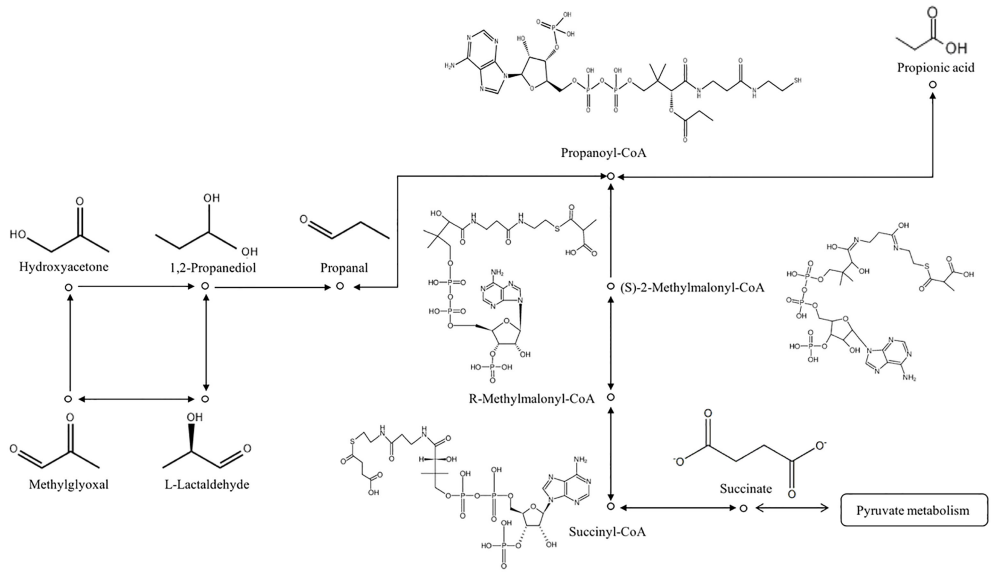

FIGURE 2
Metabolic pathway of propionic acid.

There were several limitations in this study. The sample size was relatively small. Postpartum follow-up was not performed. Finally, the duration of labor was not considered. It is reported that decane, heptane, and other alkanes are found in patients with temporary respiratory syndrome (35). Because GDM pregnant women are relatively obese, and sleep disorders in the late pregnancy may also be one of the reasons for these substances. However, these substances may also be part of the experimental reagent (such as the kit), so the kit can also release these substances during the research process, which needs further research.

5 Conclusion

The data demonstrate for the first time that propionic acid, a volatile substance, may be a potential marker of cognitive dysfunction in pregnant women with GDM aggravated by labor pain. Epidural analgesia can improve the expression of MGO/inflammatory/oxidative stress factors induced by childbirth-related pain.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the First Affiliated Hospital of Harbin Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SS designed the study, collected data, and wrote and revised the manuscript; YL and GC interpreted and analyzed the data; LG collected the data; EL designed 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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Cord blood myostatin concentrations by gestational diabetes mellitus and fetal sex

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Introduction: Myostatin is a member of the transforming growth factor β superfamily, and is mainly secreted from skeletal muscle. Animal studies have demonstrated that deficiency in myostatin promotes muscle growth and protects against insulin resistance. In humans, gestational diabetes mellitus (GDM) affects fetal insulin sensitivity. Females are more insulin resistant and weigh less than males at birth. We sought to assess whether cord blood myostatin concentrations vary by GDM and fetal sex, and the associations with fetal growth factors.

Methods: In a study of 44 GDM and 66 euglycemic mother-newborn dyads, myostatin, insulin, proinsulin, insulin-like growth factor (IGF)-1, IGF-2 and testosterone were measured in cord blood samples.

Results: Cord blood myostatin concentrations were similar in GDM *vs*. euglycemic pregnancies (mean ± SD: 5.5 ± 1.4 *vs*. 5.8 ± 1.4 ng/mL, P=0.28), and were higher in males *vs*. females (6.1 ± 1.6 *vs*. 5.3 ± 1.0 ng/mL, P=0.006). Adjusting for gestational age, myostatin was negatively correlated with IGF-2 (r=-0.23, P=0.02), but not correlated with IGF-1 (P=0.60) or birth weight (P=0.23). Myostatin was strongly correlated with testosterone in males (r=0.56, P<0.001), but not in females (r=-0.08, P=0.58) (test for difference in r, P<0.001). Testosterone concentrations were higher in males *vs*. females (9.5 ± 6.4 *vs*. 7.1 ± 4.0 nmol/L, P=0.017), and could explain 30.0% (P=0.039) of sex differences in myostatin concentrations.

Discussion: The study is the first to demonstrate that GDM does not impact cord blood myostatin concentration, but fetal sex does. The higher myostatin concentrations in males appear to be partly mediated by higher testosterone concentrations. These findings shed novel insight on developmental sex differences in insulin sensitivity regulation relevant molecules.

KEYWORDS

gestational diabetes mellitus, myostatin, testosterone, insulin-like growth factor, sex difference

Introduction

Myostatin or growth differentiation factor 8 is a member of the transforming growth factor β superfamily, and is mainly secreted from skeletal muscle (1). Myostatin is a strong negative regulator of skeletal muscle growth (1, 2), while inhibition of myostatin or its signaling prevents fat accumulation and improves insulin sensitivity in mice (3–8). In humans, elevated myostatin levels in skeletal muscle or circulation have been associated with obesity and insulin resistance in adults (9–12). So far, there have been no studies on whether gestational diabetes mellitus (GDM) - a common pregnancy complication (13) that has been associated with impaired fetal insulin sensitivity but enhanced fetal growth (14), may affect cord blood myostatin concentration. Little is known about whether myostatin is associated with fetal growth or fetal growth factors.

Females weigh less than males at birth, despite higher concentrations of major fetal growth factors (insulin and IGF-1), suggesting that females are intrinsically more insulin resistant than males *in utero* (15–17). Given sex differences in fetal growth and insulin sensitivity, we hypothesized that myostatin may exhibit sex differences and correlate with fetal growth. We are aware of only one small study (n=83) on cord blood myostatin which reported no association with fetal sex, and a negative correlation with birth weight (18).

Testosterone promotes protein synthesis, skeletal and muscle growth (19, 20). Higher cord blood testosterone concentrations have been reported in males *vs.* females (21). Decreased testosterone concentrations have been associated with insulin resistance and type 2 diabetes in men (22). Testosterone treatment has been associated with increased myostatin concentrations in men (23). We thus hypothesized that testosterone may mediate any potential sex difference in fetal myostatin concentration.

In view of the above discussed knowledge gaps, we sought to assess whether cord blood myostatin concentrations are affected by GDM and fetal sex, the associations with fetal growth and fetal growth factors, and the role of testosterone in mediating potential sex difference in cord blood myostatin.

Methods

Study design, participants and specimens

We recruited pregnant women at the last prenatal visit before delivery at Mount Sinai Hospital in Toronto between January 2019 and February 2020. Participants met the following inclusion criteria: (1) maternal age 20-45 years; (2) single term pregnancy (gestational age ≥37 weeks); (3) Caucasian or Asian (to limit the potential confounding effects of ethnicity). Exclusion criteria were: (1) *in-vitro* fertilization; (2) any known birth defects or congenital anomalies; (3) family history of type 1 diabetes; (4) any major pre-pregnancy illnesses (e.g., type 1 or 2 or unknown diabetes, hypertension); (5) preeclampsia or other severe pregnancy complications. The study was approved by the Research Ethics Board of Mount Sinai Hospital (approval number: 18-0224-E). Written informed consent was obtained from all study participants.

All pregnant women underwent a 1-hour 50 g oral glucose challenge test between 24 and 28 weeks of gestation, and those who failed the test (1-h plasma glucose ≥7.8 mmol/L) were required to undertake a 75 g 2-h oral glucose tolerance test. GDM was diagnosed if any plasma glucose value was abnormal (fasting ≥5.3 mmol/L, 1-h ≥10.6 mmol/L, 2-h ≥9.0 mmol/L) according to the Canadian Diabetes Association's diagnostic criteria (24). Mothers who had normal values in the 50 g oral glucose challenge test were considered to be euglycemic.

A total of 44 GDM and 66 euglycemic women were recruited. Data on sociodemographic characteristics and clinical risk factors were obtained by in-person interviews and reviews of medical charts using a standardized questionnaire by a trained research staff. Cord blood samples were collected by a trained research staff in the Research Centre for Women's and Infants' Health BioBank at Mount Sinai Hospital immediately following birth, kept on ice, and centrifuged within 2 hours after the specimen collection. Serum (without anti-coagulant) and EDTA plasma samples were stored in multiple aliquots at -80°C until assays.

Biochemical assays

In our research lab (Dr Lye), cord plasma myostatin (pg/mL) was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Cat# DGDF80, R & D Systems, Minneapolis, USA). Cord plasma IGF-2 (ng/mL) was measured by an ELISA kit (Cat# 22-IG2HU-E01, ALPCO Diagnostics, Salem, USA). Plasma proinsulin (pmol/L) was measured by an ELISA kit (Cat# 80-PINHUT-CH01, ALPCO Diagnostics, Salem, USA). In the clinical biochemistry laboratory of Mount Sinai Hospital, cord serum glucose (in mmol/L, 1 mmol/L=18 mg/dL) was determined by an enzymatic (hexokinase) method (Roche Diagnostics, Switzerland), serum insulin (in μU/mL, 1 μU/mL=6 pmol/L) and C-peptide (in ng/mL, 1 ng/mL= 333 pmol/L) were determined by chemiluminescence immunoassays (Roche Diagnostics, Switzerland), serum IGF-1 (ng/mL) was determined by a chemiluminescent immunoassay (Liaison XL, DiaSorin, Italy). Cord serum testosterone (nmol/L) was measured by a chemiluminescent immunoassay, and cross reactivity was 18% with 11-β-hydroxy-testosterone, less than 0.16% with estradiol and progesterone, and less than 6% for other testosterone-like hormones according to the manufacturer's instructions (Roche Diagnostics, Switzerland). The limits of detection were 0.922 pg/mL for myostatin, 0.11 mmol/L for glucose, 0.2 mU/L for insulin, 0.01 ng/mL for C-peptide, 0.455 pg/mL for proinsulin, 10 ng/mL for IGF-1, 0.02 ng/mL for IGF-2, and 0.087 nmol/L for testosterone, respectively. Intra- and inter-assay coefficients of variation were in the ranges of 1.8-6.0% for myostatin, 0.5-1.3% for glucose, 0.8-4.9% for insulin, 0.5-2.3% for C-peptide, 4.0-9.7% for proinsulin, 2.37-8.5% for IGF-1, 3.1-7.2% for IGF-2, and 2.1-18.1% for testosterone, respectively. All biomarkers were assayed in duplicates, and the average values were taken. The assay technicians were blinded to the clinical characteristics of study subjects.

Abbreviations: BMI, body mass index; GDM, Gestational diabetes mellitus; IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2.

Statistical analysis

The primary outcome was cord blood myostatin concentration. Birth weight z score was calculated according to the Canadian sex- and gestational age-specific birth weight standards (25). Mean ±standard deviation (SD) or median (interquartile range) were presented for continuous variables. Frequency (percentage) was presented for categorical variables. Student's t tests were conducted to compare continuous variables, and Chi-square tests or Fisher's exact tests (where appropriate) were conducted to compare categorical variables between two groups. Pearson correlation coefficients were calculated to examine the correlations of cord blood myostatin with testosterone, fetal growth (birth weight z score) and fetal growth factors (insulin, C-peptide, proinsulin, IGF-1 and IGF-2). Log-transformed data were used for all cord blood biomarkers in t tests, correlation and regression analyses. Generalized linear regression was used to examine the determinants of cord blood myostatin. GDM status and fetal sex were the primary exposures of interest. Other covariates included maternal age, pre-pregnancy BMI (calculated from self-reported height and weight, kg/m^2), ethnicity (Caucasian/Asian), education (university, yes/no), family history of type 2 diabetes (yes/no), smoking before pregnancy (yes/no), primiparity (yes/no), cesarean section (yes/no) and gestational age at delivery. Only a few mothers reported smoking (n=1) or alcohol drinking (n=2) during pregnancy, and thus not considered in data analyses. Covariates with P>0.2 that did not affect the comparisons were excluded in the parsimonious final regression models to obtain more stable effect estimates. In the presence of sex difference in cord blood myostatin concentrations, we examined the mediation effect of testosterone using the product ("Baron and Kenney") method (26). P<0.025 was considered statistically significant in testing the primary hypothesis on the difference in cord blood myostatin concentrations by GDM status and fetal sex (Bonferroni correction for 2 tests). With Bonferroni correction for multiple tests, P<0.025 was considered statistically significant in examining the primary correlation of interest between cord blood myostatin and testosterone in sex-specific analyses. P<0.05 was considered statistically significant in other exploratory analyses. All data analyses were conducted in R Studio (Version 1.4.1717).

Results

Compared with male *vs.* female newborns, the mothers were more likely to be Caucasian and had higher gestational weight gain (mean ± SD: 17.4 ± 7.2 *vs.* 13.2 ± 6.8 kg) (Table 1). As expected, males were heavier than females at birth (3458 ± 363 *vs.* 3239 ± 358 g). There were no significant differences in maternal age, pre-pregnancy BMI,

TABLE 1 Characteristics of mothers and newborns by infant sex (n=110).

	Male (n=53)	Female (n=57)	P*
Mothers			
Age, years	34.0 ± 3.3	35.0 ± 3.9	0.15
>=35	20 (37.7)	29 (50.9)	0.23
Ethnicity			0.03
Caucasian	35 (66.0)	25 (43.9)	
Asian	18 (34.0)	32 (56.1)	
Primiparity	17 (32.1)	25 (44.6)	0.25
Education, less than university	9 (18.8)	7 (12.7)	0.57
Smoking before pregnancy	8 (15.4)	4 (7.1)	0.23
Pre-pregnancy BMI (kg/m^2)	23.2 ± 4.16	24.3 ± 4.74	0.20
Overweight/obesity	13 (25.0)	22 (38.6)	0.19
Gestational weight gain (kg)	17.4 ± 7.2	13.2 ± 6.8	0.006
Family history of type 2 diabetes	12 (24.0)	13 (22.8)	1.00
Gestational diabetes mellitus	18 (34.0)	26 (45.6)	0.29
Newborns			
Cesarean section	37 (69.8)	40 (70.2)	1.00
Birth weight (g)	3458 ± 363	3239 ± 358	0.002
Birth weight z score	0.15 ± 0.82	-0.04 ± 0.81	0.23
Gestational age (weeks)	38.9 ± 0.79	38.9 ± 0.89	0.90

Data presented are mean ± SD or n (%).
*P values from Chi-square test or Student's t test where appropriate.

GDM, primiparity, family history of type 2 diabetes, smoking before pregnancy, caesarean section and gestational age at delivery between male and female newborns.

Comparing GDM *vs.* euglycemic pregnancies, there were no significant differences in maternal age, education, primiparity, family history of type 2 diabetes, smoking before pregnancy, caesarean section, infant sex and gestational age (Table 2). Mothers with GDM had higher pre-pregnancy BMI (25.5 *vs.* 22.3 kg/m^2) but lower gestational weight gain (12.9 *vs.* 16.8 kg), and were less likely to be Caucasian (39% *vs.* 65%). Unexpectedly, the newborns of GDM mothers had lower average birth weight than those of euglycemic mothers (3245 ± 369 *vs.* 3410 ± 365 g), partly due to more Asians (61% *vs.* 35%) who had lower birth weights than Caucasians (3250 ± 399 *vs.* 3422 ± 334 g). In Caucasian subjects (n=60), the newborns of GDM mothers had similar average birth weight *vs.* those of euglycemic mothers (3423 ± 332 *vs.* 3421 ± 339, P=0.99). In Asian subjects, the newborns of GDM mothers had lower average birth weight *vs.* those of euglycemic mothers (3133 ± 351 *vs.* 3387 ± 416, P=0.026).

Adjusting for maternal and infant characteristics, male newborns had significantly higher cord blood concentrations of myostatin (mean: 6.07 *vs.* 5.29 ng/mL, adjusted P=0.006) and testosterone (9.53 *vs.* 7.14 nmol/L, adjusted P=0.017) (Table 3 and Figure 1). As expected, female newborns tended to have higher cord blood concentrations of insulin (102.8 *vs.* 62.9 pmol/L, adjusted P=0.074) and proinsulin (23.3 *vs.* 17.7 pmol/L, adjusted P=0.066). Cord blood glucose/insulin ratio - a surrogate indicator of fetal insulin sensitivity (14, 27), was higher in males *vs.* females (7.87 *vs.* 5.46, adjusted P=0.048). Cord blood myostatin and testosterone concentrations were similar in Caucasian *vs.* Asian newborns (all P>0.5, data not shown). There were no significant interactions between fetal sex and GDM status, or between fetal sex and ethnicity in relation to cord blood myostatin or testosterone (all P>0.1).

Cord blood myostatin concentrations were similar in GDM *vs.* euglycemic pregnancies (5.50 *vs.* 5.77 ng/mL, adjusted P=0.28, Table 4). There were no significant differences in cord blood concentrations of testosterone, IGF-1 and IGF-2 comparing the newborns of GDM *vs.* euglycemic mothers. Similarly, in male newborns, there were no significant differences in cord blood concentrations of myostatin (5.94 *vs.* 6.13 ng/mL, adjusted P=0.57) and testosterone (9.74 *vs.* 9.43 nmol/L, adjusted P=0.56) comparing GDM *vs.* euglycemic pregnancies. The newborns of GDM mothers tended to have higher insulin (120.4 *vs.* 60.1 pmol/L, adjusted P=0.07) and proinsulin (24.5 *vs.* 18.0 pmol/L, adjusted P=0.14) concentrations, although the differences did not reach statistical significance. Similarly, cord blood concentrations tended to be

TABLE 2 Characteristics of mothers and newborns by GDM status (n=110).

	GDM (n=44)	Non-GDM (n=66)	P*
Mothers			
Age, years	35.0 ± 4.1	34.3 ± 3.3	0.37
>=35	23 (52.3)	26 (39.4)	0.26
Ethnicity			0.01
Caucasian	17 (38.6)	43 (65.2)	
Asian	27 (61.4)	23 (34.8)	
Primiparity	18 (41.9)	24 (36.4)	0.71
Education, less than university	9 (21.4)	7 (11.5)	0.27
Smoking before pregnancy	4 (9.3)	8 (12.3)	0.76
Pre-pregnancy BMI (kg/m^2)	25.5 ± 5.2	22.3 ± 3.3	<0.001
Overweight/obesity	21 (47.7)	12 (18.5)	0.002
Gestational weight gain (kg)	12.9 ± 7.1	16.8 ± 7.0	0.02
Family history of type 2 diabetes	14 (32.6)	11 (17.2)	0.11
Newborns			
Cesarean section	27 (61.4)	50 (75.8)	0.16
Sex, male	18 (40.9)	35 (53.0)	0.29
Birth weight (g)	3245 ± 369	3410 ± 365	0.02
Birth weight z score	-0.12 ± 0.85	0.16 ± 0.77	0.16
Gestational age (weeks)	38.8 ± 0.90	39.0 ± 0.80	0.45

Data presented are mean ± SD or n (%).
*P values from Chi-square test or Student's t test where appropriate.
GDM=Gestational diabetes mellitus.

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TABLE 3 Cord blood concentrations of myostatin, testosterone and fetal growth factors comparing male *vs.* female newborns.

	Males (n=53)	Females (n=57)	Crude P*	Adjusted P*
Myostatin (ng/mL)	6.07 ± 1.62	5.29 ± 1.04	0.009	0.006
	5.77 (4.86, 7.15)	5.26 (4.42, 5.99)		
Testosterone (nmol/L)	9.53 ± 6.36	7.14 ± 4.05	0.005	0.017
	7.70 (6.05, 9.00)	6.20 (4.70, 7.80)		
Glucose (mmol/L)	3.35 ± 0.67	3.44 ± 0.75	0.54	0.66
	3.20 (2.90, 3.70)	3.40 (2.90, 3.95)		
Insulin (pmol/L)	62.9 ± 36.4	102.8 ± 102.4	0.014	0.074
	58.0 (42.0, 71.0)	80.0 (47.0, 105.0)		
Glucose/Insulin ratio	7.87 ± 5.46	5.46 ± 3.69	0.008	0.048
(mg/dL/μU/mL)[£]	6.16 (5.12, 8.77)	4.75 (3.60, 6.93)		
Proinsulin (pmol/L)	17.7 ± 8.74	23.3 ± 14.6	0.009	0.066
	15.0 (12.6, 17.5)	18.9 (14.6, 26.1)		
C-peptide (pmol/L)	410 ± 145	453 ± 228	0.41	0.30
	369 (324, 465)	407 (317, 518)		
IGF-1 (ng/mL)	110 ± 29	111 ± 29	0.93	0.29
	105 (90, 136)	109 (91, 134)		
IGF-2 (ng/mL)	387 ± 59	393 ± 71	0.74	0.96
	366 (343, 410)	376 (343, 448)		

Data presented are Mean ± SD and Median (Q25, Q75).
IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2.
Glucose, insulin, C-peptide, IGF-1 and testosterone were measured in cord blood serum, myostatin, proinsulin and IGF-2 were measured in cord blood EDTA plasma samples.
[£] Glucose/Insulin ratio in mg/dL/mU/mL (glucose: 1 mmol/L=18 mg/dL; insulin: 1 μU/mL=6 pmol/L); higher cord blood glucose/insulin ratios indicate higher fetal insulin sensitivity.
*Crude P values were from t-tests in log-transformed biomarker data. Adjusted P values were from generalized linear models in the comparisons of log-transformed biomarker data adjusting for maternal (age, ethnicity, pre-pregnancy BMI, gestational weight gain, gestational diabetes mellitus) and neonatal (cesarean section) characteristics; other factors were excluded since they were similar and did not affect the comparisons (all P>0.2).
Tests for interaction between fetal sex and GDM in relation to cord blood biomarkers, all P>0.2 (data not shown).

higher for insulin (90.5 *vs.* 52.2 pmol/L, P=0.026) and C-peptide (462 *vs.* 385 pmol/L, P=0.08) comparing GDM and euglycemic pregnancies in male newborns,

Adjusting for gestational age at blood sampling, cord blood myostatin was positively correlated with testosterone in males (r=0.56, P <0.001), but not in females (r=-0.076, P=0.58) (Fisher's z test for difference in correlation coefficients, P<0.001) (Table 5 and Figure 2). In the total study sample, cord blood myostatin was negatively correlated with IGF-2 (r=-0.23, P=0.02), but not correlated with birth weight (z score), IGF-1, insulin, proinsulin or C-peptide. Cord blood myostatin was not correlated with glucose/insulin ratio in males (P=0.46) or females (P=0.35).

Adjusting for gestational age at blood sampling, cord blood testosterone was positively correlated with IGF-2 (r=0.28, P=0.004),

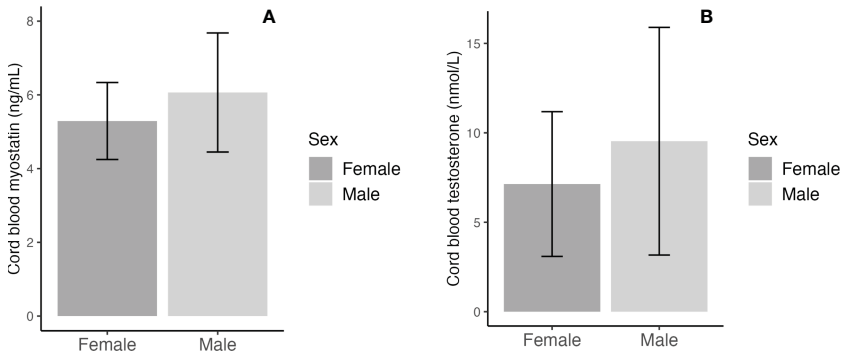

FIGURE 1
Sex differences in cord blood concentrations of myostatin **(A)** and testosterone **(B)**; the error bars represent the mean and 95% confidence intervals.

TABLE 4 Cord blood concentrations of myostatin, testosterone and fetal growth factors comparing GDM *vs.* euglycemic pregnancies.

	GDM (n=44)	Control (n=66)	Crude P*	Adjusted*
Myostatin (ng/mL)	5.50 ± 1.37	5.77 ± 1.42	0.29	0.28
	5.36 (4.44, 6.30)	5.57 (4.56, 6.57)		
Testosterone (nmol/L)	8.77 ± 6.74	7.98 ± 4.38	0.70	0.89
	6.80 (5.30, 8.10)	7.40 (5.20, 8.80)		
Glucose (mmol/L)	3.64 ± 0.83	3.24 ± 0.58	**0.01**	0.13
	3.60 (3.10, 4.10)	3.20 (2.80, 3.60)		
Insulin (pmol/L)	120.4 ± 114.3	60.1 ± 29.7	**0.002**	0.07
	80.0 (64.0, 144.0)	58.0 (41.5, 74.3)		
Glucose/Insulin ratio	5.53 ± 4.22	7.39 ± 5.05	**0.02**	0.25
(mg/dL/mU/mL) [£]	4.34 (2.73, 7.13)	5.80 (4.92, 7.94)		
Proinsulin (pmol/L)	24.5 ± 16.3	18.0 ± 8.2	0.04	0.14
	18.7 (13.9, 26.4)	15.6 (12.7, 21.0)		
C-peptide (pmol/L)	494 ± 261	393 ± 120	0.054	0.33
	460 (324, 620)	375 (313, 462)		
IGF-1 (ng/mL)	107.1 ± 29.3	112.6 ± 29.9	0.35	0.71
	104 (89, 122)	111 (91, 137)		
IGF-2 (ng/mL)	392 ± 67	388 ± 65	0.80	0.57
	366 (343, 448)	375 (342, 425)		

Data presented are Mean ± SD and Median (Q25, Q75).
IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2.
Glucose, insulin, C-peptide, IGF-1 and testosterone were measured in cord blood serum, myostatin, proinsulin and IGF-2 were measured in cord blood EDTA plasma samples.
[£]Glucose/Insulin ratio in mg/dL/mU/mL (glucose: 1 mmol/L=18 mg/dL; insulin: 1 mU/mL=6 pmol/L); higher cord blood glucose/insulin ratios indicate higher fetal insulin sensitivity.
*Crude P values were from t-tests in log-transformed biomarker data. Adjusted P values were from generalized linear models in the comparisons of log-transformed biomarker data between the two groups adjusting for maternal (age, ethnicity, pre-pregnancy BMI, gestational weight gain and family history of diabetes) and neonatal (fetal sex, cesarean section) characteristics; other factors were excluded since they were similar and did not affect the comparisons (all P>0.2). P values in bold: P < 0.025.

but not correlated with IGF-1 (r=-0.10, P=0.30) or birth weight (r=-0.075, P=0.45) in the total study sample (Table 5). Cord blood testosterone was negatively correlated with insulin in males (r=-0.43, P=0.011), but not in females (r=0.11, P=0.55) (Fisher's z test for difference in correlation coefficients, P=0.053). Similarly, testosterone was negatively correlated with C-peptide (r=-0.32, P=0.025) in males, but not in females (r=0.16, P=0.26) (Fisher's z test for difference in correlation coefficients, P=0.025).

There were no significant differences in the correlations of cord blood myostatin and testosterone with birth weight and fetal growth factors by GDM status or ethnicity (data not shown).

In the total study sample, birth weight z score was positively correlated with cord blood proinsulin (r=0.24, P=0.01) and IGF-1 (r=0.31, P=0.001), and tended to be positively correlated with C-peptide (r=0.17, P=0.08), but not correlated with IGF-2 (r=0.01, P=0.94).

Overall, male sex was associated with a 0.60 increase in cord blood myostatin concentration z score (P=0.002). Mediation analysis demonstrated that cord blood testosterone could explain a 0.18 increase in cord blood myostatin z score (30.0%, P=0.039) in males *vs.* females (Table 6). Cord blood testosterone or myostatin could not explain the sex difference in cord blood glucose/insulin ratio or birth weight (all P>0.1 for mediation effect, results not shown).

Discussion

Main findings

Our study is the first to demonstrate that GDM does not affect cord blood myostatin concentration, whereas fetal sex does. Cord blood myostatin concentrations were significantly higher in males *vs.* females. Approximately 30% of the sex difference in cord blood myostatin concentrations can be explained by testosterone. Interestingly, cord blood testosterone and myostatin was positively correlated in males only, suggesting a male-specific androgen up-regulation of myostatin secretion in early life in humans.

GDM and cord blood myostatin

Cord blood myostatin concentrations were similar in GDM *vs.* controls. Skeletal muscle is the dominant source of circulating myostatin (1); skeletal muscle-specific expression of myostatin is about 50-100 fold higher than adipose tissue-specific expression (28). Whether GDM affects skeletal muscle mass remains controversial; similar or lower lean mass has been reported in the neonates of GDM *vs.* controls (29, 30). Although GDM has been associated with increased fat mass in the

TABLE 5 Correlations of cord blood myostatin with testosterone, birth weight (z), glucose/insulin ratio and fetal growth factors.

	All		Males		Females		*P for difference
	r	P	r	P	r	P	
Myostatin with:							
Testosterone	0.34	<0.001	0.56	<0.001	-0.076	0.58	<0.001
Birth weight (z)	0.06	0.53	-0.024	0.87	0.10	0.44	0.51
Insulin	-0.14	0.25	-0.16	0.35	0.04	0.83	0.41
Glucose/Insulin	0.071	0.57	0.13	0.46	-0.17	0.35	0.28
Proinsulin	-0.01	0.90	0.017	0.90	0.09	0.51	0.71
C-peptide	-0.11	0.27	-0.15	0.32	-0.05	0.71	0.64
IGF-1	-0.05	0.60	-0.21	0.14	0.15	0.28	0.07
IGF-2	-0.23	0.02	-0.18	0.20	-0.29	0.03	0.55
Testosterone with:							
Birth weight (z)	-0.075	0.45	-0.14	0.33	0.21	0.13	0.84
Insulin	-0.26	0.03	-0.43	0.011	0.11	0.55	0.053
Glucose/Insulin	0.18	0.15	0.33	0.054	-0.19	0.30	0.06
Proinsulin	-0.10	0.32	-0.15	0.31	0.044	0.75	0.32
C-peptide	-0.07	0.47	-0.32	0.025	0.16	0.26	0.025
IGF-1	-0.10	0.30	-0.24	0.10	0.03	0.84	0.21
IGF-2	0.28	0.004	0.22	0.13	0.37	0.005	0.35

Data presented are Pearson partial correlation coefficients adjusting for gestational age at delivery/cord blood sampling. Log-transformed data were used for all biomarkers in the partial correlation analyses.
IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2.
*P values in Fisher's z tests for differences in correlation coefficients in males and females.

offspring (29, 30), myostatin expression in both visceral and subcutaneous fat was similar in obese *vs.* lean subjects (11), and circulating myostatin concentration does not appear to be correlated with fat mass (12).

Cord blood myostatin, fetal sex and testosterone

We observed significantly higher cord blood myostatin concentrations in males *vs.* females. In contrast, a smaller study (n=83) reported no significant difference in cord blood myostatin concentrations in males *vs.* females (18). The reasons for the discrepant findings may be partly due to the differences in sample size and detection method (Sandwich ELISA in our study *vs.* competitive ELISA kit in their study). Our study is consistent with an adult study reporting higher myostatin concentrations in males *vs.* females (4.3 ng/mL *vs.* 3.3 ng/mL) using the same ELISA kit as in our study (R&D Systems) (12).

Interestingly, cord blood myostatin was positively correlated with testosterone in males but not in females. Testosterone is an anabolic

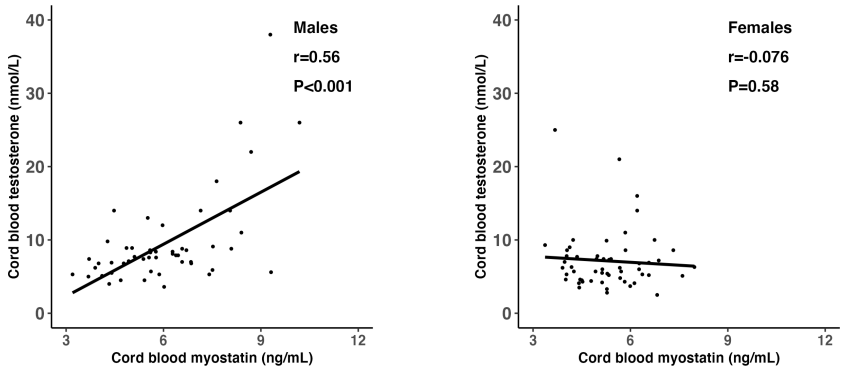

FIGURE 2
Scatter plots illustrating the differential correlations (r) between cord blood myostatin and testosterone in males and females; Fisher's Z test for difference in r (correlation coefficient): P<0.001.

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TABLE 6 Mediation analysis in the association of fetal sex with cord blood myostatin (n=110).

	Myostatin (z score)	
	β (95% CI)	P
Fetal sex, male	0.60 (0.23, 0.96)	**0.002**
*Mediation by testosterone	0.18 (0.072, 0.28)	**0.039**
Maternal age (per SD)	0.15 (-0.03, 0.33)	0.10

Data (β) presented are the standardized changes in cord blood myostatin (z score) from generalized linear models. Only fetal sex and maternal age were predictive of cord blood myostatin at P<0.2; other maternal and infant characteristics did not affect the comparisons and were thus not included in the final model. The SDs for calculating the z scores were 1.40 ng/mL for myostatin, 5.40 nmol/L for testosterone and 3.63 years for maternal age.
*The mediation effect presented is the change (95% CI) in cord blood myostatin (z score) per SD increment in cord blood testosterone that could account for the effect of fetal sex on cord blood myostatin. P values in bold: P < 0.05.

hormone promoting protein synthesis and skeletal muscle growth (19). In contrast, myostatin inhibits skeletal muscle growth (1, 2). Our data support the hypothesis that testosterone may up-regulate myostatin (and thus may counteract the effect of testosterone) in males. This observation is in line with a study in male mice reporting that the inhibition of testosterone production or androgen receptor signaling could down-regulate myostatin gene expression and protein synthesis in androgen responsive muscles (31). An adult study showed that both testosterone and myostatin concentrations were higher in young *vs.* old men, and testosterone treatment resulted in higher myostatin concentrations (23), indicating that testosterone may up-regulate myostatin secretion in men. Our data suggest that testosterone may up-regulate fetal myostatin secretion in males, and may partly mediate the higher fetal (cord blood) myostatin concentrations in males in humans.

Myostatin, testosterone and fetal insulin sensitivity

Our study confirmed that GDM and female sex were associated with lower fetal insulin sensitivity as indicated by lower cord blood glucose/insulin ratios and higher insulin and proinsulin concentrations (14, 15). Neither myostatin nor testosterone could explain the differences in glucose/insulin ratios by fetal sex or GDM in mediation analyses, suggesting neither may explain such differences. GDM was not associated with cord blood testosterone, consistent with a previous study (32). Testosterone tended to be positively correlated with fetal insulin sensitivity (glucose/insulin) in males (P=0.054). This is in line with a previous study reporting that testosterone replacement therapy improved insulin resistance in adult men (22).

Myostatin and testosterone in relation to fetal growth and fetal growth factors

We observed a negative correlation between cord blood myostatin and IGF-2, but no correlation with IGF-1 or birth weight. Myostatin appears to be regulated by growth hormone in hypophysectomised mice (33) and hypopituitary adults (34), suggesting myostatin may play a role in fetal growth. A previous study reported an inverse correlation between cord blood myostatin and birth weight in 83 newborns (r=-0.40, P=0.001) (18). The reasons for the different findings may be partly due to the differences in study population and detection methods (Sandwich *vs.* competitive ELISA). Their study included 23 large-for-gestational-age (LGA, birth weight z score >2) and 60 appropriate-for-gestational-age infants (birth weight z score from -1 to 1) (18), and the larger differences in birth weight may render a greater power to identify a significant correlation between cord blood myostatin and birth weight. None of our newborns could be identified as LGA if we used the same definition as in their study. On the other hand, we did observe a negative correlation between cord blood myostatin and IGF-2, suggesting a possible negative effect on fetal growth. IGF-2 plays a pivotal role in fetal growth (35, 36). Our observation is in line with two animal studies reporting that IGF-2 expression was greater in mice with myostatin mutation (37), and IGF-2 expression was inhibited in myoblast cultures with treatment of recombinant myostatin (38). Overall, our data are somewhat uncertain concerning the role of myostatin in fetal growth. We could not rule out the possibility of a false negative finding, and larger studies are warranted to clarify the role of myostatin in fetal growth.

Testosterone was positively correlated with IGF-2, but not correlated to IGF-1 or birth weight. IGF-2 is a fetal growth factor important for early embryonic fetal growth, and its correlation with birth weight tends to be much weaker than IGF-1 (35, 36). We failed to detect a positive correlation between IGF-2 and birth weight, probably due to the relative small sample size. The lack of correlation between cord blood testosterone and birth weight is consistent with the results in a previous study (32). As expected, birth weight was positively correlated with cord blood proinsulin, C-peptide and IGF-1, consistent with the findings in previous studies (39, 40).

Interestingly, we observed a negative correlation between cord blood testosterone and insulin or C-peptide in males but not in females. More studies from other independent cohorts are warranted to confirm this novel observation suggesting that testosterone may play a sex dimorphic role in insulin secretion during fetal life in humans.

Limitations

There are some study limitations. Firstly, the modest sample size allowed for the detection of modest/large differences, and was underpowered to detect small differences. With the study sample size (44 GDM, 66 controls; 53 males, 57 females) with alpha error at 0.025, we had a power of >=91% to detect a 0.7 SD or greater difference in cord blood myostatin concentrations between GDM and controls, or between males and females. The study power was >78% to detect an absolute correlation coefficient of 0.4 or greater in sex-specific analyses with alpha error at 0.025. Secondly, cord serum testosterone was measured by chemiluminescence immunoassay rather than mass spectrometry - the golden standard (much more costly) method. Due to cross reactivity with testosterone-like molecules, the observed cord serum testosterone concentrations could have been inflated to some extent. However, such noise random variations would only tend to decrease the probability of

detecting a significant association. Lastly, the observational nature of the study precluded the possibility of conclusive causal inference.

In conclusion, GDM does not affect cord blood myostatin concentration, but fetal sex does. The higher myostatin concentrations in males may be partly mediated by testosterone. The male-only positive correlation between cord blood testosterone and myostatin suggests a male-specific role of androgen in up-regulating fetal myostatin secretion in humans.

Data availability statement

The datasets presented in this article are not readily available because Access to deidentified research data must be approved by the research ethics board. Requests to access the datasets should be directed to zcluo@lunenfeld.ca.

Ethics statement

The studies involving human participants were reviewed and approved by Research Ethics Board of Mount Sinai Hospital University of Toronto. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RH, LB, ZP, KM, JK, SL and Z-CL conceived the study. RH, MK, KM, JK, SL and Z-CL contributed to the acquisition of research data. RH conducted the literature review, data analysis and drafted the article. All authors contributed in revising the article critically for important intellectual content, and approved the final version for publication. Z-CL is the guarantor of this work, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors 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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Interactive effect of prepregnancy overweight/obesity and GDM history on prevalence of GDM in biparous women

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Background: Prepregnancy overweight/obesity (OWO) and gestational diabetes mellitus (GDM) history may increase the prevalence of GDM in parous women, but little is known about their potential combined effect on the prevalence of GDM in biparous women.

Objective: This study aims to explore the interactive effect of prepregnancy overweight/obesity (OWO) and GDM history on the prevalence of GDM in biparous women.

Methods: A retrospective study was conducted on 16,282 second-birth women who delivered a single neonate at ≧28 weeks of gestation twice. Logistic regression was used to assess the independent and multiplicative interactions of prepregnancy overweight/obesity (OWO) and GDM history on the risk of GDM in biparous women. Additive interactions were calculated using an Excel sheet that was made by Anderson to calculate relative excess risk.

Results: A total of 14,998 participants were included in this study. Both prepregnancy OWO and GDM history were independently associated with an increased risk of GDM in biparous women (odds ratio (OR) = 19.225, 95% confidence interval (CI) = 17.106, 21.607 and OR = 6.826, 95% CI = 6.085, 7.656, respectively). The coexistence of prepregnancy OWO and GDM history was associated with GDM, with an adjusted OR of 1.754 (95% CI, 1.625, 1.909) compared to pregnant women without either condition. The additive interaction between prepregnancy OWO and GDM history was found to be not significant with regard to GDM in biparous women.

Conclusions: Prepregnancy OWO and GDM history both increase the risk of GDM in biparous women and have multiplicative interactions but not additive interactions.

KEYWORDS

gestational diabetes mellitus history, pre-pregnancy overweight/obesity, multiplicative interaction, additive interaction, recurrent gestational diabetes mellitus

Introduction

Gestational diabetes mellitus (GDM) is defined as carbohydrate intolerance during pregnancy that is increasing worldwide, including in China (1–3). GDM is associated with adverse pregnancy and birth outcomes, such as preeclampsia, macrosomia, birth trauma, shoulder dystocia and fetal hypoglycemia, respiratory distress, and even stillbirth (4–6). The prevalence of GDM ranged from 2.1% to 37.5% in the global population (7, 8) and 14.8% to 19.4% in the Chinese population (9, 10).

With the successful implementation of the two-child policy and three-child policy, the number of multipara women is increasing in China (11, 12). A large number of studies reported that the risk of GDM in multipara women is significantly higher than that in primipara women (13, 14). Previous studies have indicated that a variety of factors, including advanced maternal age, prepregnancy overweight or obesity, family history of GDM and T2DM, history of GDM, and subfertility or infertility was associated with GDM (15, 16). Compared with primipara, multipara women have the characteristics of old age and high BMI, which may be the possible reasons for the high incidence of GDM in multipara women. Given that most of the risk factors for GDM persist or become worse in subsequent pregnancies, it is not surprising that GDM has a high recurrence rate. A systematic review by Kim et al. (17) reported that the risk for recurrent GDM in subsequent pregnancy was as high as 30%–84% in women with prior GDM. Our previous study also reported that it was nearly 50% (18).

Maternal overweight and obesity are growing global public health concerns. The increasing prevalence of prepregnancy overweight/obesity has increased the risk of adverse maternal and neonatal outcomes, including increased rates of GDM, preeclampsia, cesarean section, and preterm delivery (19–21). The resulting increased incidence of GDM is associated with a series of adverse pregnancy outcomes.

Based on the background discussed above, both histories of GDM and overweight or obesity are independently associated with an increased risk of GDM. However, it was unclear whether women who were overweight or obese before pregnancy and had a history of GDM had a higher risk of developing GDM because the combined effect of a history of GDM and prepregnancy overweight or obesity on the prevalence of GDM in biparous women is still unknown. Therefore, in this study, we aimed to investigate whether the history of GDM and prepregnancy OWO synergistically affect the risk of GDM in biparous women.

Methods

The study population

The retrospective study included second-birth women who delivered a second single neonate at a gestation age of ≧28 weeks at Fujian Maternity and Child Health Hospital between January 2017 and December 2021. The eligibility criteria include all women who received perinatal care and performed a 75-g OGTT between 24 and 28 weeks of gestation. The current study was approved by the Ethics Committee of Fujian Maternity and Child Health Hospital (2020–2049). Informed consent was not required since the current study was conducted through a retrospective review of medical records.

Data collection

All data were collected from the clinical electronic medical record and extracted into Microsoft Excel for later analysis. Data regarding demographic and obstetric characteristics were collected. The main pregnancy outcome in this study is the incidence of GDM.

Definition

Prepregnancy body mass index (BMI) was obtained from clinic records and calculated by dividing maternal prepregnancy weight by maternal squared height. According to the adult weight standard published by the Ministry of Health of China, BMI < 18.5 kg/m^2 is defined as underweight, 18.5 kg/m^2 ≤ BMI < 24 kg/m^2 is defined as normal weight, 24 kg/m^2 ≤ BMI < 28 kg/m^2 is defined as overweight, and BMI ≥ 28 kg/m^2 is defined as obesity (22). All participants took a 75-g, 2-h oral glucose tolerance test. GDM was defined according to the International Association of Diabetes and Pregnancy Study Groups; a diagnosis of GDM was made when one or more of the test parameters equaled or exceeded the following cut points: fasting 5.1 mmol/L, 1-h 10.0 mmol/L, or 2-h 8.5 mmol/L (23). In this study, gestational hypertensive disorders were classified as gestational hypertension, preeclampsia, chronic hypertension with preeclampsia, and eclampsia (24). After 20 weeks of gestation, blood pressure (BP) is ≥ 140 mmHg and/or diastolic BP is ≥ 90 mmHg, but there is no proteinuria. Preeclampsia was defined as the presence of gestational hypertension with proteinuria (urine protein content > 300 mg/24 h or protein/creatinine ratio ≥ 0.3) or a systemic symptom. Eclampsia was defined as seizures that cannot be attributable to other causes in a woman with preeclampsia.

Statistical analysis

Continuous variables were presented as median (interquartile range (IQR)), and categorical variables were presented as frequency (percentage). We compared baseline characteristics between GDM cases and non-GDM cases using the Wilcoxon two-sample test, Chi-squares test, or Fisher's exact test.

Binary logistic regression was performed to explore the associations of prepregnancy BMI and IMH with the prevalence of GDM in multipara women. A logistic regression model was used to calculate the regression coefficients and covariance matrix for two factors at first. An Excel sheet provided by Andersson was then used to calculate relative excess risk due to interaction (RERI), attributable proportion due to interaction (AP), interaction index (synergy index (SI)), and their 95% confidence intervals (CIs) (25). The 95% CI of RERI and AP includes "0," and the 95% CI of SI includes "1," indicating that there is no summation interaction.

Statistical analysis was performed using IBM SPSS Statistics 26. Statistical tests were conducted on a two-sided basis, with a p-value of <0.05 considered statistically significant.

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Results

Basic characteristics of the participants

A total of 16,282 women delivered their second single neonate after the gestation age of 28 weeks at Fujian Maternity and Child Health Hospital between January 2017 and December 2021. We excluded 1,284 women from the following: 277 pregnant women with prepregnancy gestational diabetes, 228 pregnant women with twin pregnancies, and 779 women who are missing data on key variables. Of the remaining 14,998 women, 5,738 were diagnosed with gestational diabetes mellitus and 9,260 were without gestational diabetes mellitus (Figure 1). The comparisons of baseline characteristics between gestational diabetes mellitus and nongestational diabetes mellitus groups are listed in Table 1. There were no significant differences in maternal age, maternal age ≧35 years, assisted reproductive techniques (ART), gestational hypertensive disorders, intrahepatic cholestasis of pregnancy, week of delivery, and mode of delivery between the two groups. Pregnant women with gestational diabetes mellitus were likely to have a higher prepregnancy BMI, birth weight, and rate of GDM history.

Multiplicative interaction of prepregnancy BMI and history of GDM on prevalence of GDM in biparous women

Study participants were calculated based on their BMI values. The ORs of prepregnancy BMI and history of GDM for the risk of GDM in biparous women were 1.103 (95% CI, 1.086, 1.121) and 8.040 (95% CI, 4.703, 13.746) in the crude models, respectively. In the adjusted models, there were no multiplicative interactions between prepregnancy BMI and history of GDM on the prevalence of GDM in biparous women (adjusted odds ratio (aOR), 0.987; 95% CI, 0.962, 1.011). We found this combined model achieved 72.4% accuracy, 61.4% sensitivity, and 79.3% specificity (Table 2).

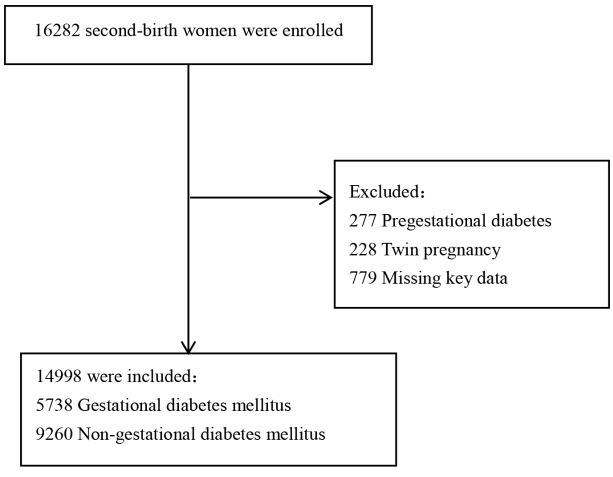

FIGURE 1
Flow chart of the study population.

Women were classified as underweight or normal weight (BMI < 24 kg/m^2), overweight (24 kg/m^2 ≤ BMI < 28 kg/m^2), and obese (BMI ≥ 28 kg/m^2) based on their prepregnancy BMI. The ORs of prepregnancy overweight, obesity, and history of GDM for the risk of GDM in biparous women were 0.309 (95% CI, 0.226, 0.424), 7.164 (95% CI, 5.223, 9.826), and 4.758 (95% CI, 2.974, 7.613) in the crude models, respectively. In the adjusted models, there were no multiplicative interactions between prepregnancy overweight with a history of GDM and prevalence of GDM in biparous women (aOR, 1.434; 95% CI, 0.884, 2.327) as well as prepregnancy obesity with a history of GDM and prevalence of GDM in biparous women (aOR, 1.071; 95% CI, 0.651, 2.327). We found this combined model achieved 82.1% accuracy, 70.8% sensitivity, and 89.0% specificity (Table 3).

Women were then further classified as underweight or normal weight (BMI < 24 kg/m^2) and overweight or obese (BMI ≥ 24 kg/m^2) based on their prepregnancy BMI. The ORs of prepregnancy overweight or obesity and history of GDM for the risk of GDM in biparous women were 19.225 (95% CI, 17.106, 21.607) and 6.826 (95% CI, 6.085, 7.656) in the crude models, respectively. In the adjusted models, there were significant multiplicative interactions between prepregnancy overweight or obesity with a history of GDM and the prevalence of GDM in biparous women (aOR, 1.754; 95% CI, 1.625, 1.909). We found this combined model achieved 81.2% accuracy, 71.7% sensitivity, and 87.1% specificity (Table 4).

Additive interaction of prepregnancy overweight or obesity and history of GDM on prevalence of GDM in biparous women

When prepregnancy overweight or obesity and a history of GDM exist at the same time, no additive interaction was found for the prevalence of GDM in biparous women (Table 5).

Discussion

In this study, we demonstrated that prepregnancy overweight or obesity and a history of GDM significantly increase the risk of GDM in biparous women separately. After accounting for confounders, the composite outcome of prepregnancy overweight or obesity and history of GDM appears to be multiplicative. However, when prepregnancy overweight or obesity and a history of GDM exist at the same time, no additive interaction was found for the risk of GDM in biparous women.

Maternal overweight or obesity before pregnancy and during pregnancy are both risk factors for GDM (26, 27). Studies suggest that it may be related to inflammation that is stimulated by a high concentration of adipokines (28). In addition, overweight or obesity may result in elevated plasma free fatty acid (FFA) levels, which leads to increased intracellular lipid accumulation in nonadipose cells like cardiomyocytes, β-cells, and hepatocytes (29). Thus, lipid accumulation in these cells induced insulin resistance *via* the activation of protein kinase C and several pathways, including diacylglycerol pathways. Clustering of these metabolic abnormalities is correlated with an increased risk of type 2 diabetes mellitus in the future (29, 30). During early pregnancy, the clustering of these

TABLE 1 Baseline characteristics according to the occurrence of GDM in biparous women.

Characteristics	GDM (n = 5,738)	Non-GDM (n = 9,260)	p-value
Maternal age (median [IQR], years)	33 [31, 36]	33 [31, 36]	0.316
Maternal age ≧35 years (No. (%))	3,594 (38.81)	2,308 (40.22)	0.086
Prepregnancy BMI (median [IQR], kg/m^2)	21.218 [19.470, 23.310]	20.324 [18.939, 22.032]	0.000
Prepregnancy BMI (No. (%))			0.000
Underweight (No. (%))	350 (6.100)	1,764 (19.050)	
Normal weight (No. (%))	1,271 (22.150)	6,299 (68.024)	
Overweight (No. (%))	3,991 (69.554)	964 (10.410)	
Obesity (No. (%))	126 (2.196)	233 (2.516)	
GDM history (No. (%))	3,520 (61.345)	1,921 (20.745)	0.000
The interval between pregnancies (months)	29.82 ± 13.20	32.83 ± 14.64	−0.505
Gravity (No. (%))			
2	2,633 (45.887)	4,133 (44.633)	0.134
3	1,855 (32.328)	3,248 (35.076)	0.000
≧4	1,250 (21.785)	1,879 (20.292)	0.0287
ART (No. (%))	146 (2.544)	285 (3.077)	0.057
Gestational hypertensive disorders (No. (%))	519 (9.045)	324 (3.499)	0.913
Intrahepatic cholestasis of pregnancy (No. (%))	82 (1.429)	127 (1.371)	0.770
Week of delivery (median [IQR], weeks)	39.00 [38.29, 39.71]	39.00 [38.14, 39.57]	0.413
Mode of delivery (No. (%))			0.308
Vaginal birth	3,022 (52.667)	4,956 (53.521)	
Cesarean section	2,716 (47.334)	4,304 (46.490)	
Birth weight (median [IQR], weeks)	3,303 [3,019, 3,580]	3,290 [2,985, 3,560]	0.010

A Chi-square test was used to compare categorical variables, and Wilcoxon two-sample test was performed to compare continuous variables.
GDM, gestational diabetes mellitus; BMI, body mass index; OWO, overweight/obesity; ART, assisted reproductive techniques.

TABLE 2 The multiplicative interaction between prepregnancy BMI and GDM history for the risk of GDM in biparous women.

Category	β	Wold value	p-value	OR (95% CI)
Prepregnancy BMI	0.098	144.938	0.000	1.103 (1.086~1.121)
GDM history	2.084	58.037	0.000	8.040 (4.703~13.746)
Prepregnancy BMI by GDM history	−0.014	1.131	0.288	0.987 (0.962~1.011)

GDM, gestational diabetes mellitus; BMI, body mass index; OWO, overweight/obesity; ART, assisted reproductive techniques.

TABLE 3 The multiplicative interaction between stratified prepregnancy BMI and GDM history for the risk of GDM in biparous women.

Category	β	Wold value	p-value	OR (95%CI)
Prepregnancy BMI				
Underweight or normal	NA			NA
Overweight	−1.173	53.202	0.000	0.309 (0.226~0.424)
Obesity	1.969	149.133	0.000	7.164 (5.223~9.826)
GDM history	1.560	42.037	0.000	4.758 (2.974~7.613)
Overweight by GDM history	0.361	2.136	0.144	1.434 (0.884~2.327)
Obesity by GDM history	0.069	0.074	0.786	1.071 (0.651~1.762)

GDM, gestational diabetes mellitus; BMI, body mass index.

TABLE 4 The multiplicative interaction between stratified prepregnancy BMI and GDM history for the risk of GDM in biparous women.

Category	β	Wold value	*p*-value	OR (95%CI)
Prepregnancy BMI				
Underweight or normal	NA			NA
Overweight or obesity	2.956	2,461.092	0.000	19.225 (17.106~21.607)
GDM history	1.921	1,075.559	0.000	6.826 (6.085~7.656)
Overweight or obesity by GDM history	0.283	8.704	0.003	1.754 (1.625~1.909)

GDM, gestational diabetes mellitus; BMI, body mass index; OWO, overweight/obesity.

TABLE 5 Additive interaction between prepregnancy OWO and GDM history.

	RR	95% CI	SE
RERI	−1.714	−4.8970~1.469	1.624
API (%)	−36.020	−115.74~43.70	40.671
S	0.687	0.333~1.418	0.370

OWO, overweight/obesity.

metabolic risk factors has been reported to be another pathophysiology for the link between obesity and GDM.

A recent systematic review provided evidence that a history of GDM was associated with morbidity other than T2DM or cardiovascular disease and with long-term mortality (30). However, after the "One-Child Family" policy restriction was abolished and the "Two-Child Family" policy even the "Third-Child Family" policy were permitted in China, the rate of recurrent GDM may be a more important problem during follow-up after the first pregnancy for women with a history of GDM. Studies revealed that elevated circulating markers of endothelial dysfunction are present in young women with a history of GDM. Muhli et al. recently found that women with a history of GDM had a low dietary quality score and a light physical activity level during subsequent pregnancy (31). In fact, it was reported that women with a history of GDM still had a lower dietary quality and lower intensity of physical activity several years later after pregnancy (32, 33). These all explain why the history of GDM was significantly associated with a higher maternal risk for GDM recurrence.

Evidence from previous studies shows that higher prepregnancy BMI is associated with lower dietary quality, and dietary quality may decline with advancing gestation in pregnant women with obesity (34). In addition, inadequate physical activity may lead to weight gain and eventually becoming overweight and obese. Furthermore, obesity and GDM are both chronic low-grade inflammatory states (28, 35, 36). All these may be possible reasons for the multiplicative interaction, but there is no additive interaction between prepregnancy overweight or obesity and a history of GDM. For women both with a history of GDM and overweight or obesity before pregnancy, it is recommended to control their weight before pregnancy not just after pregnancy to reduce GDM risk and improve adverse pregnancy outcomes. In addition, for women with GDM, weight management should be started after the termination of pregnancy to avoid becoming overweight or obese.

In conclusion, this study explored the independent and combined effects of prepregnancy overweight/obesity and the history of GDM on the risk of GDM in biparous women, as well as their potential multiplicative and additive interactions. We found that the composite outcome of prepregnancy overweight or obesity and history of GDM appears to be multiplicative after accounting for confounders. However, when prepregnancy overweight or obesity and a history of GDM exist at the same time, no additive interaction was found for the risk of GDM in biparous women. To reduce the risk of GDM and improve perinatal outcomes, it is recommended that women with a history of GDM should try to control their weight to a normal level before pregnancy. Nevertheless, our conclusions still need to be further verified by well-designed and pregnant woman-based cohort studies.

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 Ethical approval was obtained from the Fujian Maternity and Child Health Hospital Ethics Committee (2020-2049). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

All authors contributed to manuscript editing and approved the final manuscript. JY contributed to the study design. The analysis was made by XX and FH. XX and FH drafted the manuscript. YG and LZ contributed to data collection.

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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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Serum anti-Müllerian hormone levels are associated with perinatal outcomes in women undergoing IVF/ICSI: A multicenter retrospective cohort study

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Introduction: Anti-Müllerian hormone (AMH) level has long been considered as a serum biomarker of ovarian reserve clinically, while emerging data suggest that serum AMH level may also predict pregnancy outcomes. However, whether pregestational serum AMH levels are related to perinatal outcomes among women undergoing *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles is unknown.

Objective: To explore the association between different AMH levels and perinatal outcomes in women with live births in IVF/ICSI.

Methods: This multicenter retrospective cohort study was conducted among three different provinces in China, from January 2014 to October 2019. A total of 13,763 IVF/ICSI cycles with 5657 live-delivery pregnant women and 6797 newborns were recruited. Participants were categorized into three groups according to the <25th (low), 25 to 75th (average), and >75th (high) percentile of serum AMH concentration. Perinatal outcomes were compared among groups. Subgroup analyses were conducted based on the number of live births.

Results: Among women with singleton deliveries, low and high AMH levels increased the risk of intrahepatic cholestasis of pregnancy (ICP) (aOR1 = 6.02, 95%CI: 2.10-17.22; aOR2 = 3.65, 95%CI:1.32-10.08) and decreased the risk of macrosomia (aOR1 = 0.65, 95%CI:0.48-0.89; aOR2 = 0.72, 95%CI:0.57-0.96), while low AMH reduced the risk of large for gestational age (LGA, aOR=0.74, 95% CI:0.59-0.93) and premature rupture of membrane (PROM, aOR=0.50, 95% CI:0.31-0.79)compared with the average AMH group. In women with multiple deliveries, high AMH levels increased the risks of gestational diabetes mellitus (GDM, aOR=2.40, 95%CI:1.48-3.91) and pregnancy-induced hypertension (PIH,

aOR=2.26, 95%CI:1.20-4.22) compared with the average AMH group, while low AMH levels increased the risk of ICP (aOR=14.83, 95%CI:1.92-54.30). However, there was no evidence of differences in preterm birth, congenital anomaly, and other perinatal outcomes among the three groups in both singleton and multiple deliveries.

Conclusions: Abnormal AMH levels increased the risk of ICP regardless of the number of live births for women undergoing IVF/ICSI, while high AMH levels increased the risks of GDM and PIH in multiple deliveries. However, serum AMH levels were not associated with adverse neonatal outcomes in IVF/ICSI. The underlying mechanism warrants further investigation.

KEYWORDS

anti-Müllerian hormone, *in vitro* fertilization, intracytoplasmic sperm injection, perinatal outcomes, intrahepatic cholestasis of pregnancy, gestational diabetes mellitus, pregnancy-induced hypertension

Introduction

Anti-Müllerian hormone (AMH), mostly secreted by granulosa cells of preantral and early antral follicles, is a dimeric glycoprotein belonging to the family of transforming growth factor beta (TGF-β) (1, 2). During follicular development, AMH can inhibit the recruitment of initial follicles as well as participate in the regulation of follicular selection (3, 4). Lines of evidence demonstrated serum AMH is linearly related to the number of developing follicles as well as remaining relatively stable during the menstrual cycle. Thus, AMH is widely used as a serum marker of ovarian reserve *in vitro* fertilization (IVF) (1, 5, 6). However, the relationship of AMH to the quality of the oocyte pool and pregnancy outcomes remains unclear (7).

The interest in the impact of serum AMH levels on pregnancy outcomes has emerged in the last few years. Despite several retrospective cohorts pointing to serum AMH as a weak predictor of live birth after assisted reproductive technology (ART) (low AMH level is associated with decreased live birth), only a few studies focus on pregnancy complications and neonatal outcomes (8, 9). A cohort study based on the serum AMH collected in the first trimester has demonstrated that low maternal level of AMH is a predictor of pregnancy-induced hypertension (PIH) in naturally conceived women, while associations in other complications included gestational diabetes (GDM), preterm birth and small for gestational age (SGA) were not identified (10). It is interesting to note that recent studies have reported a significant association between AMH and preterm delivery in patients with polycystic ovarian syndrome (PCOS) after IVF (11, 12), suggesting its potential to be a marker of preterm delivery.

Considering the discrepancies and limited sample size, we want to elucidate if AMH is related to pregnancy outcomes, especially in women conceived with ART. ART has been increasingly used for infertile couples thanks to the advances in technology and provision of services, resulting in more than 300 thousand infants born through it each year in China (13). While ART affords patients the opportunity to have biologically-related children, potential risks including GDM, PIH, preterm birth and low-birth-weight (LBW) exist as results of the laboratory procedures and genetic background (14–18). Given the general use of AMH to assess ovarian reserve before ART, we hope the test will be given new insights as a marker of perinatal outcomes in specific aspects.

To further analyze the effect of AMH on adverse perinatal outcomes among ART pregnancies, we conducted a multi-center retrospective cohort study of women who underwent IVF/intracytoplasmic sperm injection (ICSI) cycles in different AMH groups.

Methods

Study design and participants

This retrospective, multi-center cohort study was conducted on women who underwent IVF/ICSI cycles and achieved live births from January 2014 to October 2019 in three study centers among different provinces in China, including International Peace Maternity and Child Health Hospital (Shanghai), Ningbo Women and Children's Hospital (Zhejiang Province), Suzhou Municipal Hospital (Jiangsu Province). The study was approved by the research ethics board of each center and written informed consent forms (ICFs) were obtained from all the participants before inclusion.

Subjects were identified from the database in three centers from January 2014 to October 2019 using the following inclusion and exclusion criteria. The inclusion criteria were set as follows: 1) female participants aged between 20 and 45 years, 2) participants with serum AMH measurement within 12 months before undergoing IVF/ICSI cycles. The participants were excluded if they met the following criteria: 1) participants who underwent

pre-implantation genetic testing (PGT), 2) participants using donor semen or donor oocyte, 3) mixed transfers with embryos retrieved from different oocyte retrieval cycles, 4) women with severe chronic diseases, 5) women for whom main data were missing or who were lost to follow-up. The participants were categorized into three groups according to the <25th(low), 25th to 75th(average), and >75th(high) percentile of serum AMH concentration (0.01-1.76, 1.76-5.41, 5.41-25.00ng/ml). The subgroup analysis was conducted based on the number of live births.

AMH measurement

Serum samples were collected from all participants and measured directly after arriving in the laboratory. In two of our study centers, the serum AMH was measured with chemiluminescent immunoassay (CLIA) by Kaeser 1000 chemiluminescence analyzer of Guangzhou Kangrun Biotechnology Co., Ltd. and its corresponding kit according to the manufacturer's instructions. The intra-assay and inter-assay coefficient of the variation (CV%) was <8% and <15%. The limit of detection (LoD) was <0.06 ng/ml. And in the other study center, the electrochemiluminescence method with DXI800 chemiluminescence analyzer of Beckman Company and its corresponding kit was adopted for AMH measurement. The total CV% was <8% in the analytical measure range of 0.02 to 24 ng/ml, and the limit of detection was 0.02 ng/ml.

IVF/ICSI procedures

The process of IVF or ICSI was conducted according to the standard protocols of our study centers. We performed different types of controlled ovarian hyperstimulation (COH) protocols (gonadotropin-releasing hormone (GnRH)-agonist protocol, GnRH-antagonist protocol, micro-flare protocol or others) according to the state of each patient (age, ovarian reserve and others). After COH, when the leading follicle reached 20mm in diameter or at least two follicles reached 18 mm, ovulation was induced by giving human chorionic gonadotropin (HCG) or gonadotropin-releasing hormone agonists (GnRH-a). Oocyte retrieval was performed 34-38 hours later and oocytes were fertilized by either conventional IVF or intracytoplasmic sperm injection after the assessment of semen quality. Subsequently, viable embryos were transferred in fresh embryo transfer cycles or frozen-thawed embryo transfer (FET) cycles after oocyte retrieval and routine corpus luteum support was performed after transplantation if conceived.

Outcome measurements

Maternal baseline information was derived from the electronic database of the hospitals, including sociodemographic characteristics and reproductive history. We further abstracted the ART procedures and most of the perinatal outcomes from the database of the hospitals, while the neonatal morbidity and mortality were followed up and recorded by well-trained clinical personnel. The pregnancy outcomes assessed included hypertensive disorders in pregnancy (HDP), GDM, Intrahepatic cholestasis of pregnancy (ICP), placental abruption, placenta previa, oligohydramnios, premature rupture of membrane (PROM), postpartum hemorrhage (PPH) and mode of delivery. While neonatal outcomes were assessed including the gender of neonates, birth weight, preterm birth (PTB), weight for gestational age, neonatal infection, admission to the neonatal intensive care unit (NICU), neonatal asphyxia, neonatal jaundice, and congenital anomaly. Preterm birth was defined as delivery at less than 37 weeks, and very preterm was defined as delivery of baby between 28 and 32 gestational weeks of pregnancy. LGA or SGA was defined as a birth weight more than 90th centile or less than 10th centile of our population for a specific gestational age and sex, respectively (19, 20). Diagnoses were coded according to the International Classification of Diseases version 10(ICD-10).

Statistical analysis

Continuous variables were presented as mean (standard deviation (SD)) or median (inter-quartile range) as appropriate. Comparisons of the continuous variables among three AMH groups were performed with the use of the Analysis of Variance (ANOVA) test or Kruskal-Wallis test. Categorical variables were represented as frequencies with proportions, while the Pearson Chi-square test or Fisher's exact test was used to compare the distribution of demographics between categorical variables. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using logistic regression to evaluate the association between serum AMH levels and each perinatal outcome following IVF/ICSI. To analyze the pregnancy and neonatal outcomes in singleton pregnancies, multinomial logistic regression was used to adjust ORs for potential confounding factors. While analyzing the neonatal outcomes of multiples, we performed multilevel logistic regression and adjusted for potential confounding factors (21). Those factors were selected according to baseline analysis and published literature.

The statistical analyses were performed using R software version 3.4.4 (R Foundation for Statistical Computing, Vienna, Austria). All of the statistical analyses were two-sided with a 5% level of significance.

Results

The flowchart of the study cohort was shown in Figure 1. A total of 13,763 cycles met the eligibility criteria and were included in the cohort (3440 cycles in the low AMH group, 6882 cycles in the average AMH group, and 3441 cycles in the high AMH group). 5657 women with live-born babies (6797 live births with 4519 singletons and 1138 multiples) were further included in the analysis of perinatal outcomes.

The baseline characteristics of the participants with live birth deliveries stratified by AMH levels were presented in Table 1. Socio-demographic characteristics including pre-gestational BMI,

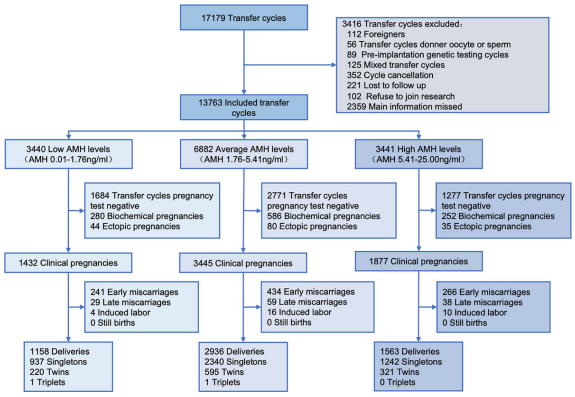

FIGURE 1
Flow chart of the study cohort.

TABLE 1 Baseline characteristics of participants with live birth deliveries according to AMH levels.

	Singleton delivery				Multiple deliveries			
	Low AMH (N=937)	Average AMH (N=2340)	High AMH (N=1242)	*P* value	Low AMH (N=221)	Average AMH (N= 596)	High AMH (N=321)	*P* value
	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	
Socio-demographic characteristics								
Maternal age (years)	32.18±4.02	30.58±3.74	29.64±3.37	<0.001	31.45±3.53	29.68±3.36	29.89±3.40	<0.001
Paternal age (years)	33.78±5.29	32.25±4.78	31.24±4.38	<0.001	32.50±4.35	31.47±4.40	31.15±4.10	<0.001
Pre-gestational BMI (kg/m2)	22.02±2.95	21.98±3.00	22.04±3.06	0.897	22.29±3.27	22.06±2.84	22.05±2.99	0.814
Race								
Han	859 (98.5)	2153 (99.6)	1159 (99.7)	<0.001	206 (99.5)	562 (99.6)	304 (99.7)	0.956
Minority	13 (1.5)	9 (0.4)	3 (0.3)		1 (0.5)	2 (0.4)	1 (0.3)	
Residence								
Residents	830 (88.6)	2042 (87.3)	999 (80.4)	<0.001	194 (87.8)	523 (87.8)	249 (77.6)	<0.001
Immigrants/ Nonresidents	107 (11.4)	298 (12.7)	243 (19.6)		27 (12.2)	73 (12.2)	72 (22.4)	
Education attainment								
Primary school or lower	21 (2.2)	37 (1.6)	15 (1.2)	0.415	4 (1.8)	9 (1.5)	5 (1.6)	0.98
Middle or high school	365 (39)	907 (38.8)	474 (38.3)		82 (37.4)	212 (35.8)	113 (35.2)	
Collage or above	550 (58.8)	1391 (59.6)	750 (60.5)		133 (60.7)	372 (62.7)	203 (63.2)	
Occupation								
Employed	629 (71.2)	1514 (70.8)	709 (69.1)	0.838	154 (72.3)	379 (69.5)	179 (70.8)	0.055
Self-employed	115 (13)	290 (13.6)	147 (14.3)		35 (16.4)	69 (12.7)	25 (9.9)	
Unemployed	139 (15.7)	333 (15.6)	170 (16.6)		24 (11.3)	97 (17.8)	49 (19.4)	

(Continued)

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TABLE 1 Continued

	Singleton delivery				Multiple deliveries			
	Low AMH (N=937)	Average AMH (N=2340)	High AMH (N=1242)	*P* value	Low AMH (N=221)	Average AMH (N= 596)	High AMH (N=321)	*P* value
	n (%)	n (%)	n (%)		n (%)	n (%)	n (%)	
Smoking status								
No	75 (97.4)	240 (98.8)	251 (98.8)	0.625	12 (92.3)	59 (98.3)	80 (98.8)	0.288
Yes	2 (2.6)	3 (1.2)	3 (1.2)		1 (7.7)	1 (1.7)	1 (1.2)	
History of reproduction								
Parity								
0	473 (50.5)	1214 (51.9)	713 (57.4)	<0.001	128 (57.9)	383 (64.3)	211 (65.7)	0.236
1	223 (23.8)	609 (26.0)	302 (24.3)		57 (25.8)	120 (20.1)	70 (21.8)	
≥2	241 (25.7)	517 (22.1)	227 (18.3)		36 (16.3)	93 (15.6)	40 (12.5)	
Gravidity								
0	816 (87.1)	2106 (90)	1168 (94)	<0.001	207 (93.7)	569 (95.5)	305 (95.0)	0.673
1	114 (12.2)	218 (9.3)	69 (5.6)		14 (6.3)	25 (4.2)	15 (4.7)	
2	7 (0.7)	16 (0.7)	5 (0.4)		0 (0)	2 (0.3)	1 (0.3)	
Number of previous abortions								
0	606 (64.7)	1519 (64.9)	848 (68.3)	0.239	151 (68.3)	435 (73.0)	230 (71.7)	0.353
1-2	300 (32.0)	744 (31.8)	363 (29.2)		66 (29.9)	144 (24.2)	86 (26.8)	
≥3	31 (3.3)	77 (3.3)	31 (2.5)		4 (1.8)	17 (2.8)	5 (1.6)	
Previous ectopic pregnancy								
No	763 (81.4)	1917 (81.9)	1041 (83.8)	0.262	187 (84.6)	513 (86.1)	292 (91.0)	0.048
Yes	174 (18.6)	423 (18.1)	201 (16.2)		34 (15.4)	83 (13.9)	29 (9.0)	
Duration of infertility (years)								
1-2	265 (28.5)	642 (27.7)	349 (28.4)	0.015	56 (25.9)	175 (29.5)	79 (24.8)	0.588
3-4	287 (30.9)	851 (36.7)	447 (36.3)		83 (38.4)	223 (37.6)	125 (39.2)	
≥5	378 (40.6)	826 (35.6)	434 (35.3)		77 (35.6)	195 (32.9)	115 (36.1)	
Primary infertility								
No	464 (49.5)	1126 (48.1)	529 (42.6)	0.001	93 (42.1)	213 (35.7)	110 (34.3)	0.149
Yes	473 (50.5)	1214 (51.9)	713 (57.4)		128 (57.9)	383 (64.3)	211 (65.7)	
Causes of infertility								
Tubal infertility	262 (28.1)	563 (24.2)	289 (23.4)	<0.001	82 (37.3)	131 (22.2)	78 (24.4)	<0.001
PCOS	10 (1.1)	35 (1.5)	128 (10.4)		0 (0)	10 (1.7)	34 (10.6)	
Anovulation (not PCOS)	14 (1.5)	25 (1.1)	23 (1.9)		6 (2.7)	7 (1.2)	12 (3.8)	
Endometriosis	53 (5.7)	130 (5.6)	49 (4.0)		6 (2.7)	26 (4.4)	14 (4.4)	
Male-factor infertility	100 (10.7)	434 (18.7)	166 (13.4)		19 (8.6)	107 (18.1)	47 (14.7)	
Unexplained infertility	12 (1.3)	29 (1.2)	6 (0.5)		1 (0.5)	7 (1.2)	0 (0)	
Combined	483 (51.7)	1111 (47.7)	575 (46.5)		106 (48.2)	303 (51.3)	135 (42.2)	

AMH, anti-müllerian hormone; BMI, body mass index; PCOS, polycystic ovary syndrome. Variables containing missing data were retained in the analyses.

education attainment, occupation and smoking status were similar among the three groups. However, the distribution of maternal age, paternal age and residence were different among groups (p<0.001). Significant differences were found in the race only in singleton delivery (p<0.001). Differences in the reproductive history of the participants were found in the parity, gravidity, duration of infertility, primary infertility, and causes of infertility (p<0.05), while no statistically significant differences were found between levels of AMH regarding times of abortion and history of ectopic pregnancy. In women with multiple deliveries, the history of ectopic pregnancy and causes of infertility were different among groups, the history of ectopic pregnancy is more frequent in women with low AMH levels. Additionally, gravidity, parity, times of abortion, and duration of infertility were comparable among the three groups. Characteristics of ART procedures (oocyte retrieval and embryo transfer cycles) according to AMH levels were presented in Table S1.

The pregnancy outcomes of different groups stratified by serum AMH levels were shown in Figure 2, we presented the crude and adjusted odds ratios assessing the risks in the low AMH group and high AMH group compared with the average AMH group. After adjusting for confounding factors in logistic regression analyses, an increased risk of ICP was found to be associated with low and high levels of AMH in singleton delivery (aOR1 = 6.02, 95%CI: 2.10-17.22; aOR2 = 3.65, 95%CI: 1.32-10.08). In multiple deliveries, the low AMH group was also found to have an increased risk of ICP compared with the average AMH group (aOR1 = 14.83, 95%CI: 1.92-54.30). In addition, low levels of AMH compared to average levels of AMH were associated with a lower risk of PROM in women with singleton delivery (aOR1 = 0.50, 95%CI:0.31-0.79). Although not found in singleton delivery, high levels of AMH were associated with a higher risk of gestational diabetes mellitus and gestational hypertension in multiple deliveries (gestational diabetes mellitus: aOR2 = 2.40, 95%CI:1.48-3.91; gestational hypertension: aOR2 = 2.26, 95%CI: 1.20-4.22), while low levels of AMH were also associated with increased risk of oligohydramnios in women with multiple deliveries compared to average levels of AMH (aOR1 = 37.75, 95%CI: 5.17-145.24). There were no significant differences in risks regarding other pregnancy outcomes among the three groups.

Figure 3 presented neonatal outcomes among three groups of serum AMH in women with singleton delivery and multiple deliveries. In singleton delivery, an decreased risk of macrosomia was found in the low AMH group compared with the average AMH group (aOR1 = 0.65, 95%CI: 0.48-0.89), while the high AMH group showed a similar effect (aOR2 = 0.72, 95%CI: 0.57-0.96). Additionally, there was an decreased risk of large for gestation age (LGA) in a group with lower levels of AMH compared with the average AMH group (aOR1 = 0.74, 95%CI: 0.59-0.93). There was no evidence of differences in preterm birth, congenital anomaly, and other neonatal complications among the three groups in both singleton delivery and multiple deliveries.

Further analyses were conducted in women with single embryo transfer and singleton live birth delivery. A total of 1985 cycles were included (341 fresh transfer cycles and 1644 frozen transfer cycles). The baseline and characteristics of ART procedures in fresh/frozen single embryo transfer cycles according to AMH levels were

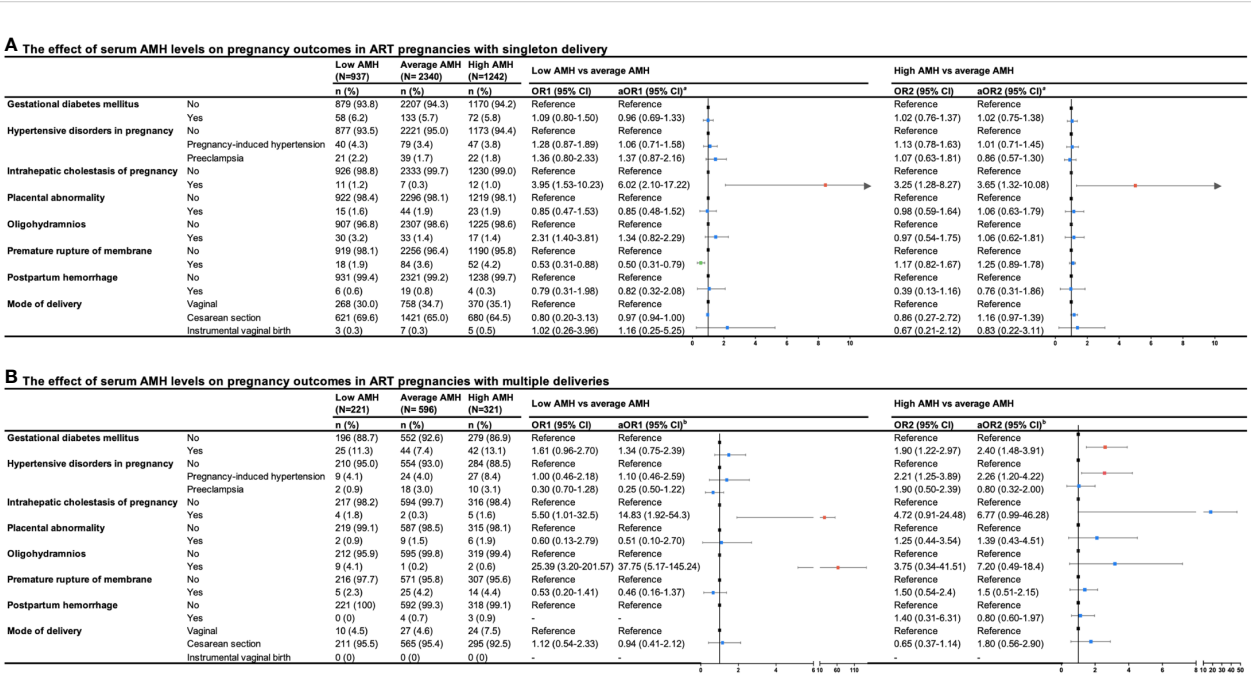

FIGURE 2
Forest plot summary of logistic regression analysis for risks of pregnancy outcomes in ART pregnancies with **(A)** singleton and **(B)** multiple deliveries. OR, odd ration; CI, confidence interval; aOR, adjusted odds ratio. aaOR was adjusted maternal age, paternal age, race, residence, gravidity, parity, duration of infertility, primary infertility, causes of infertility, study center, controlled ovarian stimulation protocol, type of insemination, transfer cycle types, embryo types, number of embryos transferred. baOR was adjusted maternal age, paternal age, residence, gravidity, parity, primary infertility, causes of infertility, study center, controlled ovarian stimulation protocol, type of insemination, transfer cycle types, embryo types.

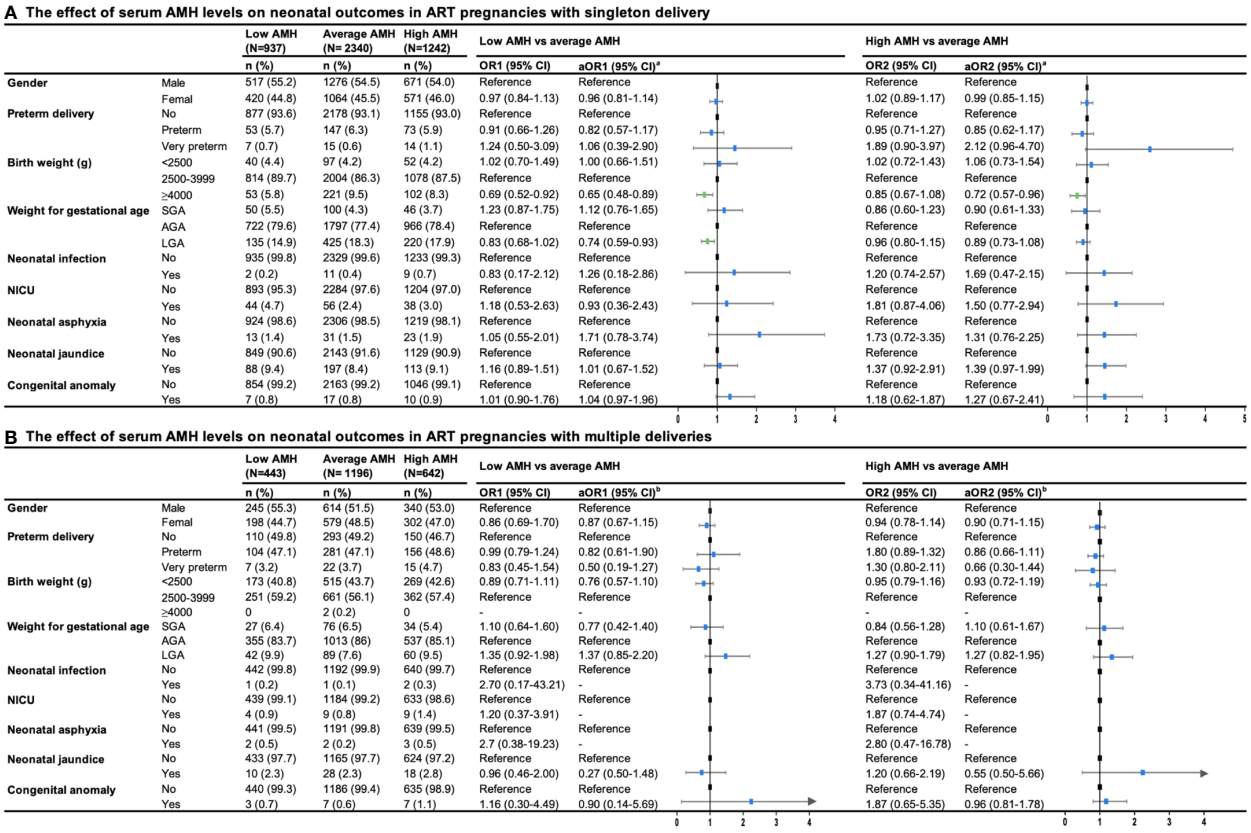

FIGURE 3
Forest plot summary of logistic regression analysis for risks of neonatal outcomes in ART pregnancies with **(A)** singleton and **(B)** multiple deliveries. OR, odds ration; CI, confidence interval; aOR, adjusted odds ration; SGA, small for gestational age; AGA, appropriate for gestational age; NICU, neonatal care unit. aaOR was adjusted for maternal age, paternal age, race, residence, gravidity, parity, duration of infertility, primary infertility, causes of infertility, study center, controlled ovarian stimulation protocol, type of insemination, transfer cycle types, embryo types, number of embryos transferred. baOR was adjusted maternal age, paternal age, residence, gravidity, parity, primary infertility, causes of infertility, study center, controlled ovarian stimulation protocol, type of insemination, transfer cycle types, embryo types.

provided in Tables S2, S3. The effect of serum AMH levels on pregnancy and neonatal outcomes with fresh/frozen single embryo transfers were generally consistent with those of the primary analysis in singleton delivery, except that the risk of GDM increased in the low AMH group with fresh cycles, the risk of cesarean section decreased in the high AMH group with frozen cycles and the difference in the risk of ICP, macrosomia and LGA was no longer significant. Details are provided in Tables S4 through S7 in the Supplementary Materials.

Discussion

In this multi-center retrospective cohort study of ART patients, we highlight in women with singleton delivery, low AMH levels increased the risk of ICP. There are also some protective factors, for instance, among women of singleton delivery, high AMH levels are associated with a lower risk of macrosomia as well as low levels of AMH are less likely to have PROM and LGA. Moreover, in women with multiple deliveries, we demonstrated that high levels of AMH increased the risk of ICP, GDM and PIH, while low AMH levels are associated with an increased risk of ICP and oligohydramnios. The findings of our study suggest an association between AMH and pregnancy outcomes among women undergoing IVF/ICSI.

The safety of ART procedures has long been a major concern among people who received the treatment, several meta-analyses of cohort studies have demonstrated adverse pregnancy outcomes among ART pregnancies, including GDM and PIH (16, 22). Although characteristics of infertility, advanced age and underlying polycystic ovary syndrome might result in confounders of the association, some prospective studies provide significant associations between ART and adverse pregnancy outcomes after adjusting for various confounders (23, 24). Thus, plasma markers as a screen for adverse outcomes are quite in need. Interestingly, AMH, a clinical marker of ovarian reserve, several studies have suggested its potential relation to specific pregnancy complications (such as preterm birth and PIH), while the relationship remains unclear concerning their limited sample size and conflicting results (10–12).

Transfer of multiple embryos in ART procedures used to bring a large number of multiple pregnancies and related risks in the last few years (25). Despite single-embryo transfer (SET) has been accepted as the best practice in clinical use, the ratio of twin delivery among total deliveries in ART was 27.9% in 2016

(Chinese mainland) (13). Our study demonstrated that high AMH levels increased the risk of PIH and GDM in multiple deliveries after ART (Gestational hypertension: aOR2 = 2.26, 95% CI: 1.20-4.22; Gestational diabetes mellitus: aOR2 = 2.40, 95% CI: 1.48-3.91), low AMH levels increase the risk of oligohydramnios. Nonetheless, we failed to observe a similar association in singleton deliveries. Hypertensive disorder of pregnancy, which affect up to 10% of all pregnancies, is one of the leading causes of pregnancy-related deaths (26, 27). The relationship between AMH and HDP has been a controversial topic in recent studies. A case-control study conducted by Birdir et al. observed the median multiple of the expected median value of AMH was comparable between the PE (Preeclampsia) group and the controls (1.040, IQR 0.941–1.081 versus 0.995, IQR 0.939–1.065, p = 0.147), indicating AMH might not be a suitable marker for prediction of PE (28). However, several studies have observed that low levels of AMH are associated with a higher risk of HDP (10, 29). As for GDM, the association between AMH and GDM was not identified in previous studies (10). In the present study, we measure maternal AMH levels before pregnancy instead of measurement during pregnancy in other studies, which might result in the discrepancy. In addition, previous studies have not performed similar research in multiple deliveries. Mechanisms underlying the effects on pregnancy complications need more investigation. Detection of AMH receptors in cardiac tissue suggests the linkage of AMH with the circulatory system (30). Skałba et al. (31) documented that plasma AMH level is associated with insulin resistance (IR) both in PCOS (group) and control group, while Tokmak et al. (32) proved a similar correlation in non-obese adolescent females with PCOS. Considering IR is closely related to the development of GDM, AMH might play a role in the development of GDM. In summary, this study suggests that we should put more attention to abnormal AMH levels in women with multiple pregnancies. More specifically, abnormal AMH levels should be concerned when we determine the number of embryos transferred, single-embryo transfer is relatively more recommended.

Our study illustrates the association between abnormal AMH levels and ICP for the first time (low AMH levels are associated with increased risk of ICP in singleton and multiple deliveries). ICP is the most common hepatic disorder related to pregnancy, which usually develops within the third trimester of pregnancy and presents with pruritus as well as elevated levels of bile acid and/or alanine aminotransferase (33). Estrogen-bile acid axis was thought to play a dominant role in the pathogenesis of ICP (34), yet AMH was proved to decrease FSH-induced CYP19a1 expression, leading to reduced estradiol (E2) levels (1, 35), we could assume that the association between AMH and E2 might attribute to the effects of AMH on the risk of ICP. While the molecular mechanisms need more investigations.

Notably, through the analysis of neonatal outcomes, we also observed that circulating levels of AMH influence the risk of macrosomia and LGA in singleton deliveries, indicating some underlying nutritional and metabolic alterations in the offspring. An increasing number of studies have supported the theory of developmental origins of health and disease (DOHaD), which refers to the theory that predisposing factors to chronic diseases are established in early life, specifically by the intrauterine environment (36). Both human and animal studies have confirmed that the developing fetus is susceptible to *in-utero* exposures, including air pollution, high-fat diet and hyperglycemia (37, 38). Additionally, recent studies also demonstrated that high AMH levels *in utero* might induce metabolic and reproductive alterations in rodent animals, which suggested the potential effects of AMH on perinatal outcomes (39). Our results also demonstrated that AMH levels are not associated with the risk of preterm birth in women undergoing IVF/ICSI, which is consistent with a previous study that is also based on women undergoing IVF/ICSI cycles (40). However, recent studies suggested AMH level as a risk factor of preterm birth in PCOS patients (11, 12). The differences might attribute to the heterogeneity of the population thanks to the higher AMH level in PCOS patients compared with non-PCOS patients (41). Future studies including long-term follow-up studies are needed to illustrate the long-term effects and potential mechanisms.

The strengths of our study include the novelty as the first research to present the association between maternal AMH levels and pregnancy outcomes after ART, as well as the size of the cohort (largest to our knowledge). Additionally, maternal levels of AMH before pregnancy give us a more advanced vision to assess the risk of complications compared with measurement in the first or second trimester of pregnancy. Moreover, in this retrospective cohort study, the confounding factors were also adjusted for analysis, either previously reported to have effects on AMH levels or varied significantly among groups stratified by AMH. Despite the limited knowledge of the pathophysiology of AMH, we provide distinctive insights on its potential to be a marker of pregnancy outcomes. Similarly, we recognize that there are still limitations in our study. First, missing data regarding clinical and follow-up information was inevitable thanks to the retrospective cohort, which resulted in information bias. Second, the discrepancy of AMH measurement methods in different centers is also a source of bias, although study center was adjusted as a confounding factor in the logistic regression. Third, the relatively low morbidity restricts us to achieve a more accurate confidence interval, thus leading to limitations in our conclusions.

In conclusion, this is the first multi-center retrospective cohort study to indicate the association between maternal AMH levels and adverse perinatal outcomes in IVF/ICSI. Our results proved the potential role of AMH as a predictive marker for adverse pregnancy outcomes. Abnormal AMH levels increased the risk of ICP regardless of the number of live births, while high AMH levels are associated with risks of GDM and PIH only in women with multiple deliveries. In addition, AMH can also be used as a protective factor concerning PROM, macrosomia and LGA. Fortunately, serum AMH levels were not associated with adverse neonatal outcomes in IVF/ICSI. The findings of our study will extend the application of AMH during pregnancy and provide clinicians with some clues for practice. The association between high AMH levels and pregnancy complications among multiple pregnancies also supports the use of SET in these 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

The studies involving human participants were reviewed and approved by The Institutional Review Board of the International Peace Maternal and Child Health Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

H-FH, Y-TW and Y-CH designed the study concept. Y-CH and K-ZS conducted the statistical analysis and drafted the manuscript. Y-CH, Y-TW, JC and Q-XM were responsible for data collection and data curation. H-FH and Y-TW critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1081069/full#supplementary-material

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Thyroid hormones and carnitine in the second trimester negatively affect neonate birth weight: A prospective cohort study

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Background: Maternal thyroid hormones and carnitine are reported to affect neonate birth weight during the second trimester, which is one of the most important markers for fetal growth and perinatal mortality and morbidity. Nevertheless, the effect of thyroid hormone and carnitine in the second trimester on birth weight has yet to be understood.

Method: This was a prospective cohort study with 844 subjects enrolled during the first trimester. Thyroid hormones, free carnitine (C0), neonate birth weight, as well as other related clinical and metabolic data were collected and assessed.

Results: Pre-pregnancy weight and body mass index (BMI) as well as neonate birth weight were significantly different among different free thyroxine (FT4) level groups. Maternal weight gain and neonate birth weight varied significantly when grouped by different thyroid-stimulating hormone (TSH) levels. There was a significantly positive correlation between C0 and TSH ($r = 0.31$), free triiodothyronine (FT3) ($r = 0.37$), and FT4 ($r = 0.59$) (all $P < 0.001$). In addition, a significantly negative influence was found between birth weight and TSH ($r = -0.48$, $P = 0.028$), so as C0 ($r = -0.55$, $P < 0.001$) and FT4 ($r = -0.64$, $P < 0.001$). Further assessment detected a stronger combined effect of C0 and FT4 ($P < 0.001$) and of C0 and FT3 ($P = 0.022$) on birth weight.

Conclusion: Maternal C0 and thyroid hormones are of great importance in neonate birth weight, and routine examination of C0 and thyroid hormones during the second trimester has a positive effect on the intervention of birth weight.

KEYWORDS

free carnitine, thyroid hormones, neonate birthweight, the second trimester, gestation

1 Introduction

Adequate thyroid hormones (THs) are crucial for the fetal growth and metabolism and play an important role in neurodevelopment (1, 2). The fetal thyroid grand starts to develop at 12th week of gestation and is functionally mature around the 18th to 20th week, whereas, for the first half of pregnancy, the fetus relies entirely on the supply of maternal TH through the transplacental passage (2–4). Abnormal maternal thyroid function during pregnancy is associated with adverse obstetrical and offspring outcomes, such as spontaneous abortion, anemia, preeclampsia, placental abruption, congenital anomalies, preterm birth and/or low birth weight, fetal distress in labor, stillbirth and/or perinatal death, and postpartum hemorrhage (3).

Carnitine exists as free carnitine (C0) and acylcarnitine fractions in blood and plays an important role in fatty acid oxidation during the gestational metabolism (4). Carnitine mainly comes from food, especially red meat, fish, and dairy products (5). Carnitine is critical for the transfer of activated long-chain fatty acids from the cytoplasm to the mitochondria for β-oxidation, resulting in the esterification of carnitine to form acylcarnitine derivatives (6). Some evidence suggests that carnitine deficiency is manifested in gestational diabetes mellitus (GDM), leading to the development of macrosomia and small for gestational age (SGA). Clinical studies showed that applying C0 (1 g/day) for a few weeks could relieve hyperthyroidism symptoms in patients. Carnitine was hypothesized to act in the periphery by antagonizing TH action. However, the correlation between C0 and THs in mid-pregnancy remains unknown.

Birth weight is one of the most important markers for fetal growth and development *in utero*, which reflects fetal adaptations to the intrauterine environment. SGA newborns have an increased risk of prenatal mortality (7), whereas large for gestational age (LGA) newborns have a higher risk of obesity and diabetes mellitus in later life (2). Various studies have shown that a higher maternal free thyroxine (FT4) level is associated with a lower birth weight (3, 8, 9). However, studies undertaken so far provide conflicting evidence concerning the impact of C0 on TH and birth weight.

In the present study, we investigated the associations between maternal serum thyroid parameters and carnitine-related metabolites during the second trimester of pregnancy. Furthermore, we examined whether the birth weight was modified by maternal serum TH and carnitine.

2 Material and methods

2.1 Study subjects

This was a prospective cohort study. Pregnant women who received regular perinatal healthcare in the Outpatient Department of the Women's Hospital School of Medicine Zhejiang University and delivered in the hospital between June 2017 and April 2019 were recruited. A total of 844 pregnant women with complete demographic and obstetric data were included for analysis. This study was approved by the Ethics Committee of Women's Hospital School of Medicine Zhejiang University.

All pregnant women were enrolled during the first trimester, all of whom had measured thyroid-stimulating hormone (TSH), FT4, free triiodothyronine (FT3), and total thyroxine (TT4) concentration in the second trimester, and neonatal birth weight data were available. Women with multiple pregnancies, accompanied with pregnancy complications such as abortion, GDM, and hypertensive disorders, using medication known to interfere thyroid hormones, or had a history of thyroid diseases were excluded.

Maternal clinical characteristics—including age, height, pre-pregnancy and prenatal weight and body mass index (BMI), gravity, parity, mode of delivery, educational level, and neonatal birth weight—were obtained from hospital information system and child care system.

The blood samples were collected after overnight fasting, and samples were centrifuged within 6 h. The concentrations of TSH, FT4, FT3, and TT4 were determined according to the measurement instructions.

2.2 Metabolic profiling detection by LC-MS/MS

We aim to investigate the 31-carnitine-related plasma metabolite level of pregnant women at the second trimester. We also obtain their neonate blood plasma sample. The blood samples were stored at −20°. Furthermore, the samples were prepared by tandem mass spectrometry (4000 QTrapTM; AB Sciex, Darmstadt, Germany) to test the concentration. The method used in the present study was essentially a modification of the procedure described elsewhere. Amino acid (AA) and acylarnitine (AC) were quantified using appropriate isotope-labeled standards. Liquid Chromatogram (LC) separation was performed on an Acquity UPLC HSS T3 column (2.1 × 100 mm, 100A°, 1.8-μm particle size; Waters Corporation, MA) using water with 0.1% formic acid (0.1% methanol and 5 mM ammonium acetate) detected with a Xevo-G2-QTOF MS (Waters Corporation) operating in a positive mode. Raw data were processed using TargetLynx as described previously. Accuracy of quantification was below 6% for all quantified metabolites except glutamic acid (13.9%). Quantitative data were obtained using MetIDQTM software.

2.3 Statistical analysis

The data were expressed as mean ± standard deviation (SD). The baseline characteristics of the subjects were described, and the p-values are indicated. Binary variables were presented as frequency and percentage and were compared using the Chi-squared test. A nonparametric test or a t-test was used to compare the medians of continuous variables. The heatmap was available from the package "ggplot" as an enhanced version or its basic function stats in R. We used the liner regression model, as well as multiple regressions to investigate the association of C0, FT3, FT4, TSH, and TT4 with birth weight. We assessed the combined effects of C0 and FT4 on birth weight by adding a product interaction term of the C0 × FT4 to the model. The same analysis was done on the effect of other hormone on birth weight. A heatmap was constructed to display the differences in

birth weight. All statistical analyses were performed using R statistical software version 3.4.1 (package rms, ggplot, visreg, and mass) or Statistical Package of Social Sciences version 20.0 for Windows (IBM Corp., Armonk, NY). In all analyses, $P < 0.05$ was considered statistically significant.

3 Results

3.1 Clinical characteristics of subjects grouped by thyroid hormone

The maternal characteristics grouped by FT4 quartile are shown in Table 1. The different ranges of FT4 are as follows: Q1: 8.33–9.72 pmol/L; Q2: 9.73–10.65 pmol/L; Q3: 10.66–11.57 pmol/L; and Q4: 11.58–15.06 pmol/L. We found that pre-pregnancy weight and BMI as well as weight gain were significantly different among the four groups with a higher level in the lower FT4–level group. We also found that compared with the higher FT4–level group, birth weight was significantly heavier in the lower groups. There were significantly statistically differences in height and gestational week at delivery among different groups, but no difference in maternal age, nulliparous rate, and ART rate among these four groups.

Table 2 presents clinical and biochemical characteristics of participants grouped by TSH. In the group with TSH < 2.5 mIU/L, pre-pregnancy BMI, weight gain, and birth weight were significantly higher; whereas, in the higher TSH–level group, pre-pregnancy weight and gestational week at delivery were higher. There was no difference in maternal age, nulliparous rate, and ART rate between the two groups.

3.2 Biochemical characteristics of subjects grouped by thyroid hormone

Among 31 carnitine-related metabolites, we selected the metabolites with statistically significant differences and listed them in Table 3. Our results showed that carnitine-related AA [alanine (ALA), tyrosine (TYR), and valine (VAL)], short-chain AC (C2, C3, C3DC+C4OH, and C5:1), medium-chain AC (C6DC and C12), and long-chain AC (C14, C16:1) were significantly different among the four groups. As the FT4 level increased, ALA, TYR, C14, and C16:1 decreased, whereas VAL, C0, C2, C3, C3DC+C4OH, C5, C6DC, and C12 increased. There were also statistical differences in LEU+ILE +PRO-OH, SA, C4, C8, C14OH, C16, C18, and C18OH among these four groups.

Carnitine-related metabolites grouped according to the TSH level are shown in Table 4. Compared with the lower TSH–level group, ALA and glycine (GLY) significantly decreased in the higher TSH–level group. LEU+ILE+PRO-OH, VAL, C0, C2, C3, C3DC+C4OH, C5, C6DC, C8:1, C10, C12, and C14 were significantly higher when TSH increased. There was a statistical difference in C4 between groups. On the basis of previous studies, C0 has a vital role in metabolism.

3.3 A clustering heatmap illustrating the relationship between thyroid hormones and carnitine metabolites

We used a clustering heatmap to describe the relationship between THs and 31 carnitine-related metabolites (Figure 1). In the row clustering step, pregnant women were grouped according to TH levels. We defined the low TH level and the high TH level as less than 10th centile and as more than 10th centile, respectively, of all participants' full range. In addition, every biomarker was clustered into different subgroups on the column side according to their color patterns in the center grids of heatmap. Red color indicates a high expression content, and blue color indicates a low expression content. The level of C0 is higher in the high FT4–level and high TSH–level groups. Heatmaps provided a systematic and clustered visualization of the analyzed data, facilitating monitoring of TH levels and carnitine-related metabolites.

TABLE 1 Clinical characteristics of pregnancy by FT4 quartile.

FT4 groups (pmol/L)	Q1 (n = 211)	Q2 (n = 211)	Q3 (n = 211)	Q4 (n = 211)	*P*
	8.33–9.72	9.73–10.65	10.66–11.57	11.58–15.06	
Maternal age (years)	31.48 ± 2.42	31.28 ± 2.62	31.65 ± 3.05	31.07 ± 2.79	0.149
Height (cm)	162.87 ± 5.49	159.65 ± 5.14	162.63 ± 2.27	160.44 ± 1.98	**<0.001**
Pre-pregnancy weight (kg)	65.58 ± 4.83	64.52 ± 3.91	63.48 ± 3.65	64.18 ± 2.97	**<0.001**
Pre-pregnancy BMI (kg/m^2)	22.65 ± 4.01	21.86 ± 3.74	21.24 ± 3.19	21.74 ± 3.26	**<0.001**
Weight gain (kg)	22.38 ± 4.21	21.65 ± 4.01	20.88 ± 2.45	19.78 ± 2.67	**<0.001**
Gestational week at delivery (weeks)	38.3 ± 0.67	38.4 ± 0.78	39.4 ± 0.72	38.7 ± 0.87	**<0.001**
Nulliparous (%)	166 (78.7)	164 (77.7)	167 (79.1)	165 (78.2)	0.987
ART (%)	13 (6.2)	12 (5.7)	9 (4.3)	12 (5.7)	0.843
Birth weight (g)	3633.23 ± 99.78	3527.79 ± 86.63	3404.56 ± 100.45	3217.93 ± 77.94	**<0.001**

Bold values are presented as mean ± SD or number (%).
FT4, free thyroxine; BMI, body mass index; ART, artificial reproductive technology.

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TABLE 2 Clinical and biochemical characteristics of pregnancy by TSH.

TSH groups (mIU/L)	TSH<2.5 (n = 762)	TSH ≥ 2.5 (n = 82)	*P*
Maternal age (years)	31.18 ± 2.42	31.65 ± 2.05	0.091
Height (m)	163.27 ± 5.49	162.63 ± 3.72	0.3032
Pre-pregnancy weight (kg)	63.58 ± 4.83	65.48 ± 3.65	**0.001**
Pre-pregnancy BMI (kg/m^2)	22.65 ± 4.01	21.24 ± 3.19	**0.002**
Weight gain (kg)	21.38 ± 4.21	18.23 ± 2.45	**<0.001**
Gestational week at delivery (weeks)	38.3 ± 0.67	39.4 ± 0.72	**<0.001**
Nulliparous (%)	674 (88.5)	73 (89.02)	0.877
ART (%)	73 (9.6)	4 (4.9)	0.160
Birth weight (g)	3543.23 ± 99.78	3304.56 ± 100.45	**<0.001**

Bold values are presented as mean ± SD or number (%).
TSH, thyroid-stimulating hormone; BMI, body mass index; ART, artificial reproductive technology.

TABLE 3 Carnitine-related metabolites grouped by FT4 quartile (n = 844).

AA and AC profiles (μmol/L)	Q1	Q2	Q3	Q4	*P*
ALA	399.64 ± 21.46	368.87 ± 35.12	343.56 ± 24.14	322.77 ± 28.62	**<0.001**
ARG	9.16 ± 2.29	8.74 ± 2.94	8.76 ± 1.82	8.81 ± 2.38	0.231
GLY	225.64 ± 25.11	226.66 ± 25.32	224.28 ± 18.34	227.45 ± 28.40	0.580
LEU+ILE+PRO-OH	135.38 ± 24.42	134.16 ± 31.72	130.85 ± 23.85	139.89 ± 23.96	**0.005**
MET	10.47 ± 3.32	10.06 ± 3.15	10.81 ± 2.87	10.42 ± 2.85	0.095
ORN	52.63 ± 12.76	52.86 ± 12.16	52.33 ± 11.44	53.37 ± 12.99	0.850
PHE	48.76 ± 8.49	48.65 ± 9.84	49.03 ± 8.43	49.93 ± 7.88	0.420
PRO	85.74 ± 16.32	86.21 ± 17.71	83.31 ± 14.06	86.34 ± 14.82	0.166
SA	0.77 ± 0.14	0.79 ± 0.13	0.78 ± 0.11	0.81 ± 0.12	**0.009**
TYR	41.73 ± 9.16	39.73 ± 7.86	37.63 ± 4.65	35.63 ± 4.16	**<0.001**
VAL	118.72 ± 17.35	123.72 ± 18.15	125.15 ± 19.27	127.20 ± 29.27	**0.001**
C0	16.35 ± 3.39	18.00 ± 2.95	20.51 ± 2.68	22.35 ± 3.62	**<0.001**
C2	2.67 ± 0.82	2.86 ± 0.76	3.04 ± 0.78	3.14 ± 0.84	**<0.001**
C3	0.30 ± 0.18	0.42 ± 0.14	0.50 ± 0.21	0.60 ± 0.19	**<0.001**
C3DC+C4OH	0.23 ± 0.12	0.28 ± 0.11	0.33 ± 0.09	0.45 ± 0.14	**<0.001**
C4	0.09 ± 0.01	0.08 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	**<0.001**
C5	0.12 ± 0.01	0.23 ± 0.09	0.27 ± 0.10	0.34 ± 0.17	**<0.001**
C5DC+C6OH	0.38 ± 0.12	0.36 ± 0.13	0.39 ± 0.11	0.38 ± 0.13	0.085
C6	0.018 ± 0.011	0.017 ± 0.012	0.017 ± 0.011	0.018 ± 0.013	0.678
C6DC	0.018 ± 0.012	0.21 ± 0.17	0.28 ± 0.11	0.36 ± 0.13	**<0.001**
C8	0.50 ± 0.13	0.49 ± 0.18	0.56 ± 0.19	0.54 ± 0.16	**<0.001**
C8:1	0.063 ± 0.022	0.060 ± 0.025	0.062 ± 0.028	0.062 ± 0.024	0.654
C10	0.056 ± 0.019	0.056 ± 0.022	0.058 ± 0.021	0.059 ± 0.020	0.337
C12	0.12 ± 0.182	0.14 ± 0.038	0.16 ± 0.034	0.18 ± 0.056	**<0.001**
C14	0.28 ± 0.045	0.26 ± 0.052	0.24 ± 0.048	0.23 ± 0.092	**<0.001**
C14OH	0.014 ± 0.0055	0.012 ± 0.0052	0.013 ± 0.0058	0.013 ± 0.0064	**0.005**

(Continued)

TABLE 3 Continued

AA and AC profiles (μmol/L)	Q1	Q2	Q3	Q4	*P*
C16	0.023 ± 0.012	0.026 ± 0.013	0.026 ± 0.011	0.024 ± 0.015	**0.035**
C16:1	0.22 ± 0.056	0.20 ± 0.034	0.18 ± 0.067	0.13 ± 0.033	**<0.001**
C18	0.57 ± 0.20	0.53 ± 0.22	0.59 ± 0.24	0.56 ± 0.21	**0.040**
C18:1	0.55 ± 0.17	0.54 ± 0.18	0.53 ± 0.12	0.56 ± 0.22	0.335
C18OH	0.015 ± 0.0047	0.016 ± 0.0043	0.012 ± 0.0069	0.015 ± 0.0026	**<0.001**

Bold values are presented as mean ± SD.
FT4, free thyroxine; AA, amino acid; AC, acylarnitine; ALA, alanine; ARG, arginine; GLY, glycine; LEU, leucine; ILE, isoleucine; MET, methionine; ORN, ornithine; PHE, phenylalanine; PRO, proline; SA, salicylic acid; TYR, tyrosine; VAL, valine.

TABLE 4 Thirty-one carnitine-related metabolites by TSH (n = 844).

AA and AC profiles (μmol/L)	TSH<2.5	TSH ≥ 2.5	*P*
ALA	378.64 ± 35.52	367.89 ± 41.67	**0.011**
ARG	8.93 ± 2.52	8.87 ± 3.01	0.841
GLY	0.81 ± 0.10	0.64 ± 0.12	**<0.001**
LEU+ILE+PRO-OH	35.63 ± 4.16	41.73 ± 9.16	**<0.001**
MET	10.56 ± 3.44	10.72 ± 3.56	0.690
ORN	53.65 ± 11.64	54.06 ± 12.08	0.763
PHE	48.78 ± 6.72	49.23 ± 7.78	0.571
PRO	86.34 ± 12.29	85.89 ± 15.89	0.760
SA	0.76 ± 0.15	0.77 ± 0.18	0.574
TYR	38.87 ± 6.87	39.12 ± 7.08	0.755
VAL	118.72 ± 17.35	127.20 ± 29.27	**<0.001**
C0	16.35 ± 3.62	20.00 ± 3.39	**<0.001**
C2	2.78 ± 0.62	3.41 ± 0.78	**<0.001**
C3	0.32 ± 0.18	0.62 ± 0.21	**<0.001**
C3DC+C4OH	0.03 ± 0.011	0.05 ± 0.013	**<0.001**
C4	0.09 ± 0.02	0.08 ± 0.02	**<0.001**
C5	0.02 ± 0.015	0.04 ± 0.014	**<0.001**
C5DC+C6OH	0.39 ± 0.13	0.37 ± 0.14	0.189
C6	0.017 ± 0.011	0.018 ± 0.013	0.443
C6DC	0.32 ± 0.12	0.44 ± 0.124	**<0.001**
C8	0.52 ± 0.19	0.50 ± 0.16	0.359
C8:1	0.38 ± 0.027	0.45 ± 0.056	**<0.001**
C10	0.37 ± 0.062	0.46 ± 0.041	**<0.001**
C12	0.31 ± 0.031	0.42 ± 0.023	**<0.001**
C14	0.28 ± 0.025	0.33 ± 0.032	**<0.001**
C14OH	0.014 ± 0.0054	0.015 ± 0.0048	0.108
C16	0.026 ± 0.013	0.025 ± 0.012	0.505
C16:1	0.19 ± 0.064	0.20 ± 0.058	0.175
C18	0.58 ± 0.19	0.60 ± 0.21	0.370

(Continued)

TABLE 4 Continued

AA and AC profiles (μmol/L)	TSH<2.5	TSH ≥ 2.5	*P*
C18:1	0.56 ± 0.17	0.57 ± 0.15	0.609
C18OH	0.014 ± 0.0045	0.014 ± 0.0039	0.999

Bold values are presented as mean ± SD.
TSH, thyroid-stimulating hormone; AA, amino acid; AC, acylarnitine; ALA, alanine; ARG, arginine; GLY, glycine; LEU, leucine; ILE, isoleucine; MET, methionine; ORN, ornithine; PHE, phenylalanine; PRO, proline; SA, salicylic acid; TYR, tyrosine; VAL, valine.

3.4 Relationship between C0 and TSH, FT3, FT4, and TT4

The relationships between C0 and TSH, FT3, FT4, and TT4 of all pregnant women are shown in Figure 2. We observed that C0 was positively correlated with TSH (r = 0.31, $P < 0.001$), FT3 (r = 0.37, $P < 0.001$), and FT4 (r = 0.59, $P < 0.001$). There was no significant correlation between C0 and TT4.

3.5 Effects of C0, TSH, FT3, FT4, and TT4 on birth weight

In the second trimester, a higher TSH (r = −0.48, P = 0.028) level was associated with a lower birth weight (Figure 3D). There was also a negative association between C0 (r = −0.55, P < 0.001) and FT4 (r = −0.64, P < 0.001) and birth weight (Figures 3A, B). There was no statistically significant difference between TT4 and birth weight (Figures 3C, E).

Linear multiple regression model demonstrated that TSH, FT4, and C0 were negatively associated with birth weight. The birth weight decreased by 12.079 g (95% CI: −18.642, −8.532), by 29.203 g (95% CI: −33.149, −15.511), and by 21.079 g (95% CI: −29.842, −13.316) for every unit increase in C0, FT4, and TSH concentrations, respectively (Table 5).

3.6 Combined effects of C0 and TSH, FT3, FT4, or TT4 on birth weight

We found that the level of TSH, FT3, FT4, and C0 in the second trimester would have greater effects on birth weight. Meanwhile, we found a relationship between C0 and thyroid function parameters. Therefore, we assessed the combined effects of C0 and THs, including TSH, FT3, FT4, and TT4, on birth weight. Figure 4 shows the heatmaps (fulfilled contour plot) for the combined association of C0 (x-axis) and TSH, FT4, or FT3 in the second trimester (y-axis) with birth weight (z-axis: red indicates higher birth weight, and blue indicates lower birth weight). For the low level of C0, the birth weight will be decreased, with a gradual increase in the FT4/FT3 level. For the high level of C0, the effect on birth weight was more obvious. When carnitine is increased to an appropriate level and the thyroid concentration is high (FT4: 18.4 pmol/L; C0: 17.5 μmol/L; FT3: 4.3 pmol/L; and C0: 16.3 μmol/L), the birth weight would be at the average level. When the FT4//FT3 level is high, even if the carnitine level decreases, the birth weight may remain at the present level. Free thyroxine (FT4/FT3) may play a greater role in regulating birth

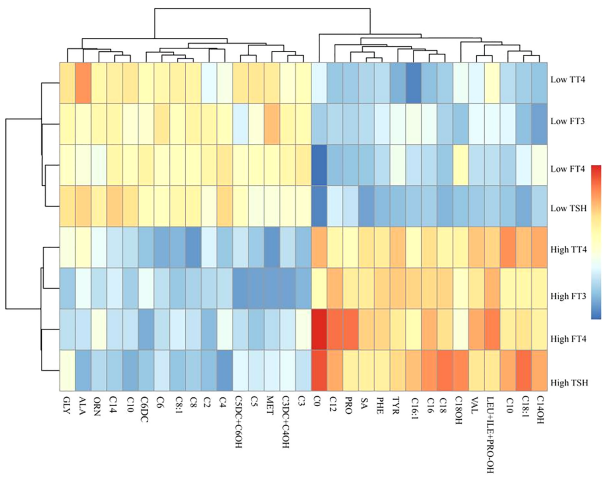

FIGURE 1
A clustering heatmap illustrating the classification between carnitine-related metabolites and thyroid hormones. Both the rows of thyroid hormones and the columns of carnitine-related metabolites have been clustered, respectively.

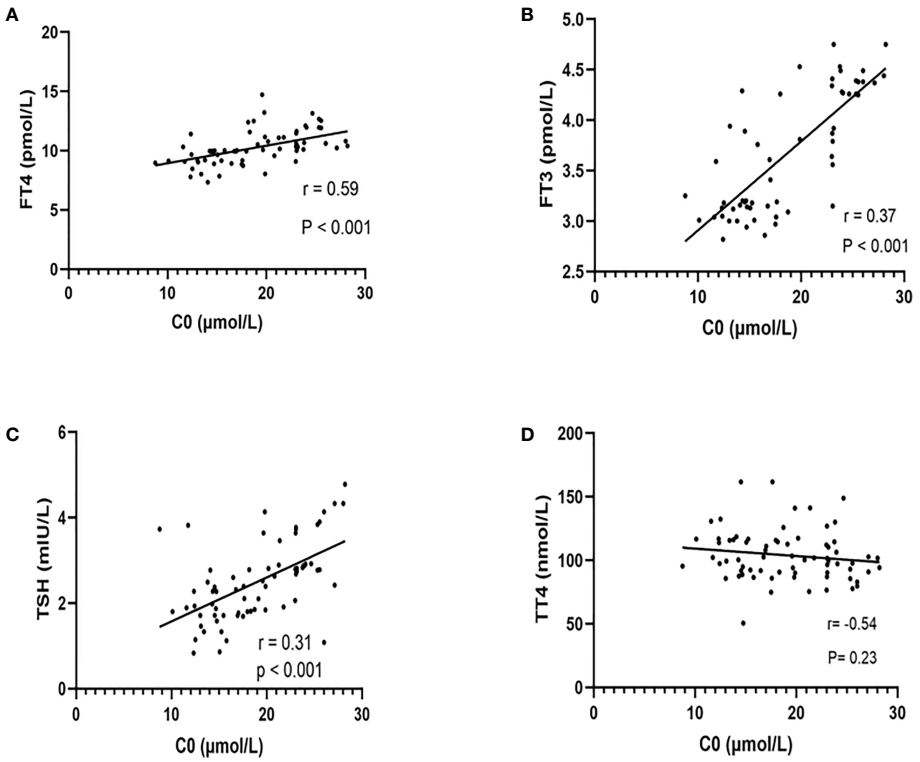

FIGURE 2
The relationship between free carnitine and thyroid hormones (FT4, FT3, TSH, and TT4). The correlation between C0 and FT4 **(A)**, FT3 **(B)**, TSH **(C)**, and TT4 **(D)** was shown above. There were significant positive correlations between C0 and FT4, FT3, and TSH rather than TT4. r = Spearman's correlation coefficient.

weight than C0. There was a considerable difference according the combination of FT4 and C0 in the second trimester ($P < 0.001$, Figure 4A). Lower FT4 and C0 levels were associated with a 0.8 SD higher birth weight. A lower FT4 level but a median C0 level had an influence of a 0.5 SD higher birth weight. A low FT4 level and a higher C0 level had no effect on birth weight. A low FT3 level and a low C0 level were associated with a 0.35 SD higher birth weight. A low FT3 level but a median C0 level were associated with a 0.2 SD higher birth weight. A low FT3 level and a higher C0 level had no effect on birth weight. In line with these analyses, a low FT4 level and a low C0 level were associated with more pronounced effects on birth weight. However, the effects estimate of birth weight did not different when combinations of TSH and C0, TT4, and C0.

4 Discussion

Our research showed that there was a significantly positive correlation between C0 and TSH, FT3, and FT4. In addition, C0, FT4, and TSH all had significantly negative influence on newborn birth weight. Therefore, we evaluated the co-effects of C0 and THs on birth weight and found that the combinations of FT4 and C0 and of FT3 and C0 were significantly associated with birth weight, whereas that of TSH and C0 and that of TT4 and C0 were not.

Our study showed that FT4 and TSH had a significantly negative effect on neonate birth weight, whereas FT3 and TT4 had not. These results are consistent with that of Zhang et al., who found that higher TSH or FT4 concentrations throughout pregnancy were associated with lower birth weight (2). Other studies also showed that babies born to mothers with higher serum FT4 levels had an elevated risk for SGA, whereas those with lower serum FT4 levels had a higher risk for LGA (10, 11). Low FT4 levels, which may lead to an increase in circulating glucose, are associated with an increased risk of GDM, resulting in a higher placental glucose transport to the fetus and subsequent fetal weight gain (10, 12). Leon et al. also reported that subjects with low FT4 levels were significantly associated with a higher insulin resistance index, thus leading to an increased risk of LGA (12). Another potential mechanism is that higher TSH and FT4 levels accelerate the degradation of lipids and proteins, resulting in chronic energy deficiency in pregnant women, which has been shown to have a negative impact on neonate birth weight (2). FT4 is the active component of TT4, the most abundant TH in the body. Because the rate of conversion of TT4 to FT4 *in vivo* is limited by enzymes, there is no significant relationship between TT4 and birth weight. FT3 is three to five times more active than FT4. It is unclear whether maternal T3 actually crosses the placenta. FT3 is a TH that plays a direct biological role. Activation of deiodinase leads to a high rate of conversion of FT4 to FT3, resulting in low FT4 levels and high FT3 levels. High FT3 levels increase fetal weight directly through anabolic effects on fetal metabolism and stimulation of fetal oxygen consumption (9). A study showed that FT3 levels were positively associated with gestational weight gain in pregnant women (13). It

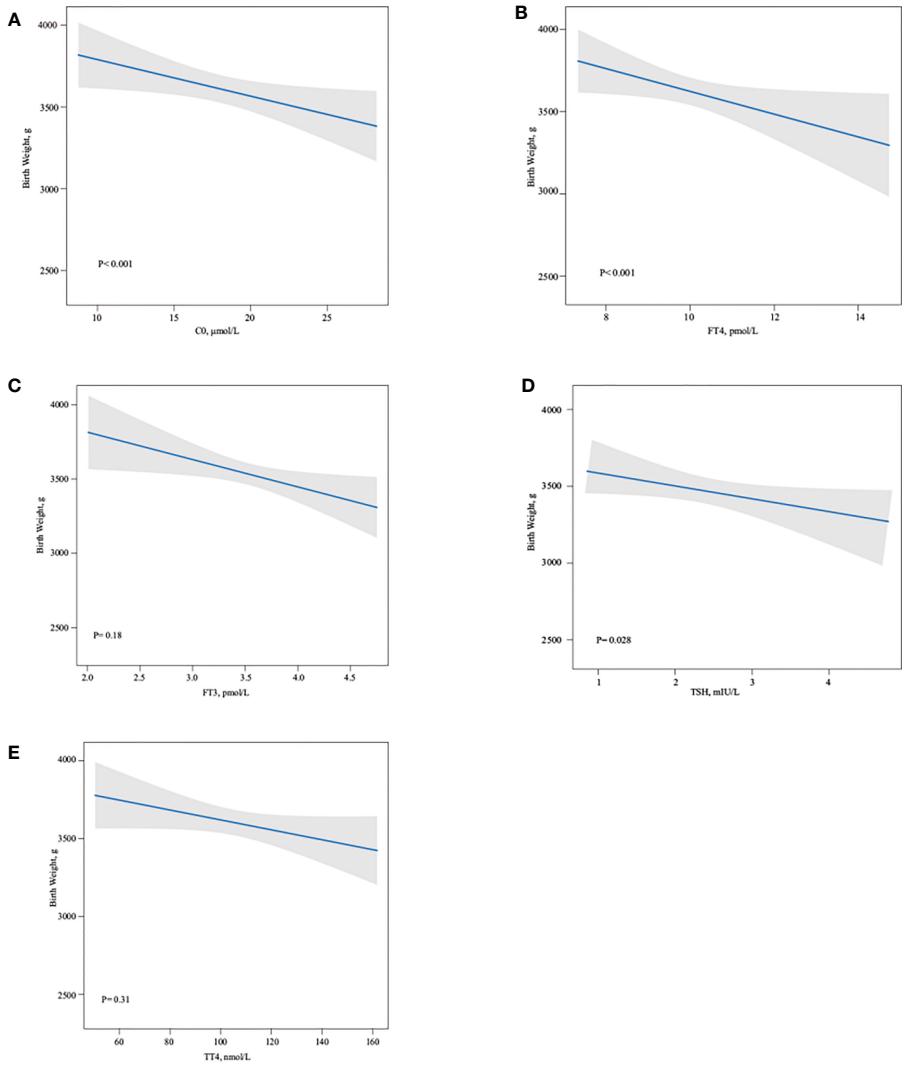

FIGURE 3
Association of maternal free carnitine and thyroid hormones in the second trimester pregnancy and neonate birth weight. Linear regression models for C0 **(A)**, FT4 **(B)**, FT3 **(C)**, TSH **(D)**, TT4 **(E)** with birth weight, as predicted mean with 95% CI, were shown above. C0, FT4, and TSH negatively influenced neonate birth weight (all $P < 0.05$), whereas there were no statistically association between FT3 and TT4 and neonate birth weight. Analyses were adjusted for maternal age, BMI, parity, and fetal sex.

has been reported that higher FT3 levels are associated with neonatal obesity, but the mechanism by which T3 affects fetal weight is unclear (9). Notably, THs are necessary for fetal cell differentiation and triggering organ development events in early pregnancy, and both high and low maternal FT4 levels were associated with adverse effects on birth weight (2). Korevaar et al. found an inverted U-shaped association of maternal FT4 with child IQ and gray matter volume (14).

The mitochondrial matrix enzyme, carnitine acetyltransferase, catalyzes the conversion of A-CoA and C0 to acetyl-carnitine and free Co-A, which plays a vital role in the production of energy in skeletal muscle, whereas TH is known to regulate several enzymes in this pathway (15). Maebashi et al. (16) reported that urinary carnitine excretion was positively correlated with serum thyroxine concentrations, with a significantly higher mean carnitine excretion in patients with hyperthyroidism and a lower carnitine excretion in patients with hypothyroidism compared with that in the control group. After correction of thyroid status, urinary carnitine excretion returned to normal in both groups (16). Another study examined total, free, and esterified carnitine levels in the skeletal muscle of patients with hyperthyroidism and hypothyroidism before and after drug treatment (17). A significant decrease was observed in total muscle carnitine concentrations in patients with hyperthyroidism compared with that in control subjects, which largely attributed to a decrease in the esterified carnitine portion. Total muscle carnitine levels were reduced in patients with hypothyroidism yet did not reach statistical significance, and no significant differences were found in esterified carnitine concentrations compared with control values. Meanwhile, Wong et al. (15) found that the level of acylcarnitine was relatively unremarkable in thyroid diseases. Other researchers argued that carnitine impairs the access of TH to the nucleus, thus decreasing the activity of TH (5, 18). An observational pilot study

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TABLE 5 Multivariable analysis of thyroid hormone, C0 in relation to birth weight.

Variables	β coefficient	P-value
TSH	**−21.079 (−29.842, −13.316)**	**0.013**
FT4	**−29.203 (−33.149, −15.511)**	**0.004**
FT3	**−22.728 (−37.836, 11.456)**	**0.125**
TT4	**−18.185 (−13.947, 36.511)**	**0.403**
C0	**−12.079 (−18.642, −8.532)**	**0.038**

Bold values are presented as mean ± SD. TSH, thyroid-stimulating hormone; FT4, free thyroxine; FT3, free triiodothyronine; TT4, total thyroxine; C0, free carnitine.

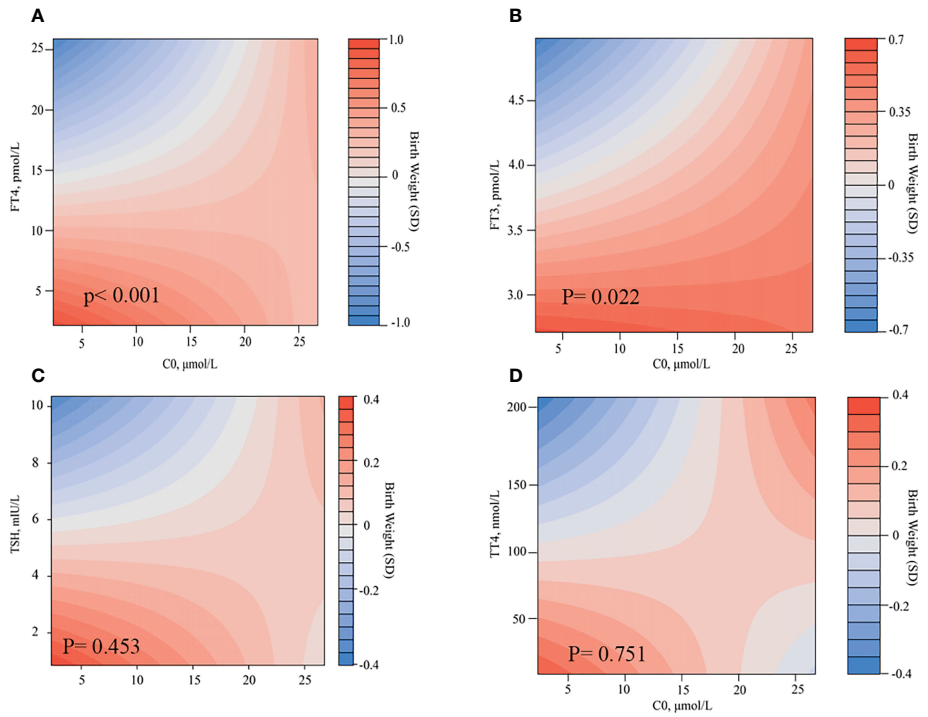

FIGURE 4
Combined effects of C0 and thyroid hormones in the second trimester on birth weight. Heatmap (filled contour plot) for the correlation of birth weight (red indicates increased gestational age–adjusted birth weight, and blue indicates decreased gestational age–adjusted birth weight) according to the interaction of C0 and FT4 **(A)**, FT3 **(B)**, TSH **(C)**, and TT4 **(D)** in the second trimester. Analyses were adjusted for maternal age, BMI, parity, and fetal sex.

found that the symptoms of patients with subclinical hyperthyroidism relieved obviously after taking L-carnitine and selenium without any significant changes of their endocrine status (19). C0 was found to be negative on birth weight, and the combination of C0 and FT4 and of C0 and FT3 would significantly decrease the birth weight in this study, which reflected that C0 had a synergistic effect with THs, and assessment of total serum carnitine and changes in urinary carnitine excretion might help to find underlying mechanisms.

The present study has certain limitations. First, neonate birth weight could be influenced by many internal and external factors, including sex hormones, diet, medicine, and heredity. With so many confounding factors, subjects enrolled in this study could not be matched completely. Second, we did not assess the total serum carnitine and urinary carnitine, which might help explain the changing and transition of carnitine during pregnancy and the underlying mechanisms. We do find the co-effect of C0 and THs in the second trimester and their influence on neonate birth weight.

In conclusion, C0 and TH are of great importance in neonate birth weight, and routine examination of C0 and TH in the second trimester has a positive effect on the intervention of birth weight.

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 IRB-20220254-R. The patients/participants provided

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

Author contributions

MY contributed to the collection, analysis, and interpretation of data as well as manuscript preparation. MS and QW contributed to the data collection and analysis, and YJ contributed to the interpretation of data. CJ contributed to the language editing. JX and QL contributed to the study design, data interpretation, and manuscript preparation. QL is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors 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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Association between aberrant amino acid metabolism and nonchromosomal modifications fetal structural anomalies: A cohort study

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Background: More than half of the cases of fetal structural anomalies have no known cause with standard investigations like karyotype testing and chromosomal microarray. The differential metabolic profiles of amniotic fluid (AF) and maternal blood may reveal valuable information about the physiological processes of fetal development, which may provide valuable biomarkers for fetal health diagnostics.

Methods: This cohort study of singleton-pregnant women had indications for amniocentesis, including structural anomalies and a positive result from maternal serum screening or non-invasive prenatal testing, but did not have any positive abnormal karyotype or chromosomal microarray analysis results. A total of 1580 participants were enrolled between June 2021 and March 2022. Of the 1580 pregnant women who underwent amniocentesis, 294 were included in the analysis. There were 137 pregnant women in the discovery cohort and 157 in the validation cohort.

Results: High-coverage untargeted metabolomic analysis of AF revealed distinct metabolic signatures with 321 of the 602 metabolites measured (53%) (false discovery rate, $q < 0.005$), among which amino acids predominantly changed in structural anomalies. Targeted metabolomics identified glutamate and glutamine as novel predictive markers for structural anomalies, their vital role was also confirmed in the validation cohort with great predictive ability, and the area under the receiver operating characteristic curves (AUCs) were 0.862 and 0.894 respectively. And AUCs for glutamine/glutamate were 0.913 and 0.903 among the two cohorts.

Conclusions: Our results suggested that the aberrant glutamine/glutamate metabolism in AF is associated with nonchromosomal modificantions fetal structural anomalies. Based on our findings, a novel screening method could be established for the nonchromosomal modificantions fetal structural anomalies. And the results also indicate that monitoring fetal metabolic conditions (especially glutamine and glutamine metabolism) may be helpful for antenatal diagnosis and therapy.

KEYWORDS

fetal structural anomalies, amniotic fluid, metabolic, maternal, pregnancy, amino acid

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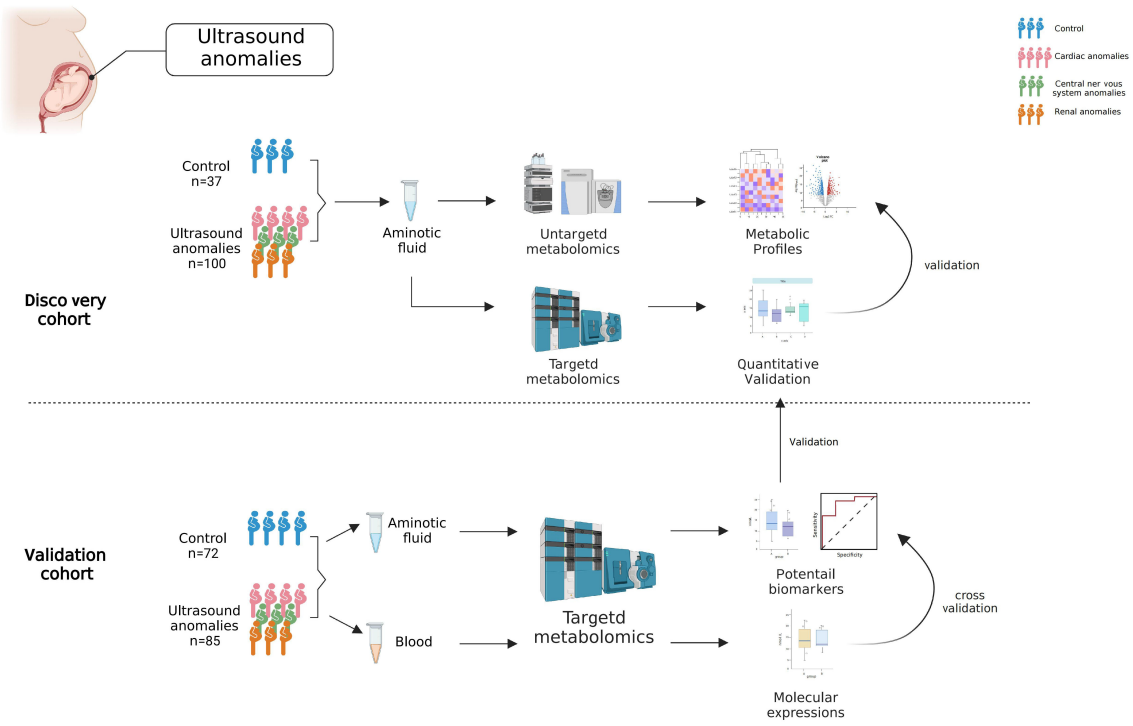

GRAPHICAL ABSTRACT
General view of aberrant glutamate and glutamine metabolism in fetal ultrasound anomalies.

Introduction

Fetal structural anomalies, which can range from minor deficiencies in a single organ to severe multi-organ system malformations, have a considerable impact on fetal morbidity and mortality (1). Prenatal ultrasound is now regarded as a routine analysis in obstetrical care, and with increasingly high resolution, fetal structural anomalies are identified in approximately 3% of pregnancies. Fetal structural anomalies have various genetic causes, including chromosomal aneuploidy, copy number variations (CNVs), and pathogenic sequence variants in developmental genes (2). Genetic investigations are essential for the assessment and clinical triage of fetal structural anomalies. Clinically, when fetal anomalies are identified, further prospective evaluations included karyotype testing and chromosomal microarray analysis (CMA) to detect aneuploidies and CNVs (3, 4). Overall, approximately 32% of fetuses with a structural anomaly identified by ultrasound have a clinically relevant abnormal karyotype, and 6.5% of them have a causative CNV (1, 3–5). Additionally, where karyotype testing and CMA failed to determine the underlying cause, whole-exome sequencing was reported to identify a well-described genetic cause in 8.5-10% of fetuses with structural anomalies (2, 6). However, more than 50% of fetal structural anomalies are left without a prospectively screening or identification method.

Pregnancy is related to the onset of many adaptation processes that change throughout gestation (7). Maternal blood constantly exchanges with the fetus's blood through the placenta to provide the nutrients needed for fetal growth and development. Amniotic fluid (AF) can also be considered a pool of metabolites reflecting the biological process of anabolism and catabolism (8, 9). The biochemical nature of AF and maternal blood makes them extremely valuable materials for fetal health diagnostics.

Spurred by tremendous technological advancements, the metabolome has become widely acknowledged as the dynamic and sensitive expression of biological phenotypes at the molecular level, placing metabolomics at the forefront of biomarker and mechanistic discoveries associated with pathophysiological processes (10). Untargeted metabolomics is applied to measure the most comprehensive range of compounds or putative metabolites present in an extracted sample without prior knowledge of the metabolome (11). In contrast, targeted metabolomics focuses on a small group (50–500) of compounds of interest; here, methods are generated and optimized for the investigation of specific metabolites and metabolic pathways with higher sensitivity and selectivity than untargeted metabolomics (12). The targeted analysis is also outstanding for hypothesis validation and expanding upon the results of untargeted analysis (13).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a current, routine, highly accurate application in newborn screening (14, 15). Similarly, metabolomics can be applied to fetal malformations by exploring the AF metabolome, and several studies have reported promising results (16, 17), revealing the possibility of using this technology in clinical practice. Since AF can reflect both maternal and fetal health, linking AF metabolic

profiles with structural anomalies is conducive to biomarker discovery, and will better guide clinical practice.

The present study aimed to characterize the metabolic signature of AF in fetal structural anomalies. Also, we tried to investigate whether metabolic changes reflect maternal or fetal conditions. In this study, we measured AF metabolites in the structural anomalies and control groups from two independent cohorts using both untargeted and targeted metabolomics. First, a high-coverage untargeted metabolomic assay based on ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was applied to 137 participants (the discovery cohort). To assay the changes in metabolites more quantitatively, we performed targeted metabolomic analysis using the UHPLC-MS/MS system and isotope-labeled internal standards. The findings in the discovery cohort were confirmed by targeted metabolomic analysis of a validation cohort of 157 participants. At the same time, we analyzed maternal serum metabolites using targeted metabolomics, which reflected the amino acid metabolism of the mothers.

Materials and methods

Study design and participant enrollment

This study was approved by the medical ethics committee of Jiangxi Maternal and Child Health Hospital (Approval number: EC-KT-202210). All the participants provided written informed consent. All participants were recruited from the prenatal diagnosis center of Jiangxi Maternal and Child Health Hospital from June 2021 to March 2022. Inclusion criterion: Pregnant women who had an indication for amniocentesis, including structural anomalies and a positive result from maternal serum screening or non-invasive prenatal testing. Exclusion criteria: (1) abnormal karyotype or chromosomal microarray analysis results; gestational age beyond 140-154 days; (3) multiple pregnancies; (4) other risk factors for prenatal diagnoses. Finally, 294 participants were included and separated into the discovery (n= 137, from June 2021 to October 2021) and validation (n= 157, from November 2021 to March 2022) cohorts. Fetuses with structural anomalies were categorized into three phenotypic groups based on abnormalities in different organ systems detected by ultrasound, including cardiac, central nervous systems, and renal anomalies. The control group in this study included women with singleton pregnancies whose fetuses had no structural malformations, but who had indications for amniocentesis, including a positive result from maternal serum screening or non-invasive prenatal testing.

Collection and processing of samples

20-25 mL of AF and 3-5 mL of blood were obtained from the pregnant women at the time of amniocentesis. The AF was centrifuged at 1200 rpm for 10 min at 4°C, and the supernatant was collected. Blood was placed at 4°C for 1 h and centrifuged at 3000 rpm at 4°C for 10 min, and serum was collected from the upper layer. All samples were stored at -80°C before analysis, and their use for research was approved by the ethical committee. In the validation cohort, AF and blood samples were obtained from the same pregnant woman.

Untargeted LC-MS metabolomics profiling

Broad-based metabolomic profiling was performed using UHPLC-MS/MS platform. Further details are provided in the Supplementary Material.

Targeted LC-MS metabolomics data collection and processing

Fifty-four amino acids and their derivatives were quantified using a Shimadzu LC-20ADXR (Shimadzu, Kyoto, Japan) coupled with a Sciex 5500+ triple quadrupole mass spectrometer (AB Sciex, Singapore). Further details are provided in the Supplementary Material.

Statistical analysis

The metabolites included in the statistical analyses were those which were consistently detected in at least 80% of the samples. The metabolome data derived from different methods were normalized. Data scaling was assessed using Pareto scaling. Multivariate statistical analyses, partial least squares discrimination (PLS-DA), functional enrichment, metabolic pathway analysis of metabolites and lipids, and receiver operating characteristic (ROC) analysis, and the respective area under the ROC curve (AUC) were performed using an online data analysis platform- MetaboAnalyst 5.0 (https://www.metaboanalyst.ca). Unit statistical analyses, such as t-tests, were performed using SPSS software (version 26.0; IBM, USA). Bar and line plots were drawn by GraphPad Prism 8.0 (GraphPad Software Inc., USA). Chemical similarity enrichment analysis was conducted using ChemRICH R package (18), and significant metabolites alterations were visualized in an enhanced heat map in gplots package using the in R (version 3.6). All p-values involved in this study were two-tailed probabilities and were adjusted by false discovery rate (FDR). Differences were considered statistically significant at FDR <0.05.

Results

High-coverage untargeted metabolomics analysis revealed distinct metabolic signatures with amino acids predominantly changed in the structural anomalies group

To comprehensively detect the metabolic profiles of structural anomalies, we implemented a high-coverage untargeted metabolomic analysis of AF samples by integrating five different analytical methods that could cover both hydrophobic and hydrophilic metabolomes. Between June 2021 and March 2022, 1580 pregnant women whose fetuses were diagnosed with structural anomalies were screened for

their eligibility for inclusion in our study (Figure 1). Finally, 137 and 157 participants prospectively enrolled in the study as described in Table 1. The untargeted metabolomic analysis enabled the detection and relative quantification of 602 metabolites in all AF samples. As shown in the PLS-DA score plot, the structural anomalies group was separated from the control group in the direction of the first principal component (Figure 2A). Running 10-fold cross-validation showed that the accuracy of one component was 0.87 (0.54 for R2 and 0.50 for Q2) (Supplementary Table 1). Moreover, 321 metabolites were identified as differential metabolites between the structural anomalies and control groups (FDR, q <0.05) (Supplementary Table 2, Supplementary Figure 1A). Among these, differential amino acids were the most abundant (Figure 2B). The KEGG pathway enrichment analysis of these differential metabolites also showed that amino acid metabolic pathways, such as glutamine (Gln) and glutamate (Glu) metabolism; alanine, aspartate and Glu metabolism; and phenylalanine, tyrosine and tryptophan biosynthesis, were the most significant changed (Figure 2C). Among all amino acids, Gln (32% increase, FDR, q<1×10−13) and Glu (84% decrease, FDR, q<1×10−11) were the significantly the significantly altered metabolite in structural anomalies group (Figure 2D, Supplementary Table 2).

Based on the above results, we focused on the amino acid changes among different structural anomalies, including cardiac, central nervous system, and renal system anomalies. Compared to the control group, each type of structural anomaly demonstrated a distinct metabolic profile, with 172 overlapping differential metabolites (Figure 2E). There were 14 amino acids in the 172 overlapping metabolites. Surprisingly, Glu levels were dramatically lower while Gln levels were significantly higher in the cardiac,

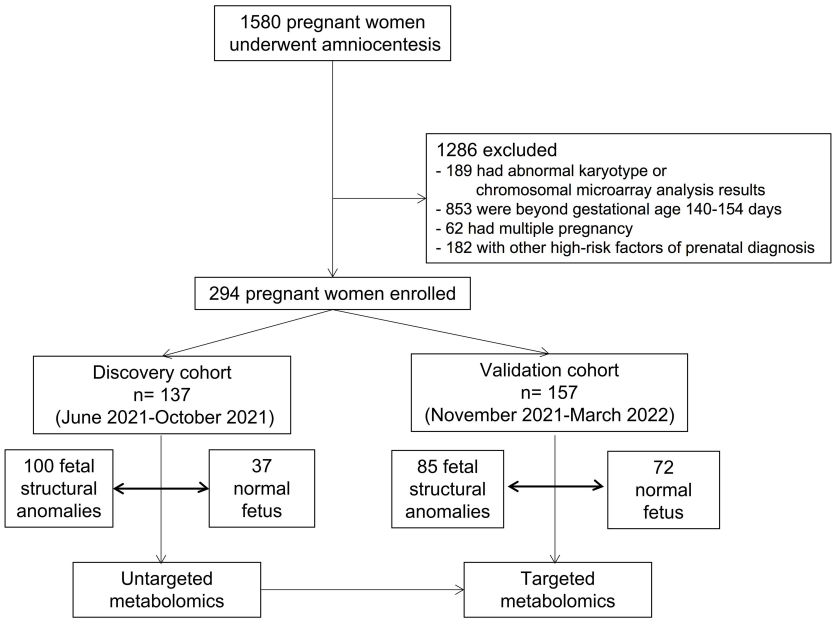

FIGURE 1
Study outline of workflow.

TABLE 1 Characteristics of cases with structural anomalies and matched controls.

	Structural anomalies		Control	
	Discovery cohort	Validation cohort	Discovery cohort	Validation cohort
AF samples, No.(%)	100 (34%)	85 (29%)	37 (13%)	72 (24%)
blood samples,No.(%)	0	85 (54%)	0	72 (56%)
gestational age when underwent amniocentesis (days)	146.76 ± 4.82	147.14 ± 4.61	145.82 ± 3.96	146.36 ± 4.68
Maternal age (years)	27.36 ± 2.12	25.68 ± 1.85	27.97 ± 2.32	29.07 ± 2.64
Smoke during pregnancy	0	0	0	0
Fetal karyotype analysis results	normal	normal	normal	normal
Fetal chromosomal microarray analysis results	normal	normal	normal	normal
Maternal ethnicity	Asian	Asian	Asian	Asian

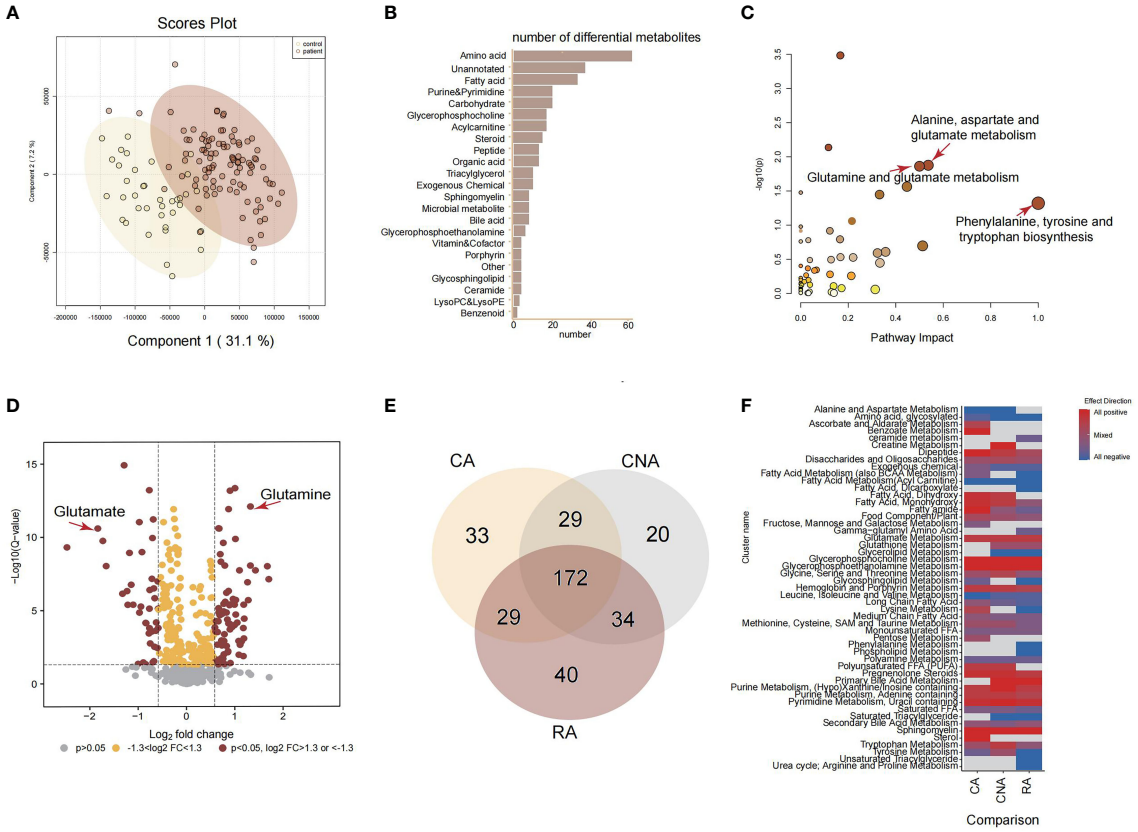

FIGURE 2
Amniotic fluid metabolic landscape for fetal ultrasound anomalies. **(A)** PLS-DA score plot for untargeted metabolomics data. **(B)** Classed enrichment analysis for differential metabolites between ultrasound anomalies group and the control group. **(C)** Pathway enrichment analysis for significantly different metabolites between ultrasound anomalies group and the control group. **(D)** Volcano plot for all metabolites from untargeted metabolomics. **(E)** Venn plot of differential metabolites from three kinds of ultrasound anomalies compared with the control group. **(F)** Chemical similarity enrichment analysis for differential metabolites from three kinds of ultrasound anomalies. CA, cardiac anomalies; CNA, central nervous system anomalies; RA, renal anomalies.

central nervous system, and renal anomalies (Supplementary Table 2). Gln-Glu exchange is important in placental amino acid transport, and Gln and Glu are the most utilized amino acids in fetuses during late gestation. Therefore, we hypothesized that Gln and Glu are vital for the early diagnosis of fetal structural anomalies.

In addition to the significant changes in Glu metabolism in the three types of structural anomalies, it is worth noting that fetuses with renal anomalies uniquely showed significantly inhibited urea cycle (arginine and proline metabolism), and that creatine metabolism was positively regulated in fetuses with central nervous system anomalies subjects (Figure 2F). These metabolic pathway changes may be typical responses to different structural anomalies.

Amniotic fluid-targeted metabolomics of identified glutamate and glutamine as novel predictive markers for structural anomalies

We performed a targeted metabolomic assay of 54 amino acids and their derivatives to quantify the metabolite changes in the structural anomalies and control groups more precisely. We first quantified AF amino acids obtained from 137 participants in the discovery cohort, confirming that aberrant amino acid metabolism occurred in the structural anomalies group (Supplementary Table 3). Gln and Glu were significantly altered in targeted metabolomics.

To further validated these results, we applied targeted metabolomics to the validation cohort. Based on the concentrations presented in the different groups, 33 amino acids, including Gln and Glu, showed significant differences between the structural anomalies and control groups (Supplementary Table 4). Twenty amino acids were shared by the three types of structural anomalies (cardiac, central nervous system and renal anomalies), and significant differences existed between the structural anomalies and control groups (Supplementary Figures 1B, C). We found that Glu levels in the AF were significantly lower (Figure 3A), while Gln levels were significantly higher in the structural anomalies group than in the control group (Figure 3B). Using Gln/Glu as a metric indicating Gln-Glu conversion, we found that this ratio fell approximately 14-fold on an average among participants in the structural anomalies group (Figure 3C). Notably, regardless of the types of anomaly present, the Gln/Glu ratio was significantly

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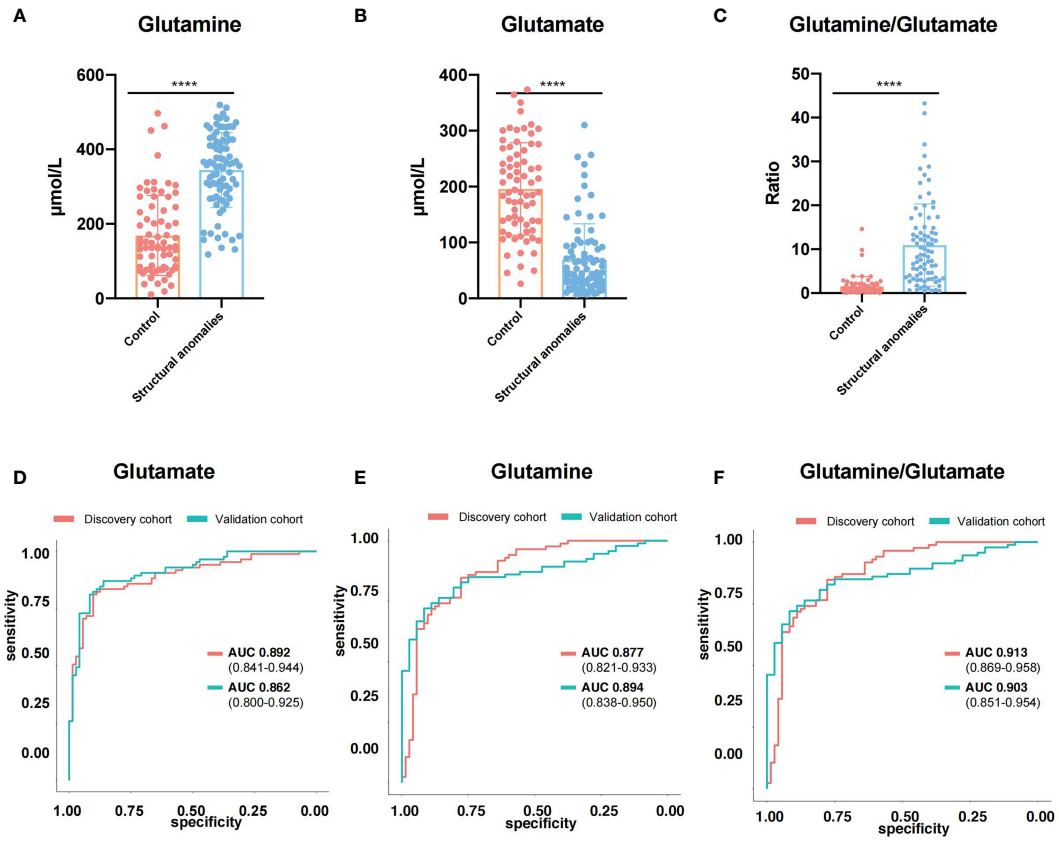

FIGURE 3
Glutamine and Glutamate were novel predictive markers for ultrasound anomalies. **(A–C)** Expression of glutamine **(A)**, glutamate **(B)** and glutamine/glutamate **(C)** in amniotic fluid of validation cohort. **(D, E)** ROC curves of glutamine **(D)**, glutamate **(E)** and glutamine/glutamate **(F)** in discovery cohort (red line) and validation cohort (blue line). ****, P<0.0001.

reduced in the structural anomalies group than in the control group (Supplementary Figure 1D). These results were consistent with our findings in the discovery cohort (Supplementary Figure 1E). In addition, Glu (AUC=0.862, 95%CI: 0.800-0.925), Gln (AUC=0.894, 95%CI: 0.838-0.950) and Gln/Glu (AUC=0.903, 95%CI: 0.851-0.954) had great prediction ability in distinguishing structural anomalies from the control group in the validation cohort (Figure 3D–F).

We then investigated whether the Gln/Glu in AF correlated with maternal metabolic conditions. Serum samples were collected from women in the validation cohort and analyzed using the same amino acid-targeted metabolomic assay. Notably, maternal serum Glu (Supplementary Figure 1F) and Gln levels (Supplementary Figure 1G) did not differ significantly between the structural anomalies and control groups. Gln/Glu ratio also did not differ between the two groups (Supplementary Figure 1H). In addition, almost all the quantified amino acids demonstrated no big differences between the structural anomalies group and control group (Supplementary Table 5), except for threonine (Supplementary Figure 1I) and leucyl-leucine (Supplementary Figure 1J). Taken together, these results suggest that changes in Gln/Glu ratio in the AF of the structural anomalies group are associated with the fetal condition rather than the maternal condition (Figure 4).

Discussion

Despite the use of karyotype testing and chromosomal microarray as routine investigations in obstetric care, a large proportion of fetal structural anomalies still have no proven cause. Herein, we explored the underlying causes of fetal malformations using AF metabolomics study. First, we performed an untargeted metabolomic assay on AF samples, starting with the discovery cohort. The results demonstrated that AF metabolic signatures were remarkably altered in the structural anomalies group compared to the control group. The most apparent alterations were observed in amino acids and their derivatives. These amino acid changes were further confirmed using targeted metabolomics, and we found 23 amino acids that were differentially expressed in the three types of structural anomalies (cardiac, central nervous system, and renal anomalies). Among these amino acids, Glu and Gln were the most significantly altered metabolites. The structural anomalies group was characterized by a significantly lower Gln/Glu ratio than the control group. To strengthen this finding, the results were validated using samples from an independent validation cohort. The results of the validation cohort were consistent with those of the discovery cohort; aberrant Glu and Gln metabolism was found in fetal structural anomalies. In addition, analysis of maternal blood samples through targeted metabolomics demonstrated no significant difference in Gln/

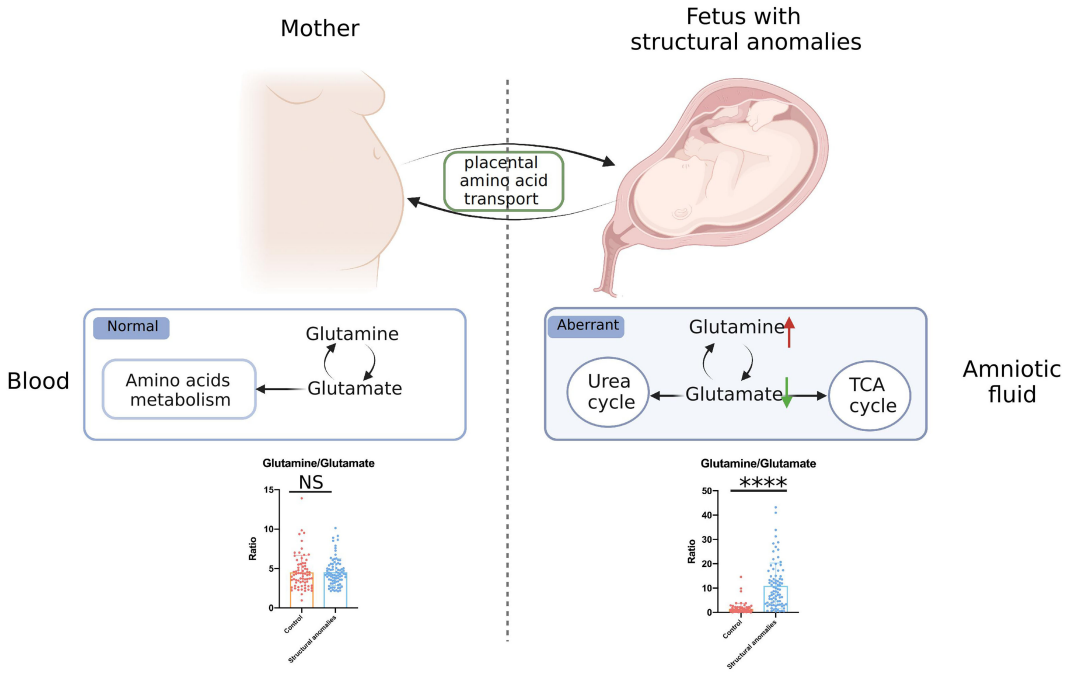

FIGURE 4
General view of aberrant glutamate and glutamine metabolism in fetal ultrasound anomalies. NS, no significance; ****, P<0.0001.

Glu ratio between the fetal structural anomalies and the control groups, suggesting that the contributors to these Glu-Gln changes in AF were closely related to fetal metabolic conditions rather than maternal metabolic status. It is also worth noting that most amino acids in maternal blood did not show significant changes in the structural anomalies and the control groups.

During pregnancy, amino acids serve as important precursors for the biosynthesis of macromolecules, including proteins and nucleotides, which are involved in fetal development and growth (19–21). Glu and Gln are among the most abundant and most utilized amino acids in the fetus during late pregnancy (19). The human placenta mediates the net transfer of amino acids to the fetus, with amino acid concentrations being generally higher in the fetus than in the mother, indicating an active transfer process across the placenta (22, 23). One notable exception to this process is Glu, which is the net placental uptake from the fetus (23). To meet the acquisitive demand for nutrients, Gln, a non-essential amino acid, is essential when fetal demand for amino acids exceeds maternal supply during pregnancy (24, 25). This demand is met through the interorgan recycling of Gln and Glu. In the fetal liver, the deamination of Gln produces Glu. Glu is transported across the syncytiotrophoblast microvillous membrane and basal membranes by high-affinity excitatory amino acid transporters and is converted to Gln in the placenta (26, 27). Glu is also an important nitrogen resource and a precursor of γ-aminobutyric acid, a key inhibitory neurotransmitter (28, 29). Therefore, the Glu-Gln cycle and exchange in the placenta-fetus unit likely play important roles in fetal growth and development.

In our study, the significant increase in Gln/Glu ratio in the AF observed in the fetal structural anomalies group suggested a disturbing Glu-Gln cycle in the fetus rather than in the mother, since no obvious changes were detected for either Glu or Gln in maternal blood. Decreased levels of Glu and increased levels of Gln in AF have also been reported in the studies of fetal malformations, prediagnostic gestational diabetes, preterm delivery and early rupture of membranes (16, 30). The underlying cause may be the dysfunction of transporters utilized by Glu and Gln. The amino acids that the fetus requires for metabolic processes and biosynthesis pathways can only be obtained from the placenta and delivered by different amino acid transporters (23, 31). For example, in fetal growth restriction, the initial rate of uptake of Gln and Glu into placental villous fragments is reportedly reduced but increases with the expression of their transporter proteins (Gln: LAT1, LAT2, SNAT5, Glu: EAAT1) (32, 33). Transporter activity is not simply determined by the protein expression levels; it is also influenced by factors that regulate substrate levels on both sides of the membrane. Interestingly, a study demonstrated that Glu efflux down its transmembrane gradient drives placental uptake *via* OAT4 and OATP2B1 from the fetal circulation and that the reuptake of Glu maintains this driving gradient, although OAT4 and OATP2B1 are not currently understood Glu transporters (26).

In the group with renal anomalies, we also found inhibited urea cycle metabolism. Arginine is the precursor for the synthesis of ornithine, proline, and nitric oxide (34), detecting the levels of arginine and its metabolites may provide insight into discriminating fetal renal anomalies and monitoring fetal urinary development.

However, there are some limitations to our study. First, this study was limited to one center: the Prenatal Diagnosis Center of Jiangxi Maternal and Child Health Hospital. UHPLC-MS/MS analysis is simple and sensitive, and it uses only a small amount of AF for metabolic analysis, AF acquisition is still invasive. Additionally, details of clinical examination results were not available in our study, so the study did not reveal the correlations between changed metabolites and the clinical data.

Data availability statement

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

Ethics statement

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

Author contributions

BY, YL, PY conceived and designed the experiment. HY, XW, TH,DL and SH collected the samples and clinical information. HY, CL, ZW performed the experiments and analyzed the data. CL, ZW drafted the manuscript, BY, YL, PY reviewed the manuscript, and BY, PY edited the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

ZW and PY are co-founders of iphenome Yun Pu Kang biotechnology Inc. Author ZW is employed by PY.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1072461/full#supplementary-material

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Thyroid autoimmunity and adverse pregnancy outcomes: A multiple center retrospective study

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Background: The relationship between thyroid autoimmunity (TAI) and adverse pregnancy outcomes is disputable, and their dose-dependent association have not been fully clarified.

Objective: To investigate the association and dose-dependent effect of TAI with multiple maternal and fetal-neonatal complications.

Methods: This study is a multi-center retrospective cohort study based on singleton pregnancies of three medical college hospitals from July 2013 to October 2021. The evolution of thyroid function parameters in TAI and not TAI women were described, throughout pregnancy. The prevalences of maternal and fetal-neonatal complications were compared between the TAI and control group. Logistic regression was performed to study the risk effects and dose-dependent effects of thyroid autoantibodies on pregnancy complications, with adjustment of maternal age, BMI, gravidity, TSH concentrations, FT4 concentrations and history of infertility.

Results: A total of 27408 participants were included in final analysis, with 5342 (19.49%) in the TAI group and 22066 (80.51%) in control group. TSH concentrations was higher in TAI women in baseline and remain higher before the third trimester. Positive thyroid autoantibodies were independently associated with higher risk of pregnancy-induced hypertension (OR: 1.215, 95% CI: 1.026-1.439), gestational diabetes mellitus (OR: 1.088, 95%CI: 1.001-1.183), and neonatal admission to NICU (OR: 1.084, 95%CI: 1.004-1.171). Quantitative analysis showed that increasing TPOAb concentration was correlated with higher probability of pregnancy-induced hypertension, and increasing TGAb concentration was positively correlated with pregnancy-induced hypertension,

small for gestational age and NICU admission. Both TPOAb and TGAb concentration were negatively associated with neonatal birthweight.

Conclusion: Thyroid autoimmunity is independently associated with pregnancy-induced hypertension, gestational diabetes mellitus, neonatal lower birthweight and admission to NICU. Dose-dependent association were found between TPOAb and pregnancy-induced hypertension, and between TGAb and pregnancy-induced hypertension, small for gestational age and NICU admission.

KEYWORDS

thyroid autoimmunity, maternal and fetal outcomes, dose dependent effect, pregnancy-induced hypertension, gestational diabetes mellitus, birth weight

Introduction

Thyroid autoimmunity (TAI) is defined as the presence of antibodies to thyroperoxidase (TPOAb) or thyroglobulin (TGAb) (1). The prevalence of TAI is 5-15% in reproductive aged women (2) and even higher in pregnant women (5-20%) (3–6).

Compared to thyroid dysfunction, the impact of TAI on pregnancy might be underestimated. Available evidence predominantly links the adverse pregnancy outcomes in TAI women to hypothyroidism (7), and clinical guidelines recommended levothyroxine supplement as the only treatment method (8). However, euthyroidism was found in the majority of (75%) pregnant women with TAI (9). Increasing studies showed that the association of TAI with miscarriage and preterm birth remained significantly after adjustment for thyroid dysfunction (10), and the pregnancy outcomes has not found to be improved by thyroid hormone replacement. Dhillon-Smith reported that adverse neonatal outcomes were not different after levothyroxine supplement in TPOAb positive women (11). Similarly, a recent multicenter RCT (T4LIFE trial) showed that, supplement of levothyroxine did not improve pregnancy outcomes in euthyroid TAI women (12). These results indicated that TAI in itself may induce adverse pregnant outcomes besides *via* mediating thyroid destruction.

Although with different results, the association between TAI and recurrent miscarriage and preterm birth were identified by prospective cohort studies and meta-analysis (10, 13, 14). Recently, increasing studies focus on the impact of TAI on other pregnancy complications, including pregnancy-induced hypertension (15, 16), gestational diabetes (17, 18), and adverse fetal-neonatal outcomes (16, 19, 20), but the relationship had not been fully clarified to draw any conclusions. The possible reason of this is that most of the studies included small sample and only focused on single outcome and without adjustment of confounders. In addition, as the reflection of thyroid autoimmunity process, there is a dose-dependent association of thyroid autoantibodies with TSH and free thyroxine level in pregnant women (21), however, study assessing dose-dependent association of thyroid autoantibodies with adverse pregnancy outcomes were seldom to date. To accessing their dose-dependent associations should provide insights toward distinguishing low-risk from high-risk individuals and optimizing clinical decision-making strategies.

Therefore, based on our large multicenter cohort, the purpose of the present study is to verified the association, as well as the dose-dependent effect of TAI with various maternal and fetal-neonatal complications.

Methods

The study was registered in Chinese Clinical Trial Registry (ChiCTR2200064466) and was approved by the ethical committees of Sun Yat-sen Memorial Hospital of Sun Yat-sen University with a waiver of informed consent (SYSEC-KY-KS-2020-200).

Study design and participants

This is an observational cohort study based on the electronic medical record in three college hospitals, the Sun Yat-sen Memorial Hospital, the First Affiliated Hospital, and the Sixth Affiliated Hospital of Sun Yat-sen University. Data of pregnant women delivered in the Department of Obstetrics in these three hospitals from July 2013 to October 2021 were included for primary screening base on the following criteria: (1) with thyroid autoantibodies (TPOAb or TGAb) results obtained in the first and second trimester or within one year before pregnancy; (2) 18-55 years old; (3) with complete records of pregnant outcomes. Participants with the following criteria were excluded: (1) with medical history of thyroid diseases before pregnancy (i.e. hyperthyroidism, thyroid cancer, surgical history on thyroid, and pituitary diseases); (2) termination due to fetal abnormality, chromosomal abnormality, maternal chronic diseases or personal reasons; (3) with multiple pregnancy.

Collection of information

From the medical records, we collected basial and gestational characteristics (age, height, weight at admission for delivery, gestational age, gravidity, parity, delivery mode, past medical history and family medical history), gestational complications and adverse outcomes (preterm birth, gestational diabetes mellitus, pregnancy-induced hypertension, postpartum hemorrhage and premature rupture of membrane), neonates (birthweight, Apgar score, need for intensive neonatal care, neonatal death). For each participant, the serum concentrations of TPOAb, TGAb, thyroid stimulating hormone (TSH), free triiodothyronine (FT3), free thyroxine (FT4) during pregnancy were all obtained and the mean gestational weeks of the first blood sample detection were 15.4 ± 6.4 gestational weeks. These thyroid parameters were examined by chemiluminescent immunoassay, and the detail of laboratory measurements from each hospital was summarized in Supplementary Table S1.

Definition of TAI and pregnancy complications

TPOAb and TGAb positivity was defined according to the cutoffs provided by the manufacturers. Participants with positive results of either TPOAb or TGAb in the first or second trimester during pregnancy, or within one year before pregnancy were included in the TAI group, while those with both TPOAb and TGAb in normal range were in control group. The definition of thyroid function parameters was according to the reference range for each trimester of each hospital.

Adverse pregnancy outcomes comprised maternal and fetal-neonatal outcomes and the definition were according to the practical guidelines of each disease. Preterm birth (PTB) was defined as termination of pregnancy between 28 and <37 gestational weeks. Gestational diabetes mellitus (GDM) diagnosed *via* 75g glucose OGTT test when one or more plasma glucose (PG) meet or exceed the thresholds: fasting PG 5.1 mmol/L, 1h-PG 10.0 mmol/L, and 2h-PG 8.5 mmol/L, according to the International Association of Diabetes and Pregnancy Study Groups-2010 guidelines (22), and women diagnosed with overt diabetes before pregnancy were excluded. Pregnancy-induced hypertension (PIH) was defined as maternal blood pressure exceeding 140/90 mmHg induced by pregnancy after 20 weeks' gestation with or without proteinuria and edema (23). Premature rupture of membrane (PROM) was defined as abruption of the amniotic sac before labor onset. Postpartum hemorrhage was defined as a cumulative blood loss of greater than or equal to 500mL for vaginal delivery or 1,000 mL for cesarean. Large for gestational age (LGA) and small for gestational age (SGA) which determined according to the National Institute of Child Health and Human Development (NICHD) standard for Asian population (24). Macrosomia was defined as neonatal birthweight heavier than 4000 grams and Apgar Score ≤7 at 1 to 10 minute after born were defined as Low Apgar Score.

Study outcomes and data analysis

The primary outcome was the association between TAI and various pregnancy complications. Maternal outcomes including PTB, PIH, GDM, PROM and postpartum hemorrhage. Fetal-neonatal outcomes including need for intensive neonatal care, Low Apgar Score, macrosomia, LGA and SGA.

Research data was analyzed by Python 3.8. Continuous variables were reported with mean and standard deviation, and categorical variables were reported with number and percentage. The difference between group were compared by Chi-squared test for categorical variables. Independent Student's t test and Mann-Whitney test were applied to compared continuous variables with normally and normally distributions, respectively. Two-sided *P* values less than 0.05 were considered statistically significant.

The association between TAI and pregnancy complications were firstly assessed by chi-squared test. Then, multiple logistic regression analysis was applied to adjusted confounders and build multiple models: (1) Model1: adjusted for maternal age and BMI; (2) Model2: Model 1+gravidity; (3) Model3: Model 2+TSH and FT4 concentration; (4) Model4: Model3 + history of infertility. For the risk of GDM and PIH, family medical history of diabetes and hypertension were considered, respectively. For the risk of preterm birth, history of recurrent miscarriage was considered. The concentrations of TPOAb and TGAb were first compared in participants with and without each maternal and fetal-neonatal complications, and those complications with TPOAb or TGAb difference were further studied for their dose-dependent effect. The associations between pregnancy complications with TPOAb or TGAb concentrations were analyzed *via* logistic regression with the same adjustment above respectively. The association between neonatal birthweight and TPOAb or TGAb concentrations were analyzed by ANCOVA with adjustment of confounders in the Model 4.

Results

Basal characteristics of participants

A total of 33589 pregnancies records met the inclusion criteria. Of these, a total of 6181 were excluded due to maternal thyroid diseases before pregnancy (n=4303), pituitary diseases or ectopic endocrine tumor (n=26), terminated due to fetal abnormality (n=179), chromosomal abnormality (n=104), fetal tumor (n=8), maternal chronic diseases or personal reason (n=30), and multiple pregnancy (n=1531). The final study cohort comprised 27408 pregnancies. Figure 1 demonstrated the flow chart of data selection.

Of all 27408 participants, 5342 (19.5%) pregnancies which have at least one record showed positive TPOAb or TGAb (TAI group) while 22066 (80.5%) pregnant women with negative TPOAb and TGAb results in all detected records (control group). In the TAI group, 2641(49.4%) were only TPOAb positive, 917 (17.2%) were only TGAb positive, and 1784 (33.4%) were positive for both

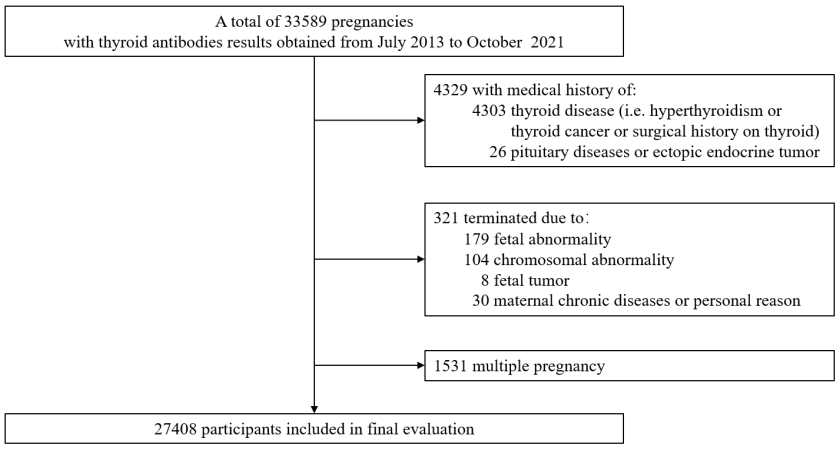

FIGURE 1
Flow chart of research population selection.

antibodies. In TAI groups, the mean concentration of TSH first tested in pregnant period was higher (1.94 ± 2.42μIU/ml *vs.* 1.43 ± 1.01μIU/ml, P <0.001), and ratio of pregnancies with TSH concentration between 2.5 and 4μIU/ml (17.8% *vs.* 10.3%, P <0.001) or exceeded 4.0μIU/ml (7.0% *vs.* 2.0%, P <0.001) were increased significantly (Table 1). That means pregnant women in TAI group have lower ratio of meeting treatment target and higher probability of subclinical hypothyroidism.

The basal characteristics of the participants were summarized in Table 1. Pregnant women in the TAI group were older (32.1 ± 4.6 years *vs.* 31.7 ± 4.6 years, P <0.001), with more gravidity (multigravida: 68.5% *vs.* 64.1%, P <0.001), but less parity (multipara: 39.6% *vs.* 43.9%, P <0.001). Participants in the TAI group were with higher proportion of recurrent miscarriage history (16.0% *vs.* 5.6%, P <0.001) and infertility history (14.8% *vs.* 11.7%, P<0.001). The gestational age at terminate was mild lower in TAI group (267 ± 25 days *vs.* 270 ± 21 days, P <0.001) and the neonates born to mother with TAI were with lower birthweight (3089.1 ± 457.3 gram *vs.* 3139.4 ± 453.0 gram, P <0.001). The differences of BMI, family histories of diabetes and hypertension were not statistically significant between groups.

Evolution of maternal thyroid parameters during pregnancy

To describe the evolution of thyroid function parameters in TAI and not TAI women, throughout pregnancy. We obtained all results of TSH, FT4, and FT3 during pregnant period. In both TAI and control group, the concentration of TSH decreased in the first eight to twelve gestational weeks and gradually increase from then on (Figure 2A), however, FT4 and FT3 both continuously decreased during the pregnant period (Figure 2B, C). Compared to control group, the concentrations of TSH in the TAI group were significantly higher in both first and second trimester (first trimester: 1.91 ± 1.05 μIU/ml *vs.* 1.31 ± 1.05 μIU/ml, P<0.001; second trimester: 1.86 ± 1.54 μIU/ml *vs.* 1.56 ± 1.04 μIU/ml, P<0.001; third trimester: 1.92 ± 1.71 μIU/ml *vs.* 1.85 ± 1.29 μIU/ml, P=0.155) (Figure 2A), and the concentrations of FT3 were lower during period (first trimester: 4.81 ± 0.70 μIU/ml *vs.* 4.88 ± 0.69 μIU/ml, P<0.001; second trimester: 4.41 ± 0.78 μIU/ml *vs.* 4.50 ± 0.63 μIU/ml, P<0.001; third trimester: 4.12 ± 0.62 μIU/ml *vs.* 4.23 ± 1.08 μIU/ml, P=0.001) (Figure 2C) and FT4 were higher (first trimester: 14.37 ± 4.00 μIU/ml *vs.* 12.76 ± 3.56 μIU/ml, P<0.001; second trimester: 11.67 ± 3.36 μIU/ml *vs.* 11.06 ± 2.94 μIU/ml, P<0.001; third trimester: 11.01 ± 3.01 μIU/ml *vs.* 10.36 ± 2.96 μIU/ml, P<0.001) (Figure 2B).

Then, we described the variation of thyroid function indicators with TPOAb concentration increasing. Because the reference ranges were different among kits, we used population-based percentiles of TPOAb concentrations for each kit to investigate the quantitative effect on thyroid function, and the 85 percentiles represent positive results of all kits. As seen in Figure 2D, we found TSH increased in the cases with TPOAb concentrations higher than 85 percentiles, and FT4 decrease with TPOAb concentrations in the cases with TPOAb concentrations higher than 90 percentiles, but FT3 did not have dose–response effect of TPOAb.

Thyroid autoimmunity and pregnancy complications

The prevalence of pregnancy complications was compared between TAI and control group (Table 2). Pregnant women in the TAI group were with higher proportion of PIH (4.06% *vs.* 3.45%, P=0.037), GDM (20.39% *vs.* 18.45%, P=0.001) and PTB (8.95% *vs.* 7.56%, P=0.001). Neonates born to mothers in the TAI group were with less macrosomia (1.55% *vs.* 2.20%, P=0.003) and LGA (4.04% *vs.* 4.77%, P=0.045), but more incidence of NICU admission (24.11% *vs.* 21.77%, P<0.001). In addition, no difference was found in other outcomes.

Then, we investigated the association between TAI and complications in logistic regression (Table 3). After adjusted for maternal age, BMI, gravidity, TSH and F4 concentrations, and

TABLE 1 Baseline characteristics of research population.

Characteristics	TAI Positive	TAI Negative	*P* value
Number	5342 (19.5%)	22066 (80.5%)	
Maternal Age (years)	32.1 ± 4.6	31.7 ± 4.6	< **0.001**
BMI (kg/m^2)	26.1 ± 3.0	26.1 ± 3.0	0.331
Gravidity			
Primigravida	1682 (31.5%)	7913 (35.9%)	< **0.001**
Multigravida	3653 (68.5%)	14125 (64.1%)	
Parity			
Nullipara	3185 (60.4%)	12277 (56.1%)	< **0.001**
Multipara	2090 (39.6%)	9616 (43.9%)	
History of Recurrent Miscarriage			
Yes	850 (16.0%)	1224 (5.6%)	< **0.001**
No	4446 (84.0%)	20633 (94.4%)	
History of Infertility			
Yes	792 (14.8%)	2575 (11.7%)	< **0.001**
No	4550 (85.2%)	19491 (88.3%)	
Family History of Diabetes			
Yes	395 (7.4%)	1680 (7.6%)	0.607
No	4947 (92.6%)	20386 (92.4%)	
Family History of Hypertension			
Yes	726 (13.6%)	3197 (14.5%)	0.097
No	4616 (86.4%)	18869 (85.5%)	
Free T4 Concentrations (pmol/L)	12.58 ± 3.80	11.88 ± 3.30	< **0.001**
TSH Concentrations (μIU/ml)	1.94 ± 2.42	1.43 ± 1.01	< **0.001**
TSH Concentrations Classification			< **0.001**
≤ 2.5 μIU/ml	3973 (75.2%)	19143 (87.7%)	
2.5-4.0 μIU/ml	941 (17.8%)	2244 (10.3%)	
> 4.0 μIU/ml	371 (7.0%)	451 (2.0%)	

P values <0.05 were shown in bold.

history of infertility, TAI were positively associated with PIH (OR: 1.206, 95%CI: 1.019-1.428, *P*=0.030), GDM (OR: 1.088, 95%CI: 1.001-1.183, *P*=0.046), PTB (OR: 1.129, 95%CI: 1.001-1.273, *P*=0.048) and admission of NICU (OR: 1.084, 95%CI: 1.004-1.171, *P*=0.040), and negatively associated with macrosomia (OR: 0.768, 95%CI: 0.599-0.985, *P*=0.038) and large for gestational age (OR: 0.833, 95%CI: 0.695-0.999, *P*=0.049). The results of PIH and GDM were remain statistically significant after additional adjustment by family history of hypertension and diabetes respectively (PIH: OR: 1.215, 95% CI 1.026-1.439, *P*=0.024; GDM: OR: 1.088, 95% CI 1.001-1.183, *P*=0.048). The result of preterm birth was not statistically significant after additional adjustment by history of recurrent miscarriage (OR=1.082, 95% CI 0.958-1.222, *P*=0.205).

Quantitative association between thyroid autoantibodies and complications

To investigate the quantitative association with complications, we first analyze the concentrations (as percentiles) of TPOAb and TGAb in participants with and without each maternal and fetal-neonatal complications. As shown in Supplementary Table S2, TPOAb concentrations were higher in pregnant women with pregnancy induced hypertension, gestational diabetes mellitus and preterm birth, and lower in women who given birth to macrosomia. TGAb concentrations were higher in pregnant women with pregnancy induced hypertension, preterm birth, and in mother who given birth to babies with small for gestational age or needed therapy in NICU (Supplementary Table S2).

Then, the associations between TPOAb concentrations and pregnancy induced hypertension, gestational diabetes mellitus, preterm birth, and macrosomia, as well as the associations between TGAb concentrations and pregnancy induced hypertension, preterm birth, SGA and NICU admission were analyzed *via* logistic regression (Figure 3). After adjusted for maternal age, BMI, gravidity, TSH and FT4 concentrations, and history of infertility, the probability of PIH rise in paralleled with increasing TPOAb (OR: 1.284, 95%CI: 1.008-1.635, *P*=0.043) and TGAb concentration (OR: 1.450, 95%CI: 1.104-1.905, *P*=0.008) respectively. The effect remains significant even after adjustment with family history of hypertension (TPOAb: OR: 1.290, 95%CI: 1.012-1.644, *P*=0.040; TGAb: OR: 1.479, 95%CI: 1.125-1.945, *P*=0.005). In addition, increasing TGAb concentration were associated with rising positivity of SGA (OR: 1.272, 95%CI: 1.067-1.516, *P*=0.007) and NICU admission (OR: 1.140, 95%CI: 1.004-1.294, *P*=0.043).

We also performed an ANCOVA analysis on the association between thyroid autoantibodies and birthweight (Figure 4A, B), and found that neonatal birthweight was decreased with TPOAb and TGAb level elevating (TPOAb: *P*=0.002; TGAb, *P*=0.005).

Discussion

In this large multi-center cohort study comprised 27408 pregnancies, we investigated the impact of TAI on multiple types of gestational complications and adverse pregnant outcomes. We verified that pregnant women with positive thyroid autoantibodies had increased risks of pregnancy-induced hypertension, gestational diabetes mellitus, and neonatal admission of NICU. Besides, we

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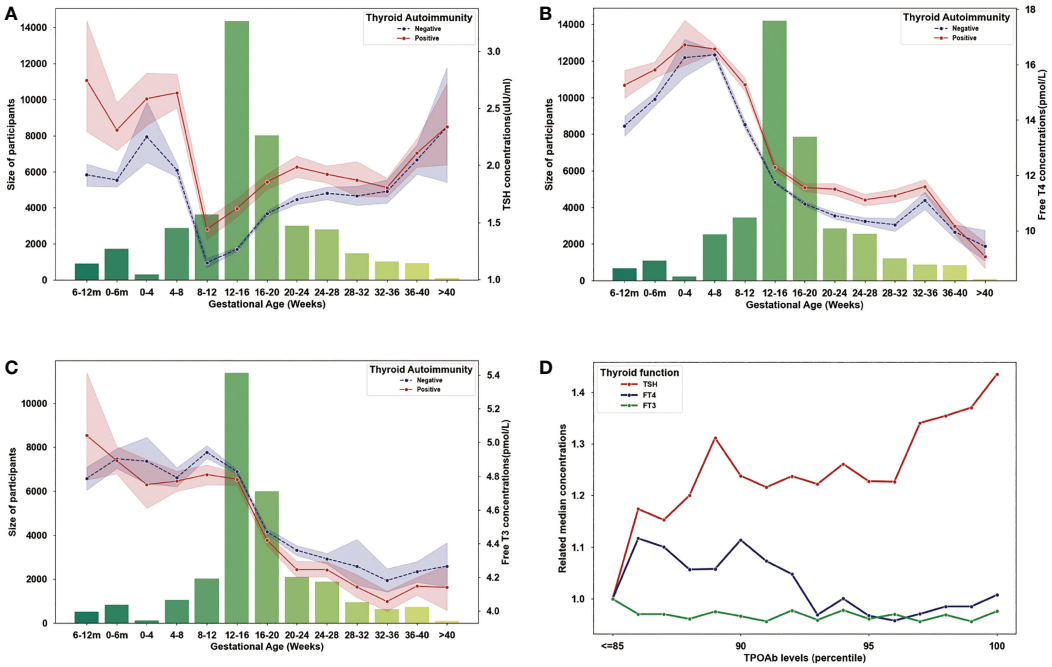

FIGURE 2
Evolution of maternal thyroid parameters during pregnancy. **(A)** Variation of TSH concentrations (lines) and sample size (bars) in each gestational month (the first and second bars represent 6-12 months and 0-6 months before pregnancy). **(B)** Variation of Free T4 concentrations (lines) and sample size (bars) in each gestational month (the first and second bars represent 6-12 months and 0-6 months before pregnancy). **(C)** Variation of Free T3 concentrations (lines) and sample size (bars) in each gestational month (the first and second bars represent 6-12 months and 0-6 months before pregnancy). **(D)** Evolution of TSH, Free T4 and Free T3 level by TPOAb concentrations (related to the concentrations of TSH, Free T4 and Free T3 in cases with TPOAb concentration lower or equal to the 85 percentile).

found the dose-dependent effect of TPOAb concentrations on pregnancy-induced hypertension, and TGAb concentration on pregnancy-induced hypertension, small for gestational age, neonatal birthweight and NICU admission. In addition, we describe the evolution of maternal serum TSH, FT4 and FT3 and determined their dose-dependent association with thyroid autoantibody. This result may provide insights toward better understanding the difference between TAI women and their control individuals.

Our present study showed that TAI in pregnancy was associated with increasing risk of PIH by approximate 20%, independent from thyroid function. Although hypertensive disorder is increased in patients with hypothyroidism (25–27), the association of thyroid autoimmunity with pregnancy-induced hypertension is disputable. Our result supported the finding of the recent Ma'anshan cohort (the MABC study) in Chinese pregnant women that TAI was positively associated with gestational hypertension (15). Saki's study also observed higher systolic blood pressure and a higher incidence of preeclampsia in pregnant women with either TPOAb or TGAb positive pregnant women (28). Still, there were some other studies shown no association between TAI and PIH, potentially because a relatively low incidence of individuals with both TAI and PIH in general pregnancies and small sample size did not have the power to find the association. Additionally, some studies in which thyroid autoantibodies were tested in the third trimester did not show association with PIH (20, 29), potentially because that, thyroid autoantibody levels fall and reaching its nadir in the third trimester (6) and the impact of thyroid autoantibodies on blood pressure may exist before this period. Family history contribute a lot to PIH, but previous study did not consider it as confounder. After adjusted for parameters including hypertension family history, we determined the association between thyroid autoantibodies and PIH, more importantly, with a dose-dependent manner. The quantitative association of pregnancy outcomes with thyroid antibodies, especially TGAb were seldom reported, and our result provide evidence that the probability of PIH rise by increasing TPOAb and TGAb level respectively. Taken together, the present results indicated the importance of monitoring blood pressure during pregnancy period in women with TAI, both TPOAb and TGAb positive in early pregnant period should be concerned, especially who with high concentration.

Although increasing studies show the relationship between TAI and GDM (17, 18, 30), it remains not wildly concerned in clinical practice. Thyroid dysfunction may impact the regulation glucose metabolism and increase risk of GDM (17) and current evidence predominantly links it to hypothyroidism. However, Huang's study found that TAI in itself has higher risk of GDM (31). In the present study, we also identified TAI as independent risk factor of GDM in pregnant women, the result was not impacted by thyroid function even after adjustment with family history of diabetes. That means blood glucose monitoring is important for TAI pregnant women no matter thyroid dysfunction or not. The attention to sugar metabolism even should last for postpartum by reason of Tang's study also reported that TAI may increase the risk of diabetes

TABLE 2 Proportions of pregnant complications between groups.

	TAI Positive	TAI Negative	*P* value
Maternal Outcomes			
Pregnancy Induce Hypertension	212 (4.06%)	748 (3.45%)	**0.037**
Gestational Diabetes Mellitus	1059 (20.39%)	3974 (18.45%)	**0.001**
Preterm Birth	469 (8.95%)	1645 (7.56%)	**0.001**
Premature Rupture of Membrane	1060 (19.84%)	4454 (20.19%)	0.589
Postpartum Hemorrhage	377 (7.06%)	1711 (7.75%)	0.090
Fetal-neonatal Outcomes			
Neonatal Outcome			< **0.001**
Normal	3962 (75.74%)	16965 (78.08%)	
NICU Admission	1261 (24.11%)	4730 (21.77%)	
Neonatal Death	8 (0.15%)	33 (0.15%)	
Apgar Score			0.546
0-3	25 (0.48%)	94 (0.43%)	
4-7	103 (1.97%)	477 (2.20%)	
8-10	5096 (97.55%)	21131 (97.37%)	
Macrosomia	83 (1.55%)	486 (2.20%)	**0.003**
Large for Gestational Age	174 (4.04%)	844 (4.77%)	**0.045**
Small for Gestational Age	545 (11.64%)	2036 (10.77)	0.094

Data are presented as mean ± SD or n (%). P values <0.05 were shown in bold.

mellitus after pregnancy (17). Similar to PIH, studies on GDM in which thyroid autoantibodies positive in the third trimester did not observed a significantly association. Notably, the majority of studies shown positive association between TAI and GDM were perform in Asian population. The underlining mechanism remain uncleared and it needs further investigation.

The association between TAI and preterm birth has been found in previous studies (14, 32–35). In the present study, we found a higher proportion of preterm birth in pregnant women with TAI, and the association between TAI and preterm birth was statistically significant after adjusted for maternal age, BMI, gravidity, TSH and F4 concentrations, and history of infertility. However, after adding history of recurrent miscarriage as confounder, the association between TAI and preterm birth was not found. This result indicated that the impact of TAI on preterm birth needed further investigation.

The impact of thyroid autoantibodies on other outcomes of the developing fetus and neonates is far from elucidated. Although the risk of SGA in TAI group did not meet statistical significance, its probability rises by increasing TGAb level. In addition, neonates born to TAI women tend to be with lower birthweight and higher risk of NICU admission. This result support the point in previous studies that thyroid autoantibodies affected fetal growth (29, 31). The mechanism of lower birthweight and higher risk of fetal adverse outcomes is not fully clarified, but we learn from one report that placenta weight is lower in TAI group (19). Thus, parameters of fetal growth and development, as well as maternal nutrition supplement should be aware in pregnant women with TAI.

To better understanding the difference between TAI women and their control individuals, we describe the evolution of maternal TSH, FT4 and FT3 throughout pregnancy base on this large multi-center cohort study. Our result shown that TSH concentrations were higher in TAI women in baseline and remain higher before the third trimester. TAI pregnancies also have higher probability of subclinical hypothyroidism, which is consistent with previous studies (21). Higher FT4 concentration in the TAI group seems to be controversial with elevated TSH concentrations. However, the lower concentration of FT3 in TAI group may explain this phenomenon, since the negative feedback efficiency of FT3 was stronger than FT4 (36). Increase level of both FT4 and TSH was also reported in previous study (37). The potential reason of high FT4 in TAI group might because of the supplementation of levothyroxine in TAI women, but the detail information was not able to obtained in the present retrospective study. As biologically active hormone, FT3 was not elevated sync with FT4 maybe the potential reasons that levothyroxine supplement did not improve pregnancy outcomes in TAI women in previous study (11, 12). To determine the reason of higher FT4 and lower FT3 concentration in TAI pregnant women, further study is needed to study the impact of thyroid antibodies on deiodinase.

There were several strengths of the present study. First, we used a large cohort of pregnant women from three centers in our study to obtain robust results. To the best of our knowledge, the number of participants in this original cohort study was largest on the topic of TAI and pregnancy complications. And based on this cohort, we were able to analyze a number of complications and adverse outcomes both maternal and fetal-neonates by adjusting multiple cofounders. Second, the quantitative association of thyroid autoantibody with pregnancy outcomes was seldom reported. Our result of their dose-dependent association adds to the limited knowledge on the complicated and multifactorial mechanisms underlying pregnancy outcomes. Third, we described the evolution of maternal TSH, FT4 and FT3 in TAI pregnancies and their variation by increasing thyroid autoantibody level. This result may provide insights toward better understanding the difference between TAI women and their control individuals.

Our study also had some limitation. First, the medicine history of levothyroxine (LT-4) supplementation was not obtained due to the retrospective design, and thus the impact of LT-4 supplementation on pregnancy complications were not able to analyze in the present study. Second, although consist of a large number of pregnant women from three centers, the present study is performed in South China where is iodine rich area, and may not represent pregnant women in general population. Third, the underlining mechanism of thyroid autoantibodies and complications were not analyzed in this study. Considering the high prevalence and clinical significance of TAI in pregnancy, further study is needed to determine how thyroid autoantibodies affect related complications.

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TABLE 3 Logistic regression of thyroid autoimmunity and pregnant complications.

	Model 1		Model 2		Model 3		Model 4	
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*
Maternal Outcomes								
Pregnancy Induce Hypertension	**1.186 (1.005-1.400)**	**0.043**	**1.195 (1.012-1.410)**	**0.036**	**1.215 (1.026-1.438)**	**0.024**	**1.206 (1.019-1.428)**	**0.030**[a]
Gestational Diabetes Mellitus	**1.090 (1.004-1.183)**	**0.039**	**1.089 (1.003-1.181)**	**0.042**	**1.093 (1.006-1.188)**	**0.036**	**1.088 (1.001-1.183)**	**0.046**[b]
Preterm Birth	**1.144 (1.017-1.286)**	**0.025**	**1.138 (1.012-1.280)**	**0.031**	**1.140 (1.011-1.285)**	**0.032**	**1.129 (1.001-1.273)**	**0.048**[c]
Premature Rupture of Membrane	1.022 (0.944-1.105)	0.596	1.032 (0.954-1.117)	0.431	1.055 (0.974-1.144)	0.188	1.056 (0.975-1.144)	0.183
Postpartum Hemorrhage	0.920 (0.815-1.038)	0.176	0.923 (0.818-1.042)	0.195	0.926 (0.819-1.046)	0.217	0.920 (0.814-1.041)	0.186
Fetal-neonatal Outcomes								
NICU Admission	**1.134 (1.052-1.223)**	**0.001**	**1.140 (1.057-1.230)**	**0.001**	**1.091 (1.010-1.179)**	**0.027**	**1.084 (1.004-1.171)**	**0.040**
Low Apgar Score	0.902 (0.731-1.113)	0.336	0.904 (0.733-1.116)	0.347	0.925 (0.747-1.144)	0.471	0.920 (0.744-1.139)	0.446
Macrosomia	**0.735 (0.575-0.940)**	**0.014**	**0.732 (0.572-0.936)**	**0.013**	**0.769 (0.599-0.986)**	**0.038**	**0.768 (0.599-0.985)**	**0.038**
Large for Gestational Age	**0.813 (0.680-0.972)**	**0.023**	**0.804 (0.672-0.961)**	**0.016**	**0.834 (0.696-1.000)**	0.050	**0.833 (0.695-0.999)**	**0.049**
Small for Gestational Age	1.096 (0.984-1.219)	0.095	1.111 (0.998-1.237)	0.054	1.098 (0.985-1.225)	0.092	1.097 (0.984-1.223)	0.096

Model 1: Adjusted for Age and BMI; Model 2: Model 1+ Gravidity; Model 3: Model 2+ TSH and FT4; Model 4: Model 3+ history of infertility.

[a]The association between TAI and pregnancy induce hypertension were further adjusted by the confounders in Model 4 and family history of hypertension, and the OR is 1.215(95% CI 1.026-1.439), P=0.024.

[b]The association between TAI and gestational diabetes mellitus were further adjusted by the confounders in Model 4 and family history of diabetes, and the OR is 1.088(95% CI 1.001-1.183), P=0.048.

[c]The association between TAI and preterm birth were further adjusted by the confounders in Model 4 and history of recurrent miscarriage, and the OR is 1.082(95% CI 0.958-1.222), P=0.205.

Data are presented as n (%). P values <0.05 were shown in bold.

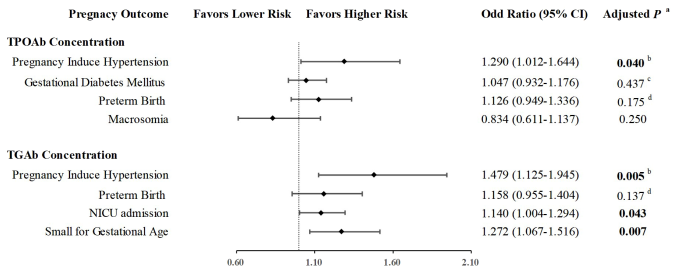

FIGURE 3
Quantitative association of thyroid autoimmunity and pregnant complications. [a]Adjusted for Age, BMI, Gravidity, TSH level, Free T4 level, and history of infertility. [b]Adjusted for Age, BMI, Gravidity, TSH level, Free T4 level, history of infertility, and family history of hypertension. [c]Adjusted for Age, BMI, Gravidity, TSH level, Free T4 level, history of infertility, and family history of diabetes. [d] Adjusted for Age, BMI, Gravidity, TSH level, Free T4 level, history of infertility, and history of recurrent miscarriage.

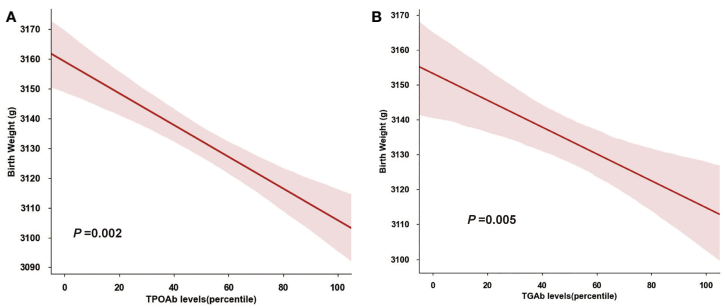

FIGURE 4
Quantitative Association of Birthweight and Thyroid Autoantibody. **(A)** Birthweight decreased with increasing TPOAb concentrations (percentiles). **(B)** Birthweight decreased with increasing TGAb concentrations (percentiles).

Conclusion

We illustrated the independent association between TAI and adverse pregnancy outcomes, including PIH and GDM. We also found neonates born to women with TAI were with lower birthweight and at higher risk for NICU admission. The quantitative association found in the present study between TPOAb and PIH, and between TGAb and PIH, SGA and NICU admission indicates that the dose-dependent effect of thyroid autoimmunity on pregnancy complications should be taken into account in future research and clinical practice.

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, Sun Yat-sen Memorial Hospital, Sun Yat-sen University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YX designed the study, analyzed the data and drafted the manuscript. HC and YG provided the clinical data. MR, KS and DL critical review of the study design. HW and RD provided laboratory data. JW and ZL provided clinical data. ZW and LY contributed to the design and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1081851/full#supplementary-material

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Mechanism of leptin-NPY on the onset of puberty in male offspring rats after androgen intervention during pregnancy

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Objectives: The time of onset of puberty has been increasingly earlier, but its mechanism is still unclear. This study aimed to reveal the mechanism of leptin and NPY in the onset of puberty in male offspring rats after androgen intervention during pregnancy.

Methods: Eight-week-old specific pathogen-free (SPF) healthy male Sprague –Dawley (SD) rats and 16 female SD rats were selected and caged at 1:2. The pregnant rats were randomly divided into the olive oil control group (OOG) and testosterone intervention group (TG), with 8 rats in each group. Olive oil and testosterone were injected from the 15th day of pregnancy, for a total of 4 injections (15th, 17th, 19th, 21st day). After the onset of puberty, the male offspring rats were anesthetized with 2% pentobarbital sodium to collect blood by ventral aorta puncture and decapitated to peel off the hypothalamus and abdominal fat. Serum testosterone (T), free testosterone (FT), dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), sex hormone binding globulin (SHBG), and leptin were detected by ELISA, and then the free androgen index (FAI) was calculated. The mRNA levels of androgen receptor (AR), estrogen receptor α (ERα), NPY, leptinR, and NPY2R in the hypothalamus and abdominal fat were detected by RT–PCR. Protein expression levels of AR, ERα, NPY, leptinR, and NPY2R in the arcuate nucleus (ARC) of the hypothalamus were detected by immunohistochemistry.

Results: The time of onset of puberty was significantly earlier in the TG than in the OOG ($P< 0.05$) and was positively correlated with body weight, body length, abdominal fat, and leptinR mRNA levels in adipose tissue in the OOG ($P< 0.05$), while it was positively correlated with serum DHT and DHEA concentrations and FAI and AR mRNA levels in the hypothalamus in the TG ($P< 0.05$). The NPY2R mRNA level and protein expression levels of ERα, NPY2R, and leptinR in the TG were significantly higher than those in the OOG, while the protein expression levels of AR and NPY in the TG were significantly lower than those in the OOG ($P< 0.05$).

Conclusions: Testosterone intervention during pregnancy led to an earlier onset of puberty in male offspring rats, which may render the male offspring rats more sensitive to androgens, leptin, and NPY at the onset of puberty.

KEYWORDS

leptin, NPY, puberty onset, androgen, GnRH

1 Introduction

Puberty is defined as the "first reproductive period", which marks the maturity of the genitals, the development of secondary sexual characteristics, the acceleration of linear growth, emotional changes, and the occurrence of first spermatorrhea or menarche (1). The onset time of puberty has shown an increasing trend of being earlier worldwide (2). In the past 150 years, the average age of menarche of European children has declined from approximately 16–17 years in the middle of the 19th century to 12 years in the middle of the 20th century (3, 4), which is estimated to be advanced by 3 years every 100 years (5). A cohort study of Swedish boys showed that the starting age of puberty was 1.5 months earlier for every decade increase in birth year (6). Moreover, the average age at which Danish boys initiated puberty was 3 months earlier from 2006 to 2008 than before (7), and that of Chinese urban boys was 2 years earlier from 2003 to 2005 than 1979 (8). The early onset of puberty not only affects mental health in children and adolescents but also increases the risk of certain diseases in adulthood, such as metabolic syndrome, cardiovascular disease, osteoporosis, and testicular cancer (9, 10), which has developed into a serious public health problem.

With the development of 'Fetal Origin of Adult Disease' (FOAD) and 'Development Origin of Health and Disease' (DOHaD) (11, 12), an increasing number of studies have linked the endocrine environment during pregnancy to the long-term effects on future generations (13–16). With lifestyle changes and the effects of exogenous endocrine disruptors (17), the proportion of women with high androgen levels during pregnancy was significantly increased (18–20). Exposure to prenatal high androgen environments may lead to changes in hormone levels and pubertal development in offspring (21).

Leptin is a protein product of the obesity (Ob) gene and plays an important role in pubertal development and reproduction (22). Recent studies have found that leptin can regulate the synthesis and release of GnRH in the hypothalamic arcuate (ARC) nucleus (23). GnRH secretion is insufficient in mice lacking the leptin gene (24). Studies have directly shown that leptin can trigger the beginning of puberty in boys (25). However, studies have found that there is no leptin receptor expression or very little expression in ARC nucleus GnRH neurons (26). Therefore, leptin cannot directly act on GnRH neurons, but it is involved in regulating puberty onset (27, 28). However, leptin may indirectly affect the activity of GnRH neurons by affecting NPY neurons (29). NPY was identified as the main regulator of GnRH pulse secretion (30), which is the 'gatekeeper' of adolescence (31). As a regulator, NPY neurons are located in the ARC (32), which can directly exert an inhibitory effect on GnRH neurons (33). This effect is achieved by the direct binding of NPY to NPY1R on GnRH neurons (34). Moreover, recent studies have demonstrated that leptin receptors exist in NPY neurons (24), which indicates that leptin can directly regulate NPY neurons (35) and thus play an important role in regulating reproduction (36).

However, whether high androgen exposure during pregnancy can participate in puberty onset by affecting the levels of leptin and NPY in offspring has rarely been reported. Therefore, this study administered androgen intervention to maternal rats during pregnancy to shed light on the mechanism of leptin and NPY in puberty onset of offspring male rats after androgen intervention during pregnancy, which provides a powerful clue for exploring the relationship between prenatal androgen and the onset of puberty in male offspring.

2 Participants and methods

2.1 Rats, diet, and experimental procedures

All animal care and experimental procedures were approved by the Ethics Committee of Bengbu Medical College ([2018] No.032). A total of 16 eight-week-old healthy SD female rats (200–250 g) and 8 male rats (300–330 g) were purchased from Jinan Peng Yue Experimental Animal Reproduction Co., Ltd. Animals were raised in a clean animal room with relative temperature (25 ± 2°C), relative humidity (40–70%), free light, and free access to water and diet. After a week of adaptive feeding, the female and male rats were caged at 9 o'clock every night at a ratio of 1:1, and vaginal plugs were observed at 9 o'clock the next morning to determine whether the rats were pregnant. Then, the pregnant female rats were randomly divided into the olive oil control group (OOG) and testosterone intervention group (TG), with 8 rats in each group. The pregnant female rats were treated with subcutaneous injection on the back of the neck starting from the 15th day of pregnancy, for a total of 4 injections (15th, 17th, 19th, 21st day). TG was given 2 mL of 2.5 g/ml testosterone solution (dissolved in olive oil), and OOG was given the same dose of olive oil. After the birth of the offspring, 36 male offspring rats were taken, which were raised to 21 days (PND21) and then weaned. From PND21, the body weight of the rats was recorded daily, and the genitals were observed. The foreskin separation of the male rats was used as a sign of the onset of puberty.

The number of days taken to initiate puberty was recorded in all experimental rats. Twelve rats (TG : OOG = 1:1) were used for the detection of body shape indicators, serum hormone levels, and the mRNA of hypothalamic neuroendocrine function genes. Then, 6 rats (TG : OOG = 1:1) were used to detect the expression of hypothalamic neuroendocrine functional proteins by immunohistochemistry.

2.2 Sample collection

After anesthesia with 2% pentobarbital sodium solution at a dose of 3 ml/kg, we punctured the abdominal aortas of the rats to collect blood, and the blood was centrifuged to obtain the upper serum, which was then stored at -80 °C. We decapitated the rats, stripped their hypothalamus and abdominal fat, immediately placed them in liquid nitrogen and transferred them to the refrigerator at -80 °C at the end of the experiment. The above samples were all operated on ice.

2.3 Body shape indices and wet weight of abdominal fat in rats

When signs of puberty onset were observed, the rats' body weight, body length, anal-genital distance (AGD), abdominal fat, and abdominal fat coefficient were measured. The body weight was measured with an electronic scale and was accurate to 0.1 g; body length refers to the distance from the tip of the nose to the superior margin of the anus, which was measured accurately to 0.1 cm; the AGD was measured by a Vernier caliper after anesthesia in rats and was accurate to 0.01 mm. Finally, abdominal fat was measured using an analytical balance and was accurate to 0.01 g; abdominal fat coefficient (%) = [abdominal fat weight (g)/body weight (g)] × 100%.

2.4 Detection of serum biochemical indices

The concentrations of T, FT, DHT, DHEA, SHBG, and leptin in the male rat serum were determined using ELISA with rat T (cat. no. CSB-E05100r, CUSABIO, Wuhan, China), rat FT (cat. no. CSB-E05097r, CUSABIO), rat DHT (cat. no. CSB-E07879r, CUSABIO), rat DHEA (cat. no. CSB-E08227r, CUSABIO), rat SHBG (cat. no. CSB-E12118r, CUSABIO) and rat leptin ELISA kits (cat. no. CSB-E07433r, CUSABIO). FAI = [T (ng/ml)/SHBG (ng/ml)] × 100.

2.5 Isolation of RNA and real-time PCR

Total RNA was extracted from the hypothalamus and abdominal adipose tissue by the TRIzol method (Thermo Scientific, Shanghai, China). For the detection of RNA concentration, we used 1 μg total RNA reverse transcription cDNA according to the instructions of the kit manufacturer (Thermo Scientific), and the mRNA levels of AR, ERα, NPY, leptinR, and NPY2R in the above tissues were detected by quantitative real-time PCR. The gene-specific primer sequences were designed by Shanghai Generay Biotech Co., Ltd., and all the primer sequences are shown in Table 1. The polymerase chain reaction mixture was 15 μl, and the reaction system contained 7.5 μl of 2×qPCR Mix, 1.5 μl of 2.5 μM gene primer, 2.0 μl of reverse transcription product, and 4.0 μl of ddH2O. The three-step PCR amplification protocol was as follows: predenaturation, 95°C 10 min; cycle, 95°C 15 s → 60°C 60 s, a total of 40 cycles; melting curve, 60°C → 95°C, heating 0.3°C every 15 s.

2.6 Immunohistochemical analysis

Hypothalamic tissue was fixed with 10% formalin and embedded in paraffin. The hypothalamus paraffin blocks were cut into 4 μm thick sections and placed in a repair box filled with citric acid antigen repair buffer (PH 6.0) for antigen retrieval in a microwave oven. After natural cooling, the slides were placed in PBS (PH 7.4), washed on a decolorized shaker 3 times for 5 minutes each time and then incubated in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase. Subsequently, 3% BSA was added to cover the tissue uniformly and sealed at room temperature for 30 min. The blocking solution was removed, and

TABLE 1 PCR primer sequences.

Primer name (-S upstream primer; -A downstream primer)	Primer sequence (5′-3′)
AR-S	TGGGACCTTGGATGGAGAACT
AR-A	GGCACATAGATACTTCTGTTTCCC
ERα-S	GTTTGCTCCTAACTTGCTCTTGG
ERα-A	TCAAGGTGCTGGATAGAAATGTG
NPY-S	CTCTGCGACACTACATCAATCTCA
NPY-A	CCTTGTTCTGGGGGCATTT
Leptin Receptor-S	GGAAACACAGGGGTCCA
Leptin Receptor-A	TAGCAGCATCAACACCGA
NPY Receptor-S	ACAGTGAACTTTCTCATAGGCAACC
NPY Receptor-A	CAGAACTGACACACATTGAAGGAAC

PBS was dropped in a certain proportion of the primary antibody (rabbit polyclonal antibody, Servicebio, Wuhan, China) on the slice. The samples were then placed in a wet box and incubated overnight at 4°C. Then, the cells were incubated with a biotinylated secondary antibody (rabbit polyclonal antibody, Servicebio, Wuhan, China) and placed at room temperature for 50 min. A DBA kit was used to observe the immune response at room temperature for 2 minutes. Finally, hematoxylin staining was used for microscopic examination. Integrated optical density (IOD) was used to describe the relative expression of AR, ERα, leptinR, NPY, and NPY2R proteins. The IOD was repeated three times for each sample using Image-Pro Plus software, and the average value was taken.

2.7 Statistical analysis

The statistical analysis was performed in SPSS 23.0 software. First, because of the inconsistency of the sampling time of onset of puberty, we standardized the data according to the sampling time [standardized index = (index before standardization/time of onset of puberty) × average time of onset of puberty] and carried out logarithmic conversion of the data. Measurement data are described as the mean ± standard deviation. The differences in morphological development indices, sex hormones, and mRNA between TG and OOG were analyzed by independent-sample *t test*, while the differences in immunohistochemical indices between TG and OOG were analyzed by the mixed linear model. Pearson correlation analysis was used to analyze the correlation. $P< 0.05$ was considered statistically significant.

3 Results

3.1 Effect of testosterone intervention during pregnancy on the time of onset of puberty in offspring male rats

Figure 1 shows that the time of onset of puberty of male offspring in the TG (44.11 ± 2.14 days; $n = 18$) was significantly lower than that in the OOG (46.89 ± 2.14 days; $n = 18$), which suggested that testosterone intervention during pregnancy may lead to earlier time of onset of puberty of male offspring ($P< 0.05$).

3.2 Effects of testosterone intervention during pregnancy on morphological development and blood circulating sex hormone levels in offspring male rats

Table 2 shows that there were no significant differences in body weight, body length, abdominal fat, abdominal fat coefficient, T, FT, DHT, DHEA, SHBG, FAI, or leptin between TG and OOG ($P > 0.05$). The results of correlation analysis showed that the time of onset of puberty in the OOG was positively correlated with body weight, body length, and abdominal fat ($P< 0.05$), while in the TG, the time of onset of puberty was positively correlated with serum DHT and DHEA concentrations and FAI ($P< 0.05$) and negatively correlated with SHBG concentration ($P< 0.05$). See Table 3 for details.

3.3 Effects of testosterone intervention during pregnancy on mRNA levels of AR, ER-α, leptinR, NPY, NPY2R

Testosterone intervention during pregnancy resulted in markedly elevated mRNA levels of NPY2R in the hypothalamus compared with the OOG, whereas the mRNA levels of leptinR in the fat were significantly downregulated ($P< 0.05$; Table 4). There was a statistically positive correlation between the time of onset of puberty and leptinR mRNA levels in adipose tissue in the OOG ($R = 0.840$, $P< 0.05$) but not with other indicators (Table 5). Nevertheless, after testosterone intervention, there was only a statistically positive correlation between the time of onset of puberty and the level of AR mRNA in the hypothalamus ($R = 0.883$, $P< 0.05$; Table 5).

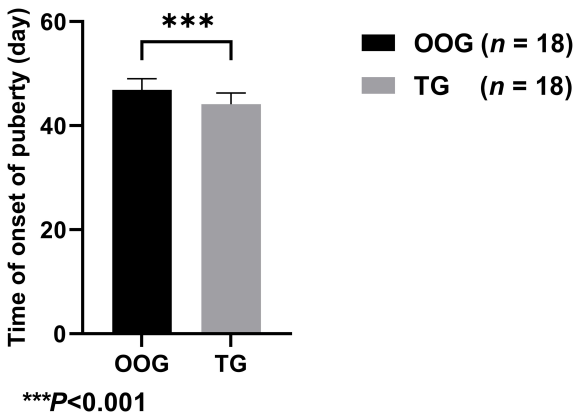

FIGURE 1
Comparison of the time of onset of puberty between OOG and TG.

TABLE 2 Comparison of body shape indices and blood circulating sex hormone indices in male offspring between OOG and TG ($\bar{x} \pm s$).

Indices	OOG (n = 6)	TG (n = 6)	t	P
S Body weight (g)	194.762±15.998	191.768±38.97	0.17	0.87
S Body length (cm)	18.775±0.459	18.768±1.951	0.01	0.99
S Abdominal fat (g)	1.388±0.492	1.023±0.382	1.43	0.18
S Abdominal fat coefficient	0.700±0.208	0.520±0.138	1.77	0.11
Sln T (ng/ml)	1.303±0.854	0.651±0.503	1.61	0.14
Sln FT (pg/ml)	2.360±0.785	1.662±0.465	1.88	0.09
Sln DHT (pg/ml)	5.699±0.691	5.031±0.323	2.15	0.06
Sln DHEA (ng/ml)	0.158±0.762	-0.475±0.618	1.58	0.15
Sln SHBG (ng/ml)	6.592±0.250	6.679±0.578	-0.34	0.74
Sln Leptin (ng/ml)	-2.264±1.290	-2.979±0.264	1.33	0.21
Sln FAI	-0.716±0.820	-1.365±0.712	1.46	0.17

TABLE 3 Correlation between body shape indices, serum sex hormone levels and the time of onset of puberty in male offspring of OOG and TG.

Variables	Body weight	Body length	Abdominal fat	T	FT	DHT	DHEA	SHBG	FAI	Leptin
OOG										
Puberty onset time	0.821*	0.891*	0.844*	0.414	0.364	0.398	0.394	0.695	0.286	-0.299
Body weight	1	0.819*	0.985**	0.47	0.384	0.455	0.454	0.448	0.432	0.258
Body length		1	0.764	0.753	0.710	0.740	0.744	0.763	0.655	-0.148
Abdominal fat			1	0.353	0.264	0.338	0.333	0.389	0.308	0.204
T				1	0.987**	1.000**	0.998**	0.429	0.984**	0.077
FT					1	0.987**	0.981**	0.468	0.970**	0.067
DHT						1	0.998**	0.413	0.986**	0.078
DHEA							1	0.424	0.983**	0.066
SHBG								1	0.301	-0.347
FAI									1	0.230
TG										
Puberty onset time	-0.747	-0.718	-0.595	0.789	0.800	0.815*	0.812*	-0.887*	0.951**	0.029
Body weight	1	0.908*	0.830*	-0.277	-0.282	-0.252	-0.286	0.936**	-0.624	0.278
Body length		1	0.957**	-0.307	-0.305	-0.339	-0.262	0.790	-0.583	0.204
Abdominal fat			1	-0.162	-0.17	-0.203	-0.092	0.679	-0.427	0.007
T				1	0.998**	0.954**	0.981**	-0.467	0.919**	0.238
FT					1	0.953**	0.981**	-0.48	0.921**	0.278
DHT						1	0.955**	-0.465	0.877*	0.192
DHEA							1	-0.518	0.924**	0.155
SHBG								1	-0.776	0.188
FAI									1	0.067

TABLE 4 Comparison of the content of functional gene mRNA in male offspring between OOG and TG ($\bar{x} \pm s$).

Indices	OOG (n = 6)	TG (n = 6)	t	P
Hypothalamus				
S AR	1.006±0.609	0.809±0.415	0.653	0.529
S ER-α	1.015±0.997	0.485±0.436	1.195	0.272
S LeptinR	0.990±0.153	1.248±0.252	-2.149	0.057
S NPY	0.995±0.486	1.442±1.010	-0.978	0.351
S NPY2R	0.997±0.315	2.719±1.070	-3.779	<0.05
Fat				
S Leptin	0.997±0.498	0.671±0.372	1.283	0.228
S LeptinR	0.981±0.300	0.650±0.093	2.584	<0.05

TABLE 5 Correlation between hypothalamic functional gene mRNA levels and the time of onset of puberty in offspring of OOG and TG.

Variables	Hypothalamic AR	Hypothalamic ERα	Hypothalamic LeptinR	Hypothalamic NPY	Hypothalamic NPY2R	Fat Leptin	Fat LeptinR
OOG							
Puberty onset time	-0.411	-0.546	0.42	-0.092	-0.555	-0.209	0.840*
Hypothalamic AR	1	0.960**	-0.446	-0.103	0.72	-0.013	-0.168
Hypothalamic ERα		1	-0.365	0.137	0.609	0.085	-0.409
Hypothalamic LeptinR			1	0.568	-0.521	0.538	0.202
Hypothalamic NPY				1	-0.58	0.208	-0.505
Hypothalamic NPY2R					1	0.176	-0.065
Fat Leptin						1	-0.223
TG							
Puberty onset time	0.883*	-0.098	0.155	0.037	0.727	-0.256	0.377
Hypothalamic AR	1	-0.073	0.193	0.043	0.807	-0.278	0.044
Hypothalamic ERα		1	0.544	0.911*	-0.419	0.745	-0.361
Hypothalamic LeptinR			1	0.838*	0.245	0.388	0.282
Hypothalamic NPY				1	-0.164	0.652	-0.06
Hypothalamic NPY2R					1	-0.285	0.441
Fat Leptin						1	-0.100

3.4 Effects of testosterone intervention during pregnancy on the expression of AR, ERα, NPY, NPY2R, and leptinR

The expression of AR, ERα, NPY, NPY2R, and leptinR in the ARC of the offspring male rats was observed by quantitative analysis of immunohistochemical images. The results showed that the protein expression levels of ERα, NPY2R, and leptinR in the TG were significantly increased compared with those in the OOG, while the expression levels of AR and NPY in the TG were significantly lower than those in the OOG ($P< 0.05$; Table 6). Moreover, the results based on immunohistochemical sections further revealed that the expression of NPY2R and leptinR in the TG was higher than that in the OOG, while the expression of NPY in the TG was lower than that in the OOG. See Figure 2 for details.

4 Discussion

The early onset of puberty has become a trend. The role of the intrauterine environment during pregnancy in the onset of puberty in offspring has received increasing attention. In the current study, we found that testosterone intervention in pregnant rats leads to an early time of onset of puberty in male offspring rats, a positive association between the time of onset of puberty and AR mRNA, an increase in hypothalamic NPY2R and leptinR protein and a decrease in AR and NPY protein expression. This showed that male offspring rats after androgen intervention during pregnancy might be more sensitive to androgens, leptin, and NPY, thus leading to early puberty.

It has become a consensus that puberty onset is correlated with body shape indicators (37); that is, puberty begins when body weight reaches a certain threshold. In this study, we found positive associations between the time of onset of puberty and weight, body length, and abdominal fat in OOG, which was consistent with previous studies. However, there were positive correlations between the time of onset of puberty and serum DHT, DHEA levels, and FAI in TG, which showed that offspring male rats after androgen intervention during pregnancy might be more sensitive to androgens. At the same time, the protein expression of AR decreased in the TG, which also suggested that the time of onset of puberty of male offspring rats was more sensitive to androgen after testosterone intervention during pregnancy. It follows that the control of puberty onset of male offspring may no longer be the feedback of body shape changes but more through the feedback regulation of sex hormones in offspring male rats after androgen intervention during pregnancy (38).

It is well known that leptin sends a negative feedback signal to the brain center when the body has sufficient energy storage, thereby maintaining the body's energy balance by reducing food intake and increasing energy expenditure (39). Moreover, related studies have shown that androgen can inhibit the expression of leptin (40). Likewise, in a study on adipocytes of 3T3-L1 mice, it was found that dihydrotestosterone (DHT) significantly decreased leptin transcription and protein expression (41). In normal men, leptin levels may be limited by androgen, resulting in lower leptin levels than in women (42), and if serum leptin levels are above a certain threshold, leptin will in turn inhibit testicular function (43). Taken together, these signs suggest that androgen has an inhibitory effect on leptin. It was found that testosterone intervention reduced the protein expression of AR and upregulated the protein expression of leptinR at the onset of puberty in male offspring rats, which may be caused by the weakening of the inhibitory effect of low levels of androgens on leptin after testosterone intervention during pregnancy. In addition to its role in regulating energy balance, leptin also plays an important role in puberty onset by stimulating gonadotropin expression by upregulating the mRNA levels of GnRHR, activin, and FSHB (44). In this study, the expression of hypothalamic leptinR in the TG was higher than that in the OOG, indicating that the binding of hypothalamic leptin to leptinR may be enhanced after testosterone intervention, which affects the onset time of puberty.

Interestingly, related studies (45) have shown that there is no leptinR protein expression on GnRH neurons; therefore, by which mediator is leptin's regulation of puberty onset time achieved? NPY, a 36-amino acid orexin-producing protein, is a key feeding center in the hypothalamic arcuate nucleus (46) and binds to its receptors Y1 and Y2 to regulate reproduction and food intake, respectively (47), in which binding to NPY1R can directly inhibit the release of GnRH (28). Moreover, kisspeptin and POMC neurons are also regulated by leptin (48, 49). However, a relevant study showed that increased Kiss1 mRNA levels were detected only after the onset of puberty (50), and Kiss1 does not express substantial amounts of leptin receptors (51), indicating that kisspeptin neurons may play a role after puberty. Another study also noted that the leptin-POMC pathway has sex specificity in terms of reproductive control, that is, it is difficult to play a role in male mice (52). However, for NPY neurons, related studies (29, 53) have provided evidence that serum leptin and hypothalamic leptin receptors are involved in the regulation of GnRH release by NPY and that NPY levels are

TABLE 6 Comparisons of proteins levels between OOG and TG in the hypothalamus of male offspring ($\bar{x} \pm s$).

Indices	OOG	TG	*F*	*P*
AR	0.273±0.040	0.247±0.032	4.538	<0.05
ERα	0.323±0.030	0.394±0.017	76.953	<0.05
NPY	0.302±0.048	0.273±0.027	4.695	<0.05
NPY2R	0.234±0.027	0.287±0.022	43.167	<0.05
LeptinR	0.179±0.044	0.243±0.037	22.574	<0.05

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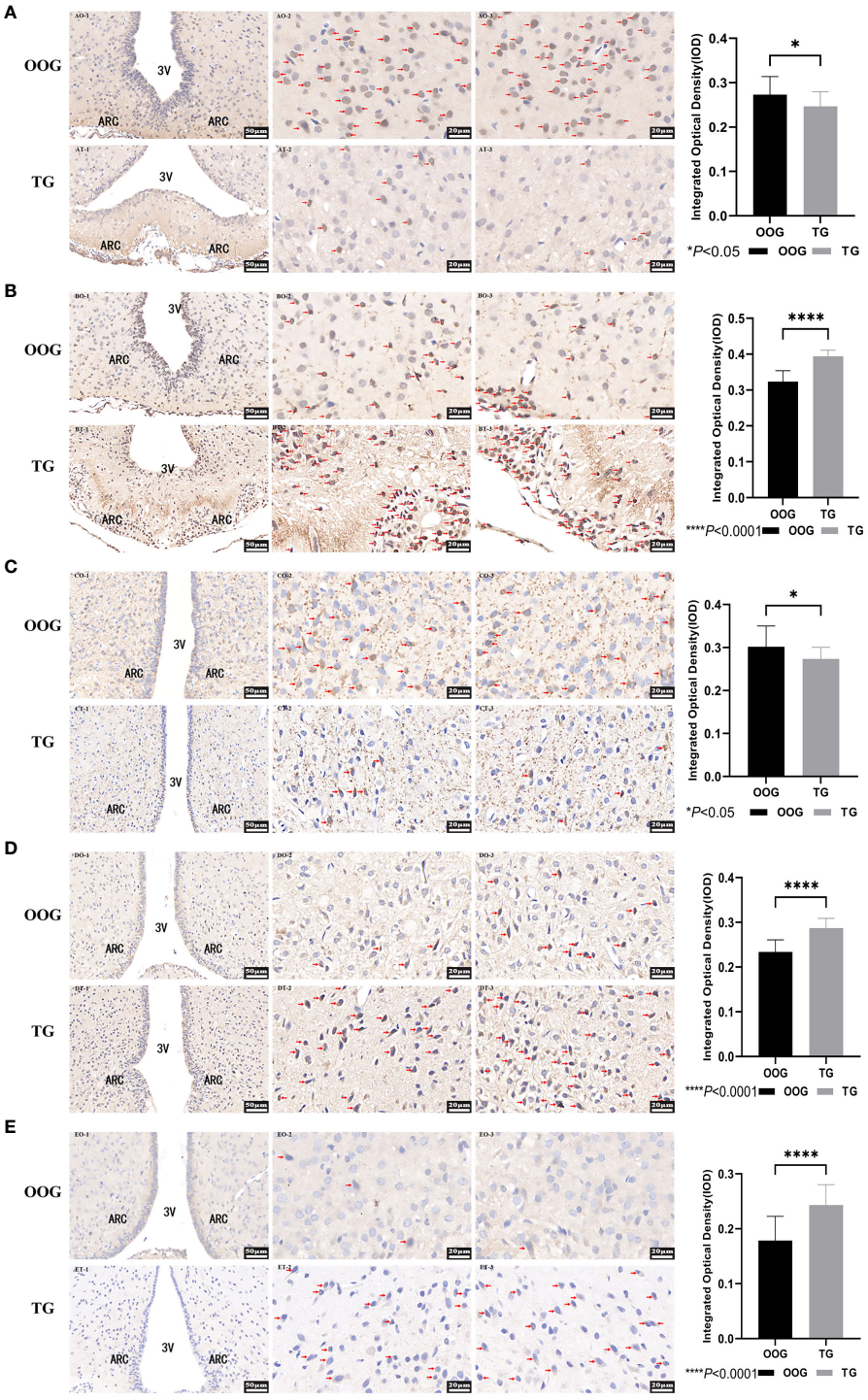

FIGURE 2
Protein expression of AR, ERα, NPY, NPY2R, and LeptinR in the hypothalamus. **(A)**, the immunohistochemical slice of AR; **(B)**, the immunohistochemical slice of ERα; **(C)**, the immunohistochemical slice of NPY; **(D)**, the immunohistochemical slice of NPY2R; **(E)**, the immunohistochemical slice of LeptinR. *P< 0.05, ****P< 0.0001.

elevated in leptin-deficient (ob/ob) mice (54). As shown in the results of this study, at the onset of puberty, the protein expression of leptinR and NPY2R in the hypothalamus increased, whereas the expression of NPY protein decreased in the TG, which may be due to the increased sensitivity of offspring male rats to leptin, thus suggesting that the binding of leptin to the leptin receptor was enhanced in the hypothalamus. The signal was transmitted to NPY neurons to inhibit NPY mRNA encoding the NPY protein, resulting in a decrease in NPY protein expression. Furthermore, in the environment of increased expression of NPY2R protein, the binding ability of NPY2R to NPY is enhanced, which mainly plays a role in energy expenditure and locomotion (55); thus, the

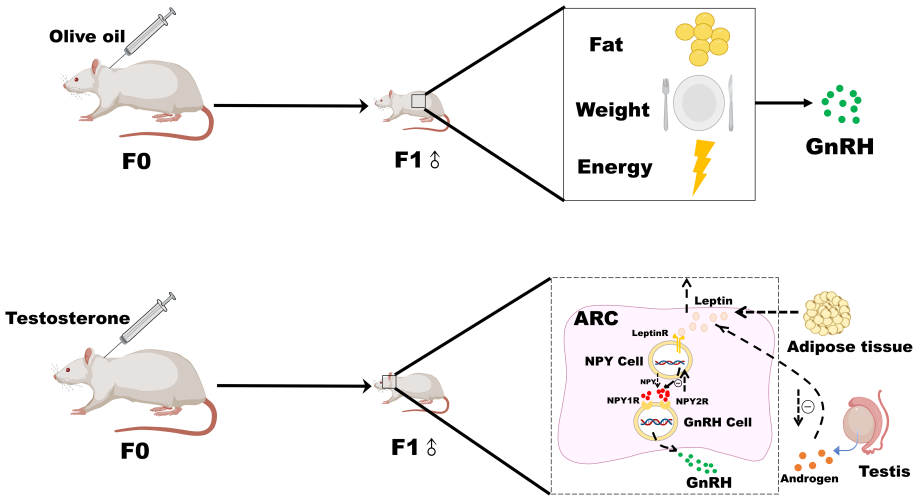

FIGURE 3
Possible mechanism of leptin-NPY on the onset of puberty in male offspring rats after androgen intervention during pregnancy.

inhibition of GnRH may be weakened followed by GnRH increasing rapidly during puberty; therefore, puberty starts earlier.

5 Limitations

In this study, we detected only NPY2R, not NPY1R, so we cannot draw specific conclusions directly through the binding of NPY and NPY1R. Moreover, the intervention of testosterone was only in the third trimester of pregnancy, and it did not reflect the effect of testosterone on the puberty onset of offspring during pregnancy. At the same time, we sampled at the onset of puberty and reflected the differences between groups in the form of standardization, which may reduce the effectiveness of reflecting differences between groups. Finally, the association results were based on too few samples, which had limitations in the interpretation of associations.

6 Conclusion

Testosterone intervention in pregnant rats led to an earlier time of onset of puberty in male offspring rats. There were positive correlations between the time of onset of puberty and serum DHT, DHEA levels, and FAI, increased NPY2R mRNA, leptinR, and NPY2R protein expression, and decreased AR and NPY protein expression in male offspring rats after testosterone intervention during pregnancy. These results indicate that high androgen during pregnancy leads to early puberty onset in male offspring, which may be related to the decreased protein expression of AR, the increased expression of leptinR in the hypothalamus, and the enhanced inhibition of NPY neurons, as well as the enhanced binding of NPY2R and NPY. This could render male offspring after testosterone intervention during pregnancy more sensitive to androgens, leptin, and NPY at the onset of puberty. Therefore, the inhibition of GnRH may be weakened, and puberty onset occurs earlier (Figure 3).

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Ethics Committee of Bengbu Medical College.

Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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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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Hyperglycemia disturbs trophoblast functions and subsequently leads to failure of uterine spiral artery remodeling

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Uterine spiral artery remodeling is necessary for fetal growth and development as well as pregnancy outcomes. During remodeling, trophoblasts invade the arteries, replace the endothelium and disrupt the vascular smooth muscle, and are strictly regulated by the local microenvironment. Elevated glucose levels at the fetal-maternal interface are associated with disorganized placental villi and poor placental blood flow. Hyperglycemia disturbs trophoblast proliferation and invasion *via* inhibiting the epithelial-mesenchymal transition, altering the protein expression of related proteases (MMP9, MMP2, and uPA) and angiogenic factors (VEGF, PIGF). Besides, hyperglycemia influences the cellular crosstalk between immune cells, trophoblast, and vascular cells, leading to the failure of spiral artery remodeling. This review provides insight into molecular mechanisms and signaling pathways of hyperglycemia that influence trophoblast functions and uterine spiral artery remodeling.

KEYWORDS

hyperglycemia, trophoblast, decidual NK cells, Hofbauer cells, uterine spiral artery remodeling

1 Introduction

Hyperglycemia (HG) is a common metabolic imbalance in pregnant women with Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), or combined with pregnancy and gestational diabetes mellitus (GDM) (1). Abnormally high blood glucose levels in the pregnancy may lead to abnormal uterine glucose concentration (2). Unhealthy dietary habits, such as a high glucose intake, are prevalent nowadays. Both HG and HG-induced cytokines releasing affect trophoblast function and uterine spiral arteries (SAs) remodeling, which can in turn increase the incidence of pregnancy complications, such as pre-eclampsia, malformations and miscarriage, and thus endanger the health of pregnant women and fetuses (3, 4).

SAs facilitate the exchange of nutrients, gases, and waste between mother and fetus (5). During SA remodeling, the original uterine SA converts into low-resistance and highly dilated vessels to meet the pregnancy blood requirements and prevent damage to the villi (6). Uterine SA remodeling has four stages. Firstly, trophoblasts and leukocytes in the vessel wall are not yet invasion, the endothelial cells (ECs) and smooth muscle cell layers of the vessel wall are intact. Secondly, the vascular structure begins to be destroyed by decidual NK (dNK) cells and macrophages in the vessel wall before trophoblast invasion (7). Thirdly, extravillous trophoblasts (EVTs) appear in the vessel wall and lumen. Finally, vascular smooth muscle cells (VSMCs) and ECs are completely lost and replaced by intravascular EVTs, and the wall matrix is replaced by fibrin-like substances. In addition, cytokines, angiogenic factors, enzymes, and extracellular matrix (ECM) also participate in regulating SA remodeling (8, 9). Trophoblasts are exposed to the maternal circulation and are influenced by the maternal endocrine, metabolic, and inflammatory environments. Hence, the influence of various maternal microenvironmental factors, such as high fat, high sugar diets, obesity and diabetes may affect trophoblast functions and the SA remodeling (10).

There are fewer blood vessels and villi in placenta of diabetic women with unexplained stillbirths than those with live births (11). Different from being replaced by trophoblasts in normal pregnancy, VSMCs in the placenta of biobreeding diabetes-prone rat were almost complete while SA remodeling was failure (12). Taken together, HG may cause insufficient SA remodeling *via* impaired trophoblasts. Herein, we will summarize the current knowledge on the possible effects of HG on trophoblast function as well as their role in uterine SA remodeling.

2 Trophoblasts and uterine SA remodeling

Trophoblasts are the first cell type to differentiate during embryogenesis. In this process, trophoblast stem cells are derived from embryonic trophectoderm. They can differentiate into various trophoblast cell lines and acquire many specialized functions, including invasion potential and endocrine activity (13). Cytotrophoblasts (CTBs) are stem cells that proliferate rapidly once embedded in maternal decidua. The outer layer of CTBs fuses into primitive syncytiotrophoblasts (STBs), which erode surrounding decidua and generate lacunae filled with blood (14). Placental villi bathed in maternal blood are floating villi in charge of placenta material transport, whereas villi, anchored in the placental basal plate, differentiate into EVTs (15).

During differentiation, trophoblasts lose adherent epithelial phenotype and acquire a mesenchymal phenotype and invasion ability through epithelial-mesenchymal transition (EMT). The E-cadherin/β-catenin complex is a calcium-dependent transmembrane protein distributing in epithelial tissues which form cell tight junctions, inhibit cell movement and maintain epithelial integrity (16). Decreased E-cadherin expression results in the upregulation of integrin α1β1, α5β1 and $\alpha_V\beta_3$, VE-cadherin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1(VCAM-1) (17, 18). EVTs invade the decidual stroma to form the interstitial extravillous trophoblasts (iEVTs) to promote the muscular layer of vessel wall degradation (19). EVTs invade the decidual blood vessels to form the endovascular extravillous trophoblasts (enEVTs) to replace ECs and VSMCs (20). VSMCs undergo morphological changes while EVTs penetrate the vessel wall *via* intravascular or interstitial pathways. They shift to a synthetic phenotype, migrate from the vessel wall, and undergo apoptosis. Trophoblasts secrete platelet derived growth factor BB (PDGF-BB) to bind the PDGF receptor β (PDGFR-β) of VSMCs to activate the PDGF signaling pathway and induce de-differentiation of VSMCs (21). EVT secretes tumor necrosis factor α (TNF-α), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and Fas ligand to induce apoptosis of VSMCs (22) (Figure 1A).

In addition, trophoblast-immune cell-vascular interactions are important determinants of adequate SA remodeling. The dNK cells, macrophages, trophoblasts, and their crosstalk are important for adequate SA remodeling, and any dysregulation may lead to remodeling obstacles (23, 24).

3 HG affects the biological functions of trophoblasts in uterine SA remodeling

Proliferation, signaling disorders, impaired placenta blood flow, and increased vascular resistance were observed in streptozotocin-induced GDM rat model (25–27). And decreased VSMC apoptosis was observed in placentas of mice with GDM (28). In addition, HG impaired the differentiation of trophoblast stem cell into an invasion phenotype and inhibited the trophoblast invasion, which further demonstrated that HG directly alters trophoblast lineage development (10). In the following paragraphs, we discuss in depth how HG influenced trophoblasts proliferation and invasion.

3.1 HG damages trophoblasts proliferation involved in uterine SA remodeling

Highly proliferating trophoblasts are necessary for placenta formation. And cell cycle control is very important in the proliferation process. *In vitro* studies have demonstrated that HG induced cell cycle arrest at G0/G1 in human trophoblast BeWo, JAR and HTR-8 cells, indicating that HG has the potential to inhibit trophoblasts proliferation (29, 30). Transcriptome and metabolome analysis showed that HG perturbed the phosphatidylinositol phosphate signaling pathways that involved in cell proliferation in BeWo cells (31). HG may inhibit cell proliferation by regulating the process of translation *via* epigenetic modifications, such as non-coding small molecule RNAs (32). HG upregulated the expression of miR-137, resulting in a negative modulatory effect on AMP-activated protein kinase, which ultimately stimulated the expression of IL-6 to inhibit cell proliferation (33). HG also promoted the expression of miR-136, inhibited the trophoblasts

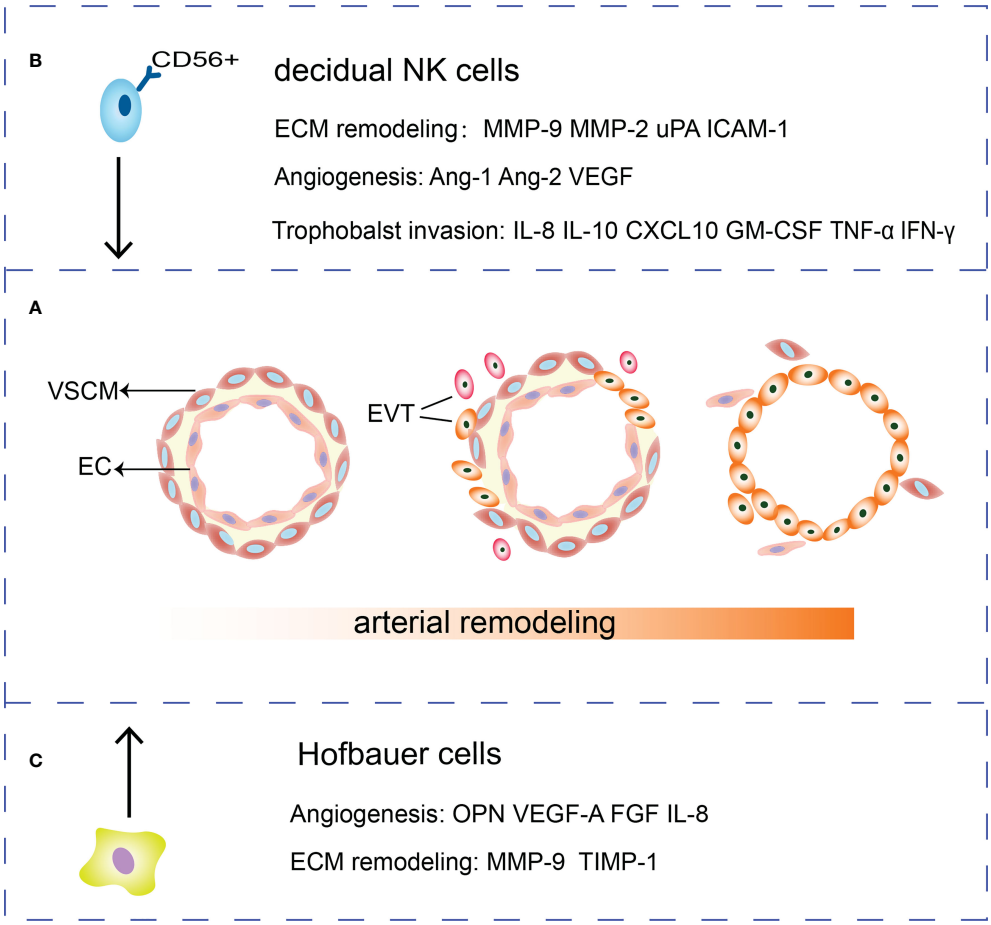

FIGURE 1
Roles of HBCs, EVTs and dNK cells in SA remodeling. **(A)** During the SA remodeling, VSMCs surrounding the arteries are removed and ECs are gradually replaced by EVTs. **(B)** dNK cells secrete cytokines such as IL-10, GM-CSF, TNF-α, and IFN-γ and chemokine IL-8, CXCL10 to regulate trophoblasts invasion. Meanwhile, dNK cells are potent sources of angiogenic factors such as Ang-1, Ang-2, VEGF. dNK cells secrete MMP-2, MMP-9, uPA and ICAM-1 that participate in ECM remodeling. **(C)** HBCs can secrete MMP and TIMP to remodel the extracellular matrix. Besides, HBCs secrete a range of factors that play a role in remodeling vessels such as OPN, FGF, VEGF-A and IL-8.

proliferation by suppressing E2F1 which is an important cell cycle regulator mediating the G1/S transition (34). MiR-362-5p was downregulated under HG conditions and inhibited the PI3K/AKT pathway by upregulating glutathione-disulfide reductase (GSR) directly, ultimately leading the inhibition of HTR-8 cells proliferation (35). MiR-520h was upregulated and inhibited cell proliferation by downregulating mTOR expression in HG-treated HTR-8 cells (36).

In vivo, immature villi in human diabetic placentas in term pregnancies suggested that HG provided more nutrition for continuous cell growth but delayed the cell differentiation and maturation (37). Upregulated Ki67 was observed in the term placenta of patients with GDM (38). However, Ki67 was downregulated in first-trimester human placental tissue with T1DM (39). In rat, the proliferative capacity of trophoblasts was weakened and the number of Ki67 positive cells decreased as the gestational day increases. At day 17 of pregnancy, Ki67 positive cells was higher in diabetic rat placentas than normal control (40). The different effects of HG on the proliferation of trophoblasts depend on gestational periods. HG provided excess nutrients for cell growth at the end of pregnancy, which also could explain why women with diabetes had higher placenta weight. Conversely, HG inhibited trophoblast proliferation in the first trimester because this period of placental development is particularly susceptible to environmental perturbations and any changes in the microenvironment may lead to impairment of trophoblast function (41).

3.2 HG damages trophoblasts invasion involved in uterine SA remodeling

In the progress of trophoblasts invasion into decidual tissue, EVTs produce proteases, such as fibrinogen activation system enzymes, matrix metalloproteinases (MMPs), and tissue inhibitor of metalloproteinases (TIMPs), to regulate the ECM remodeling and trophoblast invasion (42). The fibrinogen activation system comprises fibrinogen activators, such as urokinase-type plasminogen activator (uPA), and enzyme inhibitors such as fibrinogen activator inhibitor type 1 (PAI-1) (43). MMPs are a

family of more than 23 zinc-binding enzymes, exhibiting proteolytic activity promoting trophoblast invasion to the uterine wall. MMP activity is mainly regulated by TIMP. MMP2 and MMP9 are the most important MMP enzymes involved in trophoblasts during the first trimester (44). Pro-uPA zymogen activates uPA after binding uPAR. Activated uPA in turn cleaves and activates MMPs as well as degrades local matrix protein (45). Exposure to excess glucose may lead to shallow trophoblast migration and invasion, leading to abnormal uterine SA remodeling.

In vitro, trophoblast cell invasion is adversely disturbed under HG condition (46–51). Belkacemi et al. showed that trophoblasts invasion was reduced by approximately 62% and the activity of uPA was lower when HTR-8 cells were treated with 10mM of glucose (52). Furthermore, uPA in human early pregnancy trophoblast cell Sw.71 also decreased with the increasing glucose concentration (53). The increased E-cadherin, decreased Twist1 and Vimentin in HTR-8 cells under HG indicated a failure of EMT. EMT not only participates in EVT invasion but also balances the CTB-EVT differentiation (54). It was reported that MiR-137 was elevated in HG-treated HTR-8 cells, and the upregulation of miR-137 decreased the expression of fibronectin type III domain-containing 5, thereby inhibited the viability and migration of HTR-8 cells (55).

Some *in vitro* studies has shown that HG promoted the invasion of trophoblasts. HG induced proteoglycans alterations in 3A-Sub-E cells which is isolated from human full-term placenta, followed the increased MMP-2 and MMP-9 and decreased TIMP-2 (56). However, the HG altered proteoglycans on the surface of trophoblasts can lead to ECM deposition and complications in diabetic placenta (57). Normally, physiological levels of reactive oxygen species (ROS) promote angiogenesis, and the placental antioxidant system prevents ROS overproduction (58). HG induced the expression of the Cytochrome P450 enzyme family 1, subfamily B, polypeptide 1 (CYP1B1) which promoted trophoblast migration *via* MMP2. Inhibition of CYP1B1 may suppress ROS production under HG condition, which may provide a new method for diabetic complications caused by ROS overload (59). In placentas of diabetic rats at mid-gestation, increased ROS triggers trophoblast spreading with the increased expression of MMP-2 and MMP-9 (60).

Collectively, it is not difficult to suppose that HG inhibits the invasion and migration of trophoblasts derived from the first trimester, but promotes the invasion and migration of trophoblasts derived from the third trimester. Primary trophoblasts isolated from human placentas culture under HG could further verify our inference.

3.3 HG alters oxygen tension in placenta during uterine SA remodeling

Before the first 10 weeks of gestation, EVT forms a trophoblast plug to prevent maternal blood from entering the intervillous space and creates a physiologically hypoxic environment (2%–3% O_2) (61). The hypoxia-inducible factor 1 (HIF-1) plays a transcriptional regulatory role in hypoxic environment. There is an increased expression of TGF-β under hypoxia, thereby inhibiting trophoblasts differentiation (62). At the 12th week of gestation, the trophoblast plug dissolves and uterine SA begins to remodel, following a gradually increased oxygen concentration (8% O_2) around the trophoblast (63). Both HIF-1α and TGF-β expression decreases with increasing oxygen concentration, enabling trophoblast differentiation and ensuring extensive EVTs invasion into SA with increased MMP9 (64). However, after trophoblast differentiation into mature EVT, hypoxia and elevated HIF can promote EVT invasion (65, 66).

HG increased the thickness of trophoblast membranes and the massive collagen deposition, resulting in altered oxygen gradients in placenta and local hypoxia at the maternal-fetal interface (40). Downregulation of miR-29b in placenta with GDM promoted trophoblast invasion by upregulating the expression of HIF3A (67). Hypoxia promotes the invasion of mature EVTs. It is reasonable to suppose that HG promotes the invasion of trophoblasts in the third trimester placenta. It is also suggested that mild HG increased capillaries through negative feedback regulation of ischemia and hypoxia, however, sustained severe HG triggered hypoxia/ischemia and inhibited vascular endothelial growth factor (VEGF)/VEGFR-2 binding, thereby reducing excessive capillary formation (68, 69). What is more, HG can alter trophoblasts development by blunting trophoblast stem cell responses to low oxygen levels (10).

3.4 HG disrupts trophoblasts releasing angiogenic factors

Trophoblasts secrete angiogenic factors during uterine SA remodeling. VEGF disrupts the VSMC and ECs. Placental growth factor (PlGF), prominently expressed in villous CTBs and STBs, promotes angiogenesis under hypoxic conditions (70). Angiopoietins (Ang1, Ang2) and their receptor Tie-2 play an important role in stabilization or breakdown of blood vessel (71). Fibroblast growth factor (FGF) and PDGF-BB are involved in vasculogenesis and angiogenesis (72). Anti-angiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), are secreted. SFlt-1 is the soluble form of VEGFR-1, with a high affinity for VEGF, but no signal transduction function (73). Besides, sEng interferes with transforming growth factor β (TGF-β) and inhibits endothelial nitric oxide synthase activation, thereby disrupting angiogenesis (74).

In the first trimester trophoblasts HTR-8 and SW.71, HG decreased the secretions of VEGF, PlGF and uPA, while increased the secretions of anti-angiogenic factors sFlt-1 and sEng to inhibit artery remodeling (48, 52, 53, 75, 76). In placenta of women with GDM, increased VEGF, Ang, Eng and endothelin may lead to a collapse between angiogenic and anti-angiogenic factors (77). However, mild HG did not change the expression of VEGF (78). Persistent HG might thicken the placenta, increased the expression of HIF, thereby promoting the expression of angiogenic factors, such as VEGF and PIGF (79). FK506-binding protein like, acting as an anti-angiogenic protein and a regulator of inflammation, decreased in T1MD placenta and trophoblast cell line ACH-3P treated with HG under hypoxia condition (80). HG also promoted MT1-MMP and angiogenesis *via* PI3k signaling in GDM placenta (81). Despite these conflicting reports, it is certain that the balance between angiogenic and

anti-angiogenic factors is disrupted under HG. HG in the first trimester inhibits uterine SA remodeling by inhibiting the proliferation, invasion, and migration of trophoblasts. HG may cause hypercapillarization of villi due to collagen deposition caused by hypoxia and abnormal trophoblasts migrations in the third trimester, but these vessels are immature (82).

4 HG affects the crosstalk between immune cells and trophoblasts

Pregnant uteri are colonized by large number of immune cells, the most abundant cells of which are dNK cells and macrophages, followed by T cells and dendritic cells. Approximately 75% of decidual leukocytes are $CD56^{bright}CD16^{-}$ dNK cells and are not cytotoxic (83). Decidual macrophages, recruited from the maternal circulation, are polarized toward M1 macrophages during peri-implantation period while a profile of a mixed M1 and M2 type during EVTs invading the SA (84). Different from decidual macrophages, Hofbauer cells (HBCs) are the villous macrophages in the stroma of the first-trimester placenta arising from hematopoietic stem cells and are characterized as $CD14^{+}$ $CD68^{+}$ cells (85).

Chemokines and their receptors also play important roles in trophoblast migration and immune cells recruitment at the maternal-fetal interface (86). The dNK cells secrete IL8, CXCL10, TNF, interferon (INF) γ, TGF-b, and angiogenic factors such as VEGF-A, VEGF-C and PlGF (15, 87). Trophoblasts express the IL8 receptor CXCR1, the CXCL10 receptor CXCR3, TNF receptor TNFR1, as well as VEGFR-1 and VEGFR-3. Trophoblasts produce human leukocyte antigen to increase the levels of inhibitory receptors in dNK cells, maintaining their inactive phenotype ($CD16^{-}CD56^{+}$) (88). Meanwhile, macrophages secret IL-33, granulocyte colony-stimulating factor (G-CSF), CXCL1, TGF-b, TNF-α and Wnt5a to regulate trophoblasts invasion and migration (89). Immune cells, interacting with ECs, fibroblasts, and trophoblasts, promote the SA remodeling and placental growth. Any dysregulation of these factors may lead to remodeling obstacles (15).

In vitro, HG could mediate trophoblast releasing inflammatory factors IL-1β, IL-4, IL-8, and IL-6, IFN-γ, TNF-β, CXCL1 and G-CSF, indicating that HG created a pro-inflammatory environment at the maternal-fetal interface (76, 90, 91). High level of pro-inflammatory TNF-α was found both in GDM and TD2M placenta. Decreased IL-4 was found in T2DM and MGH placenta, promoting NK cell into active phenotype (92). This also reminds us that maternal HG caused by diabetes mellitus can lead to a disturbance in the balance of pro-inflammatory and anti-inflammatory factors at the maternal-fetal interface. In the following sections, we discuss in depth how HG disturbed immune cells and lead to the failure of SA remodeling.

4.1 HG affects crosstalk between dNK cells and trophoblasts

The dNK cells and EVTs interact with vascular ECs to promote SA remodeling (93). Firstly, dNK cells induce the apoptosis of VSMC and ECs, destruct blood vessel and secrete Ang-1, Ang-2, VEGF and MMPs to mediate angiogenesis (94, 95). The dNK cells secrete MMP-2, MMP-9, uPA, adhesion molecules such as ICAM-1 to regulate ECM remodeling (96–98). DNK cells express killer immunoglobulin receptor (KIR), CD94/NKG2A, and immunoglobulin like transcripts 2 (ILT2). These three receptors can interact with HLA-C, HLA-E and HLA-G on trophoblasts respectively, regulating trophoblast invasion (87). Cytokines, such as IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines IL-8, CXCL10 produced by dNK cells could promote EVTs invasion while the cytokines TNFα and IFN-γ inhibited trophoblast invasion by upregulating PAI expression (87, 99). IL-8 can also increase trophoblast expressing integrins α1 and β5 to gain an invasive phenotype (100) (Figure 1B).

A test for GDM peripheral blood showed a higher percent of cytotoxic NK cells ($CD16^{+}CD56^{dim)}$ in the GDM group than in controls (101). Some scholars believe that dNK cells are derived from the recruitment of peripheral $CD56^{bright}$ NK cells, which acquire dNK cells phenotype under the influence of a specific decidual microenvironment (102). Thus, changes in peripheral blood NK cells may lead to changes in the decidual NK cells. Fewer $CD56^{+}$cells adhere to decidual endothelium, while more diabetic $CD56^{+}$ cells adhere to pancreatic endothelium in pregnant women with T1DM and T2DM, indicating that HG impairs egression of $CD56^{+}$ cells into the decidua (103). Cytotoxic $CD16^{+}$ $CD56^{-}$NK cell both increased in maternal blood and placenta extravilli of GDM and T2MD. Placental $CD16^{-}CD56^{+}$ NK cells were higher in GDM and lower in T2DM, irrespective of region (92). GDM and T2MD are characterized by excessive insulin resistance, followed by maternal HG, triggering a "glucose stress" response and concurrent systemic inflammation (104). This response involves altered infiltration, differentiation, and activation of maternal innate and adaptive immune cells, which may explain increased $CD16^{+}$ NK cells. The control of blood sugar affects the expression of cytokines. Besides, cytokines differ in recent-onset DM and long-standing DM (105, 106). Thus, we don not exclude glycemic control conditions and duration of maternal HG are responsible for $CD56^{+}$ NK cells percentage and cytokine levels different in GDM and T2DM. In addition to the phenotypic changes of NK cells, the cytokines secreted by NK cells also change. Simultaneously, $CD56^{+}$ cells producing TGF-β and VEGF decreased significantly in patients with GDM (107). This secretory change may affect the regulation of trophoblast migration and invasion by dNK cells in high-risk pregnancy (108). Above all, HG may decrease dNK in the uterine wall, leading to a diminished interstitial trophoblast invasion and less SA remodeling.

4.2 HG affects crosstalk between macrophages and trophoblasts

Adopting an M2 polarity phenotype, HBCs express TIMP-1, MMP9, VEGF-A, osteopontin (OPN), and FGF to affect ECM and vascular remodeling (109–111). HBCs also secrete inflammatory factors such as IL-8, CCL-2, CCL-3, and CCL-4 with proangiogenic properties (109) (Figure 1C). Moreover, $CD14^+$ macrophages in early pregnancy decidua induce the breakdown of ECM and phagocytose apoptotic VSMCs to remodel the uterine SA (112).

HBCs treated by HG switched their M2 polarity profile towards M1 phenotype, which is not conducive to angiogenesis (113). M2 macrophages involve in anti-inflammatory processes and promote angiogenesis and tumor progression, which can produce protease to degrade the ECM (114). Thus, a reduction in the number of M2 phenotype cells may lead to impaired vascular remodeling. However, Schliefsteiner et al. showed HBCs maintain their M2 polarization to maintain a successful pregnancy, even in inflammatory states such as GDM. The co-cultivation of HBCs from GDM placentas and placental arterial endothelial cells (pAECs) did not alter ECs activation (115). Zhang et al. reported that M2a macrophages, majoring in tissue repair, increased in villi and more collagen was deposited in uncontrolled T2DM group compared with the healthy group (116). All in all, HG can disturb the balance between pro-inflammatory and anti-inflammatory subtypes of HBCs, which may cause adverse pregnancy outcomes.

5 Limitation and future direction

A recent study isolated SA from 12 to 23 weeks of gestation and found that the vascular remodeling was not complete until 23 weeks of gestation (117). Previous studies have mainly focused on pregnant women with GDM with few studies focusing on pregnant women with T1DM or T2DM. GDM is mainly screened at 24-28 weeks of gestation, but hyperglycemia occurs before 24 weeks. In addition, early GDM may has worse pregnancy outcomes (118). Therefore, the molecular and signaling pathway changes in the placenta of patients with GDM are also valuable for understanding the effect of HG on the uterine SA.

However, previous *in vivo* studies also had some limitations. First, the number of placenta cases in these studies is relatively small and the individual differences in patients are large. Secondly, no information was discussed on medication of women in the case group. Another limitation is lack of protein involved in ECM remodeling and angiogenesis such as MMP2, MMP9, uPA, PIGF and VEGF. SA remodeling occurs mainly before 24 weeks, therefore, staining of above protein in the first and second trimester placenta villi is more indicative of the effect of HG on trophoblast function.

All previous *in vitro* studies differed in terms of glucose dose, treatment time and cell line. Different or opposite conclusions have been drawn regarding the effect of HG on trophoblast function and uterine SA remodeling. For example, Basak et al. reported tube formation substantially increased at 25-30mM glucose and decreased at 40mM glucose in HTR-8 cells (119). In addition, McLeese et al. pointed out that HTR-8 cells did not survive in 5 mmol/L glucose over 48h, possibly due to the rapid glucose consumption (120). Inadera et al. pointed out that when BeWo cells were cultured at physiological levels of 5 mM glucose, the cells detached from dishes (31). Thus, the above researches remind us choosing appropriate glucose concentration is necessary to study the effects of HG on trophoblasts biological behavior and SA remodeling. The *in vitro* experiments used in this review are summarized in Table 1.

Although the relevant molecular mechanisms and signaling pathways of HG influencing trophoblast functions have been reported in these studies, the studies have mostly focused on cell lines and animal models. However, cell lines do not truly reflect *in vivo* conditions. Information obtained from animal models is also limited because SA remodeling differs between human and rats (121, 122). Therefore, it is crucial to establish a suitable model with appropriate sugar concentration for further study. *Ex vivo* model, such as human placenta-decidua co-culture, can also be used to quantify the extent of SA remodeling (123). In addition, human trophoblast organoids show similar cellular composition and biological behavior to those of immature human placentas. Despite the lack of such studies, we believe that human trophoblast organoid models cultured under HG conditions will

TABLE 1 The *in vitro* experiments used in this study are summarized.

Reference	Phenotype	cell line	key molecule	pathway	glucose concentration
proliferation					
29	HG inhibited the proliferation of first-trimester trophoblast	BeWo, JAR, JEG-3	Cyclin B1↓	/	5mM VS 25mM
30	HG inhibited the proliferation and arrested trophoblast in G0/G1 phase	HTR-8	PCNA↓	circ_FOXP1/miR-508-3p/SMAD2	5.5mM VS 30mM
31	HG perturbed biochemical networks *via* elevated oxidative stress	BeWo	/	/	11mM VS 25mM
32	HG inhibited cell proliferation	HTR-8, BeWo	/	miR-132/PENT	5mM VS 25mM
33	HG inhibited HTR-8 viability and proliferation	HTR-8	/	miR-137/PRKAA1/IL-6	5mM VS 25mM

(Continued)

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TABLE 1 Continued

Reference	Phenotype	cell line	key molecule	pathway	glucose concentration
34	HG inhibited cell proliferation	HRT-8, BeWo	E2F1↓	miR-136/E2F1	5mM VS 25mM
35	HG inhibited HTR-8 proliferation and induced apoptosis	HTR-8	/	MiR-362-5p/GSR/PI3K/AKT	5mM VS 25mM
36	HG inhibited HTR-8 proliferation and induced apoptosis	HTR-8	/	miR-520/mTOR	5.5mM VS 25mM
39	HG reduced trophoblast proliferation	Primary trophoblasts	ki67↓	/	5.5mM VS 25mM
Invasion, migration, and angiogenesis					
46	HG inhibited cell proliferation and migration	HTR-8	/	miR-134-5p/FOXP2	5mM VS 25mM
47	HG suppresses trophoblast viability,migration and induces apoptosis	HTR-8	/	circ-PNPT1/miR-889-3p/PAK1	5mM VS 25mM
48	HG inhibited HTR-8 viability, migration, and invasion	HTR-8	/	PLGF/ROS	0, 10, 15, 20, 25, 30 μM
49	HG inhibited cell migration and promoted apoptosis	HTR-8	/	FOXC1/FGF19/AMPK	5mM VS 25mM
50	HG inhibited cell viability, migration, and invasion, and promoted cell apoptosis	HTR-8	/	CTRP6/PPARγ	5.5mM(control), 10mM, 20mM, 30mM
51	HG enhanced HTR-8 autophagy and reduced invasion	HTR-8	LC3-II↑, p62↓	/	5mM VS 30mM
52	HG inhibited HTR-8 invasion	HTR-8	uPA↓	/	2.5mM VS 5mM and 10mM
53	HG inhibited Sw.71 invasive profile	Sw.71	uPA↓; VEGF, PIGF↓; sENG, sFIt-1↑	/	45(control), 135, 225, 49, 945mg/dl
54	HG inhibited HTR-8 EMT	HTR-8	E-cadherin↑, Vimentin, Twist1↓	ST2/PI3K/AKT/AMPK; ST2/P62/Twist	5mM VS 30mM
55	HG inhibited HTR-8 invasion	HTR-8	/	miR-137/FNDC5	5mM VS 25mM
56	HG stimulated trophoblast invasion and angiogenesis	3A-Sub-E	MMP-2, MMP-9↑, TIMP-2 ↓	/	5.6mM VS 30mM
59	HG stimulated trophoblast invasion and migration	HTR-8	/	/	normal medium VS 20mM
75	HG induced anti-angiogenic signaling in CTBs	Sw.71	VEGF, PIGF↓; sENG, sFIt-1, IL-6↑	/	100 (control), 150, 200, 300, or 400 mg/d
76	HG induced anti-angiogenic, and anti-migratory in first trimester trophoblast cells.	Sw.71	sENG, sFIt-1↑	/	5mM(control), 10mM, 25mM, 50mM
80	HG cause aberrant angiogenesis profile	ACH-3P	FKBPL↓, SIRT-1↓, PlGF↑	/	5mM VS 25 mM
119	HG promoted tube formation at 25 mM and inhibited tube formation at 40mM	HTR-8	MMP9 ↑	/	5.5 (control), 11, 25, and 40 mM

help us understand the effect of HG on the remodeling of uterine SAs (124).

6 Conclusion

Uterine SA remodeling requires appropriate trophoblast proliferation, invasion, and tissue remodeling, which involves a balanced MMP, TIMP and uPA. Meanwhile, trophoblasts, dNK cells and HBCs can secrete serious cytokines and angiogenic factors to regulate SA remodeling. Crosstalk between immunity cells and both trophoblast and vascular cells at maternal-fetal interface is also a part of the remodeling process. Inappropriate glucose concentrations may lead to abnormal trophoblast proliferation, migration, and invasion by disrupting the balance between MMP and TIMP. In addition, HG disrupts the balance between angiogenic factors Ang-1, VEGF, PlGF and anti-angiogenic factors sFlt-1 and sEng. Furthermore, an impaired immune cell profile under HG conditions influences SA remodeling (Figure 2). Understanding how HG affects SA

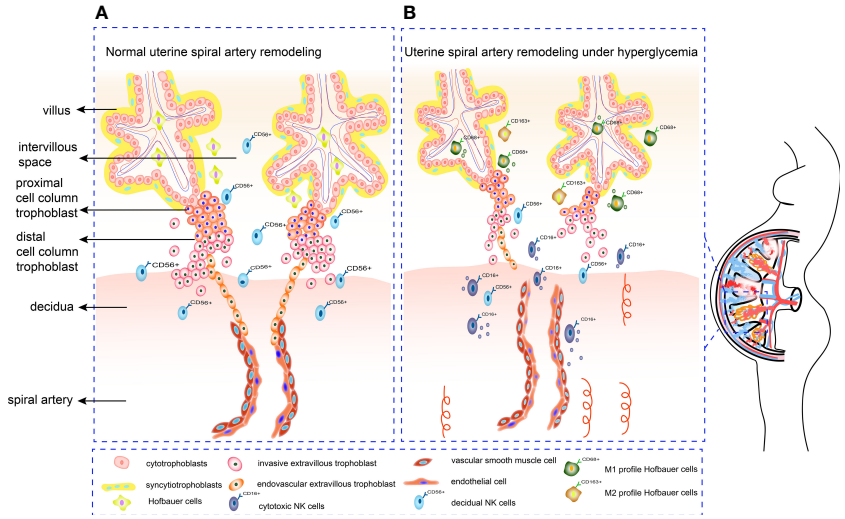

FIGURE 2
Mechanism of SA remodeling in normal pregnancy and hyperglycemia pregnancy. **(A)** CTBs proliferate rapidly once embedded in maternal decidua. The outer layer of CTBs fuses into primitive STBs, which can form proliferative proximal cell column trophoblasts. EVTs differentiate from distal cell column and break through the overlying STB layer, detaching from distal cell columns, migrating into the decidual stroma, and remodeling the SA. **(B)** Under hyperglycemia conditions, the proliferation and invasion ability of trophoblasts alters. Increased cytotoxic $CD16^{+}CD56^{dim}$ NK cells can form an inflammatory environment. Meanwhile, HBCs switch their M2 polarity profile towards M1 phenotype, which is not conducive to angiogenesis. Deficient artery transformation and immature new blood vessels can be observed in hyperglycemic placenta.

remodeling by influencing trophoblast function is crucial for revealing the mechanisms by which diabetes leads to pregnancy complications and adverse pregnancy outcomes. This review may provide a theoretical basis for future foundation and clinical research.

Author contributions

YZ: Search literature and write original draft. XL: Review, editing, project administration. YZ and XL contributed equally to this work. YX: Review and editing. YL: Funding acquisition, Resources, Supervision, Writing – review and editing. 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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Overexpression of IGF2 affects mouse weight and glycolipid metabolism and IGF2 is positively related to macrosomia

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Objective: To investigate the effects of insulin-like growth factor 2 (IGF2) on growth and glycolipid metabolism, as well as the underlying mechanism.

Methods: A mouse model of IGF2 overexpression was constructed to measure weight gain before adulthood, to obtain the values of adult glycolipid metabolism indicators in the peripheral blood and to detect the expression of genes in the IGF2 signaling pathway in different mouse tissues. The present study also explored the independent association between the IGF2 gene and macrosomia by detecting and comparing the expression levels of IGF2 mRNA/H19 RNA in maternal peripheral blood and fetal cord blood of 26 human pregnancies.

Results: In the mouse model, weights of the IGF2-overexpressing mice were significantly higher than those of the control mice at the age of 5-10 weeks. The glucose concentration, total cholesterol and high-density lipoprotein cholesterol (HDL-C) levels of IGF2-overexpressing mice were significantly lower than those of wild-type (WT) mice. Compared with the WT mice, the expression of H19 was significantly decreased in the pancreas and IGF1R was significantly decreased in the muscle of mice with IGF2 overexpression. The expression levels of STAT3 and AKT2 showed significant decrease in liver, muscle and increase in muscle of IGF2-overexpressing mice, respectively. GLUT2 expression showed significant increase in liver, kidney, muscle and decrease in pancreas of mice with IGF2 overexpression. This study also found that in normal mothers with the similar clinical characteristics, IGF2 expression in the maternal peripheral blood and fetal cord blood is an independent factor influencing macrosomia.

Conclusion: IGF2 expression was independently correlated with the occurrence of macrosomia, and overexpression of IGF2 significantly increased the weights of mice at the age of 5-10 weeks and significantly affected the values of adult glycolipid metabolism indicators, which might be the result of changes in the IGF2-IGF1R-STAT3/AKT2-GLUT2/GLUT4 pathway. These findings might suggest that IGF2 plays an important role in growth and glycolipid metabolism during both pregnancy and postnatal development.

KEYWORDS

insulin-like growth factor 2, mouse model, weight gain, glycolipid metabolism, macrosomia, STAT3/AKT axis

Introduction

Gestational diabetes mellitus (GDM) is a common complication during pregnancy, and macrosomia is one of the major adverse pregnancy outcomes of GDM (1). Insulin-like growth factor 2 (IGF2) encodes a polypeptide that is abundant in fetal tissues and circulation (2), is usually expressed in fetal tissue and invasive trophoblast cells at the placental maternal-fetal interface (3) and is the major insulin-like growth factor that plays a growth-promoting role during the process of embryonic development (4, 5). It has been reported that IGF2 regulates nutrient supplementation by the placenta, and its level in the fetal circulation reflects the growth rate of fetal tissues and nutrient demand (2, 6). The IGF2 concentration in cord serum was found to have a significantly positive effect on both birth length and weight (7). Our previous studies (8, 9) also indicated that a high glucose concentration in the context of GDM increased the expression of IGF2 and its imprinted gene (H19) by changing the levels of methylation in the IGF2 and H19 gene promoters, which might be the underlying pathogenic mechanism of macrosomia.

A study by Street et al. suggested that the fetal environment had long-term effects on growth (10). A mouse study found cases in which fetal IGF2 is misregulated (Beckwith-Wiedemann and Silver-Russell syndromes) can be diagnosed, and growth can be rescued by prenatally adjusting IGF2 or its signaling pathway (11). Compared to normal-weight children, IGF2 concentrations are increased in obese children (12). IGF2 expression in the blood shows an upward trend with increasing body mass index (BMI) in women (13). One study (14) demonstrated that children with catch-up growth in the first two years after birth had higher levels of IGF2. The IGF2 level at age five was closely correlated with five-year-old fat mass as well as the IGF2 level at birth (14). Therefore, IGF2 is also expressed postnatally in humans and may play an important role in growth and development.

IGF2 can combine with several IGF/insulin (INS) receptors (IGF1R, INSR, IGF1/INSR hybrids and IGF2R) to exert autocrine, paracrine and endocrine effects (15, 16). A case report (17) showed that patients with tumors that overproduced IGF2 developed hypoglycemia. IGF2, similar to insulin, can promote hypoglycemia by enhancing glucose uptake by skeletal muscle and inhibiting glucose release from the liver (16). At the same time, IGF2 can also suppress free fatty acid release and glucagon secretion (16).

To investigate the effects of IGF2 on mouse weight gain and glycolipid metabolism, as well as the underlying mechanism, we constructed a mouse model of IGF2 overexpression to record weight gain before adulthood, to obtain the values of adult glycolipid metabolism indicators in the peripheral blood and to detect the expression of genes in the IGF2 signaling pathway in different mouse tissues. In addition, we explored the independent association between IGF2 gene expression and macrosomia by comparing the expression levels of IGF2 mRNA/H19 RNA in maternal peripheral blood and fetal cord blood of humans.

Materials and methods

Mouse model of IGF2 gene overexpression

$IGF2^{fl/fl}$ or $IGF2^{fl/-}$ female mice were obtained by site-directed insertion of a murine IGF2 gene fragment containing the Loxp structure into H11 of C57BL/6 mice using the CRISPR/Cas9 strategy. At the same time, the CRISPR/Cas9 strategy was also used to obtain $huCYP19A1Cre^{+/-}$ male mice by inserting a cyclization recombinase (Cre) gene fragment with the promoter of huCYP19A1 (human cytochrome P450 family 19 subfamily A member 1) into Rosa26 of C57BL/6 mice. Female $IGF2^{fl/fl}$ or $IGF2^{fl/-}$ mice were mated with male $huCYP19A1Cre^{+/-}$ mice to obtain four genotypes of offspring, $IGF2^{fl/-}$. $huCYP19A1Cre^{+/-}$, $IGF2^{fl/-}$, $huCYP19A1Cre^{+/-}$ and wild type (WT). Offspring mice with both the exogenously inserted IGF2 gene and the Cre gene fragment exhibit overexpression of IGF2. The offspring mice were marked by toe clipping within one week after birth. Samples of the mouse toes were used for genotyping by agarose gel PCR electrophoresis (Figure 1A). The relevant primer sequences are shown in Table 1. The following primer was used in the real-time quantitative PCR of IGF2 gene in the livers of $IGF2^{fl/-}$. $huCYP19A1Cre^{+/-}$ and WT mice (Figure 1B): sense 5'-GGAGCTTGTTGACACGCTTCAGT-3' and antisense 5'-GAAGCAGCACTCTTCCACGATG-3'. Mice in this study were manipulated according to the principles of laboratory care and approved by the Laboratory Animal Welfare Ethics Committee of Peking University First Hospital (No. J201941).

Recording of mouse weight by week

Pregnant female $IGF2^{fl/fl}$ or $IGF2^{fl/-}$ mice were observed from the 20th day of gestation to confirm delivery. The day that offspring were found was defined as day one. The offspring mice were marked by toe clipping within one week after birth. All offspring mice were weaned at 3 weeks old and fed sterile maintenance feed for mice and rats (SPF biotechnology, China) at 3-10 weeks of age. The weights of the mice were measured and recorded at the same time every week.

Detection of glycolipid metabolism indicators in the peripheral blood of offspring mice

Ten-week-old offspring mice were anesthetized (sodium pentobarbital, 150-200 mg/kg) to collect blood by removing one of the eyeballs, and the serum was separated out. Glucose concentration, total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were detected by the glucose oxidase method, cholesterol oxidase-peroxidase (CHOD-PAP) method,

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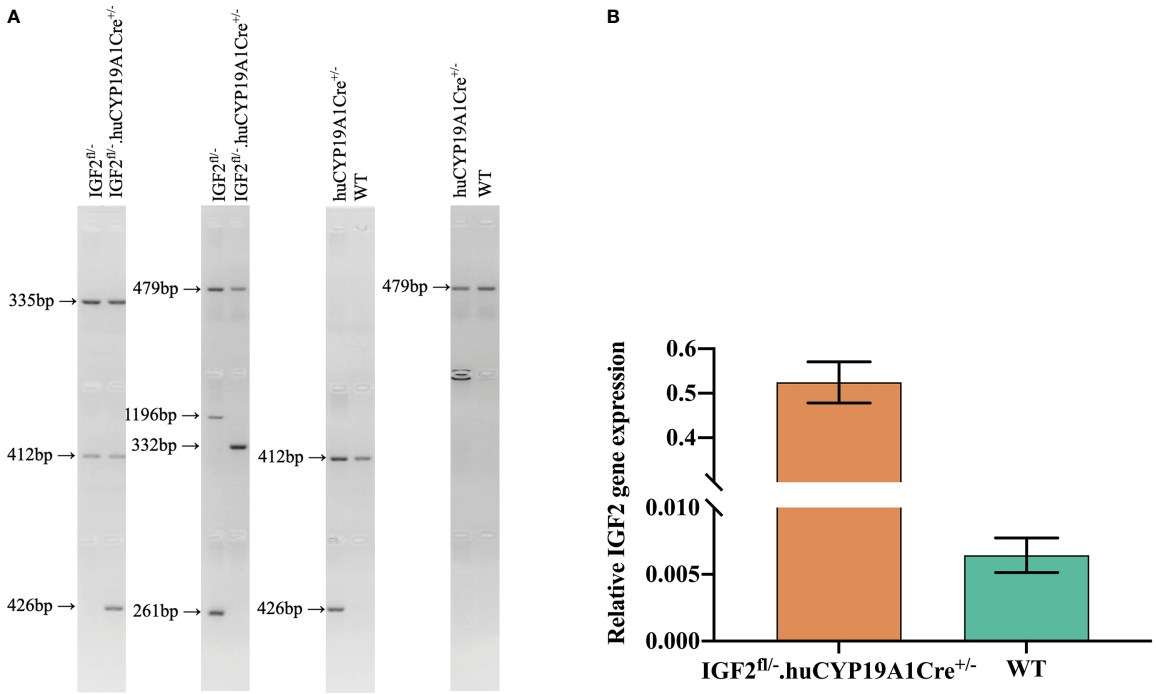

FIGURE 1
Identification of targeted mice with IGF2 gene overexpression. **(A)** Mouse genotyping by agarose gel PCR electrophoresis with the primers in Table 1. **(B)** Real-time quantitative PCR of the IGF2 gene in the livers of IGF2$^{fl/-}$. huCYP19A1Cre$^{+/-}$ and WT mice (n=5-6 mice in each group).

TABLE 1 Primers for gene identification in the mouse model of IGF2 gene overexpression.

Purpose		Forward	Reverse	Expected band size
Exogenous IGF2	CKI	GGGCAGTCTGGTACTTCCAAGCT	TGGCGTTACTATGGGAACATACGTC	335 bp
	WT	CAGCAAAACCTGGCTGTGGATC	ATGAGCCACCATGTGGGTGTC	412 bp
Cre	KI	CCTGCTGTCCATTCCTTATTCCATA	TCGGGTGAGCATGTCTTTAATCT	426 bp
	WT	CCCAAAGTCGCTCTGAGTTGTTA	TCGGGTGAGCATGTCTTTAATCT	479 bp
Effect of Cre	Pre-Cre	CTAGAGCCTCTGCTAACCATGTTC	CGGTCCGAACAGACAAACTGAAG	1196 bp
	Post-Cre			332 bp
	Pre-Cre	TCCCCATCAAGCTGATCCGG	CGGTCCGAACAGACAAACTGAAG	261 bp

IGF2, insulin-like growth factor 2; Cre, cyclization recombinase; CKI, conditional knock-in; WT, wild type; KI, knock-in; Post-Cre, after the effect of Cre was exerted; Pre-Cre, before the effect of Cre was exerted.

glycerol phosphate oxidase-peroxidase (GPO-PAP) method, direct selective inhibition method and direct protective reagent method, respectively. An iodine [^{125}I] insulin radioimmunoassay kit was used to detect insulin and insulin antibodies.

Real-time quantitative PCR of genes in the IGF2 signaling pathway in adult mouse tissues

The liver, pancreas, kidney, muscle and adipose tissues of 10-week-old offspring mice were collected into liquid nitrogen immediately after anesthesia and blood collection. An appropriate amount of tissue was placed in 1 ml TRIzol reagent and ground with a mixed refrigerated ball mill (Retsch MM400, Germany) at 30 Hz for 6 min to extract total RNA. A Nanodrop 2000 ultramicro-spectrophotometer (Thermo, USA) was used to measure the concentration of extracted RNA. Two micrograms of total RNA were used as the template to synthesize cDNA by reverse transcription, and the cDNA sample was diluted 5 times. Real-time quantitative PCR was performed with a 20 μl reaction system (including 10 μl of Powerup SYBR green master mix, 2 μl of primers, 1 μl of cDNA and 7 μl of RNase-free water). The primer sequences (18–20) used for real-time quantitative PCR of genes in the IGF2 signaling pathway (Figure 2) are shown in Table 2. The internal reference gene was glyceraldehyde phosphate dehydrogenase (GAPDH).

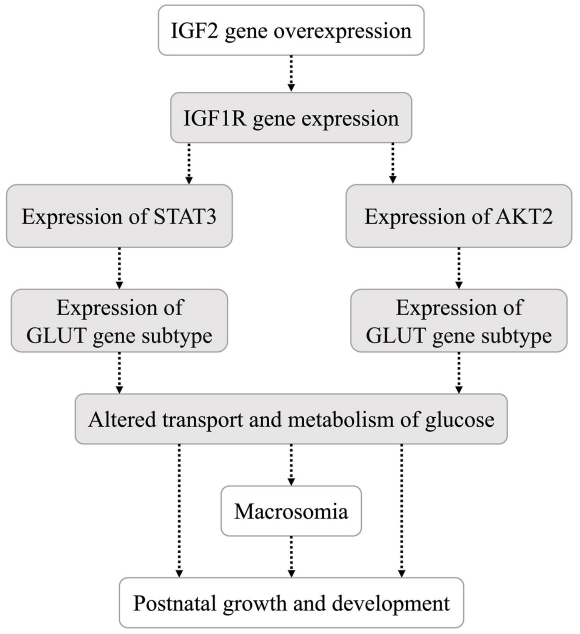

FIGURE 2
Hypothesis of the IGF2 gene downstream signaling pathway in this study.

Real-time quantitative PCR of the IGF2/H19 gene in maternal peripheral blood and fetal cord blood

A total of 26 healthy pregnant women were involved in this study, and patients with complications were excluded (including but not limited to multiple gestation, GDM, pregnancy-induced hypertension, premature birth, and fetal anomalies). Macrosomia was defined as a fetus with a birth weight > 4000 g. Blood samples from 26 pregnant women were classified into four groups based on whether the neonates had macrosomia or normal birth weight: maternal peripheral blood of macrosomia (MM, n=14), fetal cord blood of macrosomia (MF, n=14), maternal peripheral blood of neonates with normal birth weight (CTRL-M, n=12), and fetal cord blood of neonates with normal birth weight (CTRL-F, n=12). Total RNA was extracted from the blood samples and used to synthesize cDNA by reverse transcription. Then, the cDNA was used in a 480II real-time quantitative PCR system (Roche, Switzerland) to detect the expression levels of IGF2 and H19 in each group of blood samples, and the sequences of the primers (21) used are shown in Table 3. The present study was approved by the Ethics Committee of Peking University First Hospital (No. 2013-572). All participants who provided blood samples signed written informed consent forms.

Statistical analysis

SPSS Statistics 26.0 (IBM, USA) and GraphPad Prism 9 (GraphPad, CA) were used to analyze the data in this study. Data were tested by the D'Agostino & Pearson test to confirm normality. Based on whether the data conformed to a normal distribution, Student's *t* test or an independent-sample nonparametric test was used for statistical comparisons. The data for comparisons were derived from n ≥ 3 repetitions. $P < 0.05$ indicated that the differences were statistically significant.

Results

Overexpression of IGF2 affects mouse weight at 5-10 weeks of age

Among the offspring of the four genotypes ($IGF2^{fl/-}$. $huCYP19A1Cre^{+/-}$, $IGF2^{fl/-}$, $huCYP19A1Cre^{+/-}$ and WT), only the offspring mice with both the exogenously inserted IGF2 gene and the Cre gene fragment ($IGF2^{fl/-}$. $huCYP19A1Cre^{+/-}$) exhibit overexpression of IGF2. The weights of male $IGF2^{fl/-}$. $huCYP19A1Cre^{+/-}$ mice were significantly higher than those of $IGF2^{fl/-}$, WT and $huCYP19A1Cre^{+/-}$ mice at the ages of 5 weeks, 6 weeks and 7/9-10 weeks, respectively (all $P < 0.05$). Compared with $IGF2^{fl/-}$, $huCYP19A1Cre^{+/-}$ and WT mice, female $IGF2^{fl/-}$. $huCYP19A1Cre^{+/-}$ mice exhibited significant increases in body weight in mice aged 5-10 weeks (all $P < 0.05$). Regardless of sex, there was no significant difference in body weight among $IGF2^{fl/-}$,

TABLE 2 Primers for real-time quantitative PCR of mouse tissues.

Gene	Forward	Reverse
H19	GGTGTCTCGAAGAGCTCGGA	CCATGGTGTTCAAGAAGGCTGG
IGF1R	ACTGACCTCATGCGCATGTGCTGG	CTCGTTCTTGCGGCCCCCGTTCAT
STAT3	CAATACCATTGACCTGCCGAT	GAGCGACTCAAACTGCCCT
AKT2	TGACTATGGGCGAGCAGTGG	CTCCATGACCTCCTTCGCATC
GLUT2	TGGAAGGATCAAAGCAATGTTG	CATCAAGAGGGCTCCAGTCAA
GLUT4	CGTTGGTCTCGGTGCTCTTAGTA	GCAGAGCCACGGTCATCAAG
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA

IGF1R, insulin-like growth factor 1 receptor; AKT2, AKT serine/threonine kinase 2; STAT3, signal transducer and activator of transcription 3; GLUT2, glucose transporter 2; GLUT4, glucose transporter 4; GAPDH, glyceraldehyde phosphate dehydrogenase.

TABLE 3 Primers for real-time quantitative PCR of human blood.

Gene	Forward	Reverse
IGF2	TGGACACCCTCCAGTTCGTC	GCGGAAACAGCACTCCTCAA
H19	ACTCAGGAATCGGCTCTGGAA	CTGCTGTTCCGATGGTGTCTT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

IGF2, insulin-like growth factor 2; GAPDH, glyceraldehyde phosphate dehydrogenase.

huCYP19A1Cre$^{+/-}$ and WT mice from 5-10 weeks of age. The mouse weights are shown in Table 4.

Overexpression of IGF2 affects glycolipid metabolism in adult mice

The glucose concentration, total cholesterol and HDL-C levels of IGF2$^{fl/-}$. huCYP19A1Cre$^{+/-}$ male mice were significantly lower than those of WT mice (all $P < 0.05$). The levels of total cholesterol and HDL-C in female IGF2-overexpressing mice (IGF2$^{fl/-}$. huCYP19A1Cre$^{+/-}$) were significantly lower than those in WT female mice (all $P < 0.05$). In addition, overexpression of IGF2 had no significant effect on the levels of insulin, insulin antibodies and triglycerides, regardless of mouse sex. The results are shown in Figure 3.

Overexpression of IGF2 affects gene expression of the glycolipid metabolism pathway in adult mouse tissues

It was reported (16) that IGF2, similar to insulin, can promote hypoglycemia by enhancing glucose uptake by skeletal muscle and inhibiting glucose release from the liver. In addition, IGF2 can suppress free fatty acid release from adipose tissue and glucagon secretion from the pancreas (16). As shown in Figure 4, compared with that of WT mice, the expression of H19 was significantly decreased in the pancreas and IGF1R was significantly decreased in the muscle of mice with IGF2 overexpression (IGF2$^{fl/-}$. huCYP19A1Cre$^{+/-}$). The signal transducer and activator of transcription 3 (STAT3)/AKT serine/threonine kinase (AKT) axis is a classic downstream pathway of the IGF/IGF1R signaling pathway (22). The expression levels of STAT3 and AKT2 showed significant decrease in liver, muscle and increase in muscle of IGF2-overexpressing mice, respectively. At the same time, as key molecules involved in glucose metabolism, the expression of glucose transporter (GLUT) 2 and GLUT4 also showed alterations in IGF2-overexpressing mice. Notably, GLUT2 expression showed significant increase in liver, kidney, muscle and decrease in pancreas of mice with IGF2 overexpression, suggesting that it might be an important part of the pathway downstream of IGF2.

Independent association between IGF2 expression and macrosomia

As shown in Table 5, the neonatal birth weights [mean (standard deviation)] in the macrosomia and normal birth weight groups were 4192 (165) g and 3404 (299) g, respectively, which were

TABLE 4 Comparison of mouse weights at 5-10 weeks old across four genotypes.

Age (week)		IGF2$^{fl/-}$.huCYP19A1Cre$^{+/-}$(Weight, g)	IGF2fl/- (Weight, g)	huCYP19A1Cre$^{+/-}$ (Weight, g)	WT (Weight, g)
Male	5	21.6 ± 1.7^{a}	18.3 ± 2.3	18.3 ± 2.7	19.1 ± 1.1
	6	22.4 ± 1.5^{c}	21.1 ± 1.6	21.1 ± 0.8	20.6 ± 1.0
	7	23.8 ± 1.5^{b}	23.0 ± 1.5	22.6 ± 0.8	22.6 ± 1.2
	8	24.9 ± 1.2	23.8 ± 1.2	23.8 ± 0.7	23.7 ± 1.0
	9	26.0 ± 1.3^{b}	25.1 ± 1.7	24.0 ± 0.9	24.9 ± 1.1
	10	26.6 ± 1.4^{b}	26.0 ± 1.6	25.0 ± 1.1	25.7 ± 1.2
Female	5	18.5 ± 1.1^{abc}	15.8 ± 2.4	15.8 ± 1.1	16.7 ± 1.1
	6	20.0 ± 1.0^{abc}	17.5 ± 1.2	16.7 ± 1.1	17.7 ± 1.1
	7	20.7 ± 1.4^{abc}	18.2 ± 1.1	17.7 ± 1.0	18.6 ± 1.1
	8	21.4 ± 1.2^{abc}	18.7 ± 0.9	18.7 ± 0.9	19.2 ± 0.8
	9	21.9 ± 1.4^{abc}	19.5 ± 1.1	19.6 ± 1.0	19.9 ± 0.9
	10	22.7 ± 1.2^{abc}	20.2 ± 1.1	20.0 ± 0.7	20.4 ± 0.9

WT, wild type. [a]Compared with IGF2$^{fl/-}$ mice, P < 0.05; [b]Compared with huCYP19A1Cre$^{+/-}$ mice, P < 0.05; [c]Compared with WT mice, P < 0.05; n=7-17 mice in each group.

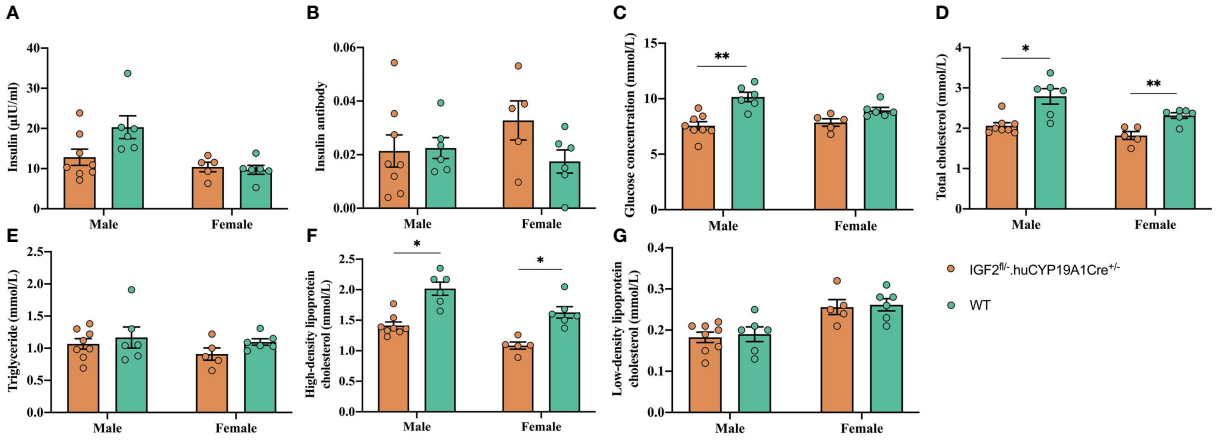

FIGURE 3
Effect of IGF2 overexpression on glycolipid metabolism in adult mice. The insulin **(A)**, insulin antibody **(B)**, glucose concentration **(C)**, total cholesterol **(D)**, triglyceride **(E)**, high-density lipoprotein cholesterol **(F)** and low-density lipoprotein cholesterol **(G)** levels of adult IGF2$^{fl/-}$.huCYP19A1Cre$^{+/-}$ and WT mice; n=5-8 mice in each group. *$P < 0.05$; **$P < 0.01$.

significantly different ($P < 0.01$). Maternal age, height, gestation age, prepregnancy weight, predelivery weight and gestational weight gain showed no statistically significant difference between the macrosomia and normal birth weight groups.

As shown in Figure 5, the mRNA expression of IGF2 showed significant decrease and the H19 RNA showed higher expression in the maternal peripheral blood of the macrosomia group (MM), compared with that in the normal birth weight group (CTRL-M) (Figures 5A, E). Compared with that in the fetal cord blood of the normal birth weight group (CTRL-F), the mRNA expression of IGF2 in the fetal cord blood of the macrosomia group (MF) was increased (Figure 5B), and the RNA expression of H19 was significantly reduced (Figure 5F). Comparisons of gene expression in the maternal peripheral blood and corresponding cord blood of the same group showed that IGF2 expression in the cord blood was significantly higher than that in the maternal peripheral blood (Figure 5C), and the fold difference was higher in the macrosomia group than that in the normal birth weight group (Figures 5C, D). In

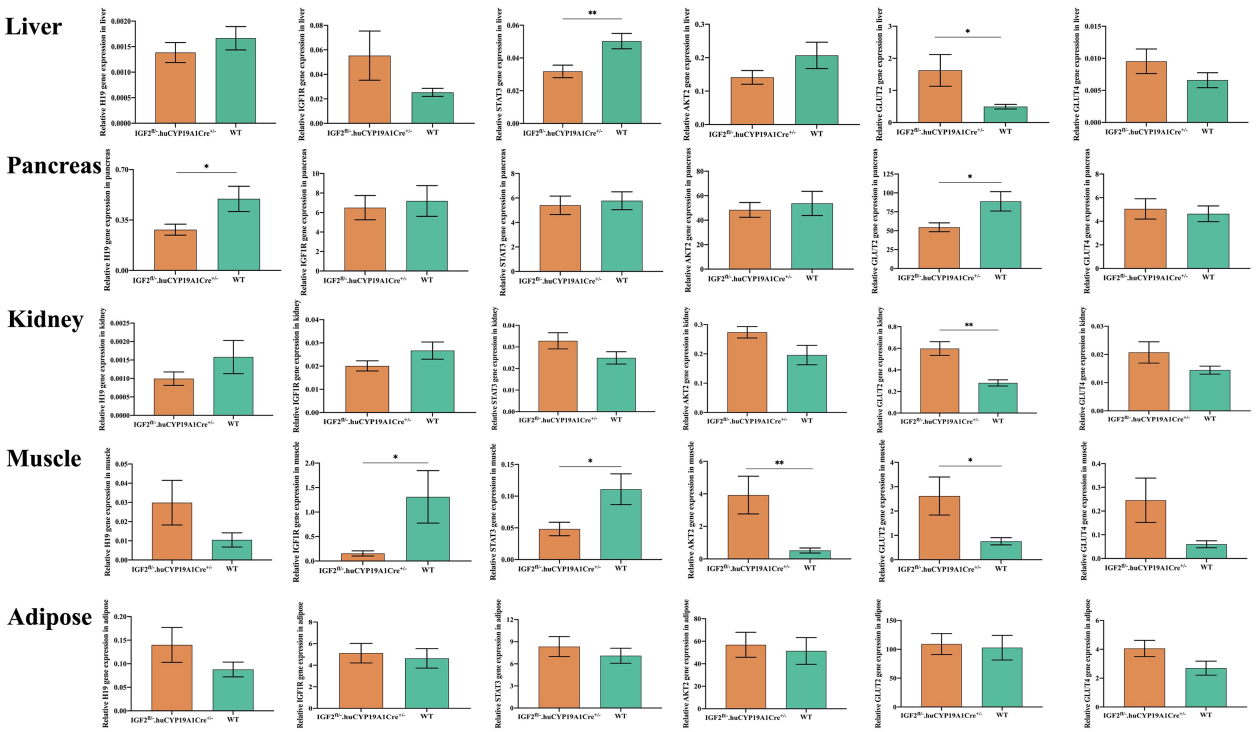

FIGURE 4
Effects of IGF2 overexpression on the genes of the pathway related to glycolipid metabolism in adult mouse tissues; n=4-6 mice in each group. *$P < 0.05$; **$P < 0.01$.

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TABLE 5 Clinical information for pregnant women who provided human blood.

	Macrosomia group (*n* =14)	Normal birth weight group (*n* =12)	*P* value
Age (year)	34.9 ± 5.0	33.2 ± 3.5	0.316
Height (cm)	161.4 ± 4.6	164.6 ± 5.7	0.120
Gestation age (wk)	39.6 ± 1.1	39.2 ± 0.7	0.215
Neonatal birth weight (g)	4192 ± 165	3404 ± 299	< 0.01
Prepregnancy weight (kg)	58.6 ± 7.7	57.7 ± 4.9	0.721
Predelivery weight (kg)	72.1 ± 8.4	71.9 ± 5.9	0.950
Gestational weight gain (kg)	13.5 ± 4.0	14.2 ± 2.4	0.576

BMI, body mass index.

addition, the RNA expression of H19 showed the opposite trend (Figures 5G, H). These results might indicate that in the case of normal mothers with similar clinical characteristics, IGF2 expression is an independent factor influencing macrosomia.

Discussion

By constructing a mouse model of IGF2 overexpression, we showed in the present study that IGF2 overexpression significantly increased the weights of mice at 5-10 weeks of age [equivalent to human puberty, which corresponds to 12-18 years of age (23)] and changed the values of adult glycolipid metabolism indicators, which might be the result of changes in the IGF1R-STAT3/AKT2-GLUT2/GLUT4 downstream pathway of IGF2. In addition, we found an independent correlation between IGF2 levels and macrosomia by comparing the expression levels of IGF2/H19 in maternal peripheral blood and fetal cord blood.

A previous study suggested that IGF2 levels at age five were closely correlated with five-year-old fat mass, and children with catch-up growth in the first two postnatal years had higher levels of IGF2 (14), which indicated that IGF2 might play an important role in the postnatal growth and development of humans. The results of this study showed that compared with WT siblings, mice with IGF2 overexpression showed significant increase in weight at 5-10 weeks of age. Previous studies (14, 24) reported that a sex bias exists in the expression of IGF2, and the IGF2 level in females is significantly higher than that in males at the age of 5 years. Moreover, another study (25) found that hypoxic conditions lead to the upregulation of IGF2 expression in the placenta of female fetuses, while the hypoxic placenta of male fetuses shows no change. In the present study, the weight increases at the age of 5-10 weeks induced by IGF2 overexpression in female mice were more significant than those in male mice. In addition, a previous study (13) evaluated blood samples from women aged 40-79 years and found that IGF2 shows an upward trend in blood as BMI increases, which means that IGF2 may also have an important effect on weight changes during and after adulthood.

Our previous study (8) demonstrated that high glucose concentrations can upregulate the expression of IGF2. However, excessive expression of IGF2 can result in the occurrence of hypoglycemia (17). The present study showed that the glucose concentration in the blood of mice with IGF2 overexpression was significantly lower than that in the blood of WT mice (Figure 3C). Therefore, the upregulation of IGF2 in response to high-glucose conditions may be a feedback loop to increase the utilization of

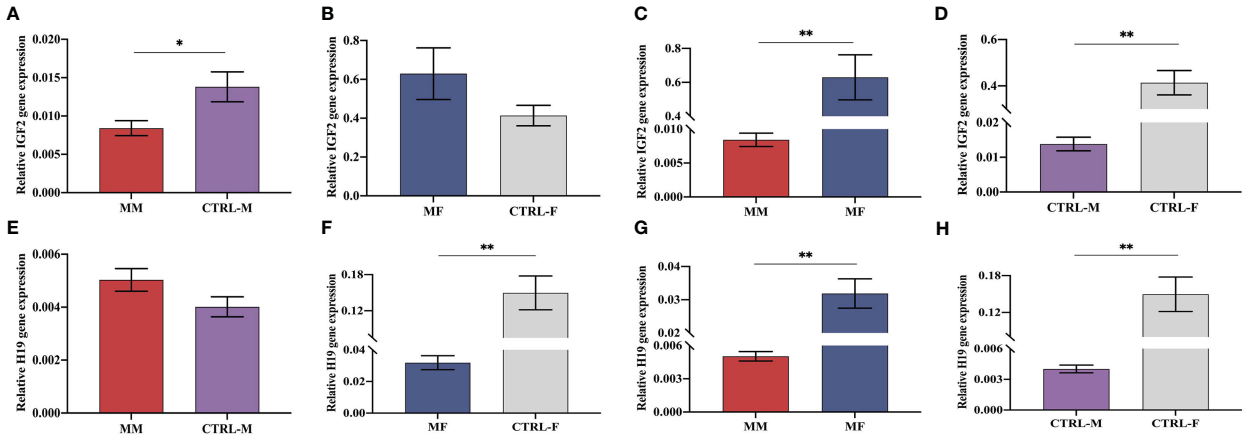

FIGURE 5
Expression levels of IGF2 mRNA **(A–D)** and H19 RNA **(E–H)** in maternal peripheral blood and the corresponding fetal cord blood of the macrosomia group (MM, MF) and normal birth weight group (CTRL-M, CTRL-F). *$P < 0.05$; **$P < 0.01$.

glucose and reduce the blood glucose level, which probably contributes to weight gain. A previous paper (26) indicated that male offspring of GDM mice have lower expression of IGF2 in the liver, and they show more severe glucose intolerance and insulin resistance than female offspring. Elevated IGF2 levels may be beneficial in stabilizing blood glucose concentrations, such as in the intrauterine hyperglycemic environment in women with GDM, but the consequence of this process affecting weight gain probably leads to macrosomia, which is an adverse pregnancy outcome. In addition, IGF2 also has the ability to reduce lipolysis and inhibit free fatty acids (16). The results of this study showed that total cholesterol and HDL-C were significantly reduced in mice with IGF2 overexpression. The interaction between IGF2 and lipid metabolism needs to be further studied.

IGF2 can combine with several IGF/insulin (INS) receptors (IGF1R, INSR, IGF1/INSR hybrids and IGF2R) to exert autocrine, paracrine and endocrine effects (15, 16). The STAT3/AKT axis is a typical downstream pathway of the IGF/IGF1R signaling pathway (22). Both STAT3 and AKT are upstream regulators of glucose transporters (27, 28). In the present study, the expression levels of underlying downstream genes (including H19, IGF1R, STAT3, AKT2, GLUT2 and GLUT4) of IGF2 were analyzed in multiple organs and tissues of mice with IGF2 overexpression. IGF2 overexpression induced altered expression of downstream genes. The hypothesis that IGF2 regulates the transport and metabolism of glucose through the IGF1R-STAT3/AKT2-GLUT2/GLUT4 pathway was preliminarily investigated. Due to the different effects of IGF2 overexpression on tissues involved in glycolipid production, storage or consumption, the specific process by which IGF2 overexpression regulates its downstream pathway in each tissue needs to be explored further.

Based on previous studies, it was determined that IGF1 has high postnatal expression (29, 30) and that the expression of IGF2 is high during embryonic development but decreases to an undetectable level after birth (30–32). Several recent studies (12–14, 24, 33) have suggested that IGF2 is also expressed postnatally in humans and may play an important role in growth and development. Due to the previous opinion, there is currently a lack of related research on human postnatal IGF2 compared to studies on postnatal IGF1 (24). The present study constructed a mouse model of IGF2 overexpression, whose postnatal level of IGF2 was higher than that of WT mice, and the effects of IGF2 overexpression on the postnatal growth and glycolipid metabolism of the mice were explored, which might provide evidence for the postnatal effects of IGF2. However, this study also had some limitations. The effects of IGF2 overexpression on the birth weights and prefive-week-old weights of offspring mice were not significant, which might be the result of the influence of different litter sizes (34, 35). Therefore, to compensate for the deficiency of the mouse model, we used maternal peripheral blood and fetal cord blood samples from normal singleton pregnancies to demonstrate the independent correlation between IGF2 expression and macrosomia.

It was reported that IGF2 cord serum concentration had a significant positive effect on both birth length and weight (7) and IGF2 content in the placental lysates was one of the most important factors associated with fetal growth restriction (36). Our previous study (9) indicated that in normal pregnant women, the expression of IGF2 in the placenta and cord blood was significantly higher for women with macrosomia than for women with normal birth weight neonates. However, the maternal prepregnancy and predelivery BMIs of women with macrosomia were significantly higher than those of women with normal birth weight neonates (9). By controlling for maternal age, height, gestation age, prepregnancy weight, predelivery weight and gestational weight gain, the present study demonstrated that IGF2 expression was an independent factor connected with macrosomia.

In conclusion, by constructing a mouse model of IGF2 overexpression, we showed that IGF2 expression was independently correlated with the occurrence of macrosomia, and overexpression of IGF2 significantly increased the weights of mice at the age of 5-10 weeks and significantly affected the values of adult glycolipid metabolism indicators, which might be the result of changes in the IGF2-IGF1R-STAT3/AKT2-GLUT2/GLUT4 pathway. These findings might suggest that IGF2 plays an important role in growth and metabolism during both pregnancy and postnatal development.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking University First Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Laboratory Animal Welfare Ethics Committee of Peking University First Hospital.

Author contributions

This study was conceived and designed by YW. The data were collected by QZ, SQ and JH with material and technical support from HY. The data were analyzed by QZ and YW. The paper was written by QZ and YW. 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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Nomogram for predicting the risk of preterm delivery after IVF/ICSI treatment: an analysis of 11513 singleton births

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Background: There is a higher risk of preterm delivery (PTD) in singleton live births conceived after *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) compared with spontaneously conceived pregnancies. The objective of our study was to build a predictive nomogram model to suggest the possibility of PTD in singleton pregnancies after IVF/ICSI treatment.

Method: 11513 IVF/ICSI cycles with singleton live births were enrolled retrospectively. These cycles were randomly allocated into a training group (80%) and a validation group (20%). We used the multivariate logistics regression analysis to determine prognostic factors for PTD in the training group. A nomogram based on the above factors was further established for predicting PTD. Receiver operating characteristic curves (ROC), areas under the ROC curves (AUC), concordance index (C-index), and calibration plots were analyzed for assessing the performance of this nomogram in the training and validation group.

Results: There were fourteen risk factors significantly related to PTD in IVF/ICSI singleton live births, including maternal body mass index (BMI) > 24 kg/m^2, smoking, uterine factors, cervical factors, ovulatory factors, double embryo transferred (DET), blastocyst transfer, FET, vanishing twin syndrome (VTS), obstetric complications (placenta previa, placenta abruption, hypertensive of pregnancies, and premature rupture of membrane), and a male fetus. These factors were further incorporated to construct a nomogram prediction model. The AUC, C-index, and calibration curves indicated that this nomogram exhibited fair performance and good calibration.

Conclusions: We found that the occurrence of PTD increased when women with obesity, smoking, uterine factors, cervical factors, ovulatory factors, DET, VTS, and obstetric complications, and a male fetus. Furthermore, a nomogram was constructed based on the above factors and it might have great value for clinic use.

KEYWORDS

nomogram, prediction, preterm birth, *in vitro* fertilization, intracytoplasmic sperm injection

Introduction

Infertility is a global medical condition that is estimated to affect 8%-12% of women worldwide (1). An increasing number of infertile couples of reproductive ages seek treatments, and thus, the use of assisted reproductive technology (ART) is climbing. In recent decades, many reproductive clinicians have paid more attention to the safety of offspring in ART (2). Unfortunately, they found a higher incidence of adverse perinatal outcomes in neonates born after *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI), even in singleton pregnancies, than those conceived naturally (3, 4). It has been reported that preterm delivery (PTD), a leading determinant of neonatal mortality, is also more common in IVF infants than in those conceived with spontaneous conception (5–7).

PTD is defined as delivery before 37 weeks of gestation (8). As mentioned, it is a key cause of newborn deaths, which can bring up new couples' significant socioeconomic burden (5, 6, 8). There is still no consensus on which factors are associated with the increasing risk of PTD in IVF/ICSI cycles (9). In an analysis of 144018 IVF cycles, many patients' inherent risk factors, such as infertility duration, cause of infertility, and donor oocytes, were responsible for a higher incidence of PTD (10). After that, in a study analyzing more than 20,000 singleton infants revealed that blastocyst transfer and frozen-thawed embryo transfer (FET) were also risk factors for PTB in addition to female inherent risk factors (11). Besides, a meta-analysis conducted by Pinborg et al. showed that PTD was also related to IVF procedures (12). Thereinto, researchers have demonstrated that IVF singletons had an increasing risk of PTD compared to ICSI singletons (12). However, data available by Keyhan et al. did not support this finding (9). No increasing risk of PTD was observed by Keyhan et al. between IVF babies and ICSI babies after propensity score matching based on baseline characteristics (9). This inconsistency highlights the need for further exploring the related risk factors for PTD in IVF/ICSI singletons. It is also important to construct a predictive model based on these factors, which enables clinicians to predict the occurrence of PTD, allowing for early implementation of interventions to prevent the onset of it.

Nomogram is an intuitive graph of a predictive model, which has been used to predict incidence or prognosis of diseases by integrating important prognostic factors (13, 14). It is helpful for us to create an easy-to-use predictive model for assessing the incidence of PTD in IVF/ICSI singletons. In this study, we aimed to analyze the hazardous factors of PTD to construct a nomogram predictive model for screening the risk of PTD in IVF/ICSI pregnancies.

Patients and methods

Patient enrollment

From January 2015 to February 2021, IVF/ICSI cycles with live births at the Reproductive Medicine Center of Tongji Hospital were retrospectively identified. Inclusion criteria were shown below: a) cycles resulting in singleton births; b) fresh and FET cycles; c) cycles with gonadotropin releasing hormone (GnRH) stimulation protocols in fresh cycles; d) natural cycles and cycles with hormone replacement in FET cycles. Moreover, exclusion criteria were as follows: a) cycles with donor eggs; b) cycles with non-gonadotropin releasing hormone (GnRH) stimulation protocols; c) cycles with preimplantation genetic diagnosis; d) cycles with missing baseline and follow-up data. All information was retrieved from the electronic medical record system. This study was approved by the Medical Ethics Committee of Tongji Hospital, Tongji Medical College, affiliated Huazhong University of Science and Technology (TJ-IRB20220624).

Controlled ovarian stimulation (COS) protocols in fresh cycles

As mentioned above, fresh cycles with GnRH stimulation protocols were included in our analysis. In light of ovarian reserve, female age, and other characteristics, the individualized COS protocol for each patient was determined. Generally, women with a normal ovarian response were provided with a long GnRH agonist (GnRH-a) regimen, while a flexible GnRH antagonist (GnRH-ant) protocol was offered to women with a diminished ovarian reserve or a known poor response (15). There are four COS protocols, including the GnRH-a ultralong protocol, the depot GnRH-a protocol, the GnRH-a long protocol, and the GnRH-ant protocol. All these protocols were described previously (16–18).

In short, in the GnRH ultralong protocol, women were injected subcutaneously with 3.75 mg long-acting GnRH-a (Decapeptyl; Ferring, SaintPrex, Switzerland) on days 1-3 of cycles to achieve pituitary suppression (16). The depot GnRH-a protocol was performed by subcutaneously administering 3.75 mg long-acting GnRH-a (Decapeptyl; Ferring, Saint-Prex, Switzerland) on Day 2 of the cycle. Moreover, in the GnRH-a long protocol, patients were provided with a daily subcutaneous injection of 0.1 mg GnRH-a (Decapeptyl; Ferring, Saint-Prex, Switzerland), initiating from the mid-luteal phase. In GnRH-a protocols, when complete pituitary desensitization was confirmed (a low serum estradiol (E_2) level of ≤30 pg/mL and a serum luteinizing hormone level of ≤ 2 IU/L), gonadotropins (Gn) were applied. While in the GnRH-ant protocols, the stimulation process starts with the administration of recombinant follicle stimulating hormone (r-FSH) (Gonal-F; Merck-Serono, Geneva, Switzerland) from Day 2 or 3 of the cycle. Then, the GnRH-ant cetrorelix acetate (Cetrotide; Merck-Serono) was injected at 0.25 mg/d to prevent premature ovulation when one of the criteria was met (E_2 levels >300 pg/ml, LH levels >10 IU/l, and the diameter of at least one follicle >14 mm).

When two leading follicles reached a mean diameter of 18 mm or three follicles reached a mean diameter of 17 mm, 250 μg human chorionic gonadotrophin (hCG) (Ovidrel; Merck-Serono, Geneva, Switzerland) was used to trigger ovulation. Oocytes were retrieved 34-36 h after triggering.

IVF/ICSI-embryo transfer (ET) procedures

The processes of oocyte fertilization, embryo culture, and embryo transfer have been described previously (15, 16). In brief, oocytes were fertilized by IVF, ICSI, or rescue ICSI (RICSI). RICSI was performed when there was no second polar body (PB) in oocytes or <25% of the oocytes presented a second PB after insemination with IVF (19). Normal fertilization was defined as the appearance of two pronuclei (2PN). Moreover, a maximum of two embryos were transferred.

The quality of embryos

On day 3 (D3) after oocyte retrieval, all of the cleavage embryos were checked. Good-quality embryos on D3 comprised seven to eight blastomeres without multinucleation and had < 20% fragments (16). The quality of blastocysts (D5 or D6) was evaluated using the Gardner Blastocyst Scoring System (20). In short, according to the cell number and junction, the inner cell mass and trophectoderm of blastocysts were graded from A to C. Good-quality blastocysts were defined as AA, AB, BA, and BB (20).

Embryo freezing and recovery

Based on the instructions of the vitrified freezing/resuscitation solution (Japan Kato), the embryos were frozen and thawed. The survival of thawed embryos was observed after 2H. The surviving cleavage embryos reached or exceeded half of the blastomere, while the surviving blastocyst partially or fully dilated (21).

Endometrial preparation in FET cycles

The endometrial preparation was described previously (21). In brief, in the natural cycles, the follicle, endometrium and ovulation were monitored during the menstrual cycle. When the peak of luteinizing hormone (LH) was observed, progesterone (P) was given. The cleavage embryos and blastocysts were thawed on D3 and D5 respectively after ovulation. As for the hormone replacement cycles, estradiol valerate (Bayer, Germany) was taken orally from D2 to D12 during the menstrual cycle. When the endometrium was ≥7 mm and the estrogen action time was ≥10 days, P was administrated. The cleavage embryos and blastocysts were thawed on D4 and D6 respectively after ovulation.

Data collection

Data were collected on female age, female BMI, women smoking, duration of infertility (years), antral follicle counts (AFC), baseline FSH, type of infertility (primary or secondary infertility), and infertility factors (tubal, pelvic, endometriosis, uterine, cervical, ovulatory, diminished ovarian reserve (DOR), male, or unexplained factors) in fresh or FET cycles.

In fresh cycles, the following factors were included in the analysis: stimulation protocols (GnRH-a ultralong, GnRH-a depot, GnRH-a long, or GnRH-ant protocols), days of Gn, total dose of Gn, E_2 and P level on the triggering day, endometrial thickness (EMT) on the triggering day, No. of oocyte retrieved, type of fertilization (IVF, ICSI, or RICSI), No. of embryo transferred (double or single embryo transferred, DET or SET), type of embryo (cleavage embryo or blastocyst), and morphology of the embryos transferred.

In FET cycles, the factors as follows were included: type of endometrial preparation, such as natural cycles and hormone replacement cycles (HRT), EMT on progesterone administration, type of fertilization (IVF, ICSI, or RICSI), DET or SET, type of embryo (cleavage embryo or blastocyst), and morphology of the embryos transferred.

During pregnancies, gestational sacs, obstetric complications, such as placenta previa (PP), placenta abruption (PA), hypertensive of pregnancies (HDP), or premature rupture of membrane (PROM), neonatal gender, birth weight and gestational age were all collected. In our study, PTD was defined as delivery with gestational age reached 28 weeks but less than 37 weeks. All relevant information in the electronic medical record system was collected and checked by two authors (Z.L. and L.C.).

Nomogram

All selected risk factors were enrolled to establish the nomogram model using the R package “rms”. The predictive

model was calculated using the R package "lrm" and displayed as an interactive nomogram by "regplot". The multivariate logistic regression coefficient (β) generated a risk score with each factor. Each factor had its point (corresponding line is β(X-m) terms), and the total scores were calculated by adding all the points together. The total scores corresponded to the probability of PTD occurring.

Statistical analysis

In this study, continuous data with normal distribution are presented as the mean ± SD, while non-normal distribution data are displayed as median (IQR). Categorical data are presented as numbers (percentages). T tests or Mann-Whitney U tests were utilized to compare the continuous data, while chi-square tests or Fisher's exact tests were used to analyze the categorical data. Univariate values with P value < 0.1 were screened for the next step of multivariate regression analysis. In the multivariate logistic regression analysis, adjusted odds ratios (AORs) and 95% confidence intervals (CIs) were used to indicate the level of association between risk factors and PTD. P value < 0.05 was considered statistically significant. SPSS 26 software was used for statistical analysis.

All statistically significant risk factors in the multivariate logistic regression analysis were further used to build a nomogram predictive model. The discrimination calibration abilities of this model were evaluated by calculating receiver operating characteristic curves (ROC), area under the ROC curve (AUC), Harrell's concordance index (C-index), and the calibration plots in the training and validation groups (14). The value of the C-index and AUC ranges from 0 to 1, with 0.5-0.7 indicating poor performance of this model, 0.7-0.8 demonstrating fair performance, and 0.8-0.9 suggesting good performance (22). R software (version 4.0.5) was used to construct the nomogram model and analyze the ROC, AUC, C-index, and calibration curve.

Results

General characteristics of patients

A total of 22809 cycles resulting in live births were identified (Figure 1). Among these cycles, 11296 cycles were not available for analysis, as one of the following exclusion criteria was met: (a) multiple live births (4527cycles), (b) donor oocyte (1913 cycles), (c) non-GnRH stimulation protocols in fresh cycles (948 cycles), (d) cycles with preimplantation genetic diagnosis (629 cycles), and (e) missing baseline or follow-up data (3279 cycles). Finally, the number of IVF/ICSI available for analysis was 11513. Then, we randomly allocated 9208 cycles (80%) to the training group and 2305 cycles (20%) to the validation group.

Table 1 described the baseline characteristics of the IVF/ICSI cycles with singleton live births between the training group and validation group. No significant difference in most characteristics was observed between the two groups (Table 1). Furthermore, Table 2 demonstrated a comparison of baseline characteristics between the PTD and non-PTD groups in the training group.

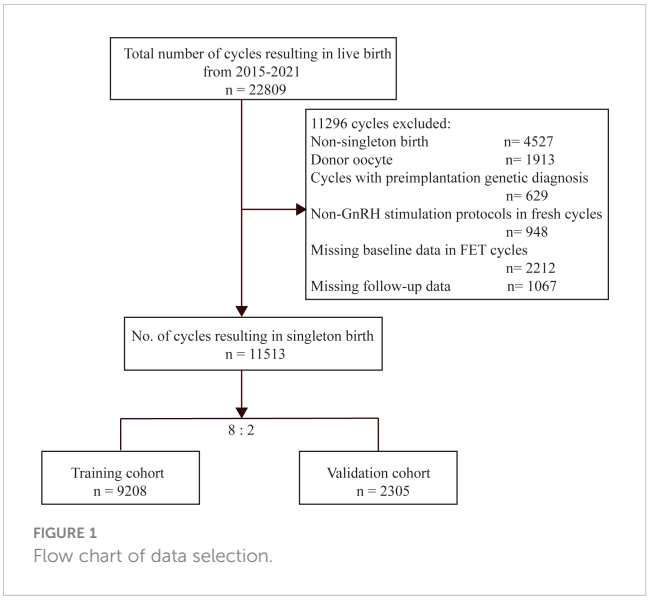

FIGURE 1
Flow chart of data selection.

There were 953 cycles (10.3%) in the PTD group and 8255 cycles (89.7%) in the non-PTD group (Table 2). As shown in Table 2, the median (IQR) of birth weight was 3050 (1750) and 2060 (1210) in non-PTD and PTD group respectively. The incidence of PTD was higher if there was one of the following factors: (a) female age ≥ 35, (b) female BMI > 24 kg/m^2, (c) smoking, (d) high baseline FSH, (e) secondary infertility, (f) uterine factors, (g) cervical factors, (h) ovulatory factors, (i) EMT ≤7 mm, (j) two embryo transferred, (k) blastocyst transfer, (l) FET, (m) non-high quality embryo transfer, (n) two gestational sacs, (o) obstetric complications (PP, PA, GDMHDP, and PROM), and (p) a male fetus (Table 2).

Clinical characteristics in fresh cycles

When only included fresh cycles, we also found the difference in female age, female BMI, smoking, cervical factors, ovulatory factors, EMT, gestational sacs, obstetric complications, and neonatal gender between PTD and non-PTD groups (Table S1). In addition to the patient's characteristics, data related to ovarian stimulation in the fresh cycle were also presented in Table S1, including stimulation protocols, average dose of Gn, E2 or P on hCG day, and No. of oocyte retrieved. The result showed that women with GnRH-agonist long protocol had lower risk to deliver a preterm baby, while women with GnRH-antagonist protocol had higher risk (Table S1).

Clinical characteristics in FET cycles

When only included FET cycles, there was also a higher risk for PTD in women with obesity, uterine factors, DET, non-high quality embryo transfer, two gestational sacs, obstetric complications, and neonatal gender between PTD and non-PTD groups (Table S2). However, no difference was observed in the type of endometrial preparation (natural cycles and HRT cycles) between PTD and non-PTD groups (Table S2).

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TABLE 1 Demographic characteristic of patients in the training and validation groups.

Variables	Training group n=9208	Validation group n = 2305	P value
Female age, y			.626
<35	7772 (84.4%)	1955 (84.8%)	
≥35	1436 (15.6%)	350 (15.2%)	
Female BMI, kg/m^2			.824
≤24	6674 (72.5%)	1676 (72.7%)	
>24	2534 (27.5%)	629 (27.3%)	
Smoking	119 (1.3%)	30 (1.3%)	.972
Duration of infertility#, y	3.00 (2.00)	3.00 (2.00)	.971
AFC			.457
5-24	7993 (86.8%)	2005 (87.0%)	
<5	410 (4.5%)	90 (3.9%)	
≥24	805 (8.7%)	210 (9.1%)	
Baseline FSH#, mIU/ml	7.21 (2.29)	7.14 (2.27)	.333
Type of infertility			
Primary	6094 (66.2%)	1543 (66.9%)	.490
Secondary	3114 (33.8%)	762 (33.1%)	
Factors of infertility			
Tubal	4924 (53.5%)	1191 (51.7%)	.120
Pelvic	1724 (18.7%)	441 (19.1%)	.653
Endometriosis	866 (9.4%)	216 (9.4%)	.960
Uterine	1779 (19.3%)	435 (18.9%)	.625
Cervical	61 (0.7%)	16 (0.7%)	.867
Ovulatory	2162 (23.5%)	558 (24.2%)	.461
DOR	1160 (12.6%)	264 (11.5%)	.136
Male	3429 (37.2%)	895 (38.8%)	.159
Unexplained	527 (5.7%)	140 (6.1%)	.520
EMT, mm			
≤7	171 (1.9%)	43 (1.9%)	.979
>7	9037 (98.1%)	2262 (98.1%)	
Treatment type			
IVF	6095 (66.2%)	1496 (64.9%)	.042*
ICSI	2607 (28.3%)	703 (30.5%)	
IVF+RICSI	506 (5.5%)	106 (4.6%)	
No. of embryo transferred			.612
1	5801 (63.0%)	1439 (62.4%)	
2	3407 (37.0%)	866 (37.6%)	
Blastocyst transfer	4085 (44.4%)	1036 (44.9%)	.615
FET	4037 (43.8%)	992 (43.0%)	.486
High-quality embryo transfer	7548 (82.0%)	1890 (82.0%)	.979
Gestational sacs			.129
1	8364 (90.8%)	2070 (89.8%)	
2	844 (9.2%)	235 (10.2%)	

(Continued)

TABLE 1 Continued

Variables	Training group n=9208	Validation group n = 2305	P value
PP	282 (3.1%)	65 (2.8%)	.542
PA	89 (1.0%)	22 (1.0%)	.958
GDM	454 (4.9%)	136 (5.9%)	.059
HDP	279 (3.0%)	55 (2.4%)	.100
PROM	127 (1.4%)	31 (1.3%)	.899
Neonatal gender			.885
Male	5018 (54.5%)	1260 (54.7%)	
Female	4190 (45.5%)	1045 (45.3%)	
Birth weight, g	2950 (1725)	3000 (1727)	.979
PTD	953 (10.3%)	223 (9.7%)	.339

BMI, body mass index; AFC, antral follicles count; FSH, follicle-stimulating hormone; DOR, diminished ovarian reserve; EMT, endometrial thickness; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; RICSI, rescue ICSI; FET, frozen-thawed embryo transfer; PP, placenta previa; PA, placenta abruption; GDM, gestational diabetes mellitus; HDP, hypertensive of pregnancies; PROM, premature rupture of membrane; PTD, preterm delivery; *P<0.05.
#: The data were presented as median (IQR).

TABLE 2 Baseline characteristics of patients with singleton live birth between PTD and non-PTD groups in the training group.

Variables	Non-PTD n = 8255	PTD n = 953	P value
Female age, y			
<35	6992 (84.7%)	780 (81.8%)	.022*
≥35	1263 (15.3%)	173 (18.2%)	
Female BMI, kg/m^2			.082*
≤24	6006 (72.8%)	668 (70.1%)	
>24	2249 (27.2%)	285 (30.0%)	
Smoking	78 (0.9%)	41 (4.3%)	<.001*
Duration of infertility#, y	3.00 (3.00)	3.00 (2.00)	.634
AFC			.699
5-24	7173 (86.9%)	820 (86.0%)	
<5	363 (4.4%)	47 (4.9%)	
≥24	719 (8.7%)	86 (9.0%)	
Baseline FSH#, mIU/ml	7.06 (2.34)	7.23 (2.28)	.013*
Type of infertility			.053*
Primary	5490 (66.5%)	604 (63.4%)	
Secondary	2765 (33.5%)	349 (36.6%)	
Factors of infertility			
Tubal	4418 (53.5%)	506 (53.1%)	.804
Pelvic	1538 (18.6%)	186 (19.5%)	.507
Endometriosis	770 (9.3%)	96 (10.1%)	.455
Uterine	1541 (18.7%)	238 (25.0%)	<.001*
Cervical	49 (0.6%)	12 (1.3%)	.016*
Ovulatory	1902 (23.0%)	260 (27.3%)	.003*
DOR	1031 (12.5%)	129 (13.5%)	.356
Male	3101 (37.6%)	328 (34.4%)	.057*
Unexplained	479 (5.8%)	48 (5.0%)	.335

(Continued)

TABLE 2 Continued

Variables	Non-PTD n = 8255	PTD n = 953	P value
EMT, mm			.035*
≤7 >7	145 (1.8%) 8110 (98.2%)	26 (2.7%) 927 (97.3%)	
Treatment type IVF ICSI IVF+RICSI	5444 (65.9%) 2351 (28.5%) 460 (5.6%)	651 (68.3%) 256 (26.9%) 46 (4.8%)	.305
No. of embryo transfer			<.001*
1	5283 (64.0%)	518 (54.4%)	
2	2972 (36.0%)	435 (45.6%)	
Blastocyst transfer	3501 (42.4%)	584 (61.3%)	<.001*
FET	3445 (41.7%)	592 (62.1%)	<.001*
High-quality embryo transfer	6822 (82.6%)	726 (76.2%)	<.001*
Gestational sacs 1 2	7665 (92.9%) 590 (7.1%)	699 (73.3%) 254 (26.7%)	<.001*
PP	220 (2.7%)	62 (6.5%)	<.001*
PA	34 (0.4%)	55 (5.8%)	<.001*
GDM	402 (4.9%)	52 (5.5%)	.428
HDP	179 (2.2%)	100 (10.5%)	<.001*
PROM	45 (0.5%)	82 (8.6%)	<.001*
Neonatal gender			.003*
Male	4455 (54.0%)	563 (59.1%)	
Female	3800 (46.0%)	390 (40.9%)	
Birth weight#, g	3050 (1750)	2060 (1210)	<.001*

PTD, preterm delivery; BMI, body mass index; AFC, antral follicles count; FSH, follicle-stimulating hormone; DOR, diminished ovarian reserve; EMT, endometrial thickness; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; RICSI, rescue ICSI; FET, frozen-thawed embryo transfer; PP, placenta previa; PA, placenta abruption; GDM, gestational diabetes mellitus; HDP, hypertensive of pregnancies; PROM, premature rupture of membrane; #, data are presented as median (interquartile range, IQR); *P<0.1.

Logistic regression analysis

To eliminate confounding factors, multivariate logistic regression analysis was used to analyze the risk factors for PTD, which were detailed in Table 3. Factors (female age, female BMI, smoking, baseline FSH, type of infertility, uterine factors, cervical factor, ovulatory factor, male factor, EMT, No. of embryo transferred, blastocyst transfer, FET, high-quality embryo transfer, gestational sacs, PP, PA, GDM, HDP, PROM, and neonatal gender) with P<0.1 in Table 2 were entered into the multivariate logistic regression analysis. Thirteen factors remained statistically significant. Although P value in cervical factor was 0.053 (P>0.05), we still included this factor in further analysis as this factor had very important clinical significance in the occurrence of PTD.

The results showed that compared to women whose BMI was ≤ 24 kg/m2, overweight patients had a significantly higher risk of PTD after IVF/ICSI (OR 1.25, 95% CI: 1.06-1.47). Women who had a smoking habit were more likely to deliver a preterm infant (OR 4.97, 95% CI: 3.21-7.67). Moreover, women with uterine, cervical, and ovulatory infertility factors displayed a rising PTD ratio (OR 1.47, 95% CI: 1.23-1.76; OR 2.02, 95% CI: 0.99-4.11; OR 1.24, 95% CI: 1.04-1.47, respectively). In comparison to the SET group, women who had DET had a higher risk of PTD (OR 1.37, 95% CI: 1.17-1.61). Besides, women with blastocyst transfer (OR 1.56, 95% CI: 1.19-2.03) and FET (OR 1.56, 95% CI:1.20-2.03) had higher risk of PTD. During pregnancies, women who had two gestational sacs had a greater chance of delivering a preterm baby (OR 4.17; 95% CI: 3.46-5.03). In addition, women who had obstetric complications, such as PP (OR 3.06, 95% CI: 2.24-4.19), PA (OR 10.22, 95% CI: 6.35-16.46), HDP (OR 4.26, 95% CI: 3.21-5.66), and PROM (OR 20.32, 95% CI: 13.55-30.47), also had an increasing risk of PTD. Interesting, we found a male fetus (OR 1.182, 95% CI: 1.019-1.371) was more likely to be born prematurely (Table 3).

Nomogram prediction model

Furthermore, the above prognostic factors were incorporated to establish a predictive model for predicting the probability of PTD

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TABLE 3 Multivariable logistic regression model for predicting occurrence of PTD in training group.

Factors	AOR (95%CI)	P value
Female age		.344
<35	1	
≥35	1.10 (0.90-1.34)	
Female BMI		.010*
≤24	1	
>24	1.25 (1.06-1.47)	
Smoking	4.97 (3.21-7.67)	<.001*
Type of infertility		.373
Primary	1	
Secondary	1.08 (0.92-1.26)	
Basal FSH	1.01 (0.97-1.04)	.695
Uterine factors	1.47 (1.23-1.76)	<.001*
Cervical factors	2.02 (0.99-4.11)	.053
Ovulatory factors	1.24 (1.04-1.47)	.016*
Male factors	0.98 (0.84-1.15)	.791
EMT		.305
≤7	1	
>7	1.28 (0.80-2.06)	
No. of embryo transfer		<.001*
1	1	
2	1.37 (1.17-1.61)	
Blastocyst transfer	1.56 (1.19-2.03)	.001*
FET	1.56 (1.20-2.03)	.001*
High-quality embryo transfer	0.88 (0.74-1.06)	.168
Gestational sacs		<.001*
1	1	
2	4.17 (3.46-5.03)	
PP	3.06 (2.24-4.19)	<.001*
PA	10.22 (6.35-16.46)	<.001*
HDP	4.26 (3.21-5.66)	<.001*
PROM	20.32 (13.55-30.47)	<.001*
Neonatal gender		.027*
Male	1.182 (1.019-1.371)	
Female	1	

PTD, preterm delivery; BMI, body mass index; FSH, follicle-stimulating hormone; EMT, endometrial thickness; FET, frozen-thawed embryo transfer; PP, placenta previa; PA, placenta abruption; HDP, hypertensive of pregnancies; PROM, premature rupture of membrane; AOR, adjusted odds ratio; CI, confidence interval; *P<0.05.

incidence in IVF/ICSI singleton live birth (Figure 2). The nomogram developed from the training group was shown in Figure 2. The usage of the nomogram is illustrated with an assumptive woman with BMI > 24, smoking, cervical factor, DET, FET, two gestational sac, and have a male fetus. The total score added up to 4.92 for this patient, which represents approximately 0.792 of probability of PTD incidence (Figure 2).

Validation of the nomogram model

The performance of this nomogram was assessed by AUC, C-index, and calibration plots. In the training and validation groups, both calibration plots indicated good agreement between the predicted probability and actual observation of PTD (Figures 3A, C). In addition, the AUC for this predictive model was 0.774 and 0.770 in the training and validation groups, respectively (Figures 3B, D). The optimal threshold was -2.239 and -2.011 in training and validation groups, respectively (Figures 3B, D). At the above thresholds, the sensitivity was 77.4% and the specificity 6 was8% in training group, while in validation group, the sensitivity was 88.1% and the specificity was 57.4% (Figures 3B, D). The C-index of the predictive model was 0.774 (95% CI: 0.757-0.791) in the training group and 0.770 (95% CI: 0.731-0.809) in the validation group (Table 4). These results suggested that the model exhibited fair performance.

Discussion

PTD is one of serious obstetric complications, which accounts for 11% of pregnancies all over the world (23). A growing number of reports have indicated that PTD was more common in IVF/ICSI pregnancies than natural pregnancies (7). There is still no effective predictive model based on risk factors to predict the incidence of PTD in IVF singleton pregnancies.

In this study, we used multiple logistic regression analysis to identify significant risk factors related with PTD in the training group, including maternal obesity, smoking, uterine factors, cervical factors, ovulatory factors, DET, blastocyst transfer, FET, double gestational sacs, obstetric complications (PP, PA, HDP, and PROM), and a male fetus. All of the above factors were utilized to develop the prognostic model, which was visualized by a nomogram. Good discrimination and calibration were shown in this model, as assessed by the AUC, C-index, and calibration plots in both the training and validation groups. Hence, this nomogram model for predicting PTD in IVF/ICSI singleton live births had fair performance and might hold promise for clinical use.

We demonstrated that PTD was affected by inherent female characteristics and infertility factors, such as BMI, smoking, uterine factors, cervical factors, and ovulatory factors. The findings of our study are in accordance with previous studies. For example, Cnattingius et al. found that overweight women in Sweden had a higher risk of PTD (24). Moreover, another systematic review also supported the above result, suggesting that there was an increasing risk of PTD for women with obesity (25). The intrauterine environment is critical to the normal function of the placenta, and the fetus develops. Maternal obesity might provide a chronic inflammatory environment, in which the development of the fetus is impaired, consequently leading to PTD (26, 27). Moreover, many studies have showed that smoking was the

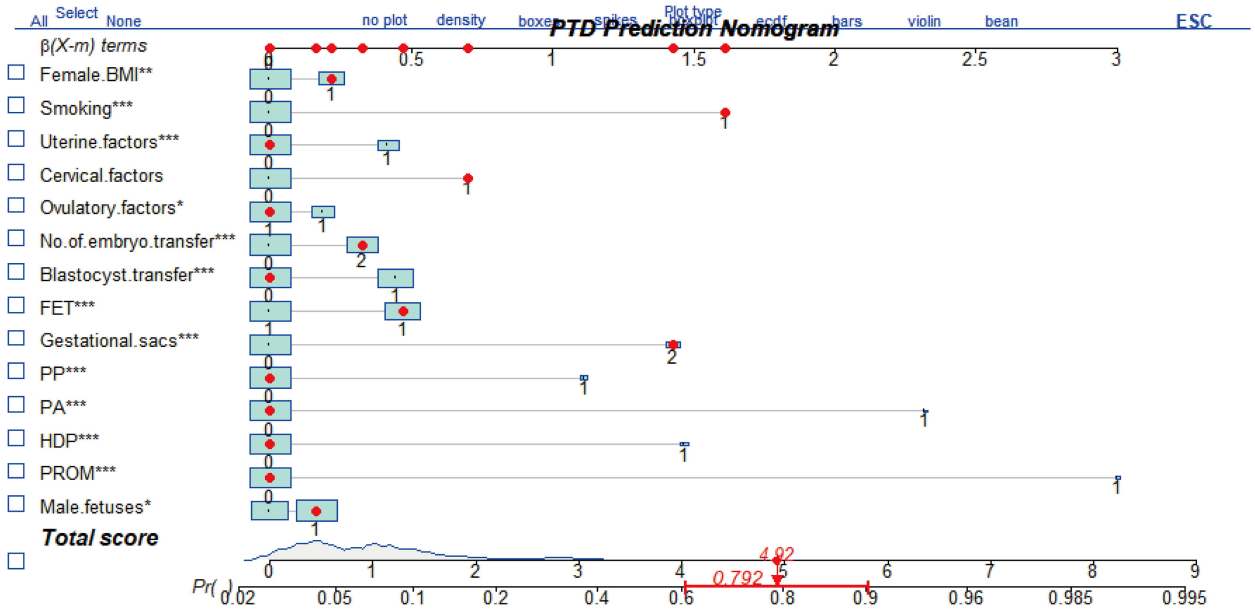

FIGURE 2
The nomogram to predict the incidence of PTD in patients after IVF/ICSI. It includes female body mass index (BMI), smoking, uterine factors, cervical factors, ovulatory factors, No. of embryo transferred, blastocyst transfer, frozen-thawed embryo transfer (FET), gestational sacs, placenta previa (PP), placenta abruption (PA), hypertensive of pregnancies (HDP), and premature rupture of membrane (PROM). The usage of this nomogram is interpreted in an assumptive woman with BMI >24 kg/m^2, smoking habit, cervical factors, two embryo transfer (DET), FET, two gestational sacs, and a male fetus. For this woman, the total point added up to 4.92, which indicated approximately 0.792 of probability of PTD incidence. *P<0.05, **P<0.01, ***P<0.001.

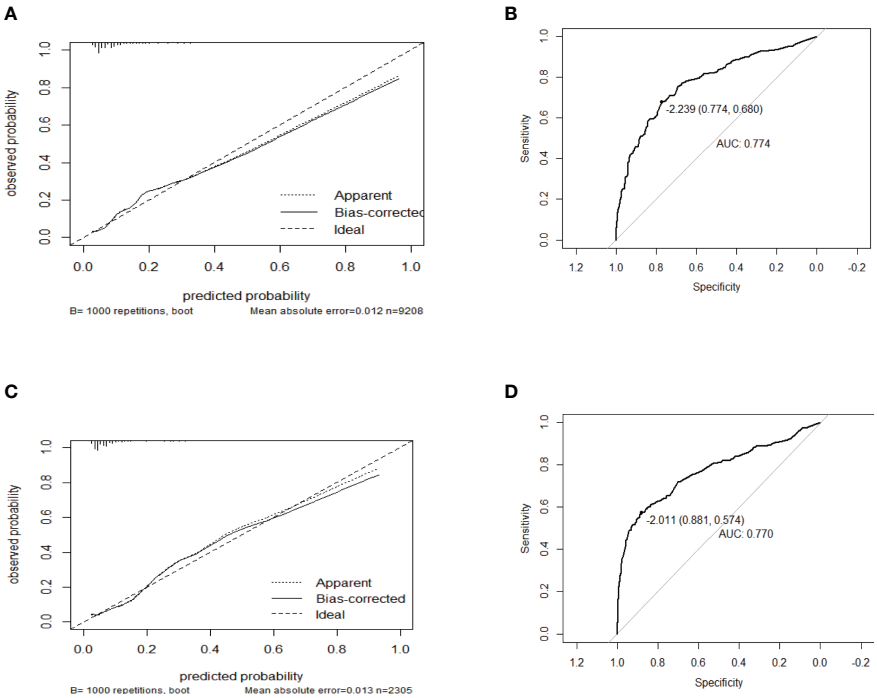

FIGURE 3
The calibration plots and receiver operator characteristic curves (ROC) of the nomogram in the training and validation group. Calibration plots in **(A)** the training group and **(C)** validation group. ROC in **(B)** the training group and **(D)** validation group.

TABLE 4 The C-index and AUC of the nomogram in the training and validation groups.

Outcome	Model	Training cohort		Validation cohort	
		C-index	AUC	C-index	AUC
PTD	Female BMI + Smoking + Uterine factors + Cervical factors + Ovulatory factors + No. of embryo transfer + Blastocyst transfer + FET + Gestational sacs + PP +PA + HDP + PROM+ Male fetus	0.774 (0.757-0.791)	0.774	0.770 (0.731-0.809)	0.770

PTD, preterm delivery; BMI, body mass index; FET, frozen-thawed embryo transfer; PP, placenta previa; PA, placenta abruption; HDP, hypertensive of pregnancies; PROM, premature rupture of membrane; C-index, concordance index; AUC, area under ROC.

most important risk factor for PTD (28, 29). Therefore, women after IVF/ICSI treatment need to control their weight and quit the smoking habit to prevent the occurrence of PTD.

In terms of infertility factors, uterine factors, such as septate uterus, can cause an increase in the risk of PTD (30). Besides, it is well-known that cervical factors, such as cervical shortening (a cervical length of less than 25 mm) and cervical incompetence, are associated with PTD (6, 31). In this occasion, progesterone support is available for women with short cervix, while cervical cerclage is required for women with cervical insufficiency (32, 33). In addition, a large study enrolling 635604 IVF/ICSI cycles in the UK reported that infertile women with ovulatory disorders also had a greater chance of having a preterm baby after adjusting for confounding factors (34). Ovulatory disorder is a cause of infertility with polycystic ovary syndrome (PCOS) (35). A higher risk of PTB after fresh IVF/ICSI cycles was observed in women with PCOS than in controls (36). One of the possible explanations may be the existence of hyperandrogenism in the environment (36). It was reported that the remodeling and ripening of the cervix could be affected by androgens; thus, the rate of PTD rose (36). For infertility women with polycystic ovary and hyperandrogenism, androgen reduction therapy should be performed first, followed by ovulation induction, and pregnancy monitoring also needs to be strengthened to prevent PTD.

In addition, embryo factors may also affect PTD, such as No. of embryo transferred, blastocyst, and frozen-thawed embryo. The results showed that infertile women with SET had a lower risk of PTD than those with DET, which was in line with previous studies (37). Hence, reproductive doctors should encourage infertility women with IVF/ICSI treatment to undergo SET. Moreover, blastocyst transfer was a risk factor for PTD in comparison with cleavage transfer, which was in line with the result of a previous study (38). As for the underlying mechanism, the *in vitro* culture might affect the potential genetic or epigenetic on the trophectoderm cells; thus, the implantation and placentation were different which might cause higher incidence of PTD (38). Besides, there was a higher risk of PTD in cycles with frozen-thawed embryos than that with fresh embryos, which was accordance with a previous study (11). The possible reasons are still unknown.

During pregnancy, the incidence of PTD was higher in women with two gestational sacs and obstetric complications. Women would be more likely to be born a preterm infant if there were double gestational sacs during pregnancy but had a singleton live birth. This phenomenon is also called vanishing twin syndrome (VTS) (39). Other researchers discovered that a higher risk of PTB only occurred when VTS occurred after gestational week 14, suggesting that it was dependent on the timing of vanishing twins (40). Therefore, women who develop VTS after 14 weeks' gestation need to strengthen pregnancy care and fetal monitoring. In addition, regardless of the methods of conception, obstetric complications, such as PP, PA, HDP, and PROM, are all related to a high incidence of PTD (6, 41). For the above different obstetric complications, different treatment measures should be performed to reduce the occurrence of PTD. If PTD is inevitable, timely treatment should be carried out to ensure the safety of mother and child.

Interesting, there was a higher risk for PTD in male fetuses compared with female fetuses. Although the underlying mechanisms are still not clear, the different trophoblast cells may explain it. Trophoblast in pregnancies with male fetuses may produce more pro-inflammatory cytokines; thus, intrauterine inflammation may cause a high incidence of PTD in male fetuses (42).

However, we did not find an association between IVF/ICSI treatment and PTD, which is different from the findings of some studies (10, 12). For instance, data available by Pinborg et al. showed that there was a lower risk of PTD in ICSI singletons than in IVF singletons, indicating that the fertilization procedure might have an effect on this perinatal outcome (12). Nevertheless, other work described that the reason behind this difference was that the high risk of PTD in IVF singletons might be secondary to female inherent risk factors instead of IVF itself (9). Although the type of IVF/ICSI treatment might not affect PTD, we found women with GnRH-antagonist protocol had higher risk to deliver a preterm baby, while women with GnRH-agonist protocol had lower risk. Zhu et al. also observed a higher PTD rate in GnRH-antagonist group (9.0%) than that in GnRH-agonist (4.0%) after propensity matching, but there was no significant difference (43). Hence, it's possible that our results may be due to women's inherent clinical characteristics, such as advanced age.

As for the advantages of our research, the first is the large sample size. 11513 cycles resulting in singleton births were included for analyzing. Besides, important data regarding clinical characteristics (smoking, the causes of infertility, and obstetric complications) and IVF/ICSI-ET procedures (different stimulation protocols, type of fertilization, and type of embryo transfer) were available in our study. Last but not least, to the best of our knowledge, this was the first study to construct a nomogram model for predicting the incidence of PTD in women after IVF/ICSI. It might also help doctors identify infertile women who are at higher risk of PTD. Therefore, those patients who had obesity, a smoking habit, uterine factors, cervical factors, ovulatory factors, DET, blastocyst transfer, FET, VTS, obstetric complications, or a male fetus after IVF/ICSI should be informed of the

possible high rate of PTD. They are also encouraged to actively cooperate with prenatal examinations and care to reduce the occurrence of this poor neonatal outcome.

Nevertheless, several limitations in our study also need to be addressed. First, this study was carried out in a single reproductive center and lacks external validation in other centers; thus, there might be inevitable bias. Additionally, potential biases cannot be excluded as it is a retrospective study. Third, all possible related factors of PTD might not be covered in this analysis. For example, some risk factors, including women smoking, previous obstetric history, genital tract infection, etc., may be significant with PTD as well, yet the data were not available. Last, two subtypes (spontaneously preterm birth and iatrogenic preterm birth) of PTD cannot be separated by us. Actually, there are different risk factors between these two subtypes.

In summary, our study explored the risk factors for PTD in women with IVF/ICSI singleton live births and found that the incidence of PTD rose when it comes to maternal obesity, infertility factors, blastocyst transfer, FET, DET, VTS, obstetric complications, or a male fetus. Furthermore, the nomogram for visualizing the predictive model was built by us and demonstrated good discrimination and calibration to some extent. Hence, it might be of great value for clinical use. Notwithstanding that, more prospective studies need to be conducted to investigate risk factors for PTD in IVF/ICSI singleton live birth and validate our predictive tool.

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 study was approved by the Medical Ethics Committee of Tongji Hospital, Tongji Medical College, affiliated Huazhong University of Science and Technology (TJ-IRB20220624).

Author contributions

ZL contributed to the design of study. ZL, LC, JL, XH, YL, and LS collected data. ZL performed data analysis. ZL drafted the manuscript, which was revised by CL, KQ, CS, and HZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1065291/full#supplementary-material

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First trimester sCD40L levels associated with adverse neonatal outcomes in euthyroid pregnant women with positive TPOAb

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Backgrounds: It remained unclear whether isolated positive thyroid peroxidative antibodies (TPOAb) were associated with adverse maternal and neonatal outcomes. The purpose of this study was to observe adverse neonatal outcomes among euthyroid pregnant women with positive TPOAb and to investigate the underlying risk factors.

Methods: Euthyroid pregnant women with TPOAb positivity were enrolled and followed up in our study. Adverse neonatal outcomes such as preterm birth, low birth weight, and fetal macrosomia were observed. Clinical data in the first trimester were collected and compared between groups with or without adverse neonatal outcomes. Maternal serum soluble CD40 ligand (sCD40L) was also measured at the same time.

Results: A total of 176 euthyroid pregnant women with TPOAb positivity were finally enrolled and analyzed in our study. Thirty-nine (22.16%) euthyroid women with TPOAb positivity were found to have adverse neonatal outcomes. Thirteen participants received assisted reproductive technology (ART) in our study, and seven participants were in the adverse neonatal outcome group. Preterm birth, low birth weight, and fetal macrosomia were the most common comorbidities. The proportion of receiving ART and the levels of sCD40L and platelet were significantly higher in the adverse neonatal outcome group (all $P < 0.05$). Multivariate regression analysis showed that sCD40L and receiving ART were the independent risk factors for adverse neonatal outcomes. The odds ratio values of sCD40L higher than 5.625 ng/ml were 2.386 [95% confidence interval (CI) = 1.017 to 5.595; $P = 0.046$] for overall adverse neonatal outcome, 3.900 (95% CI = 1.194 to 12.738; $P = 0.024$) for preterm birth, and 3.149 (95% CI = 0.982 to 10.101; $P = 0.054$) for low birth weight.

Conclusions: Approximately one of the four euthyroid women with TPOAb positivity might have adverse neonatal outcomes. Measurement of sCD40L in first trimester might have a predictive value for adverse neonatal outcomes in euthyroid pregnant women with positive TPOAb.

KEYWORDS

thyroid peroxidase antibody, pregnancy, adverse neonatal outcomes, sCD40L, platelet

Introduction

Thyroid hormones are important determinants for the development of fetuses, especially in early pregnancy (1). Thyroid autoimmunity (TAI), defined with the presence of thyroid peroxidative antibodies (TPOAb) or thyroglobulin antibodies (TgAb) in circulation, is the most common cause of thyroid dysfunction in women of childbearing age. In the worldwide, about 8%–14% women might have TAI disease (2). TAI in pregnancy could affect the stabilization of maternal thyroid hormones and is related to adverse outcomes for pregnant women and fetuses, such as gestational diabetes mellitus, pregnancy-induced hypertension, miscarriage, preterm birth, and mental development in offspring in previous epidemiological study (3, 4).

Isolated positive TPOAb women are particular phenotype that is defined as having normal thyroid function and positive TPOAb in circulation. Studies have shown that euthyroid women with TPOAb positivity were also associated with preterm birth, abnormal fetal growth, and so on (5–7). It was hypothesized that immune disorders played a central role in the mechanism of adverse pregnancy outcomes in these patients (8). The activation of autoreactive T cells and their related costimulatory molecules plays important roles in the immune response of TAI (9, 10). Thus, the changes in costimulatory molecules might be of special interest in euthyroid women with isolated positive TPOAb with adverse pregnancy outcomes.

As a pair of important costimulatory molecules, CD40 and its ligand, CD40L, were closely related to the production of antibodies (11). CD40L is expressed on the membrane of T cells and linked to CD40 on B cells, providing a signal for the differentiation, proliferation, and activation of B cells. CD40L exists in both membrane and soluble forms *in vivo*. Soluble CD40L (sCD40L) is produced by the shedding of CD40L and has similar functions to CD40L (12). sCD40L was found to be positively correlated with TPOAb (13). However, there is no study on the clinical significance of sCD40L levels in euthyroid women with positive TPOAb and its association with adverse neonatal outcomes. Given the early effects of TAI and low-grade systemic inflammation in pregnant women, we tried to determine whether there was a relationship between biomarkers of immune activation sCD40L levels in the first trimester and adverse neonatal outcomes.

The purpose of this study was to observe the incidence of adverse neonatal outcomes in euthyroid women positive for TPOAb and to investigate the association between maternal serum sCD40L levels or other underlying risk factors in early pregnancy and adverse neonatal outcomes.

Methods

Participants

The longitudinal observational study was conducted at the affiliated Suzhou Hospital of Nanjing Medical University during the period from March 2020 and July 2021. Pregnant women with positive TPOAb and normal thyroid function in the first trimester were enrolled in our study. Positive TPOAb was defined with serum TPOAb titers greater than the upper limit of the reference value. Normal thyroid function was defined with normal free thyroxine (FT4) and normal thyroid-stimulating hormones (TSH) in serum.

The 2017 American Thyroid Association (ATA) guideline advocates the use of population-based reference ranges of TSH during pregnancy, whereas if these ranges are not available, they recommended using 4.0 mIU/L as the upper reference limit of TSH for the first trimester. Thus, TSH values > 4 μIU/ml were excluded in our study to eliminate the possible effect of subtle thyroid dysfunction (14).

All participants enrolled in the first trimester and follow-up until childbirth. Inform consent was signed in each participant before the enrollment. Adverse neonatal outcomes (preterm birth, low birth weight, and fetal macrosomia) were recorded and analyzed.

Premature birth was defined with newborns whose gestational age is less than 37 weeks but more than 28 weeks. Low birth weight was defined with newborns whose birth weight is less than 2,500 g. Fetal macrosomia was defined with a newborn with a birth weight of more than 4,000 g. The flow diagram of study was summarized in Figure 1.

The exclusion criteria for the participation in the study are as follows: age > 45 years old, missing data/records during follow-up, previous history of thyroid disease, previous history of diabetes mellitus or gestational diabetes mellitus (15), medications that affect thyroid function or glucose metabolism, taking medications of immuno-suppressive therapy, personal history of metabolic or chronic systemic disease, history of drinking or smoking, family history of hereditary or congenital diseases, and recent infectious disease.

Data collection and laboratory measurements in early pregnancy

The demographical data of all participants such as age, gestational weeks, gravida, parity, pre-pregnancy body mass index (BMI), receiving assisted reproductive technology (ART) or not,

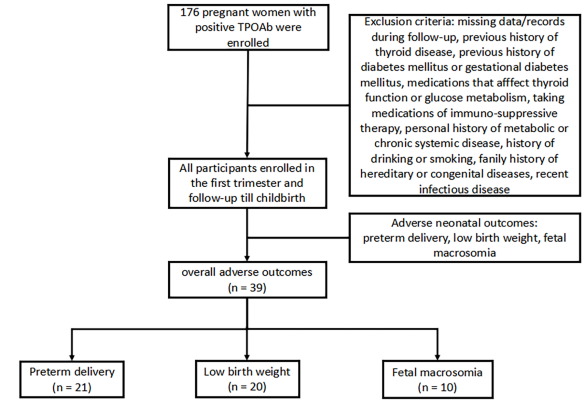

FIGURE 1
Flow diagram of study.

type of ART such as in vitro fertilization (IVF), or intracytoplasmic sperm injection (ICSI) were recorded in the early pregnancy. All participants will undergo a routine obstetric examination in our hospital. Data of glycosylated hemoglobin A1c (HbA1c), fasting plasma glucose (FPG), ferritin, uric acid (UA), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c), C-reactive protein (CRP), white blood cell (WBC), red blood cell (RBC), hemoglobin (Hb), and platelet were also collected.

Serum levels of TSH, FT4, and TPOAb of all participants in gestational weeks 10–13 were measured. Serum levels of TSH, FT4, and TPOAb were measured using automated chemiluminescent immunoassays (Architect i2000SR; Abbott Laboratories, Chicago, IL, USA). Reference ranges used in our study were as follows: TSH, 0.35~4.94 μIU/ml; FT4, 9.01~19.04 pmol/L; TPOAb, <12 IU/ml.

Measurement of sCD40L

Fasting blood was collected in all participants in the early stage of pregnancy. Serum sample was store in −80°C refrigerator for centralized measurement. A commercial ELISA kit (Xvguang Kexing Antibody Biotechnology Co., Ltd.) was used to measure serum sCD40L. The minimum detectable dose was 0.015 ng/ml, with intra- and inter-coefficients of variation of <5%.

Statistical analysis

Statistical analysis was performed using SPSS 26.0 and GraphPad Prism 8.0.2. The data were tested for normality using the Kolmogorov–Smirnov test. Data are presented as mean ± standard deviation for normally distributed variables or median (5% and 95% interquartiles) for nonnormally distributed variables. According to the adverse neonatal outcomes, the participants were divided into two subgroups. The differences between groups were compared by Student's t-test or Mann–Whitney U-test. The Fisher's exact test was used to test categorical variables. According to types of adverse outcomes, participants were divided into four subgroups. The differences among subgroups were performed with nonparametric test. Logistic regression analysis was carried out to evaluate the odds ratio (ORs) and 95% confidence interval (95% CI) of adverse neonatal outcome in euthyroid pregnant women with positive TPOAb. *P*-value of 0.05 or less was considered statistically significant.

Results

The comparison of baseline characteristics of pregnant women with or without adverse neonatal outcome

A total of 176 euthyroid pregnant women with positive TPOAb with complete data were finally enrolled and analyzed in our study. Thirty-nine of the 176 (22.16%) participants were having adverse neonatal outcomes. According to the occurrence of adverse neonatal outcomes, the participants were divided into groups: with adverse neonatal outcome (n = 39) and without adverse neonatal outcome (n = 137). The baseline and laboratory characteristics of the patients were provided in Table 1. There were no statistically significant differences between age, gestational weeks, pre-pregnancy BMI, gravida, parity, TSH, FT4, ferritin, HbA1c, FPG, UA, TG, TC, HDL-c, LDL-c, CRP, WBC, RBC, and Hb. Thirteen participants received ART were analyzed in our study. Six participants (five IVF and one ICSI) were in the group without adverse neonatal outcome, and seven participants (three IVF and four ICSI) were in the group with adverse neonatal outcomes. The proportion of receiving ART participants in adverse neonatal outcome group was higher than in the group without adverse neonatal outcome (P = 0.012, Table 1). However, there was no significant difference in the type of ART received (P = 0.265, Table 1).

Platelet and sCD40L were significantly higher in participants with the adverse neonatal outcome group (P < 0.001, P = 0.005). A positive correlation was found between sCD40L and platelet (r = 0.323, P < 0.001). There was no correlation between sCD40L and other parameters.

Serum sCD40L and platelets were higher in early pregnancy in euthyroid pregnant women with adverse neonatal outcome

We then analyzed the types of adverse neonatal outcome that occurred. A total of 39 (22.16%) participants had adverse neonatal outcome including preterm birth (21, 11.93%), low birth weight (20, 11.36%), and fetal macrosomia (10, 5.68%).

sCD40L levels of participants were 8.28 ± 4.15 ng/ml in the overall adverse neonatal outcome group, 8.60 ± 4.14 ng/ml in the preterm birth group, and 8.49 ± 4.65 ng/ml in the low birth weight group. sCD40L levels were found to be significantly higher in overall adverse outcomes, preterm birth, and low birth weight (P = 0.005, P = 0.019, and P = 0.047; Figure 2A). The sCD40L levels of participants with fetal macrosomia were 7.39 ± 3.22 ng/ml and were higher than those without in the adverse outcome group (6.25 ± 2.43ng/ml), but no statistically significances were found.

Platelet counts were also found to be significantly higher in overall adverse outcomes, preterm birth, and low birth weight (P < 0.001, P = 0.045, and P = 0.022; Figure 2B). There was no significant difference in the fetal macrosomia group. Twelve pregnant women were found to have preterm birth and low birth weight; sCD40L level was 8.43 ± 4.30ng/ml; platelet level was 246.42 ± 71.54 × 10^9/L (data not shown).

The cutoff value for sCD40L and platelet levels in early pregnancy in predicting adverse neonatal outcomes

Receiver operator characteristic (ROC) curve analysis was performed for the diagnostic performance of sCD40L and platelet levels for overall adverse neonatal outcomes (Figure 3). The best cutoff value for sCD40L and for platelet in predicting adverse neonatal

TABLE 1 Baseline and demographical characteristics and laboratory parameters of women in the first trimester between normal and abnormal neonatal outcome groups.

	Abnormal neonatal outcome (n = 39)	Normal neonatal outcome (n = 137)	*P*-value*
Age (years)	29.74 ± 3.51	29.96 ± 4.04	0.766
Gestational weeks	12.83 ± 1.55	13.10 ± 1.93	0.429
Pre-pregnancy BMI (kg/m^2)	22.73 ± 3.59	22.26 ± 2.96	0.410
Gravida	2 (1–3)	2 (1–2)	0.829
Parity	0 (0–1)	0 (0–1)	0.517
ART (n, %)	7 (17.95%)	6 (4.38%)	0.012*
IVF (n)	3	5	0.265
ICSI (n)	4	1	
TPOAb (IU/ml)	239.76 (88.7-771.36)	416.34 (161.07-642.84)	0.555
TSH (μIU/ml)	1.68 ± 0.69	1.60 ± 0.69	0.549
FT4 (pmol/L)	13.11 ± 1.40	12.74 ± 1.27	0.118
Ferritin (ng/ml)	48.96 (32.61–98.22)	54.49 (31.72–86.39)	0.763
HbA1c (%)	5.23 ± 0.22	5.19 ± 0.34	0.441
FPG (mmol/L)	4.56 ± 0.35	4.64 ± 0.46	0.362
UA (μmol/L)	218.39 ± 42.24	218.50 ± 52.48	0.990
TG (mmol/L)	1.57 ± 0.74	1.55 ± 0.68	0.837
TC (mmol/L)	4.86 ± 0.84	4.82 ± 0.79	0.795
HDL-c (mmol/L)	1.72 ± 0.35	1.80 ± 0.38	0.299
LDL-c (mmol/L)	2.56 ± 0.64	2.50 ± 0.67	0.585
CRP (mg/L)	2.84 (1.28–6.08)	2.29 (1.03–4.28)	0.412
WBC (× 10^9/L)	8.47 ± 1.81	8.61 ± 1.77	0.655
RBC (× 10^{12}/L)	4.26 ± 0.34	4.23 ± 0.37	0.609
Hb (g/L)	127.92 ± 8.19	128.31 ± 10.08	0.828
platelet (× 10^9/L)	260.62 ± 62.51	225.91 ± 49.92	<0.001*
sCD40L (ng/ml)	8.28 ± 4.15	6.25 ± 2.43	0.005*

*P < 0.05 is statistically significant.
BMI, body mass index; ART, assisted reproductive technology; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; TPOAb, thyroid peroxidative antibodies; TSH, thyroid-stimulating hormones; FT4, free thyroxine; HbA1c, glycosylated hemoglobin A1c, FPG, fasting plasma glucose; UA, uric acid; TG, triglyceride; TC, total cholesterol; HDL-c, high-density lipoprotein cholesterol; LDL-c, lipoprotein cholesterol; CPR, C-reactive protein; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin.

outcomes was 5.625 ng/ml with an average area under curve (AUC) value of 0.648 (95% CI = 0.547 to 0.750) and 207 × 10^9/L with an average AUC 0.647 (95% CI = 0.547 to 0.746), respectively.

Multivariate logistic regression analysis of adverse neonatal outcomes in euthyroid pregnant women with positive TPOAb

As we unexpectedly found that sCD40L, platelet counts, and the proportion of ART received were significantly higher in the group with adverse neonatal outcome, we attempted to evaluate the association of these risk factors with adverse neonatal outcome in euthyroid women with positive TPOAb. Our results revealed that serum sCD40L levels higher than 5.625 ng/ml and receiving ART were the independent risk factors for overall adverse outcome in euthyroid pregnant women with positive TPOAb in the first trimester (OR = 2.386, 95% CI = 1.017 to 5.595, *P* = 0.046; and OR = 7.384, 95% CI = 1.704 to 31.995, *P* = 0.008; Figure 4).

To analyze the association of risk factors and types of adverse neonatal outcome separately, we found that sCD40L level higher than 5.625 ng/ml was also a risk factor for preterm birth with an OR value of 3.900 (95% CI =1.194 to 12.738, *P* = 0.024; Figure 5). In the group with low birth weight, OR was 3.149 (95% CI = 0.982 to 10.101, *P* = 0.054; Figure 6). The OR values of receiving ART for preterm birth were 9.053 (95% CI = 1.667 to 49.169, *P* = 0.011;

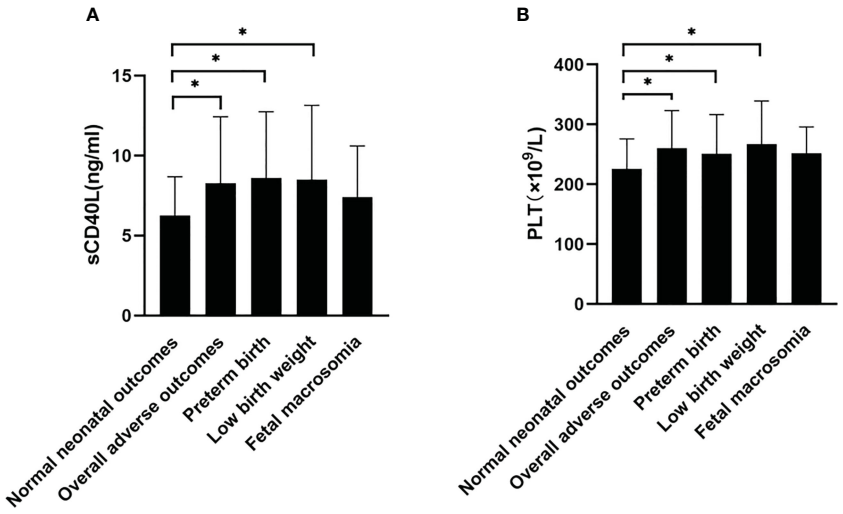

FIGURE 2
Comparison of sCD40L and platelet among groups with or without adverse neonatal outcomes. According to types of adverse neonatal outcomes, participants were divided into groups with normal neonatal outcome, overall adverse outcomes, preterm birth, low birth weight, and fetal macrosomia. sCD40L **(A)** and platelet **(B)** were compared among groups using one-way ANOVA test. *$P < 0.05$.

Figure 5) and 12.927 (95% CI = 2.197 to 76.069, $P = 0.005$; Figure 6) for low birth weight.

TSH was found to be risk factor for low birth weight (OR = 3.180, 95% CI = 1.380 to 7.327, $P = 0.007$; Figure 6) but showed no significant difference in overall adverse neonatal outcome and preterm birth.

Platelet counts higher than 207 × 10^9/L were also a risk factor for overall adverse neonatal outcome in our study with an OR value of 5.147 (95% CI = 1.582 to 16.750, $P = 0.007$; Figure 4). However, multivariate logistic analysis did not show significant difference in group of preterm birth and low birth weight (Figures 5, 6).

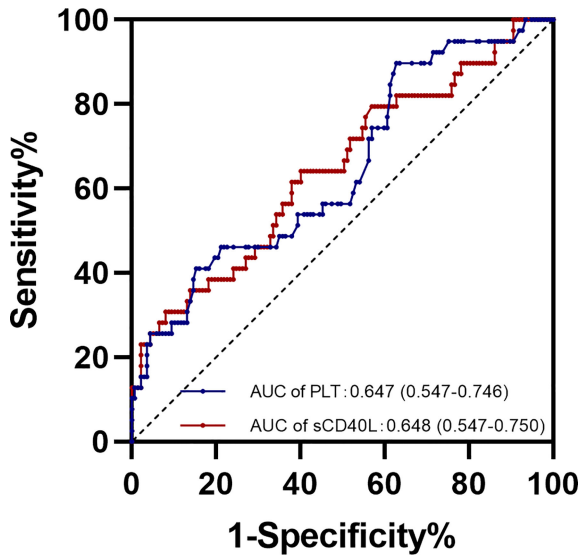

FIGURE 3
ROC curve for sCD40L and platelet levels in early pregnancy in predicting adverse neonatal outcomes.

Discussion

In the present study, a total of 39 (22.16%) euthyroid pregnant women with positive TPOAb had adverse neonatal outcomes during pregnancy. Preterm birth, low birth weight, and fetal macrosomia are the most common comorbidities. We found that serum sCD40L was significantly higher in the group with adverse neonatal outcome. Logistic regression analysis showed that sCD40L > 5.625 ng/ml in early pregnancy was independent risk factor for overall adverse neonatal outcome group and preterm birth. We proposed that the measurement of sCD40L in early pregnancy might have predictive value for adverse neonatal outcomes in euthyroid pregnant women with positive TPOAb.

Maternal thyroid hormones are dispensable for the development of the fetus, especially before the 20th gestational week, because the thyroid gland in the fetus is not well developed (1, 2). It is well documented that subclinical hypothyroidism is associated with adverse fetal outcomes (3, 4, 16–18). It remains unclear whether isolated TPOAb positivity is associated with adverse neonatal outcomes. The literature has yielded different

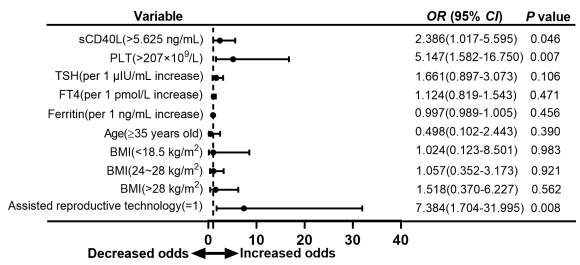

FIGURE 4
Forest plot of multivariate logistic regression analysis of risk factors for adverse neonatal outcomes in euthyroid pregnant women with positive TPOAb.

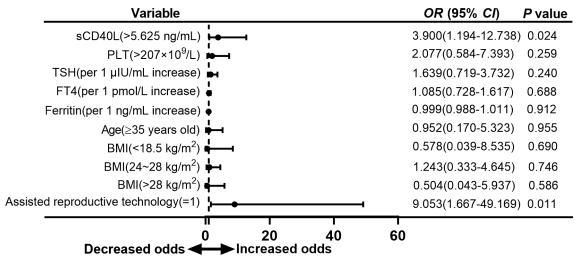

FIGURE 5
Forest plot of multivariate logistic regression analysis of risk factors for preterm birth in euthyroid pregnant women with positive TPOAb.

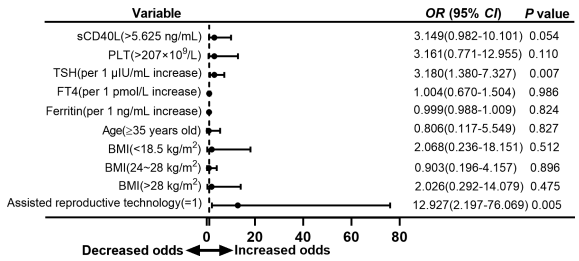

FIGURE 6
Forest plot of multivariate logistic regression analysis of risk factors for low birth weight in euthyroid pregnant women with positive TPOAb.

results regarding the association of isolated TPOAb positivity and adverse neonatal outcomes. In a longitudinal study of 7,641 euthyroid pregnant women, TPOAb positivity was associated with a low birth weight (6). Yuan et al. reported that TPOAb positivity was not associated with poor pregnancy or fetal outcome in euthyroid women but with a higher risk of low birth weight in female fetuses (18). Meta-analysis showed that TPOAb positivity was associated with a higher risk of preterm birth (5, 7, 19). In our study, we found that about one of the four euthyroid pregnant women with positive TPOAb might have adverse outcome. Preterm birth, low birth weight, and fetal macrosomia were the most common comorbidities.

There are several possible explanations for the association of isolated TPOAb positivity with adverse pregnancy outcomes. Relative insufficiency of thyroid hormones due to positive TPOAb and the presence of thyroid antibodies and potential autoimmune disorders were possible underlying mechanisms of TAI-related adverse pregnancy outcomes (7). Women with TPOAb positivity had higher TSH and lower FT4 levels than women with TPOAb negativity (20, 21). Because of the high demand in early pregnancy, subtle thyroid dysfunction might exist because of the defect of capacity to compensate for high demand in early pregnancy. We observed a similar incidence of adverse neonatal outcomes in euthyroid women with TAI. There was no significant difference in TSH and FT4 between groups with adverse neonatal outcomes and without adverse neonatal outcomes. Thus, uncompensated maternal thyroid function during pregnancy is not the only explanation for the association between TAI and adverse neonatal outcomes. Then, it was reported that TPOAb could be transported through the placenta to cause abnormal fetal thyroid function and even abnormal growth (22). We did not find any differences in the titers of TPOAb. Therefore, our data did not support the mechanism of TPOAb itself and adverse neonatal outcomes. Maternal autoimmunity disorder was the other explanation (23). The activation of T cells and its related cytokines was reported to be participated in the immune disorder in patients with TAI (9, 10). Increased sCD40L can be detected and positively correlated with antibodies in many autoimmune diseases (24–26). sCD40 was reported to be positively correlated with TPOAb in patients with autoimmune thyroid disease (12, 13). Increased sCD40L was found to be higher in positive TPOAb women with adverse neonatal outcome in our study.

CD40-CD40L is a pair of membrane molecules. Both belong to the tumor necrosis factor receptor family and play an important role in the immune inflammatory response (12, 13). sCD40L is shed from activated T cells, platelets, and tissue cells and enters body fluids. Similar to its membrane molecules, it can combine with CD40 on immune cells to transmit information and is closely related to the pathogenesis of immune-related diseases. Activated platelets can release sCD40L and promote inflammation, activate the vascular endothelium, induce the expression of cyclooxygenase 2, and synthesize prostaglandins (11). Platelet counts were found to be higher in autoimmune thyroid diseases (27). It has been reported that the platelet activation of pregnant women with preeclampsia, fetal growth restriction, and preterm birth is higher than that of normal pregnant women and nonpregnant women (28–30). Increased platelet counts in the first trimester were suggested to be useful in predicting of early fetal demise (31). Our study also found that platelets were higher in pregnant women in the adverse neonatal outcome group. In addition, we found that there was a positive correlation between sCD40L and platelet count in pregnant women with TAI. CD40-CD40L might be a bridge mediating the immune response between platelets and lymphocytes in TAI (32).

A total of 13 (7.3%) participants received ART in our study. Receiving ART was closely linked to the adverse neonatal outcome such as preterm birth and low birth weight in our study, which was consistent with previous report (8, 19, 33). Previous cohort or clinical trials showed that levothyroxine (LT4) intervention for euthyroid women with positive TPOAb did not improve the pregnancy outcome (34, 35). The specific mechanism and causal relationship need to be further investigated.

Another interesting finding was that TSH was a risk factor for low birth weight in isolated positive TPOAb women in our study (as shown in Figure 6). As previously discussed, hypothyroidism and subclinical hypothyroidism were associated with low birth weight. It was also reported LT4 therapy for SCH was beneficial to decrease the risk of low birth weight in subclinical hypothyroidism (36). However, LT4 therapy may not mandatory for positive TPOAb women with TSH > 2.5 mIU/L and TSH < 4.0 mIU/L according to the latest 2017 ATA guideline (14). In addition, there were a few studies concentrated on low birth weight and isolated positive TPOAb in euthyroid women. Thus, it remains unclear what the best TSH range for positive TPOAb women is and whether LT4 therapy was beneficial for these women in early pregnancy.

The strengths of our study were as follows. First, all participants enrolled and analyzed in our study were euthyroid according to 2017 ATA guidelines. Thus, the possible effect of thyroid dysfunction was eliminated. Second, we concentrated on the adverse outcomes of euthyroid pregnant women positive for TPOAb and found, for the first time, that costimulatory molecules had a predictive role for adverse outcomes in early pregnancy.

This study also has some limitations. First, the sample size for this longitudinal observational study was small. Larger cohort studies are needed for further investigation, and sCD40L throughout pregnancy needs to be investigated. Second, we did not have longitudinal data on thyroid hormones before and after pregnancy. Furthermore, the predictive value of the marker should be further validated in clinic.

Conclusions

In our study, we found that one of the four euthyroid women with TPOAb positivity might have adverse neonatal outcomes during pregnancy. These patients need to be carefully monitored during pregnancy. Increased sCD40L levels in the first trimester might be potential biomarker for adverse neonatal outcomes in pregnant women positive for TPOAb.

Data availability statement

The clinical data of the population used to support the findings of this study are available from the corresponding author upon request.

Ethics statement

This study was approved by the Institutional Review Board of Suzhou Municipal Hospital affiliated to Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

XXC and QW proposed and designed the study and performed the statistical analysis work. HZ, QS and XGC collected data and followed up the participants. XXC drafted the manuscript. LC reviewed and edited the manuscript and funding acquisition. 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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Normal glucose tolerant women with low glycemia during the oral glucose tolerance test have a higher risk to deliver a low birth weight infant

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Background: Data are limited on pregnancy outcomes of normal glucose tolerant (NGT) women with a low glycemic value measured during the 75g oral glucose tolerance test (OGTT). Our aim was to evaluate maternal characteristics and pregnancy outcomes of NGT women with low glycemia measured at fasting, 1-hour or 2-hour OGTT.

Methods: The Belgian Diabetes in Pregnancy-N study was a multicentric prospective cohort study with 1841 pregnant women receiving an OGTT to screen for gestational diabetes (GDM). We compared the characteristics and pregnancy outcomes in NGT women according to different groups [(<3.9mmol/L), (3.9-4.2mmol/L), (4.25-4.4mmol/L) and (>4.4mmol/L)] of lowest glycemia measured during the OGTT. Pregnancy outcomes were adjusted for confounding factors such as body mass index (BMI) and gestational weight gain.

Results: Of all NGT women, 10.7% (172) had low glycemia (<3.9 mmol/L) during the OGTT. Women in the lowest glycemic group (<3.9mmol/L) during the OGTT

had compared to women in highest glycemic group (>4.4mmol/L, 29.9%, n=482), a better metabolic profile with a lower BMI, less insulin resistance and better beta-cell function. However, women in the lowest glycemic group had more often inadequate gestational weight gain [51.1% (67) vs. 29.5% (123); p<0.001]. Compared to the highest glycemia group, women in the lowest group had more often a birth weight <2.5Kg [adjusted OR 3.41, 95% CI (1.17-9.92); p=0.025].

Conclusion: Women with a glycemic value <3.9 mmol/L during the OGTT have a higher risk for a neonate with birth weight < 2.5Kg, which remained significant after adjustment for BMI and gestational weight gain.

KEYWORDS

low glycemia, normal glucose tolerant, pregnancy outcomes, oral glucose tolerance test, low birth weight

1 Introduction

In contrast to women with gestational diabetes (GDM) who have higher glucose levels and an increased risk for large-for-gestational age (LGA) neonates (1, 2), women with low glycemia may be at increased risk to deliver neonates with a low birth weight. It has been demonstrated that infants born with a birth weight <2.5Kg are at increased risk to develop an adverse metabolic profile later in life with increased risk to develop type 2 diabetes (T2DM) and cardiovascular disease (3–6). Low birth weight can be caused by maternal conditions such as placental dysfunction, malnutrition or impaired maternal metabolism (7, 8). Glucose diffuses from the mother to the fetus by placental transport mediated by glucose transporters (GLUT)-1, GLUT-4 and GLUT-9 {Stanirowski, (9) #14}. Since the fetus' blood glucose level is proportional to the blood glucose level of the mother, hypoglycemia might increase the risk for various adverse pregnancy outcomes such as low-birth-weight or small-for-gestational age (SGA) neonates (7, 8, 10, 11).

However, only few studies have focused on the potential relationship between low glycemia during the oral glucose tolerance test (OGTT) and the impact on maternal and neonatal outcomes in normal glucose tolerant (NGT) women. Studies focused mostly on the effects of reactive hypoglycemia during an OGTT (7, 8, 12–16) and data are limited on the potential effects of lower glycemic values in general. In addition, these studies reported conflicting results concerning the impact on maternal and neonatal outcomes, especially on neonatal birth weight (7, 12–14). Moreover, most studies investigated the effects of hypoglycemia in women with GDM or obesity (12, 14, 16, 17). Data are sparse on pregnancy outcomes of NGT women with a lower glycemic value measured during the OGTT between 24-28 weeks of pregnancy.

We aimed therefore to evaluate maternal characteristics and pregnancy outcomes in a large cohort of NGT women with a low glycemic value, being less than the American Diabetes Association (ADA) cut-off for hypoglycemia outside pregnancy (<3.9mmol/L), measured at any stage (fasting, 1-hour or 2-hour) during the 75g OGTT used for screening for GDM during pregnancy (18). In addition, we also aimed to evaluate maternal characteristics and pregnancy outcomes across different groups of low glycemia [stratified according to quartiles of glycemic value (<3.9mmol/L), (3.9-4.2mmol/L), (4.3-4.4mmol/L) and (>4.4mmol/L)] during the OGTT.

2 Subjects and methods

2.1 Study design and setting

This is a sub-analysis of the Belgian Diabetes in Pregnancy-North (BEDIP-N) study. The BEDIP-N study was a multicentric prospective cohort study to evaluate different screening strategies for GDM that has previously been described in detail (19–25). The BEDIP-N study was approved by the Institutional Review Boards of all participating centers and all investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008. Before inclusion to the study, informed consent was obtained. Participants were enrolled between 6 and 14 weeks of pregnancy, when fasting plasma glucose (FPG) was measured. Women with impaired fasting glycemia or diabetes in early pregnancy according to the ADA criteria were excluded (26). Women without (pre)diabetes received universal screening for GDM between 24-28 weeks of pregnancy with a non-fasting 50g glucose challenge test (GCT) followed by a 75g 2-hour OGTT. Results of the GCT were blinded for participants and health care providers. All participants received an OGTT irrespective of the GCT result. Glucose was measured in fluoride-oxalate tubes, limiting the risk for false low glucose values as fluoride inhibits glycolysis. The OGTT was performed according to standard operating procedures provided to each participating center and blood samples were immediately sent to the laboratory for analyzes.

The 2013 World Health Organization (WHO) criteria were used for the diagnosis of GDM (19, 20, 27). The ADA-recommended glycemic targets were used for the treatment of GDM (27).

According to the ADA, hypoglycemia in pregnancy is defined as a value <3.5 mmol/L (63 mg/dl), whereas a value <3.9 mmol/L (70 mg/dl) is considered as a low glycemic value or level one hypoglycemia in pregnancy (18, 28, 29), while a value < 3.0 mmol/L (54 mg/dl) is classified as a level two hypoglycemia (18, 28, 29). In addition, we also divided the cohort into groups of low glycemia [stratified according to quartiles of the glycemic value (<3.9mmol/L), (3.9-4.2mmol/L), (4.3-4.4mmol/L) and (>4.4mmol/L)] during the OGTT to evaluate maternal characteristics and pregnancy outcomes across these different groups.

In total, 1841 women received an OGTT, of which 12.4% (n=229) were diagnosed with GDM and 1612 women were NGT. Because only four women in the GDM-group had low glycemia (<3.9 mmol/L) at fasting, 1-hour or 2-hour OGTT, we excluded the GDM-group for further analysis.

2.2 Study visits and measurements

Baseline characteristics and obstetrical history were collected at first visit (19). Anthropometric measurements were obtained, and several self-administered questionnaires were completed at first visit (6-14 weeks of pregnancy) and at the time of the OGTT (26-28 weeks of pregnancy) (19).

Blood pressure (BP) was measured using an automatic BP monitor. A BMI ≥ 25 kg/m² was defined as overweight and a BMI ≥ 30 kg/m² was defined as obesity based on the measured BMI at first prenatal visit. During this visit, a fasting blood test was taken to measure FPG, insulin, lipid profile [total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL) cholesterol and triglycerides], and hemoglobin A1c (HbA1c). The homeostasis model assessment of insulin resistance (HOMA-IR) and beta-cell function (HOMA-B) were measured in early pregnancy (30). At the visit with OGTT in pregnancy, a fasting lipid profile, HbA1c and different indices of beta-cell function [HOMA-B, the insulinogenic index divided by HOMA-IR and the insulin secretion-sensitivity index-2 (ISSI-2)] were measured (30, 31). In addition, indices of insulin sensitivity were measured, such as the Matsuda index, a measure of whole body insulin sensitivity, and the reciprocal of the HOMA-IR index (19, 30–34).

At first visit and at the time of the OGTT, a food questionnaire was used to question servings per week of different important food categories and beverages (35). Less healthy consumption was assigned 0 or -1 points. By summing up the points for all 14 food groups, the diet score could range from -12 to 15. At the time of the OGTT, the International Physical Activity Questionnaire (IPAQ) questionnaire (validated for the Belgian population) assessed physical activity (19, 36). Results of the IPAQ were reported in categories (low, moderate or high activity levels) as previously reported (37).

2.3 Pregnancy and delivery outcome data

Following pregnancy outcomes were collected: gestational age, preeclampsia (*de novo* BP ≥140/90mmHg > 20 weeks with proteinuria or signs of end-organ dysfunction), gestational hypertension (*de novo* BP ≥140/90mmHg > 20 weeks), type of labor and type of delivery, macrosomia (>4 kg), LGA defined as birth weight >90 percentile according to standardized Flemish birth charts adjusted for sex of the baby and parity (38), SGA defined as birth weight <10 percentile according to standardized Flemish birth charts adjusted for sex of the baby and parity (38), low birth weight defined as a birth weight <2.5kg, preterm delivery (<37 completed weeks), shoulder dystocia and admission on the neonatal intensive care unit (NICU) (19). A glycemic value <2.2 mmol/L was considered as a neonatal hypoglycemia across all centers, irrespective of the need for intravenous administration of glucose and admission on the NICU. The difference in weight between first prenatal visit and the time of the OGTT was calculated as early weight gain. Total gestational weight gain was calculated as the difference in weight between first prenatal visit and delivery. Excessive total gestational weight gain (EGWG) was defined according to the 2009 National Academy of Medicine [NAM, former Institute of Medicine (IOM)] guidelines (39).

2.4 Statistical analysis

Descriptive statistics were presented as frequencies and percentages for categorical variables and means with standard deviations or medians with interquartile range for continuous variables. Categorical variables were analyzed using the Chi-square test or the Fisher exact test in case of low (<5) cell frequencies, whereas continuous variables were analyzed using the Kruskal-Wallis test for data with a non-normal distribution or One-way ANOVA test for data with a normal distribution.

Women were divided into groups according to the lowest glucose value measured during the 75g OGTT. To estimate crude and adjusted odds ratios (aORs) of the effects of lowest group of glycemia (<3.9mmol/L) versus the highest group of glycemia (>4.4mmol/L) during the 75g OGTT on delivery outcomes, a conditional logistic regression was used for binary outcomes. Excessive weight gain, inadequate weight gain (less than recommended weight gain according to NAM guidelines), induction of labor, caesarean sections (CS) and LGA were corrected for the following confounding factors: maternal age, ethnic background, smoking during pregnancy, history of macrosomia, multiparity, total gestational weight gain and for early pregnancy BMI, fasting glycemia, fasting insulin, fasting HDL-cholesterol and fasting LDL-cholesterol. Macrosomia and emergency CS were corrected for age, ethnic background, total gestational weight gain, and for early pregnancy BMI, fasting glycemia, and fasting LDL-cholesterol. Gestational hypertension, preterm delivery and low weight infants <2.5kg were corrected for

BMI in early pregnancy and total gestational weight gain, while birth weight baby ≥4.5Kg was corrected for BMI in early pregnancy. A Spearman's correlation test was used to determine the relationship between birth weight and the lowest glycemia at fasting, 1-hour or 2-hour during the 75g OGTT. Logistic regression analysis was performed for the binary outcomes (birth weight <2.5kg and preterm delivery) and fasting glucose or 2-hour post load glucose as continuous predictors. Results are presented as odds ratios with 95% confidence intervals. A p-value <0.05 was considered significant. In addition, a receiver operating characteristic (ROC)-analysis was performed with an estimated area under the curve (AUC) with 95% CI for the binary outcome as response and the continuous predictor as explanatory variable. This analysis results in a sensitivity and specificity level associated with each outcome. The AUC ranges between 0.5 (discrimination no better than chance) and 1 (perfect discrimination). The optimal cut-off value can be selected as the best combination of sensitivity and specificity. If equal importance is given to sensitivity and specificity, the maximum Youden index indicates the best cut-off value. The Youden index was calculated as the sum of sensitivity and specificity minus 1 and ranges from -1 through 1. Analyzes were performed by statistician A. Laenen using SAS software.

3 Results

Of the total cohort, 1841 women received a 2-hour 75g OGTT at 26-28 weeks of pregnancy. In the total cohort (NGT and GDM women combined), 9.6% (176) women had a low glycemic value (<3.9 mmol/L) at fasting, 1-hour or 2-hour measurement during the OGTT. Because only four women with GDM had low glycemia during the OGTT, women with GDM were excluded for further analysis (Figure 1). Within the NGT-cohort, 10.7% (172) had low glycemia (<3.9 mmol/L), 2.3% (35) had glycemia <3.5 mmol/L and 0.7% (11) had glycemia <3.0 mmol/L at fasting, 1-hour or 2-hour OGTT. Most women (71.5%, n=123) had a low glycemia (<3.9 mmol/L) fasting, 9.3% (16) had a low glycemia at the 1 hour and 27.3% (47) at the 2-hour measurement. Of all NGT women, only 0.6% (10) had a low glycemia at several time points during the OGTT.

3.1 Characteristics and pregnancy outcomes of women with low glycemia (<3.9 mmol/L) during the OGTT

Compared to women with glycemia ≥3.9 mmol/L (n=1440, 89.3%), women with low glycemia [<3.9 mmol/L, n=172 (10.7%)] during the OGTT were younger, had a better metabolic profile with a lower BMI, a lower HOMA-IR in early pregnancy and at the time of the OGTT, and less impaired beta-cell function [ISSI-2: 3.3 (2.5-4.0) vs. 2.3 (1.9-2.8); p<0.0001] at the time of the OGTT (Table 1). Women with low glycemia had more often inadequate gestational weight gain (less than recommended by NAM) and less labor inductions compared to women with higher glucose values during the OGTT (Table 1). There were no differences in diet score or physical activity between both groups (Table 1). Women with a low glycemia during the OGTT had also more often low glycemia (<3.9mmol/L) and hypoglycemia (<3.5 mmol/L) at the non-fasting glycemia measurement before the GCT at 24-26 weeks of pregnancy [respectively 19.6% (33) vs. 6.6% (92); p<0.001 and 7.7% (13) vs. 1.7% (24); p<0.001] (Table 1).

3.2 Characteristics of women with hypoglycemia (<3.5 mmol/L) during the OGTT

Women with a value <3.5 mmol/L (2.3%, n=35) during the OGTT were younger, were more often single and smoked less often before pregnancy compared to women with a glycemia ≥3.5 mmol/L (Appendix I). Women with value <3.5 mmol/L during the OGTT had also more often low glycemia (<3.9mmol/L) and glycemia <3.5mmol/L at the non-fasting glycemia measurement before the

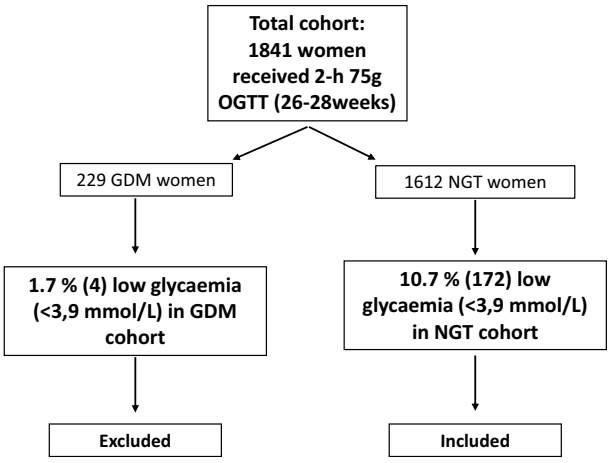

FIGURE 1
Flowchart in- and exclusion criteria. OGTT, oral glucose tolerance test; GDM, gestational diabetes mellitus; NGT, normal glucose tolerant.

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TABLE 1 Comparison of characteristics and pregnancy outcomes between women with glycemia <3.9 mmol/L and women with glycemia ≥3.9 mmol/L at fasting, 1h or 2h 75g OGTT in the normal glucose tolerance group.

NGT-group			
	Glycemia <3.9mmol/L N=172 (10.7%)	Glycemia ≥3.9 mmol/L N=1440 (89.3%)	P-value
General			
Age (years)	29.9 ± 3.9	30.7 ± 3.9	**0.007**
% Ethnic minorities	6.4 (11)	8.5 (121)	0.363
% Multiparity	45.3 (78)	46.5 (670)	0.769
6-14 weeks visit			
Week first visit with FPG	12.2 ± 1.5	11.9 ± 1.8	**0.024**
BMI (Kg/m²) % BMI <18.5 % BMI 18.5-24.9	22.7 ± 3.9 4.1 (7) 76.6 (131)	24.6 ± 4.5 2.6 (37) 26.2 (375)	**<0.001**
% Overweight % Obesity	19.3 (33) 5.8 (10)	37.9 (542) 11.7 (167)	**<0.001** **0.022**
% Waist ≥80cm	62.1 (100)	75.5 (1044)	**<0.001**
Weight gain (first visit till OGTT) (Kg)	6.8 ± 2.7	7.2 ± 3.4	0.071
Systolic blood pressure (mmHg)	113.6 ± 9.8	115.0 ± 10.5	0.098
Diastolic blood pressure (mmHg)	68.2 ± 7.1	70.6 ± 8.2	**<0.001**
Fasting glycemia (mmol/L)	4.3 (4.1-4.4)	4.6 (4.3-4.7)	**<0.001**
HOMA-IR	1.0 (0.7-1.4)	1.3 (1.0-1.9	**<0.001**
HOMA-B	133.7 (92.6-204.3)	131.8 (96.3-183.3)	0.482
HbA1c (mmol/mol and %)	30 (28-32) 4.9 (4.7-5.1)	31 (29-32) 5.0 (4.8-5.1)	**<0.001**
Fasting Total cholesterol (mmol/L)	4.6 (4.1-5.2)	4.7 (4.2-5.2)	0.171
Fasting HDL (mmol/L)	1.8 (1.6-2.1)	1.7 (1.5-2.0)	**0.011**
Fasting LDL (mmol/L)	2.3 (1.9-2.7)	2.4 (2.0-2.9)	**0.006**
Fasting TG (mmol/L)	1.0 (0.8-1.1)	1.0 (0.8-1.3)	0.518
Total Score lifestyle Physical activity Diet	1.0 (0.0-2.0) 2.0 (0.0-5.0)	1.0 (0.0-2.0) 2.0 (0.0-4.0)	0.446 0.215
24-28 weeks visit			
BMI (Kg/m²) % BMI <18.5 % BMI 18.5-25	25.2 ± 3.8 1.2 (2) 60.6 (100)	27.2 ± 4.4 0.1 (1) 36.0 (506)	**<0.001**
Systolic blood pressure (mmHg)	111.9 ± 10.5	113.3 ± 10.0	0.092
Diastolic blood pressure (mmHg)	65.9 ± 7.9	67.1 ± 7.9	**0.026**
Glucose non-fasting 0 min on GCT (mmol/L)	4.5 ± 0.7	4.9 ± 0.9	**<0.001**
% Glucose <3.9 mmol/L non-fasting 0min on GCT	19.6 (33)	6.6 (92)	**<0.001**
% Glucose <3.5 mmol/L non-fasting 0min on GCT	7.7 (13)	1.7 (24)	**<0.001**
Glucose 60 min on GCT (mmol/L)	6.2 ± 1.4	6.5 ± 1.4	**0.002**
% Glucose <3.9 mmol/L 60min on GCT	2.3 (4)	1.7 (24)	0.533
% Glucose <3.5 mmol/L 60min on GCT	0.6 (1)	0.7 (10)	1.000
Fasting glycemia (mmol/L)	3.8 (3.7-3.9)	4.3 (4.2-4.6)	**<0.001**

(Continued)

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TABLE 1 Continued

NGT-group			
	Glycemia <3.9mmol/L N=172 (10.7%)	Glycemia ≥3.9 mmol/L N=1440 (89.3%)	P-value
30 min glucose OGTT (mmol/L)	6.3 (5.6-7.1)	6.9 (6.3-7.7)	**<0.001**
1-hour glucose OGTT (mmol/L)	6.0 (4.8-7.0)	6.9 (6.0-7.9)	**<0.001**
2-hour glucose OGTT (mmol/L)	4.9 (3.8-6.0)	6.0 (5.3-6.9)	**<0.001**
HbA1c (mmol/mol and %)	29 (27-30) 4.8 (4.6-4.9)	30 (29-32) 4.9 (4.8-5.1)	**<0.001**
Matsuda insulin sensitivity	0.8 (0.6-1.1)	0.6 (0.4-0.8)	**<0.001**
HOMA-IR	1.2 (0.8-1.7)	1.8 (1.3-2.5)	**<0.001**
HOMA-B	409.5 (237.2-619.5)	220.8 (160.7-309.9)	**<0.001**
ISSI-2	3.3 (2.5-4.0)	2.3 (1.9-2.8)	**<0.001**
Insulinogenic index/HOMA-IR	0.5 (0.3-0.7)	0.3 (0.2-0.4)	**<0.001**
Fasting Total cholesterol (mmol/L)	6.2 (5.5-7.2)	6.3 (5.7-7.0)	0.795
Fasting HDL (mmol/L)	2.0 (1.6-2.4)	1.9 (1.6-2.2)	0.057
Fasting LDL (mmol/L)	3.4 (2.8-4.2)	3.4 (2.9-4.2)	0.853
Fasting TG (mmol/L)	1.7 (1.4-2.2)	1.8 (1.4-2.3)	0.147
Total score lifestyle Physical activity Diet	1.5 (0.0-2.0) 2.0 (0.0-5.0)	1.0 (0.0-2.0) 2.0 (0.0-4.0)	0.506 0.172
IPAQ low	13.9 (23)	16.6 (229)	0.373
Delivery			
Total Weight gain (first visit till delivery) (Kg)	11.3 ± 4.1	12.3 ± 5.1	**0.005**
% Excessive weight gain	15.3 (20)	29.7 (363)	**<0.001**
% Inadequate weight gain	51.1 (67)	29.7 (363)	**<0.001**
Gestational age (weeks)	39.0 ± 1.7	39.3 ± 1.6	**0.011**
% Preeclampsia	1.7 (3)	1.8 (26)	1.000
% Gestational hypertension	2.3 (4)	4.4 (64)	0.232
% Preterm delivery	7.0 (12)	5.2 (74)	0.323
% Induction labor	16.3 (28)	27.1 (388)	**0.002**
% Caesarean sections (total)	19.2 (33)	20.3 (291)	0.723
Weight baby (g)	3286.3 ± 534.4	3411.3 ± 505.4	**0.004**
% Weight baby <2.5 kg	5.8 (10)	3.9 (56)	0.225
% Macrosomia (>4Kg)	6.4 (11)	9.8 (140)	0.149
% LGA	9.9 (17)	13.2 (189)	0.227
% SGA	7.6 (13)	4.8 (68)	0.110
% Shoulder dystocia	0.6 (1)	1.2 (17)	0.712
% Neonatal hypoglycemia <2.2 mmol/L	2.8 (3)	4.1 (38)	0.514
% NICU admission	8.2 (14)	9.7 (139)	0.528

OGTT, oral glucose tolerance test; GCT, glucose challenge test; GDM, gestational diabetes mellitus; BMI, Body Mass Index; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; HOMA-B,, Homeostatic Model Assessment for B-cell secretion; ISSI-2, Insulin Secretion-Sensitivity Index-2 HDL, high-density lipoprotein; LDL, low-density-lipoprotein; TG, triglycerides; IPAQ, International Physical Activity Questionnaire; LGA, large-for-gestational age infant; SGA, small-for-gestational age infant; NICU, neonatal intensive care unit; IFG, impaired fasting glycemia; IGT, impaired glucose tolerance; Overweight, BMI ≥25-29.9 Kg/m^2; Obesity, BMI ≥30 Kg/m. Categorical variables are presented as frequencies %(n); continuous variables are presented as mean ± SD if normally distributed and as median ± IQR if not normally distributed; Differences are considered significant at p-value<0.05. Bold means a statistical significant value of p<0.05.

GCT [respectively 22.9% (8) vs. 7.6% (117); p=0.005 and 11.4% (4) vs. 2.1% (33); p=0.008]. At the time of the OGTT, these women were less insulin resistant (lower HOMA-IR), had a less impaired beta-cell function (higher ISSI-2 index), and had also more often inadequate gestational weight gain compared to women with glycemia ≥3.5 mmol/L. There were no differences in diet score or physical activity, nor differences in pregnancy outcomes between both groups (Appendix I).

3.3 Characteristics and pregnancy outcomes of women in the lowest quartile group (<3.9 mmol/L) compared to the highest quartile group (>4.4 mmol/L) of low glucose levels during the OGTT

Women in the lowest quartile group (10.7%, n=172) of glycemia (<3.9 mmol/L) measured during the OGTT were younger and had more often a paid job compared to women (29.9%, n=482) in the highest quartile group (glycemia >4.4 mmol/L) (Table 2). Overview of the four quartile groups is available in Appendix II. There were no differences in alcohol consumption or smoking before and during pregnancy between the lowest and highest quartile groups. In early pregnancy and at the time of the OGTT, women in the lowest quartile group had a lower BMI, were less insulin resistant (lower HOMA-IR) and had a less impaired beta-cell function (higher ISSI-2 index) compared to the highest quartile group (Table 2). Of all women in the lowest quartile group, 10.5% (18) had also a glycemia <3.9 mmol/L in early pregnancy compared to none of the women in the highest quartile group (p<0.001). Women in the lowest quartile group had also more often low glycemia (<3.9 mmol/L) and glycemia <3.5 mmol/L at the non-fasting glycemia measurement before the GCT [respectively 19.6% (33) vs. 2.7% (13); p<0.001 and 7.7% (13) vs. 0.6% (3); p<0.001] compared to women in the highest quartile group of glycemia during the OGTT. There was no difference in gestational weight gain between the first perinatal visit and the time of the OGTT. However, at the time of delivery, women in the lowest quartile group had more often less gestational weight gain than recommended [51.1% (67) vs. 29.5% (123); p<0.001] compared to women in the highest quartile group. There were no differences in diet score or physical activity between both groups (Table 2). Women in the lowest quartile group, had less often gestational hypertension and less need for labor inductions or emergency CS, but had more often infants with a birth weight <2.5Kg [5.8% (10) vs. 1.9% (9); p=0.009] compared to women with glycaemia >4.4 mmol/L (Table 2). However, within the group with neonates with low birth weight, there was no difference in rates of SGA, preterm delivery or intra-uterine growth restriction. The lower rate of labor inductions [aOR 0.54, 95% CI (0.30-0.96); p=0.036] and the increased rate of infants with low birth weight [aOR 3.41, 95% CI (1.17-9.92); p=0.025] remained significant after adjustments for confounders (Table 3). A birth weight <2.5Kg occurred also twice as frequently in the 2nd group (27.4%, n=441, 3.9-4.2 mmol/L) and 3rd group (32.1%, n=517, 4.25-4.4 mmol/L) compared to the highest quartile glycemia group [respectively 5.0% (22) vs. 1.9% (9); p=0.009 and 4.9% (25) vs. 1.9% (9); p=0.010] (Appendix II). This remained significant after adjustment for confounders [respectively, aOR 2.69, 95% CI (1.06-6.80); p=0.037 and aOR 3.25, 95% CI (1.33-7.97); p=0.010] compared to the highest quartile glycemia group.

There was a weak positive correlation between birth weight and glycemia during the OGTT [r(1600) = 0.13; p<0.001] (Figure 2). As fasting glycemia decreased, the risk for a low birth weight increased. An estimation of the cut-off for fasting glycemia during the OGTT with best trade-off between sensitivity and specificity (with maximum Youden index) to predict a low birth weight <2.5Kg, was seen at a FPG of 4.4mmol/L, with a sensitivity of 84.8% and specificity of 32.9% (Appendix III). The AUC on the ROC curve for fasting glycemia as a predictor for low birth weight (<2.5kg) was 0.603 (95% CI 0.534-0.672) (Figure 3).

4 Discussion

We found that 10.7% of NGT women had a low glycemic value (<3.9 mmol/L) during the 75g OGTT, most often at the fasting measurement. This is in line with a Turkish study reporting a prevalence of 11.4%, using the same cut-off of 3.9 mmol/L (ADA level for hypoglycemia outside pregnancy) measured during a 75g OGTT (7). In our study, only 2.3% of NGT women had a glycemic value <3.5 mmol/L during the 75g OGTT. This is less than reported by other studies (12–14), however cut-offs for hypoglycemia differed among the different studies.

To the best of our knowledge, we are the first to report that compared to women with glycemia values >4.4 mmol/L during the OGTT, women with glycemia <3.9 mmol/L, had a better metabolic profile with a lower BMI, less insulin resistance, and less impaired beta-cell function, but higher rates of a birth weight <2.5 Kg with an aOR of 3.41. Importantly, this increased risk remained significant after adjustment for confounders such as BMI and total gestational weight gain since women with low glycemia gained more often less weight than recommended. In addition, we excluded women with GDM, which has the advantage that in our study women did not receive any treatment influencing glycemia. Our results are in line with the Turkish study, in which they also found a higher rate of low-birth-weight neonates in women with low glycemia defined as <3.9 mmol/L (or 70 mg/dL) (7).

An association between a low birth weight and low glycemia or reactive hypoglycemia has been reported by other studies focusing on GDM-women, often using different cut-offs for glycemia (for example <2.8 mmol/L) or a different glucose load for the OGTT (for example 100g OGTT) (10, 13, 14, 17).

In pregnant women with pregestational diabetes and in women with GDM, it has been clearly demonstrated that hyperglycemia increases the risk for macrosomia and LGA infants, since the fetus is dependent on nutrients of the mother and higher glucose levels in the fetus lead to fetal hyperinsulinism (40). However, less data are available on the potential effects of low glycemia on pregnancy outcomes (40). A study with 334 women with GDM, who were matched for obesity, race and parity, showed that the rate of SGA was significantly higher in the low glycemia (<4.8 mmol/L) group compared to the non-diabetic control group (41). Our results also

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TABLE 2 Comparison of characteristics and pregnancy outcomes between women with glycemia <3.9 mmol/L (lowest quartile) and women with glycemia >4.4 mmol/L (highest quartile) at fasting, 1h or 2h 75g OGTT in the normal glucose tolerance group.

NGT-group			
	Glucose <3.9 mmol/L N= 172 (10.7%)	Glucose >4.4 mmol/L N=482 (29.9%)	P-value
General			
Age (years)	29.9 ± 3.9	31.1 ± 4.1	**<0.001**
% Ethnic minorities	6.4 (11)	10.9 (52)	0.091
% multiparity	45.3 (78)	50.8 (245)	0.217
% paid job	94.1 (161)	89.0 (427)	**0.049**
% living without partner	17.1 (29)	20.2 (97)	0.378
% smoking before pregnancy	25.3 (43)	29.2 (140)	0.335
% smoking during pregnancy	2.9 (5)	4.6 (22)	0.350
% Alcohol use before pregnancy	69.5 (119)	66.3 (317)	0.241
% Alcohol use during pregnancy	8.2 (14)	6.3 (30)	0.596
% First degree family history of diabetes	8.8 (14)	10.4 (48)	0.571
% History of GDM*	5.1 (4)	6.6 (16)	0.649
% History of macrosomia >4Kg*	4.1 (7)	7.9 (38)	0.172
6-14 weeks visit			
BMI (Kg/m²)	22.7 ± 3.9	25.9 ± 5.0	**<0.001**
% Underweight % Overweight % Obesity	4.1 (7) 19.3 (33) 5.8 (10)	2.1 (10) 51.7 (246) 17.6 (84)	0.162 **<0.001** **<0.001**
% Waist ≥80cm	62.1 (100)	81.1 (374)	**<0.001**
Systolic blood pressure (mmHg)	113.6 ± 9.9	116.0 ± 10.8	**0.008**
Diastolic blood pressure (mmHg)	68.2 ± 7.1	71.3 ± 8.6	**<0.001**
Fasting glycemia (mmol/L)	4.3 (4.1-4.4)	4.7 (4.6-4.8)	**<0.001**
Fasting glycemia <3.9 mmol/L	10.5 (18)	0 (0)	**<0.001**
HOMA-IR	1.0 (0.7-1.4)	1.6 (1.2-2.2)	**<0.001**
HOMA-B	133.7 (92.6-204.3)	124.8 (93.6-178.4)	0.243
HbA1c (mmol/mol and %)	30 (29-32) 4.9 (4.7-5.1)	31 (30-33) 5.0 (4.9-5.2)	**<0.001**
Fasting Total cholesterol (mmol/L)	4.6 (4.1-5.2)	4.7 (4.2-5.3)	0.042
Fasting HDL (mmol/L)	1.8 (1.5-2.1)	1.7 (1.5-1.9)	**<0.001**
Fasting LDL (mmol/L)	2.3 (1.9-2.7)	2.5 (2.0-2.9)	**<0.001**
Fasting TG (mmol/L)	1.0 (0.8-1.1)	1.0 (0.8-1.3)	**0.040**
Total Score lifestyle Physical activity Diet	1.0 (0.0-2.0) 2.0 (0.0-5.0)	1.0 (0.0-2.0) 2.0 (-1.0-4.0)	0.062 0.176
Weight gain (first visit till OGTT) (Kg)	6.8 ± 2.7	7.1 ± 3.4	0.315
24-28 weeks visit			
BMI (Kg/m²)	25.2 ± 3.8	28.5 ± 4.8	**<0.001**
Systolic blood pressure (mmHg)	111.9 ± 10.5	114.4 ± 10.4	**0.006**
Diastolic blood pressure (mmHg)	65.9 ± 7.9	68.7 ± 7.8	**<0.001**

(Continued)

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TABLE 2 Continued

NGT-group			
	Glucose <3.9 mmol/L N= 172 (10.7%)	Glucose >4.4 mmol/L N=482 (29.9%)	P-value
Glucose non-fasting 0 min on GCT (mmol/L)	4.5 ± 0.8	5.1 ± 0.9	**<0.001**
% Glucose <3.9 mmol/L non-fasting 0min on GCT	19.6 (33)	2.7 (13)	**<0.001**
% Glucose <3.5 mmol/L non-fasting 0min on GCT	7.7 (13)	0.6 (3)	**<0.001**
Glucose 60 min on GCT (mmol/L)	6.2 ± 1.4	6.6 ± 1.4	**<0.001**
% Glucose <3.9 mmol/L 60min on GCT	2.3 (4)	0.8 (4)	0.128
% Glucose <3.5 mmol/L 60min on GCT	0.6 (1)	0.4 (2)	0.785
Fasting glycemia (mmol/L)	3.8 (3.7-3.9)	4.7 (4.6-4.8)	**<0.001**
30 min glucose OGTT (mmol/L)	6.3 (5.6-7.1)	7.2 (6.7-7.9)	**<0.001**
1-hour glucose OGTT (mmol/L)	6.0 (3.8-6.0)	7.3 (6.4-8.3)	**<0.001**
2-hour glucose OGTT (mmol/L)	4.9 (3.8-6.0)	6.3 (5.5-7.1)	**<0.001**
HbA1c (mmol/mol and %)	29 (28-30) 4.8 (4.6-4.9)	31 (29-32) 5.0 (4.8-5.1)	**<0.001**
Matsuda insulin sensitivity	5.9 (4.1-7.6)	3.1 (2.4-4.2)	**<0.001**
HOMA-IR	1.2 (0.8-1.7)	2.4 (1.7-3.1)	**<0.001**
HOMA-B	409.5 (237.2-619.5)	189.9 (141.1-260.4)	**<0.001**
ISSI-2	3.3 (2.5-4.0)	1.9 (1.7-2.3)	**<0.001**
Insulinogenic index/HOMA-IR	0.5 (0.3-0.8)	0.2 (0.2-0.3)	**<0.001**
Fasting Total cholesterol (mmol/L)	6.2 (5.5-7.2)	6.3 (5.7-7.1)	0.852
Fasting HDL (mmol/L)	2.0 (1.6-2.4)	1.9 (1.6-2.1)	**<0.001**
Fasting LDL (mmol/L)	3.4 (2.8-4.2)	3.5 (2.9-4.2)	0.689
Fasting TG (mmol/L)	1.7 (1.4-2.2)	1.9 (1.5-2.4)	**0.002**
Total score lifestyle Physical activity Diet	 1.5 (0.0-2.0) 2.0 (0.0-5.0)	 1.0 (0.0-2.0) 2.0 (-1.0-4.0)	 0.226 0.052
% IPAQ low	13.9 (23)	17.1 (79)	0.336
Delivery			
Total Weight gain (first visit till delivery) (Kg)	11.3 ± 4.1	12.0 ± 5.6	**0.062**
% excessive weight gain	15.3 (20)	33.8 (141)	**<0.001**
% inadequate weight gain	51.1 (67)	29.5 (123)	**<0.001**
Gestational age (weeks)	39.0 ± 1.7	39.4 ± 1.5	**0.026**
% Preeclampsia	1.7 (3)	2.3 (11)	0.682
% Gestational hypertension	2.3 (4)	6.8 (33)	**0.029**
% Preterm delivery	7.0 (12)	4.2 (20)	0.143
% Induction labor	16.3 (28)	32.4 (156)	**<0.001**
% Caesarean sections (total)	19.2 (33)	24.3 (117)	0.127
% Emergency CS (during labor)	7.6 (13)	13.3 (64)	**0.045**
Weight baby (g)	3286.3 ± 534.4	3468.6 ± 515.6	**<0.001**
% Weight baby <2.5 kg Of which:	5.8 (10) 60.0 (6)	1.9 (9) 33.3 (3)	**0.009** 0.484

(Continued)

TABLE 2 Continued

NGT-group			
	Glucose <3.9 mmol/L N= 172 (10.7%)	Glucose >4.4 mmol/L N=482 (29.9%)	P-value
% SGA % preterm delivery % Intrauterine growth restriction	40.0 (4) 0 (0)	55.6 (5) 0 (0)	0.632 -
% Macrosomia (>4Kg)	6.4 (11)	12.7 (61)	**0.023**
% Weight baby ≥4.5Kg	0.6 (1)	2.3 (11)	0.151
% LGA	9.9 (17)	17.1 (82)	**0.026**
% SGA	7.6 (13)	4.2 (20)	0.079
%Shoulder dystocia	0.6 (1)	0.8 (4)	0.744
% Neonatal hypoglycemia <2.2 mmol/L	2.8 (3)	4.7 (15)	0.404
% NICU admission	8.2 (14)	9.8 (47)	0.535

OGTT, oral glucose tolerance test; GCT, glucose challenge test; GDM, gestational diabetes mellitus; BMI, Body Mass Index; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance; HOMA-B,, Homeostatic Model Assessment for B-cell secretion; ISSI-2, Insulin Secretion-Sensitivity Index-2 HDL, high-density lipoprotein; LDL, low-density-lipoprotein; TG, triglycerides; IPAQ, International Physical Activity Questionnaire; LGA, large-for-gestational age infant; SGA, small-for-gestational age infant; NICU, neonatal intensive care unit; IFG, impaired fasting glycemia; IGT, impaired glucose tolerance; Overweight, BMI ≥25-29.9 Kg/m^2; Obesity, BMI ≥30 Kg/m. Categorical variables are presented as frequencies %(n); continuous variables are presented as mean ± SD if normally distributed and as median ± IQR if not normally distributed; Differences are considered significant at p-value<0.05. *A history of GDM and a history of a macrosomic baby were calculated on the number of women with a previous pregnancy. Bold means a statistical significant value of p<0.05.

showed that fasting glycemia during the 75g OGTT can be a predictor for a low birth weight, as the risk for this outcome increases when fasting glycemia decreases. Exploratory analysis on our data showed an AUC of 0.603 (95% CI 0.534-0.672) for a fasting cut-off of 4.4 mmol/L, indicating that this has only a poor predictive value for a low birth weight. In addition, our logistic regression result suggested a correlation between a glycaemic value of 3.9 mmol/L and low birth weight, which is stronger related with the point of 3.5 mmol/L. This is in line with a recent study in the UK, which showed that fasting glycemia or a 2-hour postload glycemia <3.5mmol/L during the 75g OGTT can be a predictor for low birth weight (17). However, this study focused on a high risk population with GDM, whereas our study only included NGT women.

Our results also indicate that women with low glycemia during the OGTT, had significantly more often already low glycemia in early pregnancy and a low non-fasting glycemia in the weeks before the OGTT, suggesting that these women have more often a lower glycemia throughout pregnancy. Previous studies have reported associations between hypoglycemia on the 50g GCT and SGA infants (42, 43). This association was mainly seen on the 1-hour GCT value, which is in contrast to our results since we only found an association with the non-fasting random glucose measured before the GCT. Our results also indicate that the increased risk for a low birth weight is independent of confounders such as BMI and inadequate gestational weight gain. In addition, there were also no differences in diet nor in physical activity between the different

TABLE 3 Adjusted odds ratios for pregnancy outcomes comparing the lowest quartile of low glycemia (<3.9mmol/L) with the highest quartile of low glycemia (>4.4mmol/L) during the 75g OGTT.

Outcome	Crude OR	95%CI	P-value	Adjusted OR	95%CI	P-value
% Excessive weight gain*	0.35	0.21-0.59	<0.001	0.65	0.21-1.99	0.451
% Inadequate weight gain*	2.50	1.67-3.74	<0.001	1.00	0.37-2.71	0.995
% Gestational hypertension***	0.33	0.11-0.9.	0.037	0.41	0.12-1.40	0.155
% Preterm delivery***	1.72	0.82-3.61	0.148	1.734	0.73-4.09	0.208
% Labor induction *	**0.40**	**0.26-0.63**	**<0.001**	**0.64**	**0.30-0.96**	**0.036**
% Emergency CS **	0.53	0.29-0.99	0.048	0.45	0.18-1.16	0.099
% Weight baby <2.5 kg***	**3.22**	**1.29-8.07**	**0.012**	**3.41**	**1.17-9.92**	**0.025**
% Macrosomia (>4Kg)**	0.47	0.24-0.91	0.026	0.68	0.29-1.63	0.392
% LGA*	0.54	0.31-0.93	0.027	0.81	0.38-1.74	0.589

OR: odds ratio; CI: confidence interval; LGA: large-for-gestational age infant. Differences are considered significant at p-value<0.05.
* Adjusted for age, ethnic minority background, smoking during pregnancy, history of macrosomia, multiparity, BMI in early pregnancy, fasting glycemia in early pregnancy, fasting insulin in early pregnancy, fasting HDL)cholesterol in early pregnancy, fasting LDL-cholesterol in early pregnancy and total gestational weight gain.
** Adjusted for age, ethnic minority, BMI in early pregnancy, fasting glycemia in early pregnancy, fasting LDL-cholesterol in early pregnancy and total gestational weight gain.
*** Adjusted for BMI in early pregnancy and total gestational weight gain. Bold means a statistical significant value of p<0.05.

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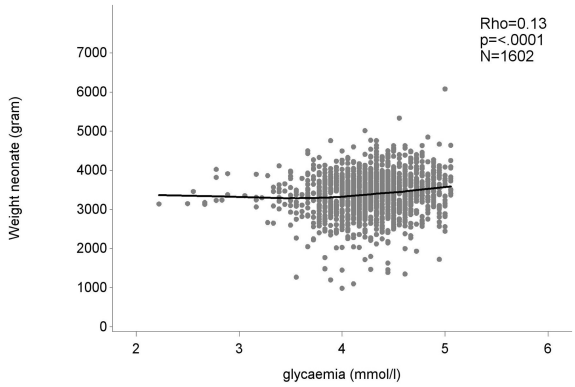

FIGURE 2
The association between the weight of the neonate and the lowest glycemia value measured at fasting, 1- or 2-hour measurement of the 75g OGTT. Weight neonate in gram, glycemia in mmol/l.

groups in our study. This suggests that a low glycemia during pregnancy might be a marker of placental insufficiency (7, 8). It is known that less severe deficiencies in arterial remodeling of the placenta result in SGA infants (44, 45). In addition, if maternal blood glucose decreases, less glucose is transferred to the fetus, leading to lower insulin production by the fetus, which might lead to growth restriction (4).However, there is currently no evidence from intervention studies that a more strict follow-up or different management strategy for women with low glycemia during the OGTT, might reduce the risk to deliver an infant with low birth weight. In addition, not only prevention of SGA infants is important, as infants with a low birth weight (<2.5 Kg) as such are also at increased risk to develop T2DM and cardiovascular disease later in life. This increased risk for an adverse metabolic profile later in life, might be related to adaptations by the fetus induced by the lower glucose levels, leading to abnormal pancreatic beta-cell function and reduced capacity to secrete insulin extending into adult life (4). Additionally, insulin secretion and insulin resistance might also be genetically determined and as such affect intrauterine growth (3, 4).

A major strength of our study is the large multicentric prospective cohort with a large, detailed dataset containing broad demographic, clinical and obstetrical outcomes. We provide the first data on the association between both maternal and neonatal outcomes in NGT women with low glycemia measured fasting or at the 1-hour or 2-hour time point during a 75g OGTT. Data on the risk for adverse pregnancy outcomes were adjusted for important confounders. In addition, women with GDM were excluded, so that we could evaluate pregnancy outcomes in a non-treated population. We used fluoride-oxalate tubes to collect blood samples for the analyses of glucose, limiting the risk for false low glucose values as fluoride inhibits glycolysis. The blood samples were also sent immediately to the

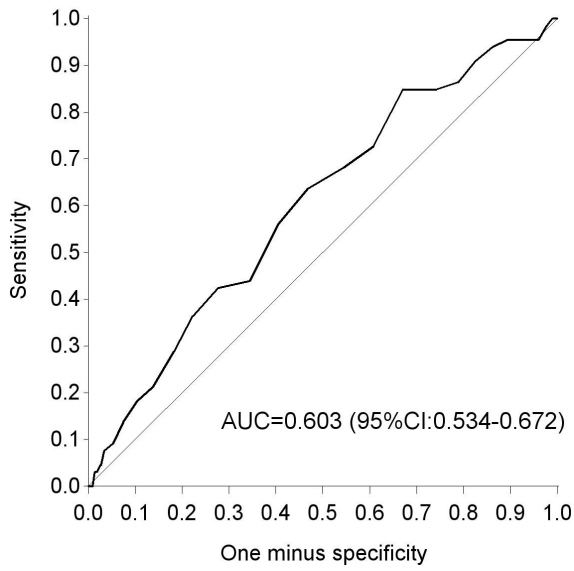

FIGURE 3
ROC curve for fasting glycemia during 75g OGTT as a predictor for a birth weight <2.5 kg. AUC, area under the curve; ROC, receiver-operating curve.

laboratory for analyzes. Furthermore, glycemia was analyzed at different time points during pregnancy (at 11 weeks, 24-26 weeks and 26-28 weeks). A limitation of the study is the mainly Caucasian population in our cohort. In addition, we had no detailed data on nutrition from food diaries and we had no follow-up data on the evolution of glycemia after the OGTT in pregnancy. As the group with low postload glycemia was small, differences in pregnancy outcomes between women with low fasting glycemia and women with low postload glycemia could not be adequately evaluated. We had also no data on placental blood flow to evaluate placental insufficiency.

5 Conclusion

In conclusion, our results suggest that women with a glycemic value (<3.9 mmol/L) during the 75g OGTT are at increased risk to deliver an infant with a low birth weight (<2.5Kg). Importantly, this increased risk remained significant after adjustment for confounders such as BMI and low gestational weight gain.

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

Author contributions

KB conceived the sub-analysis. LR and LD prepared the data and ALa did the statistical analysis. LR did the literature review. LR and KB wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version. The corresponding author LR had full access to all the data in the study and had final responsibility for the contents of the article and the decision to submit for publication.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1186339/full#supplementary-material

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Cortisol dysregulation in anxiety infertile women and the influence on IVF treatment outcome

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Introduction: Dysregulation of the stress-regulatory hormone cortisol is associated with anxiety, but its potential impact on infertile women and *in vitro* fertilization (IVF) treatment remains unclear. This prospective cross-sectional study aimed at evaluating the dysregulation of cortisol and its correlation to anxiety in infertile women. The influence of stress on IVF outcomes was also investigated.

Methods: A point-of-care test was used for the measurement of morning serum cortisol in 110 infertile women and 112 age-matching healthy individuals. A Self-Rating Anxiety Scale (SAS) was used for the anxiety assessment of infertile women, and 109 of them underwent IVF treatment starting with the GnRH-antagonist protocol. If clinical pregnancy was not achieved, more IVF cycles were conducted with adjusted protocols until the patients got pregnant or gave up.

Results: Higher morning serum cortisol level was identified for infertile patients, especially for the elder. Women with no anxiety showed significant differences in cortisol levels, monthly income, and BMI compared with those with severe anxiety. A strong correlation was found between the morning cortisol level and the SAS score. When the cutoff value is 22.25 μg/dL, cortisol concentration could predict the onset of anxiety with high accuracy (95.45%) among infertile women. After IVF treatments, women with high SAS scores (>50) or cortisol levels (>22.25 μg/dL) demonstrated a lower rate of pregnancy (8.0%-10.3%) and more IVF cycles, although the impact of anxiety was not affirmative.

Conclusion: Hypersecretion of cortisol related to anxiety was prevalent among infertile women, but the influence of anxiety on multi-cycle IVF treatment was not affirmative due to the complicated treatment procedures. This study suggested that the assessment of psychological disorders and stress hormone dysregulation should not be overlooked. An anxiety questionnaire and rapid cortisol test might be included in the treatment protocol to provide better medical care.

KEYWORDS

cortisol, anxiety, point-of-care testing, infertility, IVF outcome

Introduction

Infertility is a global burden that affects 10%–15% of couples of reproductive age (1, 2). The impairment of reproductive function and stigmatization have led to a significantly higher level of stress and a prevalence of mood disorders (3–5). The overall prevalence of anxiety among infertile women was 36.17% (4), and a higher rate (37.2-42.2%) has been reported in Chinese patients who visited the reproductive medical center for help (6, 7). Anxiety will not only affect mental health but also impair reproduction function through complicated mechanisms such as dysregulation of hormones and metabolisms (8, 9). Therefore, the diagnosis of anxiety should not be overlooked for infertility treatment.

In addition to the classical scale tests for psychological assessment (10, 11), efforts have been made to identify biomarkers that can facilitate the evaluation of anxiety in infertile women (12, 13). The hypothalamus–pituitary–adrenal (HPA) axis hormones, especially cortisol, are major stress and threat regulators (14, 15). As one of the most abundant hormones in the human serum, cortisol regulates energy metabolism in response to adverse stimulations (16) and shares a common synthetic pathway with sex hormones such as testosterone and progesterone (17, 18). The complicated role of cortisol makes it a good target for neuroendocrine studies among women of reproductive age.

The impact of cortisol dysregulation on reproductive function and *in vitro* fertilization (IVF) has been well established. In spontaneous fertilization, cortisol could act directly on granulosa lutein cells to inhibit the support of steroidogenesis by luteinizing hormone (19). It also plays an important role in follicular development and safeguards oogenesis by promoting follicular cell survival (20). Increased levels of cortisol and prolactin impair the menstrual cycle in females and reduce the chances of conception (21). Peripheral cortisol levels can reflect adrenal function, which indirectly interferes with sex hormone production in infertile patients (17). In studies of IVF treatments, long-term cortisol levels measured from hair negatively predict pregnancy, and the accumulation of salivary cortisol accounts for 26.7% of the variance of clinical pregnancy outcomes (22). Lower serum and follicular cortisol levels on the day of oocyte retrieval were found to be significantly associated with successful IVF treatment (23). Although the mechanism remained unclear, cortisol is likely an important neurohormone in the complex relationship between psychosocial stress and IVF outcome (24).

Studies of physiological biomarkers for anxiety, however, revealed heterogeneous conclusions about cortisol dysregulations (14, 25). Women with anxiety demonstrated a higher level of 24-hour urinary free cortisol (26), but hair cortisol concentration representing the long-term secretion was not related to self-reported anxiety (27). The non-invasive collection of hair or urine samples is convenient for the patients, but the operation protocol involved complicated steps and instruments, which are rarely available in clinical settings (28, 29). In the contrast, blood sample collection is invasive, but already widely used for sex hormone level determination of infertile patients in hospitals (30). Elevation of morning serum cortisol level has been reported in pregnant women with anxiety, as determined by an automated commercialized chemiluminescence analyzer (31). Thus, to better demonstrate the correlation between cortisol dysregulation and anxiety, the variation in sampling preparation and test operation should be minimized.

This study aimed to evaluate the potential of serum morning cortisol levels in facilitating the diagnosis of anxiety in infertile women and investigate the influence of physiological and psychological stress on the IVF treatment outcome. Since most studies focused on the cortisol level at different time points during treatment, we would like to investigate whether psychological and physiological stress occurred before the treatment and affect the outcome of multiple IVF cycles. A Self-Rating Anxiety Scale (SAS) with a well-established Chinese norm and a novel point-of-care test (POCT) for serum cortisol was adopted. The standardized sample collection and convenient operation of the test platform enabled the rapid onsite detection of serum cortisol, and provide insights for future clinical applications. With the retrieval of the pregnancy outcome after IVF treatment, the influence of anxiety and cortisol dysregulation was addressed. We hypothesized that dysregulation of cortisol might be prevalent and linked to anxiety or poor pregnancy outcome in infertile women.

Materials and methods

Study design

This prospective cross-sectional study was conducted in the Second Affiliated Hospital of Chongqing Medical University from December 2018 to March 2020. The study design and protocol were in accordance with the Declaration of Helsinki (1989) and approved by the institutional review board of the hospital. Two cohorts were recruited, including 120 infertile women from the reproductive medicine center (infertility group) and 112 healthy women from the medical center (control group). The sample size of the study was calculated with an online calculator for clinical studies developed by Wang and Ji (32).

After obtaining informed consent, basic information including age, blood glucose, blood pressure, and regular blood test result of patients were checked for inclusion or exclusion. The infertile patients in the reproductive medicine center were already diagnosed before they were asked to join the study, whereas the healthy patients in the medical center were visiting the hospital for regular annual examinations. Since all volunteers were planning to undergo serum sample collection for other assays, they acknowledged that the sample would be shared for the cortisol test to minimize the risk. The blood collection was conducted between 7:00 am and 9:00 am. The infertility group completed an additional background information collection form, a questionnaire

Abbreviations: AUC, Area under the curve; BMI, Body mass index; CI, Confidence interval; GAD-7, 7-item self-administered scale; HPA, Hypothalamus–pituitary–adrenal; IVF, In vitro fertilization; NPV, Negative predictive value; POCT, Point-of-care test; PPV, Positive predictive value; ROC, Receiver operating characteristic; SAS, Self-Rating Anxiety Scale; SPSS, Statistical product and service solutions.

for anxiety, and a blood test for antral follicle count (AFC), anti-Mullerian hormone (AMH), and follicle-stimulating hormone (FSH) (Roche Cobas E601., Germany).

At the reproductive medicine center, 120 patients confirmed with infertility (failed to become pregnant after 1 year of preparation) were recruited. The inclusion criteria were as follows (1): 20 - 45 years old (2), able to understand and complete the SAS test, and (3) able to visit the reproductive medicine center before 7:30 am and finish blood collection on time (4), no hypertension or hyperglycemia, and (5) no visible abnormality in the blood sample. The exclusion criteria were as follows (1): under hormone treatment such as dexamethasone in the past 3 months (2); having endocrine diseases such as hypothyroidism, hyperprolactinemia, adrenal cortical hyperplasia, and diabetes (3), a history of psychological disorder (4), history of smoking or alcohol abuse. Patients in the control group have no known medical history of infertility treatments. The same inclusion criteria were adopted for these healthy individuals, except that they were only asked for the detection of morning serum cortisol. For the infertile women, those with severe negative life events within 1 year and known gynecological diseases such as uterine malformation and severe endometriosis were also excluded.

After completing all procedures, the date was matched and checked. Women who lacked background information or failed to complete any tests were excluded (n = 10), resulting in a final number of 110 (91.7%) and 112 (100%) for infertility and control groups, respectively. Among the 110 infertile patients, 1 didn't undergo IVF treatment in our reproductive medicine center for personal reasons. A GnRH-antagonist protocol for IVF treatment was applied for all patients during the first cycle, which started within 1 month of the stress evaluation. More IVF cycles were conducted if the first one failed. The treatment continued until successful clinical pregnancy or the patient decided to give up. Finally, the number of IVF cycles performed and treatment outcomes were retrieved for the 109 participants.

Measurement of cortisol

The collection of serum samples was conducted before breakfast with a standard extraction protocol and equipment. After arrival and resting for > 30 min in the morning, 5 mL of peripheral blood was taken between 7:00 am and 9:00 am using a standard coagulation blood collection tube. The sample was centrifuged at 3500 rpm for 6 min, and the serum was isolated for testing and storage. Serum cortisol was either measured immediately after separation or stored at 4°C before being tested within 8 h. The samples were kept at −20°C for long-term storage.

A quantum-dot immunochromatography assay for cortisol was used in this study (Jiangsu NepQD Biotech Ltd.). The quantitative result of serum cortisol concentration was measured by a portable immunofluorescence analyzer designed for this POCT (NepQD-Infinity-V1, Jiangsu NepQD Biotech Ltd., China). To perform the assay, 20 μL of serum sample was mixed with the diluent, and 60 μL of diluted sample was added to the sample loading well of the test card. After 10-min incubation, the test card was inserted into the analyzer for fluorescent signal reading. The detection range of this platform is 1.00–60.00 μg/dL, with an intra- and inter-assay coefficient of variation < 15%. The performance correlation between this POCT platform and the chemiluminescent assay is > 0.975.

Measurement of SAS

A Chinese version of the widely employed SAS by Zung was used for the psychological evaluation of infertile patients (33). Basic instructions were provided by trained researchers using the same wording, and then the patients were asked to complete the 20-item questionnaire independently. The raw score was multiplied by 1.25 according to the Chinese norm, with the cutoff being 50. Scores of 50–59, 60–69, and above 70 indicate mild, medium, and severe levels of anxiety, respectively.

IVF treatment

Patients involved in this study underwent GnRH-antagonist protocol for the first cycle of IVF treatment. Embryos transfers were performed on day 3 (2 cleavage stage embryos) or day 5 (1 blastocyst). Most patients finished the first IVF cycle within 3 months. Those who were not pregnant continued with other treatment protocols. The IVF treatment continued until successful clinical pregnancy or the patient decided to give up. In the end, the number of IVF cycles performed and treatment outcomes were retrieved for each individual.

Statistical analysis

The data of 110 patients from the reproductive medicine center (infertility group) and 112 patients from the medical center (control group) were used for statistical analysis using SPSS Statistics 23.0 (IBM Corporation, Armonk, NY, USA). The distributions of age, serum cortisol level, BMI, SAS score, AFC, AMH, and FSH concentrations were examined using the Shapiro–Wilk test. Since the data were nonparametric, the differences in age and cortisol concentration between infertility and control groups were verified using the Mann–Whitney U test. The anxiety status of women with primary or secondary infertility was also compared with this method.

To investigate the level of cortisol across different ages, the participants were divided into different age groups (≤25, 26–28, 29–31, 32–34, and ≥35), and the difference was verified using the two-tailed Mann–Whitney U test. The correlation between variables was evaluated using the non-parametric hypothesis test Kendall test, with the significance level set at 0.05 and 0.01. Further comparison between age, cortisol level, monthly income, year of infertility, level of education, and BMI was performed between four anxiety groups (no, mild, medium, and severe) among the infertility participants using the Kruskal–Wallis test, which is a non-parametric test for median comparison between multiple groups.

The onset of anxiety among infertile patients was assessed based on the SAS questionnaire. Binary logistic regression was conducted to illustrate the contributing factors of anxiety. "Anxiety" and "no anxiety" was set as the dichotomous dependent variables, and age, cortisol level, year of infertility, BMI, monthly income, and education level were set as covariates. Monthly income and education level were categorical covariates, and others were numerical variants. The Forward: Likelihood Ratio model was selected for the construction of the regression equation.

To investigate the potential role of morning serum cortisol concentration in the evaluation of anxiety in the target population, we constructed the **receiver operating characteristic** (ROC) curve and determined the Youden index and cutoff value for cortisol. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for anxiety were calculated based on the cutoff concentration. A Pearson's Chi-square analysis was used to evaluate the difference in clinical pregnancy outcomes regarding the physiological and psychological approach for anxiety assessment, and the average number of IVF cycles conducted for each subgroup was calculated. The influence of infertility types and factors on pregnancy outcomes were also compared with the same method.

Results

Demographic characterization and cortisol levels

The basic information of individuals in the infertility group (N = 110) and control group (N = 112) is summarized in Table 1. The age of the infertility group ranged from 23 to 44 years with an average of 30.67 years, whereas that of the control group ranged from 21 to 44 years with an average of 31.37 years. In the infertility group, 20 (18.2%) patients were at the advanced maternal age as defined by the Chinese standard (≥35), and 15 (13.6%) were overweight according to local standards (BMI > 24.0) (34, 35). Patients at advanced maternal age generally have a decreased likelihood of natural pregnancy and a higher incidence of pregnancy complications (35). The year of infertility ranged from 1 to 8 years. Most of the patients (58.2%) that joined this study had a history of infertility of 2-4 years.

As shown in Table 1, the serum cortisol level of the control group followed a normal distribution, but that of the infertility group did not. A comparison of the two groups revealed a significant difference in stress regulatory hormone concentration ($p = 0.000$), but not in age ($p = 0.337$). The level of morning cortisol was significantly higher in the infertility group (18.39 μg/dL), with a larger standard deviation (± 7.79 μg/dL).

Correlation of variables

A positive correlation between cortisol level and age was found in the infertility group (Kendell's tau = 0.201, $p = 0.003$), but not in the control group (Kendell's tau = −0.064, $p = 0.329$). To further investigate the association between cortisol level and age, participants were divided into subgroups. The concentration gap between the two cohorts increased as their age increased, and a significant difference was observed for the 32-34 ($Z = -2.725$, $p = 0.006$) and >35 ($Z = -3.630$, $p = 0.000$) subgroups (Figure 1). The morning cortisol concentration was relatively stable across the age of interest (20–45) for the control patients.

Pairwise correlation analysis was conducted between different factors of the infertile patients (Table 2). The SAS score was strongly correlated to morning serum cortisol level, monthly income, and BMI (all $p < 0.01$). Cortisol concentration was positively related to age, SAS score, BMI (all $p < 0.01$), and year of infertility ($p < 0.05$), but was negatively related to monthly income ($p < 0.01$). Many demographic characters were correlated with each other, including age, year of infertility, BMI, and education level (all $p < 0.01$). Higher monthly income was linked to higher education levels but lower BMI ($p < 0.01$), which might be a reflection of healthier dietary habits. Interestingly, education level was negatively correlated to BMI and year of infertility ($p < 0.01$) in the infertility group. The number of cycles for IVF treatment was strongly related to age and year of infertility ($p < 0.01$), but negatively correlated to the pregnancy outcome ($p < 0.05$), which is because the treatment will continue until the patient gets pregnant or give up.

Prediction of anxiety with cortisol levels

As determined by the SAS score, 58.1% (n = 64) of infertile patients had no anxiety, whereas. Comparison between the anxiety (n = 46) and not anxiety (n = 64) groups of the infertility patients revealed that all numerical data (age, cortisol, years of infertility, BMI) of these two groups were in a skewed distribution. Further investigation of patients with different levels of anxiety (mild 15.5%, n = 17; medium 17.3%, n = 19; severe 9.1%, n = 10) demonstrated the pairwise difference in cortisol level, monthly income, and BMI (Table 3). The level of serum cortisol in patients with no anxiety was significantly different from that in patients with anxiety; meanwhile, patients with mild, medium, and severe anxiety had similar serum cortisol levels. The severe group had a significantly lower monthly income than other groups, implying that poverty might be a factor that exacerbates the disease. As the average BMI increased with the severity of anxiety, patients with no anxiety had a significantly lower BMI compared with the severe groups. This finding was generally in agreement with the percentage of overweight patients with no, mild, medium, and severe anxiety (4.7%, 17.6%, 21.1%, and 50.0%).

To identify the factors that contributed to anxiety in the infertility group, we first conducted a binary logistic regression using the Forward: Likelihood Ratio model. Among all input factors, only cortisol level was significantly related to anxiety ($\chi^2 = 93.307$, $p < 0.000$). The regression analysis gave a sensitivity, specificity, and accuracy of 93.8%, 95.7%, and 94.5% respectively. This finding was in agreement with the significant difference ($Z = -3.666$, $p = 0.000$) in cortisol concentration between infertility and control groups (Table 1; Figure 1). In addition, the pairwise correlation test and binary logistic regression indicated a significant correlation between SAS score and morning serum cortisol concentration.

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TABLE 1 Demographic characteristics of participants.

Parameters	Mean (SD)	Levels	Counts
Age			
Control group	31.38 ± 5.62*	≤25	17 (15.2%)
		26–34	63 (56.2%)
		Above 35	32 (28.6%)
Infertility group	30.67 ± 4.48*	≤25	10 (9.1%)
		26–34	80 (72.7%)
		Above 35	20 (18.2%)
Cortisol (μg/dL)			
Control group	14.38 ± 4.84		
Infertility group	18.39 ± 7.79*		
AFC	12.03 ± 5.26*		
AMH (ng/ml)	3.65 ± 2.51*		
FSH (mIU/mL)	7.81 ± 2.18		
SAS Score	38.45 ± 22.32*	0-49	64 (58.1%)
		50-59	17 (15.5%)
		60-69	19 (17.3%)
		Above 70	10 (9.1%)
BMI	21.51 ± 2.04*	<18.5	6 (5.5%)
		18.5–24.0	89 (80.9%)
		>24.0	15 (13.6%)
Years of infertility	3.74 ± 2.12*	1 Y	13 (11.8%)
		2–4 Y	64 (58.2%)
		Above 5 Y	33 (30.0%)
Monthly income (CNY)		Below 2000	17 (15.4%)
		2000–5000	51(46.4%)
		Above 5000	42 (38.2%)
Education level		Middle school/below	22 (20.0%)
		High school	41 (37.3%)
		Bachelor/above	47 (42.7%)

*Test for normal distribution, $p < 0.05$ (two-tailed).

The prediction of anxiety with morning serum cortisol levels of the infertile women was achieved through the construction of the ROC curve. As shown in Figure 2, the area under the curve (AUC) was calculated to be 0.960, with a 95% confidence interval (CI) between 0.917 and 1.000. When the cutoff value for serum cortisol concentration was set to 22.25 μg/dL, the maximum value of the Youden index (0.910) was reached. Based on the threshold calculated, the performance of the cortisol test was summarized (Table 4). A PPV of 93.6%, NPV of 96.8%, sensitivity of 95.6%, and specificity of 95.3% were obtained. The overall diagnostic accuracy for the morning serum cortisol test to the SAS score was 95.4% for infertile patients.

IVF Treatment outcomes

Among the 110 infertile patients involved in the anxiety assessment, 109 underwent IVF treatment in our reproductive medicine center and 63.3% became clinically pregnant. Miscarriage was documented for one patient with primary infertility and two with secondary infertility. After 1 IVF cycle, 44.9% of the participants conceived, and 19.3% failed even with 2 cycles or more. As shown in Table 5, no significant difference regarding the final treatment outcome was found between patients with primary or secondary infertility, or between patients with various infertility factors. The pregnancy rate was similar between

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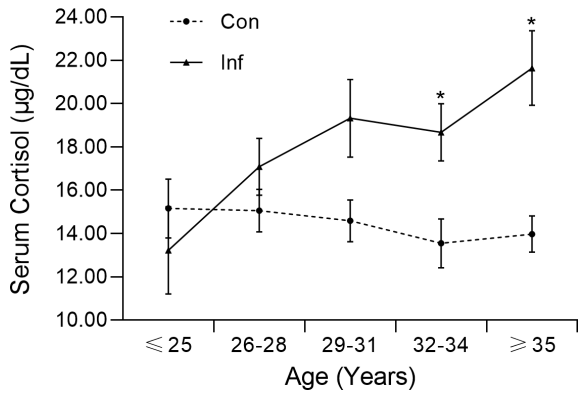

FIGURE 1
Morning serum cortisol level of infertile patients (Inf) and controls (Con) in different age groups. The error bar represents the standard error mean.

women with anxiety (58.7% and 57.4%) and without anxiety (66.7% and 67.7%), regardless of the method used for disease evaluation. When the cortisol levels and SAS scores of women who conceived during the first treatment cycle were compared, no significant differences were found. It should be noticed that after the first IVF cycle, the IVF treatment procedure varied between individuals to achieve the best outcome. Although a decrease in pregnancy rate (8.0%-10.3%) was observed for patients with high perceived anxiety (SAS > 50) or level of serum cortisol (concentration > 22.25 μg/dL), the impact of anxiety to multi-cycle IVF treatment was inconclusive.

When comparing the number of IVF cycles performed, a significant difference was found between patients with primary and secondary infertility (1.28 VS 1.90). We also identified that women with secondary infertility were elder, and their SAS score, morning cortisol level, and BMI were significantly higher ($p < 0.05$) compared with those of primary infertility. However, if the anxiety statues were used as the independent variables, the average number of IVF cycles conducted for the anxiety groups was similar to that of the not anxiety ones (1.76-1.77 VS 1.61-1.62).

Discussion

Demographic analysis between infertility and control groups revealed a similar age range and a skewed distribution. The morning serum cortisol demonstrated a normal distribution in that of the control group, which was similar to the distribution in the general population. Compare with hair cortisol which is also frequently evaluated in anxiety studies, serum sample requires fewer sample preparation steps and could be tested with commercialized platforms. More importantly, reference ranges have been proposed for serum/plasma cortisol levels of healthy individuals. As reported by the Roche Diagnostic GmbH (Roche Cobas platform, Elecsys Cortisol II), the 5^{th}-95^{th} percental of the morning (6-10 am) serum cortisol level in healthy adults ranges from 6.02-18.4 μg/dL, and the reference range reported by Beckman Coulter cortisol test is 6.7-22.6 μg/dL. The test results of the control patients in our study generally followed a similar distribution within the same reference range. However, the POCT method enabled the onsite evaluation of serum cortisol with a share of the serum sample, and the result can be obtained within 15 min. A significant increase in morning cortisol levels was identified among infertile participants, which might be due to higher stress (i.e., anxiety) or other factors related to endocrine dysregulation.

Despite the similarity in age, the infertile patients demonstrated a higher average serum cortisol concentration and an increased cortisol level with age, especially in those over 32 years old (Figure 1). Previous studies have also documented higher cortisol levels in women with anxiety or patients with depression (31, 36). The gap in cortisol levels between the same age groups was possibly due to physiological or psychological differences between the two populations, as women might feel more stressed about their infertility issues in their 30s.

The correlation analysis for the primary variables of infertile patients revealed complicated relationships between variables (Table 2). Our result demonstrated a significant correlation between the SAS score and morning serum cortisol level, which has also been reported in pregnant women with anxiety (31). These findings implied that women with a stress-induced mood disorder

TABLE 2 Correlations between demographic and clinical variables of infertility group.

Correlation	SAS score	Cortisol level	Age	Monthly income	Year of infertility	BMI	Education level	IVF cycle
Cortisol level	0.624**							
Age	0.124	0.201**						
Monthly income	−0.232**	−0.248**	−0.150					
Year of Infertility	0.070	0.156*	0.495**	−0.153				
BMI	0.233**	0.299**	0.215**	−0.283**	0.152*			
Education level	−0.024	−0.075	−0.275**	0.303**	−0.240**	−0.258**		
IVF cycle	0.071	0.050	0.356**	-0.117	0.250**	0.123	−0.236**	
Pregnancy	-0.047	-0.087	-0.041	-0.033	-0.040	0.060	0.000	−0.212*

*$p < 0.05$ (two-tailed); **$p < 0.01$ (two-tailed).

TABLE 3 Assessment of parameters among infertile patients with different levels of anxiety.

Pair of groups	Cortisol level	Age	Monthly income	Year of infertility	BMI	Education level
All groups	0.000*		0.007*		0.001*	
No-mild	0.000*		1.000		0.073	
No-medium	0.000*		1.000		0.071	
No-severe	0.000*	0.071	0.003*	0.315	0.007*	0.292
Mild-medium	1.000		1.000		1.000	
Mild-severe	1.000		0.112		1.000	
Medium-severe	1.000		0.060		1.000	

*Kruskal–Wallis test $p < 0.05$ (two-tailed); no, mild, medium, and severe represent the group of patients whose SAS score ranges from <50, 50–59, 60–69, and >70, n = 110.

were likely suffered from dysregulation of the stress-regulatory hormone as well.

The negative correlation between monthly income and SAS score was in agreement with several studies involving the socioeconomic status of patients with anxiety and depression (6, 10). A previous investigation conducted by Xu et al. in China revealed that higher education in infertile women could be a protective factor against anxiety (7). Although we did not observe a strong correlation between SAS score and education level, the significant correlation between education level and monthly income supported the same finding indirectly.

Based on the SAS score, the prevalence of anxiety in the infertility group was 41.9%, which is within the range of that of low- and middle-income countries (95% CI: 31.86%–78.62%) reported by Kiani et al. (4). The monthly income represents a typical medium income level in China, but the percentages of overweight patients in the mild, medium, and severe anxiety groups were much higher than that in the no-anxiety group, which was not related to the age difference (Table 3). The percentage of overweight women in the severe anxiety group was much higher than that of local women of childbearing age (37).

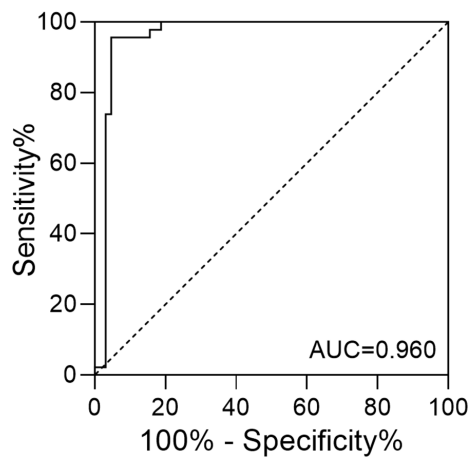

FIGURE 2
The ROC curve of serum cortisol concentration in predicting anxiety among infertile patients.

Further investigation in a larger sample size is needed to identify the mechanism.

Due to the complicated physiological conditions of infertile patients, we could not determine whether the significant correlation between anxiety and BMI was caused by any minor malfunction of the endocrine system such as cortisol hypersecretion ($p < 0.01$) or the oily diet popular in the Chongqing region. Infertile anxiety women are generally prone to chronic pressure and are more likely to have a worse diet and unhealthy lifestyles, which exacerbate the issue. Similar to our result, a positive correlation between BMI and morning cortisol level has also been reported in a previous study (38). We postulate that hypercortisolemia could potentially contribute to increasing adiposity in the setting of caloric excess, as cortisol also regulates energy metabolism. Compared with serum cortisol level, which showed an increasing trend in the infertility group (Figure 1), the SAS score was independent of age. These findings suggest that the development of anxiety disorder in our patients might be attributed to both physiological and socioeconomic factors.

Our analysis also identified a positive connection between the year of infertility, age, and BMI. These findings were in agreement with the idea that being overweight (BMI > 24.0) might cause many health issues such as infertility (39). Neither the SAS score nor the cortisol level was associated with the year of infertility, suggesting that stress or anxiety may not exacerbate with time. However, the onset of mood disorder and the impairment of reproductive function might be connected through complicated endocrine issues.

A previous study involving both sides of infertility couples indicated that sex, education level, infertility duration, and treatment failure would all contribute significantly to anxiety (4). However, the binary logistic regression analysis in the present work suggested that only the morning serum cortisol level contributes significantly to anxiety ($p = 0.000$). The difference might be due to the lack of cortisol level as a parameter, the usage of different evaluation instruments (SAS vs Generalized Anxiety Disorder-7), the selection of the target population (diagnosed female vs couples), and the social or environmental difference. Our study suggested that morning serum cortisol levels yielded a high AUC for the ROC curve and predictive accuracy comparable to the binary logistic model (Figure 2; Table 4). The application of the POCT method suggested that morning serum cortisol level could serve as a

TABLE 4 Performance of the serum cortisol level test in anxiety assessment.

		SAS score			
		Anxiety	No anxiety		
Serum cortisol	Anxiety	44	3	PPV	Sensitivity
		40.00%	2.73%	93.62%	95.65%
	Not anxiety	2	61	NPV	Specificity
		1.82%	55.45%	96.83%	95.31%
Total		46	64		

SAS cutoff value >50; Serum cortisol cutoff value > 22.25 μg/dL.

potential biomarker to facilitate the screening of anxiety among infertile women.

The effect of stress on IVF outcomes has been investigated intensively in the field. It has been reported that both anxiety and concentrations of cortisol rose during IVF treatment (40), and lower blood cortisol at the time of oocyte retrieval is associated with successful treatment (24). Since our anxiety evaluation was conducted before any sex hormone administration and IVF treatment, it should be considered as a baseline for the psychological and physiological condition of patients. At this stage, the physiological condition of patients was not affected by external hormones, and they were not influenced by the excitement or stress of IVF treatment.

To further investigate the potential influence of anxiety on multi-cycle IVF treatment, the pregnancy outcome for 109 patients was retrieved. We found that the number of IVF cycles conducted was positively correlated with age ($p < 0.01$) and year of infertility ($p < 0.01$), but negatively correlated with pregnancy outcome (Table 2). This suggested that as the patients grow older, the difficulty of treatment increases, even if multiple procedures were tried. As shown in Table 5, the types and factors for infertility did not show a strong impact on the outcomes of the multi-cycle IVF treatment. However, patients with secondary infertility underwent more IVF cycles (1.90 VS 1.28), probably because the average age of these women was elder (31.6 VS 29.2). The significantly severe anxiety status of the elder, secondary infertility women evaluated through SAS and cortisol level also supports the idea that psychological evaluation and support should become incorporated into the medical service provided by reproductive medical centers.

The pregnancy rate for the "anxiety groups" either determined through SAS or cortisol level was 8.0%-10.3% lower, but the result did not reach a significant level or affirmative conclusion. Fortunately, the pregnancy rate of the anxiety groups and no anxiety groups all lay within the success rate reported by our hospital in the past few years (53% to 64%). Compared with other reproductive medical centers in China (success rate 50% to 65%), the outcomes of the IVF treatments conducted in this study were also reasonable. The same result was confirmed for the pregnancy rate after the first IVF cycle, where the same GnRH-antagonist protocol was adopted. Previous studies involving only one treatment cycle revealed a strong correlation between lower morning cortisol levels on the transplant day and successful pregnancy (41). We speculate that the anxiety level might change during treatment, and the stress regulation on the operation day

TABLE 5 Comparison of the treatment outcome between patients with different types of infertility and infertility factors, as well as patients evaluated with SAS or morning serum cortisol.

	Group	Pregnant	Not pregnant	Total	IVF cycle	$\chi 2$	p-value
Type of infertility	Secondary	43 (61.43)	27 (38.57%)	70 (64.22%)	1.90	*0.296*	0.587
	Primary	26 (66.66%)	13 (33.33%)	39 (35.78%)	1.28		
Infertility factors	Husband	12 (57.14%)	9 (42.86%)	21 (19.27%)	1.33	*1.303*	0.728
	Wife	29 (60.42%)	19 (39.58%)	48 (44.04%)	1.58		
	Both	26 (70.27%)	11 (29.73%)	37 (33.94%)	2.03		
	Unknown	2 (66.66%)	1 (33.33%)	3 (2.75%)	1.33		
SAS	High SAS	27 (58.7%)	19 (41.3%)	46 (42.2%)	1.76	*0.727*	0.394
	Low SAS	42 (66.7%)	21 (33.3%)	63 (57.8%)	1.62		
Cortisol	High cortisol	27 (57.4%)	20 (42.6%)	47 (43.1%)	1.77	*1.220*	0.269
	Low cortisol	42 (67.7%)	20 (32.3%)	62 (56.9%)	1.61		

IVF cycle represents the average IVF cycle performed for the group of patients; SAS cutoff value > 50; Cortisol cutoff value > 22.25 μg/dL.

should have a stronger effect. In addition, variables such as the number of oocytes retrieved, mature oocytes, and the number of embryos transferred were not strictly controlled between the IVF cycles and individuals. Consequently, no affirmation could be done on the effect of anxiety on the outcome of multi-cycle IVF treatment. A slightly higher average number of IVF cycle (1.76-1.77 VS 1.61-1.62) was also observed for the anxiety patients, in agreement with the fact that more IVF cycle was required until the patient was conceived or finally gave up. Most patients who failed after the first IVF cycle were willing to try multiple times, which might lead to a higher success rate. As we observed in this study, an alternative IVF protocol might be effective and result in clinical pregnancy. It is also possible that the medical operations of the women during IVF, especially the injection of sex hormones, had overcome the influence of anxiety and cortisol dysregulation to a certain degree (42). The expectation of a positive outcome from the treatment (43), and the encouragement from doctors and nurses could also help to release the stress.

Generally, our study has several strengths such as the novel application of onset serum cortisol detection and the fast entrance to IVF treatment. The evaluation of anxiety before any IVF treatment enabled the quantitative comparison of morning cortisol concentration between the healthy control group and the infertile women, which was missing from previous studies (24, 41, 44). The analysis between these two groups of participants provided a better demonstration of morning serum cortisol levels as a reflection of physiological stress. The SAS questionnaire could be easily applied in the reproductive medical center and has a well-defined Chinese norm. Both the SAS and cortisol tests revealed the baseline psychological and physiological condition of the patients without the influence of hormone injection during treatment. However, the limitations of this study should also be mentioned. Cortisol as one of the most important stress and metabolism regulatory hormones might also relate to other stress-related mood disorders such as depression. Since the reason for infertility is often complicated, the causation between anxiety, cortisol dysregulation, and infertility is unclear. We observed a high prevalence of overweight participants with no hypertension or hyperglycemia in this study. Detailed information on the endocrine function and metabolism of these women remained unclear. Future studies with larger population sizes and better control IVF treatment protocols should be conducted to address these issues.

Conclusion

The investigation of physiological and psychological stress revealed a high prevalence of anxiety and a significant increase in morning serum cortisol levels related to the SAS score of infertile women. Although the effect of anxiety on multi-cycle IVF treatment was inconclusive due to the variations in treatment protocols, we observed a slightly lower rate of clinical pregnancy for the anxiety patients, who also showed higher levels of morning serum cortisol. A study with a larger scale, identical infertility factors, or standardized IVF treatment between patients might be needed for better evaluation of the potential effect of anxiety.

Despite that anxiety before treatment could not be viewed as a direct drawback for infertility treatment, given the high prevalence of the disease and dysregulation of cortisol secretion, evaluation and intervention of the psychological and physiological issues of anxiety are preferred. Some relaxation methods such as music therapy could be recommended, and patients with severe symptoms can be referred to a psychologist (45, 46). With the convenience of sampling and the POCT method, morning serum cortisol measurement could be a feasible approach to promote the primary evaluation of anxiety and related endocrine dysregulations among infertile women. Such assessment before IVF treatment might be beneficial to the relief of stress, restoration of the endocrine regulatory system, and improve the well-being of patients.

Trial registration

This trial was approved by the institutional review board of The Second Affiliated Hospital of Chongqing Medical University, identifier: No. 2018 (100).

Data availability statement

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

Ethics statement

This trial was approved by the institutional review board of The Second Affiliated Hospital of Chongqing Medical University, identifier: No. 2018 (100). The patients/participants provided their written informed consent to participate in this study.

Author contributions

HZ and YC conceived and designed the study. QL prepared the cortisol test platform and wrote the first draft of the paper with YC. YW, BN, and TF performed the data retrieving, processing, and analysis. HC and XK helped with questionnaire collecting, data recording, and blood sample collection in the reproductive medicine center. HZ performed the cortisol measurement and supervised all data collected in the hospital. 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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High anti-Mullerian hormone level is adversely associated with cumulative live birth rates of two embryo transfers after the first initiated cycle in patients with polycystic ovary syndrome

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Objective: Anti-Mullerian hormone (AMH) has been recently identified as a potential predictor of live birth rates (LBRs) following assisted reproductive technology (ART) treatment. This study aimed to investigate the association between AMH levels and the outcomes of *in vitro* fertilization (IVF) in patients with polycystic ovary syndrome (PCOS).

Methods: Patients with PCOS initiating their first ovarian stimulation under the gonadotropin-releasing hormone antagonist protocol at the Guangdong Women and Children Hospital, China, were enrolled from November 2014 to September 2018. A total of 157 patients who underwent fresh embryo transfer (ET) cycles were included in group A, whereas 187 patients who underwent frozen–thawed ET cycles were included in group B. After the failure of the first ET cycle, 94 patients underwent the second ET cycle with frozen–thawed embryos. Of these 94 patients, 52 had failed the first fresh ET cycle (group C) and 42 had failed the first frozen–thawed ET cycle (group D). Successful embryo transfer was defined as live birth. This retrospective cohort study addressed the association between AMH levels and pregnancy outcomes using logistic regression approaches. After adjusting for age, body mass index, antral follicle counts, baseline follicle-stimulating hormone levels and baseline progesterone levels, LBRs were compared among the four groups and the cumulative live birth rate after two embryo transfers (TCLBR) was calculated.

Results: The LBRs showed no differences among the four groups. Higher serum AMH levels were found to be associated with a lower TCLBR [adjusted OR 0.937 (0.888–0.987), *P = 0.015*]. In patients who underwent the second ET cycle, LBRs were inversely proportional to AMH levels [crude OR 0.904 (0.828–0.986), *P = 0.022* versus adjusted OR 0.845 (0.754–0.946), *P = 0.004*, respectively]. In addition, the LBR was approximately 61%–78% lower in the group with AMH

levels of >12 ng/mL [crude OR 0.391 (0.168–0.912), *P = 0.030* versus adjusted OR 0.217 (0.074–0.635), *P = 0.005*, respectively].

Conclusions: Among PCOS patients high AMH level (>12 ng/ml) is found to be associated with low TCLBR and low LBR of the second embryo transfer cycles. The results provide limited clinical inferences and warrant further investigation.

KEYWORDS

anti-Mullerian hormone, polycystic ovary syndrome, embryo transfer, live birth, pregnancy outcome, assisted reproductive technology, fitting curve

Introduction

The prevalence of polycystic ovary syndrome (PCOS) is 4%–21% globally (1) and 7.8% in China (2). Some patients with PCOS undergo *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) for the treatment of infertility caused by ovulation disorder. PCOS not only manifests as an ovulatory disorder but also may be accompanied by complex gynecological endocrine alterations and may impact the outcome of assisted reproductive technology (ART). Therefore, it is important to identify predictors for pregnancy outcomes of IVF/ICSI.

Anti-Mullerian hormone (AMH), a glycoprotein synthesized by granulosa cells of small follicles in the female ovary, can inhibit the maturation of small follicles (3), demonstrating superiority in predicting ovarian reserve and stimulation responsiveness (3–5). Some studies have suggested the role of AMH levels in predicting the live birth rate (LBR) after IVF/ICSI treatment considering the close relationship between AMH and LBR (6). In addition, scholars have compared the efficiency of follicle-stimulating hormone (FSH) levels, antral follicle counts (AFCs) and AMH levels in predicting live birth. High AMH levels in the same AFC quartile have been showed to associate with a higher cumulative live birth rate (CLBR) and an increased number of oocytes (7). Although the focus of their study was slightly different, Ligon et al. suggested that the predictive efficiency of AMH was superior to that of FSH and revealed that lower AMH levels were independently associated with lower LBRs and increased canceled cycles (8). However, some studies have challenged the capability of AMH in predicting live births after IVF (9, 10), arguing the poor accuracy of AMH in predicting the LBR and clinical pregnancy rate (CPR) in patients undergoing treatment with ART (11–13).

It is not clear yet that AMH associates with LBR or other pregnancy manifestations such as CLBR, increased risk of early miscarriage during initial IVF/ICSI treatment, etc. (14, 15). Some studies have demonstrated that CLBR decreases with an increase in AMH levels (16), especially when AHM levels exceed 5–7 ng/mL (17), whereas other studies have reported that AMH has limited predictive accuracy (18, 19).

A stratified analysis may serve as a more reasonable testing method (20, 21), as both AMH levels and LBRs vary across diseases. AMH levels are lower in patients with diminished ovarian reserve (DOR) but 2–4 times higher in patients with PCOS than those without PCOS (5). The inconsistent results of previous studies have suggested further investigations of the relationship between AMH and pregnancy outcomes after IVF/ICSI in patients with PCOS.

Given that patients with PCOS are predisposed to ovarian hyperstimulation syndrome (OHSS) (22), some ART centers dismiss fresh embryo transfer (ET) for all patients with PCOS, which may inevitably lead to prolonged live birth time and anxiety. Therefore, comparing pregnancy outcomes after fresh and frozen–thawed ET cycles in patients with PCOS is necessary. Most women with PCOS proceed with a second ET cycle after the failure of the first cycle. However, a third attempt after the failure of the second cycle is sporadic. Therefore, evaluating the CLBR after two ET cycles may benefit pregnancy outcomes in clinical settings. In this study, CLBR after two ET cycles was referred to as TCLBR. We analyzed the IVF/ICSI cycle data of patients with PCOS to detect the association between AMH levels and pregnancy outcomes, particularly LBR and TCLBR, under different ET strategies.

Materials and methods

Study design and participants

This retrospective cohort study enrolled 1181 patients with PCOS undergoing ET cycles at the Reproductive Health and Infertility Department, Guangdong Women and Children Hospital, China, from November 2014 to September 2018. PCOS was diagnosed based on the Rotterdam criteria (23). The exclusion criteria were as follows: repetitive initiated cycles; treatment without the gonadotropin-releasing hormone (GnRH) antagonist protocol; the age of <18 or ≥40 years; history of ovarian surgery; congenital or acquired reproductive malformations; recurrent miscarriages; endometriosis and uncontrolled hypertension, diabetes and thyroid diseases. The initiated cycle was defined as a cycle in which a woman receives specific medication for ovarian stimulation and attempts follicular aspiration. In addition, patients with canceled cycles, those with missing data or with outliers in AMH values and those lost to follow-up were

excluded. Canceled cycles were defined as uncompleted ETs after initiating ovarian stimulation. Couples with chromosomal abnormalities or same type thalassemia were excluded, whereas those using donor sperm because of the chromosomal abnormalities of husbands were included. Eventually, 344 patients with PCOS undergoing the first ET in their first initiated cycle under a GnRH antagonist protocol were included. Of these 344 patients, 157 patients undergoing the first fresh ET were included in group A and 187 patients undergoing the first frozen–thawed ET were included in group B. After the failure of the first ET cycle, 94 patients underwent the second ET cycle with the remaining frozen embryos. Patients who underwent the second ET cycle with frozen–thawed embryos after the failure of the first fresh ET cycle were included in group C. Patients who underwent the second ET cycle with frozen–thawed embryos after the failure of the first frozen–thawed ET cycle were included in group D (Figure 1). This study was approved by the Guangdong Women and Children Hospital Institutional Review Board, and informed consent was waived.

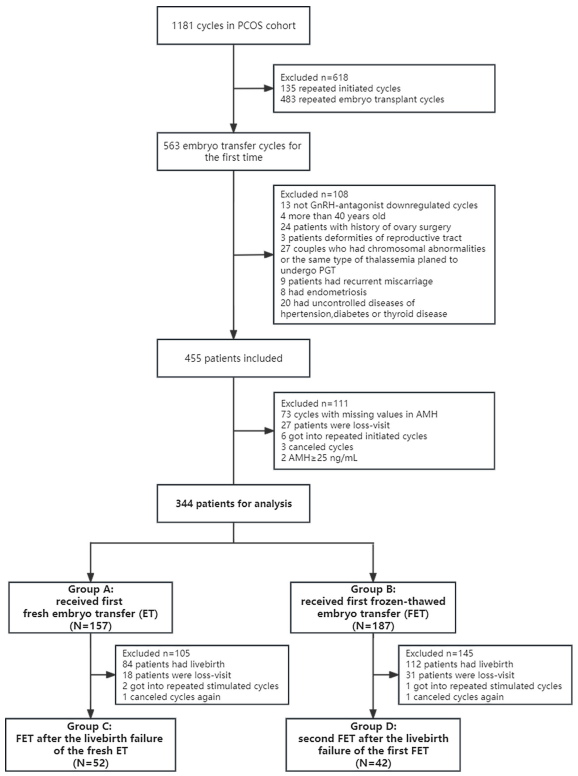

FIGURE 1
Research flowchart of women with PCOS. Group A: Patients who underwent the first fresh embryo transfer. Group B: Patients who underwent the first frozen–thawed embryo transfer. Group C: Patients who underwent frozen–thawed embryo transfer after the failure of the first fresh embryo transfer. Group D: Patients who underwent frozen–thawed embryo transfer after the failure of the first frozen–thawed embryo transfer. Patients in all four groups were in their first initiated cycle. PCOS, polycystic ovary syndrome; PGT, preimplantation genetic testing; AMH, anti-Mullerian hormone; ET, embryo transfer; FET, frozen–thawed embryo transfer.

Treatment protocol

According to the GnRH antagonist protocol, gonadotropin was injected daily in all patients from the first to the fourth day of their menstrual cycles, irrespective of natural or artificial cycles. Gonadotropin stimulation was performed using recombinant follicle-stimulating hormone (rFSH) (Gonal-F, Merck Serono, Italy, or Puregon, Organon, Oss, the Netherlands) or highly purified urinary FSH (Menopur, Ferring Pharmaceuticals Ltd, Denmark) and combined with human menopausal gonadotropin (HMG; Zhuhai Lizhu Medicine Ltd, China). The initiating dose of gonadotropin varies between 75 and 250 units per day according to age, AFCs, AMH levels, body mass index (BMI) and clinicians' verdicts. Follicular development was monitored via transvaginal ultrasonography according to serum sex hormone levels. Within 4 days of initiating ovarian stimulation, the dose of gonadotropin was adjusted based on the ovarian response. When serum luteinizing hormone levels decreased below 1.0 IU/mL, 75 IU of injectable HMG or recombinant LH (Luveris, Merck Serono) was administered daily. Ganirelix acetate (ganirelix acetate injection; Organon, Netherlands) or Cetrotide (cetrorelix acetate injection; Merck Serono, Italy) at a dose of 250–500 μg was administered from the fifth day of stimulation (fixed protocol) or when the mean diameter of dominant follicles reached 12 mm (flexible protocol) until the day of triggering.

Ovulation was considered triggered after the diameter of two or more follicles was ≥18 mm, that of at least three follicles was ≥17 mm or that of at least 60% of follicles was ≥15 mm. Human chorionic gonadotropin (hCG, Zhuhai Lizhu Medicine Ltd) at the dose of 8,000 or 10,000 IU or recombinant chorionic gonadotropin (Ovidrel, Merck Serono) at the dose of 250 mg was administered for inducing ovulation. If serum estrogen (E_2) levels were ≥5,000 pg/mL, the GnRH agonist triptorelin (Gonapeptyl, Ferring) at the dose of 0.2 mg or triptorelin combined with 2,000 IU of urinary hCG was administered to reduce the risk of OHSS. Transvaginal oocyte retrieval was performed under ultrasound guidance within 36 hours of ovulation triggering. Fertilization was accomplished via IVF, ICSI or both. Before July 2017, a self-prepared culture medium was used, which was subsequently replaced with Kato culture medium.

During treatment, the first ET, whether using fresh embryos or frozen–thawed embryos, was performed based on indicators such as fertilization, E_2 levels after oocyte retrieval, ascites, endometrial condition and patient discomfort. A maximum of two cleavage-stage embryos or blastocysts were transferred. According to the standard protocol, an assessment system was employed for monitoring the morphology of oocytes and early embryos during the culture. Cleavage-stage embryos with at least five blastomeres were considered transferable, and those with 6–10 blastomeres were considered high-quality embryos (at least six cells in an embryo with a maximum of 20% fragmentation on day 3). Blastocysts were assessed according to the Gardner criteria (24): blastocysts with a grade of 3BB and higher were considered high quality.

Embryos were frozen if the risk of OHSS was high or upon patient's requested. Frozen–thawed ET was performed when

endometrial thickness reached 0.8 cm after natural or HMG-induced ovulation or during artificial hormone replacement cycles. As luteal support from the day after oocyte recovery, progesterone or organic hormone was administered as an intravaginal capsule (800 mg per day), or progesterone was administered as a gel (90 mg per day) or via intramuscular injection (40 mg per day). Serum estradiol and progesterone levels were evaluated after 4 days of ET. If E_2 levels were <200 pg/mL or progesterone levels were <20 ng/mL, estradiol or progesterone was respectively administered. If the pregnancy test indicated positive results, luteal support was maintained until 8 weeks of gestation.

Outcomes

The primary outcomes included LBR (the ratio of the number of live births to the number of embryo transfer cycles) and TCLBR (the ratio of the total number of live births after the first two embryo transfers to the number of patients who underwent the first initiated cycle). A live birth was defined as the successful delivery of a live baby. The 2017 American Society for Reproductive Medicine (ASRM) international glossary was referenced to define the terms of ART (25), and the Golan criteria were used for classifying OHSS (26). Pregnancy outcomes were monitored through follow-up.

Statistical analysis

Based on the Shapiro–Wilk test, identified variables with normal distribution were expressed as the mean ± standard deviation, whereas other variables were expressed as the median and interquartile range. In addition, character variables were expressed as the number of counts and percent. The chi-square test was applied for categorical variables and a t-test was applied to continuous variables. Groups A and C represented two cycles of the same patient. Therefore, they could not be compared with the assumption of independence. And the comparison couldn't be conducted between group B and group D because they were two cycles from the same patient too. We can compare the LBR of group A with group B, group C with group D, and group B with group C. The comparison of the LBR of group A with group D is unsuitable, because group A is the first fresh ET but group D is the second FET. The correlation of AMH levels with LBRs and TCLBRs in the four groups was evaluated via curve fitting. For comparison, patients were grouped according to the turning point of the curve (AMH levels, 12ng/mL). After adjusting for age, BMI, AFCs, baseline FSH levels and baseline progesterone levels, logistic regression analysis was performed to examine the correlation between AMH levels and LBRs in the four groups independently and that between AMH levels and TCLBRs of all 344 patients. Statistically significant differences were indicated by odds ratio (OR) with 95% confidence intervals (95% CIs) of <1, α values of 0.05 (bilateral test) and *P*-values of <0.05. A multivariate logistic regression model based on the generalized additive model was employed to fit the splines. All statistical analyses were performed using the R (version 3.3.2) software package (http://www.R-project.org, The R Foundation) and the Free Statistics (version 1.7) software.

Results

Participant's characteristics

Data regarding the baseline characteristics, ovarian stimulation, implantation and pregnancy outcomes of all 344 patients are summarized in Table 1, with AMH levels of 12 ng/mL as the subgroup cut-off value. The number of patients with AMH levels of

TABLE 1 The characteristics of baseline, ovarian stimulation, implantation, and clinical pregnant outcome of all patients.

Variables	1 ng/mL ≤ AMH < 12 ng/mL	12 ng/mL ≤ AMH < 25 ng/mL	*P*-value
All participants	201	143	
Number of the first transfer			< 0.001
Group A	110 (54.7)	47 (32.9)	
Group B	91 (45.3)	96 (67.1)	
Number of the second transfer			0.202
Group C	34 (60.7)	18 (47.4)	
Group D	22 (39.3)	20 (52.6)	
Age of female patients (years)	29.0 (26.0, 32.0)	29.0 (27.0, 32.0)	0.451
Infertility type			0.918
Primary	124 (61.7)	89 (62.2)	
Secondary	77 (38.3)	54 (37.8)	
Infertility duration (years)	3.0 (2.0, 5.0)	3.0 (2.0, 5.0)	0.403
Infertility cause			0.259

(Continued)

TABLE 1 Continued

Variables	1 ng/mL ≤ AMH < 12 ng/mL	12 ng/mL ≤ AMH < 25 ng/mL	*P*-value
Fallopian tube factor + PCOS	78 (38.8)	61 (42.7)	
Male factor + PCOS	44 (21.9)	30 (21.0)	
PCOS only	62 (30.8)	33 (23.1)	
Compound factor	17 (8.5)	19 (13.3)	
BMI (kg/m^2)	22.8 (20.4, 25.0)	21.6 (19.9, 23.7)	0.002
AMH (ng/mL)	7.9 (6.1, 9.6)	16.0 (13.5, 18.5)	< 0.001
AFC	24.0 (19.0, 24.0)	24.0 (24.0, 25.0)	< 0.001
Baseline FSH (mIU/mL)	6.1 (5.3, 7.2)	6.0 (5.2, 6.9)	0.471
Baseline LH (mIU/mL)	6.7 (4.9, 10.0)	8.7 (6.2, 12.5)	< 0.001
Baseline LH/FSH	1.1 (0.8, 1.5)	1.4 (1.1, 2.0)	< 0.001
Baseline E_2 (pg/mL)	34.0 (26.0, 44.0)	38.1 (31.1, 47.0)	0.007
Baseline P_4 (ng/mL)	0.5 (0.3, 0.7)	0.5 (0.3, 0.7)	0.419
Baseline PRL (ng/mL)	16.7 (12.1, 22.0)	18.7 (13.6, 24.8)	0.037
Baseline T (ng/mL)	0.3 (0.2, 0.4)	0.4 (0.3, 0.5)	< 0.001
HOMA-IR	2.3 (1.6, 3.0)	1.8 (1.4, 2.5)	0.003
FPG (mmol/L)	5.06 ± 0.44	5.01 ± 0.41	0.265
FIns (mU/L)	10.3 (7.0, 13.7)	8.3 (6.2, 11.4)	0.010
FSH levels on starting day (mIU/mL)	6.23 ± 1.44	6.27 ± 1.65	0.833
LH levels on starting day (mIU/mL)	6.7 (4.6, 9.7)	8.2 (5.7, 12.2)	< 0.001
LH/FSH ratio on starting day	1.1 (0.8, 1.5)	1.3 (1.0, 1.9)	< 0.001
E_2 levels on starting day (pg/mL)	34.1 (24.3, 45.2)	39.0 (30.8, 49.1)	0.005
P_4 levels on starting day (ng/mL)	0.4 (0.2, 0.5)	0.4 (0.2, 0.6)	0.102
Duration of stimulation (day)	10.0 (9.0, 12.0)	10.0 (9.0, 12.0)	0.568
Total dose of Gn (IU)	1350.0 (1075.0, 1800.0)	1237.0 (1012.0, 1512.0)	0.002
Initiating dose of Gn (IU)	125.0 (112.5, 150.0)	112.5 (100.0, 137.5)	< 0.001
E_2 levels on the triggering day (pg/ml)	3498.0 (2504.0, 5360.0)	4718.0 (3001.0, 6724.0)	< 0.001
P_4 levels on the triggering day (ng/ml)	0.9 (0.6, 1.3)	0.9 (0.6, 1.4)	0.580
LH levels on the triggering day (mIU/ml)	2.8 (1.9, 4.3)	3.1 (1.8, 5.0)	0.472
Endometrial thickness on the triggering day (mm)	10.0 (9.0, 12.0)	10.0 (8.8, 11.0)	0.134
Number of oocytes retrieved	16.0 (11.0, 21.0)	22.0 (13.5, 29.0)	< 0.001
Endometrial thickness on the transfer day (mm)	10.0 (8.0, 11.0)	9.0 (8.0, 10.0)	< 0.001
Embryo stage on the transfer day			< 0.001
Cleavage	123 (62.1)	58 (42.0)	
Blastocyst	75 (37.9)	80 (58.0)	
Number of transferred embryos			0.669
1	33 (16.4)	26 (18.2)	
2	168 (83.6)	117 (81.8)	

(Continued)

TABLE 1 Continued

Variables	1 ng/mL ≤ AMH < 12 ng/mL	12 ng/mL ≤ AMH < 25 ng/mL	*P*-value
Embryo transfer strategies			< 0.001
Fresh embryo transfer	110 (54.7)	47 (32.9)	
Frozen–thawed embryo transfer	91 (45.3)	96 (67.1)	
Clinical pregnancy	137 (68.2)	101 (70.6)	0.625
Ectopic pregnancy	4 (2.0)	5 (3.5)	0.498
Early abortion	12 (6.0)	10 (7.0)	0.702
Ongoing pregnancy	121 (60.2)	86 (60.1)	0.991
Late abortion	5 (2.5)	6 (4.2)	0.536
Premature delivery	31 (16.1)	25 (18.4)	0.581
Multiple births	22 (10.9)	14 (9.8)	0.917
Pregnancy complications	32 (15.9)	16 (11.2)	0.456
Live births after the first embryo transfer	116 (57.7)	80 (55.9)	0.744
Live births after the second embryo transfer	35 (62.5)	15 (39.5)	0.028
Cumulative live births after the two embryo transfers	151 (75.1)	95 (66.4)	0.078

Pregnancy complications: hypertensive disorders in pregnancy, gestational diabetes mellitus and hyperemesis gravidarum.
PCOS, polycystic ovary syndrome; BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; P4, progesterone; PRL, prolactin; T, testosterone; HOMA-IR, homeostasis model assessment of insulin resistance; FPG, fasting blood glucose; FIns, fasting insulin; Gn, gonadotropin.

>12.0 ng/mL was significantly higher in group B (96/187, 67.1%) than in group A (47/157, 32.9%) *(P< 0.001)*. Age and the type, duration and cause of infertility were not significantly different between subgroups. Significant differences were observed in baseline variables, including BMI, AFCs, LH levels, LH/FSH ratio, E_2 levels, prolactin levels, testosterone levels, homeostasis model assessment of insulin resistance (HOMA-IR) levels and fasting insulin levels ($P < 0.05$). In addition, some variables associated with ovarian stimulation and implantation, such as the dose of gonadotropin, number of oocytes retrieved, endometrial thickness on the transfer day, embryo stage and embryo transfer strategies, differed between subgroups ($P <0.05$).

AMH levels may indicate the severity of PCOS to a certain extent and can be affected by the heterogeneity of PCOS. In this study, testosterone levels, HOMA-IR values and fasting insulin levels were found to be significantly different in the subgroups with AMH levels of 12 ng/mL as the turning point, which may influence the relationship between AMH and LBR. Notably, patients with higher AMH levels had higher testosterone levels but lower HOMA-IR levels (Table 1).

Correlation between AMH and LBR

AMH levels were adversely associated with LBRs in group C, with or without adjusting for age, BMI, AFCs and baseline FSH and progesterone levels [crude OR 0.881 (0.781–0.994), $P = 0.039$ versus adjusted OR 0.796 (0.653–0.971), $P = 0.024$, respectively] (Table 2). LBRs decreased with an increase in AMH levels during the second ET cycle [crude OR 0.904 (0.828–0.986), $P = 0.022$ versus adjusted OR 0.845 [0.754–0.946], $P = 0.004$, adjusted for age, BMI, AFCs and baseline FSH and progesterone levels) (Table 3). In addition, high AMH levels (>12 ng/mL) indicated a high risk of OHSS in group A [crude OR 3.02 (1.03–8.88), $P = 0.045$ versus adjusted OR 3.42 (1.1–10.65), $P = 0.034$, adjusted for age and BMI].

Turning point of the correlation between AMH and LBR

The turning point of the relationship between AMH and LBR was at approximately 12 ng/mL, that is, the intersection of curve fitting of the four groups (Figure 2A). Curve fitting demonstrated the following trend: when AMH levels exceeded 12 ng/mL, LBRs remained stable after the first ET cycle but decreased after the second ET cycle (Figure 2B). In the second embryo transfers, compared with the patients whose AMH levels were lower than 12ng/mL, LBRs of the AMH level exceeding 12 ng/mL were reduced (62.5% versus 39.5%, $P = 0.028$) (Table 1). After the second ET cycle, LBRs were approximately 61%–78% lower in patients with AMH levels of >12 ng/mL than in those with AMH levels of <12 ng/mL [crude OR 0.391 (0.168–0.912), $P = 0.030$ versus adjusted OR 0.217 (0.074–0.635), $P = 0.005$, adjusted for age, BMI, AFCs and baseline FSH and progesterone levels] (Table 3).

LBRs of the four groups

The LBRs of groups A, B, C and D were 54%, 60%, 56% and 50%, respectively. The LBR was similar between groups A and B

TABLE 2 Odds ratio between serum AMH levels and live birth rates in group C.

	Crude odds ratio	Adjusted odds ratio (I)	Adjusted odds ratio (II)
AMH (ng/mL)	0.881 (0.781–0.994)	0.848 (0.733–0.982)	0.796 (0.653–0.971)
P-value	0.039	0.028	0.024
Patients grouped based on AMH levels of 12 ng/mL			
1≤AMH<12	1	1	1
12≤AMH<25	0.239 (0.071–0.806)	0.16 (0.039–0.659)	0.086 (0.014–0.527)
P-value	0.021	0.011	0.008

Group C: Patients who underwent frozen–thawed embryo transfer after the failure of live birth after the first fresh embryo transfer. Adjusted odds ratio (I): adjusted for age and BMI. Adjusted odds ratio (II): adjusted for age, BMI, AFCs and baseline FSH and P levels.
FET, frozen–thawed embryo transfer; ET, fresh embryo transfer; BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; P, progesterone.

(*P= 0.234*), between groups B and C (*P = 0.595*) and between groups C and D (*P = 0.582*) (Figure 3).

CLBR after two embryo transfer cycles

The TCLBR of all 344 patients was 71.5%, which decreased as AMH levels increased [adjusted OR 0.937 (0.888–0.987), *P = 0.015*, adjusted for age, BMI, AFCs and baseline FSH and progesterone levels). The TCLBR was sufficiently reduced in patients with AMH levels exceeding 12 ng/mL [adjusted OR 0.499 (0.289–0.862), *P = 0.013*] (Table 4). The TCLBR was similar between groups A and B (72% versus 71%, respectively, *P = 0.862*) (Figure 3).

Comment

Correlation that is known

Previous studies have suggested that elevated AMH levels are associated with a low LBR after the second ET cycle in women with PCOS (27, 28) and are adversely associated with CLBR (16, 17). In addition, some studies have reported that high AMH levels are associated with adverse pregnancy outcomes, such as a low clinical pregnancy rate (28) and a high preterm birth rate (29). However, some studies have reported that the relationship between AMH and CLBR remains uncertain (28, 30) and that AMH is unlikely associated with adverse pregnancy outcomes (31). This study is a secondary analysis of the association between AMH levels and treatment outcomes of IVF in patients with PCOS. In the first initiated cycle of ET, AMH levels were found to be adversely associated with the LBR of the second ET cycle and the TCLBR.

In addition, an adverse association was observed between AMH levels and LBRs among patients who underwent the second ET cycle with frozen–thawed embryos after the failure of live birth after the first fresh ET.

Turning point of the correlation

We observed a nonlinear correlation between AMH levels and LBRs in women with PCOS, with a turning point of approximately 12 ng/mL AMH levels. Patients with AMH levels of >12 ng/mL had lower LBRs after the first transfer, much lower LBRs after the second transfer and lower TCLBRs than patients with AMH levels of <12 ng/mL. This finding provides a valuable reference for designing ET strategies based on AMH levels.

Other results in the context

One of the objectives of this study is to guide clinicians in selecting fresh or frozen–thawed ET based on AMH levels. This study revealed that AMH levels were not associated with the LBR after the first ET cycle. If patients exhibited a high risk of OHSS for the first fresh ET, cryopreservation of embryos was suggested to

TABLE 3 Odds ratio between serum AMH levels and live birth rate after the second embryo transfer cycles.

	Crude odds ratio	Adjusted odds ratio (I)	Adjusted odds ratio (II)
AMH (ng/mL)	0.904 (0.828–0.986)	0.893 (0.815–0.979)	0.845 (0.754–0.946)
P-value	0.022	0.015	0.004
Patients grouped based on AMH levels of 12 ng/mL			
1≤AMH<12	1	1	1
12≤AMH<25	0.391 (0.168–0.912)	0.361 (0.15–0.867)	0.217 (0.074–0.635)
P-value	0.030	0.023	0.005

Adjusted odds ratio (I): adjusted for age and BMI. Adjusted odds ratio (II): adjusted for age, BMI, AFCs and baseline FSH and P levels.
BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; P, progesterone.

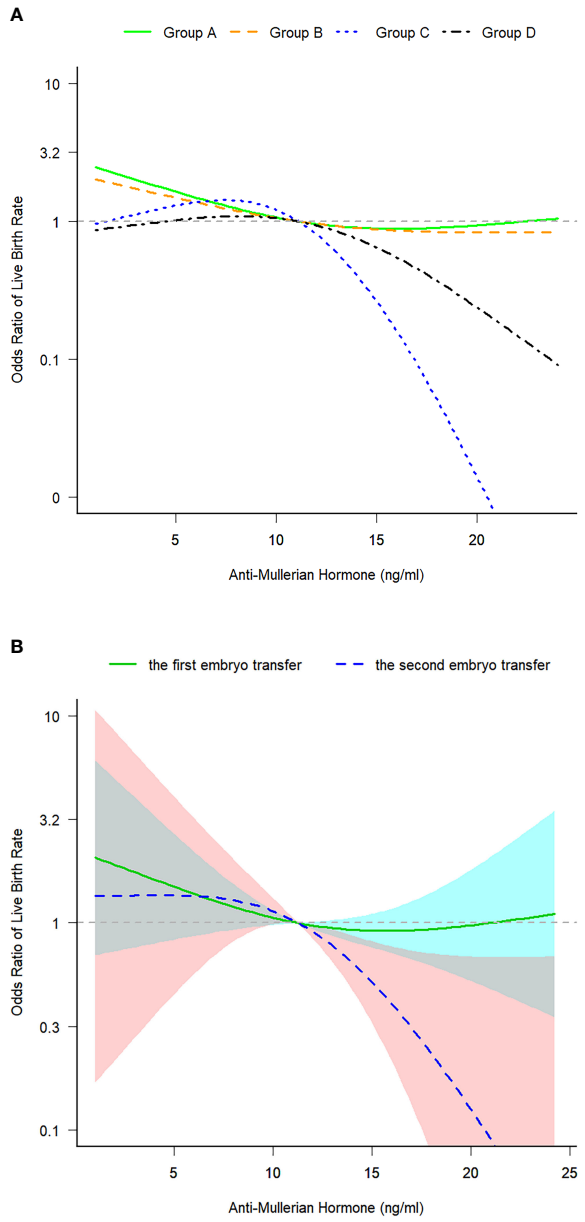

FIGURE 2
(A) Curve fitting for examining the correlation between serum anti-Mullerian hormone levels and live birth rates in the four groups. **(B)** Curve fitting for examining the correlation between serum anti-Mullerian hormone levels and live birth rates after the first and second embryo transfer cycles. Group A: Patients who underwent the first fresh embryo transfer. Group B: Patients who underwent the first frozen–thawed embryo transfer. Group C: Patients who underwent frozen–thawed embryo transfer after the failure of the first fresh embryo transfer. Group D: Patients who underwent frozen–thawed embryo transfer after the failure of the first frozen–thawed embryo transfer. Patients in all four groups were in their first initiated cycle. The 95% confidence intervals were significant only in group C: The model was adjusted for age, BMI, AFCs and baseline FSH and P levels. ET, fresh embryo transfer; FET, frozen–thawed embryo transfer; CI, confidence interval; BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; P, progesterone.

wait for an optimal transfer condition. The number of patients with AMH levels of >12.0 ng/mL who underwent the first transfer cycle with frozen–thawed embryos was significantly higher than that of patients who underwent the first transfer cycle with fresh embryos. High AMH levels (>12 ng/mL) were correlated with a high risk of OHSS in the first fresh ET cycle. However, LBRs were similar between groups A and B, indicating that AMH levels cannot serve as a criterion for choosing between fresh and frozen–thawed embryos. In addition, LBRs were similar after the first and second frozen–thawed ET cycles (group B versus group C). In summary, if serum AMH levels before the first initiated cycle are <12 ng/mL, selecting fresh embryos for the first transfer is recommended for patients with PCOS, which may save the waiting time for the transfer.

Clinical and research implications

The present study employed curve fitting to identify the turning point of the relationship between AMH levels and LBRs, thus providing a valuable reference for subsequent research. Considering the similarity of turning points in the same disease, we believe that grouping subjects based on the turning point is more reasonable in analysis compared to traditional quartile grouping, which is also a highlight of the present study.

To improve PCOS patients' ART live birth rate is a challenge to ART clinicians. The basic conditions of patients requiring the second ET cycle are very likely worse than those of patients with a successful first transfer. The primary pathological mechanisms underlying PCOS may include hyperandrogenemia and insulin resistance (IR). As an auxiliary diagnostic indicator of PCOS, AMH is associated with hyperandrogenemia and IR. Basic interventions such as pre-transfer lifestyle modifications, anti-androgenic therapy and improving IR are recommended for patients with high AMH levels, especially those with the first transfer failure. It is also recommended that to access the risk of PCOS women with high AMH levels before ART treatment to develop appropriate individualized treatment protocols and implement prenatal and follow-up examinations throughout pregnancy. AMH levels play an important role in clinical consultation, which can be used to estimate pregnancy outcomes effectively and inform patients regarding the second ET in advance.

Limitations and suggestions

This study has some limitations that should be acknowledged. The results of this study cannot be generalized. The inclusion of a limited number of samples from a single center may have led to bias. Smaller sample sizes can deal with only a few confounders, which may result in bias if important confounders are not included in the analysis. In addition, the results may differ if data from other centers are used for analysis. The impact of small sample size can be reflected in parameter estimates. For TCLBR (Table 3), the 95% CIs of crude and adjusted odds ratios for variable AMH grouped by 12 ng/ml appeared wilder owing to limited observations in the group with AMH levels of ≥12 and <25 ng/mL. If more observations are recorded in this group, variances may decrease and 95% confidence intervals may be narrower, resulting in smaller *P* values, even for

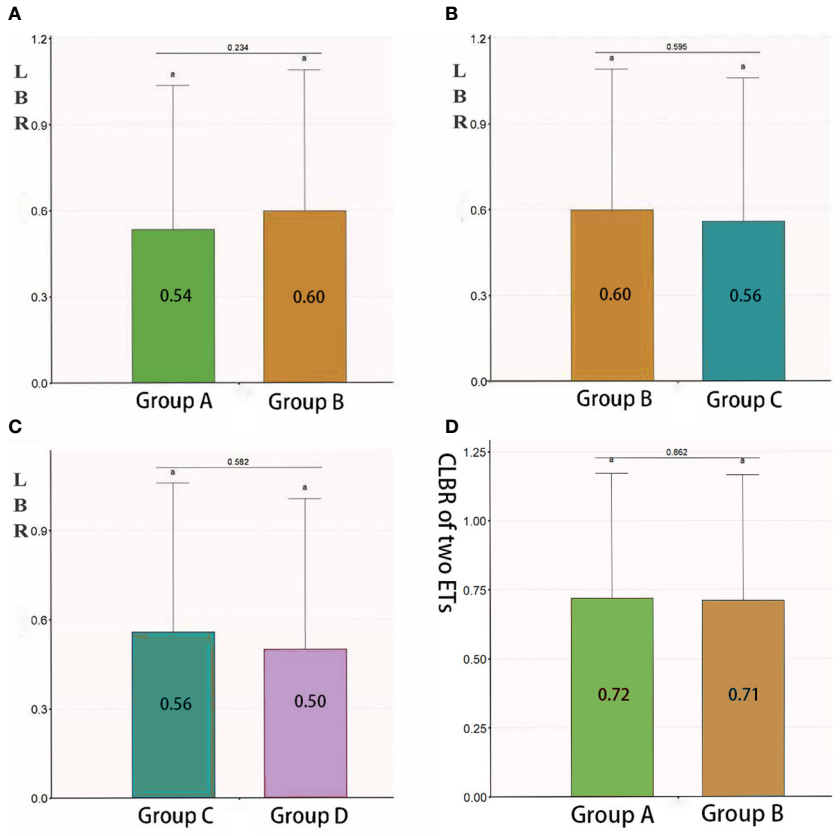

FIGURE 3
LBR and CLBR in group **(A)**, **(B)**, **(C)** and **(D)**. Patients who underwent the first fresh embryo transfer. Group B: Patients who underwent the first frozen– thawed embryo transfer. Group C: Patients who underwent frozen–thawed embryo transfer after the failure of the first fresh embryo transfer. Group D: Patients who underwent frozen–thawed embryo transfer after the failure of the first frozen–thawed embryo transfer. Groups A and C as well as groups B and D represent two cycles of the same patient. Therefore, they could not be compared with the assumption of independence. When the lowercase letter "a" was observed in both two groups, there were no statistically significant differences between the two groups. LBR, live birth rate; CLBR, cumulative live birth rate; ET, embryo transfer.

crude odds ratios. Therefore, obtaining representative randomized samples and maintaining a sufficient sample size may help to achieve unbiased results in future studies. The treatment protocol has been described in detail so that other centers can reproduce the findings of this study.

Furthermore, some potential unadjusted confounders may have altered the results of this study. AMH levels were found to be significantly higher in patients who underwent frozen–thawed ET than in those who underwent fresh ET, indicating that the selection of fresh or frozen–thawed embryos may represent a confounder interacting with AMH levels. The important confounders in this study include estrogen and progesterone levels, endometrial thickness, the number of blastocysts formed, and the number of oocytes retrieved (Table 1). Baseline covariates were statistically

TABLE 4 Odds ratio between serum AMH levels and cumulative live birth rates after two embryo transfer cycles in all patients.

	Crude odds ratio	Adjusted odds ratio (I)	Adjusted odds ratio (II)
AMH (ng/mL)	0.957 (0.915–1.002)	0.949 (0.905–0.994)	0.937 (0.888–0.987)
P-value	0.061	0.027	0.015
Patients grouped based on AMH levels of 12 ng/mL			
1≤AMH<12	1	1	1
12≤AMH<25	0.655 (0.409–1.051)	0.613 (0.378–0.993)	0.499 (0.289–0.862)
P-value	0.079	0.047	0.013

Adjusted odds ratio (I): adjusted for age and BMI. Adjusted odds ratio (II): adjusted for age, BMI, AFCs and baseline FSH and P levels.
BMI, body mass index; AFC, antral follicle count; FSH, follicle-stimulating hormone; P, progesterone.

adjusted to ensure outcome validity to the highest extent. However, covariates that changed with baseline characteristics and the dose of medication in the initiated cycle were not adjusted. Future studies should be appropriately designed to focus on the main factors contributing to the decrease in TCLBR and LBR after the second embryo transfer cycle in patients with high AMH levels and to verify the findings of this study.

In this study, the number of patients who completed all possible ET cycles was limited, primarily owing to the lack of embryos and personal reasons. Consequently, we analyzed the CLBR after two ET cycles, with the interval between the two cycles being <1 year. Moreover, the data were outmoded (most recent data collection in 2018), considering that the main article (32) has been published and this study represents a secondary analysis. We will analyze the updated data of CLBR resulting from completed ET cycles in future studies.

Conclusions

Among PCOS patients high AMH level (>12 ng/ml) is found to be associated with low TCLBR and low LBR of the second embryo transfer cycles. The results provide limited clinical inferences and warrant further investigation.

Data availability statement

The datasets presented in this article are not readily available because the data are not publicly available due to ethical restrictions. Requests to access the datasets should be directed to FL, liushine2006@163.com.

Ethics statement

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Guangdong Women and Children Hospital. The ethics committee waived the requirement of written informed consent for participation.

Author contributions

NS, JZ and FL contributed to conception and design of the study. CH, SW, XZ and LL organized the database. NS and JZ performed statistical analyses. NS, JZ and YZ interpreted the data. JZ, MX wrote the manuscript. NS, YZ and FL contributed to the critical revision of the article. 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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Iodine deficiency and real-life supplementation ineffectiveness in Polish pregnant women and its impact on thyroid metabolism

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Introduction: Iodine is a pivotal component of thyroid hormones, and its deficiency leads to negative pregnancy outcomes. Therefore, during gestation, additional iodine supplementation is recommended.

Objectives: By evaluating a group of women from western Poland, the study updated on iodine status during pregnancy and the effectiveness of iodine supplementation in relation to the maternal and neonatal thyroid function.

Patients and methods: A total of 91 women were recruited before the delivery between 2019 and 2021. During the medical interview, the patients declared their dietary supplements intake. Thyroid parameters (TSH, ft3, ft4, a-TPO, a-Tg, and TRAb) were measured in the serum of mothers and in the cord blood of newborns after birth. Urinary iodine concentration (UIC) and urine/creatinine (UIC/crea) ratio were assessed in single urine samples using a validated high-performance liquid chromatography with ultraviolet detection (HPLC-UV). Neonatal TSH screening from dried blood spot was analyzed.

Results: Pregnant women showed a median (interquartile range) UIC of 106 (69–156) µg/liter and UIC/crea ratio of 104 (62–221) µg/g, whereas approximately 20% had UIC/crea below 50 µg/g, indicating iodine deficiency. The iodine supplementation ratio was 68%. No significant differences in UIC, UIC/crea and thyroid parameters were found between iodine supplemented and non-supplemented groups; however, the highest ioduria was detected when iodine was supplemented in addition to levothyroxine in comparison with both substances administered separately. Patients with UIC/crea within 150–249 µg/g demonstrated the lowest TSH and a-TPO levels. Screening TSH was above 5 mIU/liter in 6% of children.

Conclusions: Despite the national salt iodization and the recommendation to supplement iodine during gestation, the status of the abovementioned microelement and real-life intake revealed the ineffectiveness of the current iodine-deficiency prophylaxis model in pregnancy.

KEYWORDS

urinary iodine concentration (UIC), micronutrients at pregnancy, iodine, selenium, hypothyroidism, thyroiditis, neonatal cord blood, pregnancy supplementation guidelines

1 Introduction

Thyroid dysregulation is a prevalent clinical issue, particularly relevant during pregnancy, affecting almost one-fifth of women. In the course of gestation, the requirement for thyroid hormones (THs) increases by 25–50%, due to the elevation in thyroid-binding globulin production, placental transfer of iodine and thyroxine (T4) to the fetus, TH degradation in the placenta and the enhanced renal microelement clearance (1). Moreover, THs are involved in the neuronal migration and myelination during fetal nervous system development (2). Notably, iodine represents a fundamental component of THs. Pregnant women with iodine deficiency frequently suffer from hypothyroidism (HT) as well as goiter and present an increased risk of developing thyroid autoimmunity (3). Moreover, TH deficiency results in poorer obstetric outcomes, such as higher prevalence of miscarriages, stillbirths, growth retardation, and congenital abnormalities (4). Significant iodine deficiency in prenatal life leads to the most severe form of HT, whereas mild or moderate maternal lack of iodine may cause intellectual disability, disturbed psychomotor function, poorer socialization, and a decreased IQ score in school-aged children (5, 6). In 1997 in Poland, the iodine deficiency prophylaxis program was introduced, involving mandatory iodization of household salt [20–40 mg potassium iodide (KI) per kg of salt] or neonate formulas (10 μg/100 ml of milk), and recommending additional supplementation of 100–150 μg KI per day for pregnant and breastfeeding women, which subsequently restored the optimal iodine status in the general population (7). In terms of epidemiology, it eradicated the endemic goiter in children and reduced its prevalence in pregnant women (from 80 to 19%), decreased neonatal transient HT (from 2 to 0.16%), as well as limited the thyroid cancer incidence in women over 40 years of age (8, 9). According to the latest WHO report, Poland fulfilled the requirement of > 90% of households with iodinated salt (7).

However, salt consumption may currently decrease due to the high incidence of hypertension in the population and the recommendations of the Polish Society of Hypertension, which aim to decrease the daily salt intake to 5 g/day or due to a higher proportion of imported food in the local diet (10, 11). Nevertheless, in terms of the optimal ioduria, following a 10- and almost 20-year follow-up after the introduction of the prophylaxis, the program has undoubtedly failed with regard to pregnant and lactating women (12, 13). Therefore, the necessity of additional iodine supplementation in the dose of 150–200 μg daily during pregnancy and the lactation period was particularly emphasized in the latest guidelines of the Polish Society of Endocrinology (14).

In contrast, according to the consensus of the World Health Organization (WHO), the United Nations Children's Fund (UNICEF) and the International Council for Control of Iodine Deficiency Disorders (ICCIDD), currently renamed as the Iodine Global Network (IGN), iodine supplementation is not recommended when the general population has been iodine-sufficient over the previous 2 years, as expressed by urinary iodine concentration (UIC) >/= 100 μg/liter, whereas the UIC value of < 150 μg has been defined as a deficiency in the course of pregnancy (15). The abovementioned recommendations are ambiguous, although they remain unanimous regarding the benefits of continuous monitoring and updating iodine status in pregnancy worldwide. The available national data evaluating iodine deficiency prophylaxis effectiveness were obtained from central, northern, and southern regions of Poland, in which all experts concur regarding the ineffectiveness of this model among pregnant women. However, to date, no studies have been conducted on residents of the western part of the country (12, 16–18).

The objective of the study was to evaluate iodine status in pregnant women and assess the supplementation rate, effects, and guidelines adherence in a real-life setting among pregnant women from western Poland, with a particular emphasis on maternal and neonatal thyroid function.

2 Patients and methods

The study comprised 91 Caucasian women from the area of Greater Poland (western region of Poland) who were enrolled on admission to the obstetric ward prior to their term delivery. They were randomly recruited in a public hospital, which is the leading obstetrics center in the western Poland—Gynecological and Obstetric Clinical Hospital at Poznan University of Medical Sciences (tertiary referral center) within the period between 2019 and 2021. The group consisted of women, who were healthy, euthyroid, or hypothyroid in the range adjusted to the pregnancy status, with or without antithyroid antibodies [anti-thyroid peroxidase (a-TPO), anti-thyroglobulin (a-Tg), anti–TSH-receptor (TRAb)], and either

treated or not with levothyroxine (LT4). They were divided into subgroups receiving supplementation or not, treated with LT4 or not, as well as into a subgroup with or without antithyroid antibodies. Patients were in good general condition, with a negative history of any serious chronic diseases, malignancies, or renal/liver disease (except for benign cysts). None of the patients declared a specific type of diet, including a fish-rich, vegan, or vegetarian diet, and the patients denied foreign trips lasting more than 1 month.

Patients were screened and interviewed by instructed midwives in terms of inclusion/exclusion criteria and the intake of dietary supplements during pregnancy, including product brand name, dose, frequency, and duration. A random single spot urine sample was collected before the delivery and stored in a freezer at −20°C in the amount of approximately 10 ml. Additionally, a maternal non-fasting venous blood sample was taken by venipuncture prior to the delivery. Up to 2 ml of cord blood from the neonatal part of the placenta was collected during the third phase of labor after cessation of umbilical cord pulsation. Both serum samples were stored frozen in −20°C. Ioduria, as UIC, was measured using a validated ion-pair high-performance liquid chromatography with ultraviolet detection (HPLC–UV), as described (19). Creatinine was determined in the same urine sample by a colorimetric enzyme-linked immunosorbent assay (ELISA) detection kit (ThermoFisher, EIACUN, Frederick, USA). In order to objectify the results, the urinary iodine/creatinine ratio (UIC/crea) was calculated. UIC and UIC/crea results were analyzed according to the latest WHO criteria for iodine status assessment in pregnant women (20). Concentrations of serum TSH, ft3, ft4, and antithyroid antibodies (a-TPO, a-Tg) were measured by means of electrochemiluminescence (ECLIA, Hitachi and Roche Diagnostics kits) using Cobas e601 analyzer (Indianapolis, IN, USA) and TRAb by radioimmunoassay (RIA, BRAHMS Diagnostics, Berlin, Germany). Neonatal TSH was verified on the 3rd–4th day of life (TSHs) as part of national screening for congenital HT. The measurement was performed in dried blood spots from the heel puncture by an immunoluminometric (LIA) assay.

The study was performed in accordance with the Declaration of Helsinki (21) and was approved by The Local Bioethics Committee of Poznan University of Medical Sciences (protocol no. 104/19, date of approval: 10 January 2019, annexed 4 February 2021, protocol no. 132/21).

2.1 Statistical analysis

Statistica, version 13.3 (TIBCO Software Inc., California, USA), GraphPad Prism, version 9.5.1. (GraphPad Software, LCC, Boston, USA) and Microsoft Excel (2019) from Microsoft Office (Adobe Inc., California, USA) were used for statistical calculations. The data were not normally distributed, according to Shapiro–Wilk's test, thus non-parametric statistical tests were applied. The groups were compared using the Mann–Whitney *U* or the paired Wilcoxon test (when parameters were analyzed within the mother–child pairs), as well as the analysis of variance (ANOVA)/Kruskal–Wallis tests with *post-hoc* analysis. The Spearman R test was performed to analyze correlations between the parameters. The results are presented mainly as the median and interquartile range (IQR, Q1–Q3), 95% confidence intervals (95% CIs), or mean with standard deviation (± SD). A p-value < 0.05 was considered significant.

3 Results

The patients' characteristics are presented in Table 1.

Pregnant women from the western Poland showed a median (IQR) UIC 106 (69–156) μg/liter and UIC/crea ratio equal to 104 (62–221) μg/g, which is defined as iodine deficiency (UIC or UIC/crea < 150 μg/liter or μg/g) for this particular group, according to the WHO ranges (15); see Table 2. Approximately 20% had UIC/crea below 50 μg/g, which is a borderline value for another indicator of iodine deficits. The correlation was observed between both indicators of ioduria used in the study, that is, UIC and UIC/crea ($R = 0.63$, $p < 0.001$).

Supplementation of iodine during pregnancy was declared by 62/91 pregnant women (68%). In the majority of cases, iodine was ingested as a part of multivitamin diet supplements in a median dose (IQR) of 200 (150–200) per day, with a range extending from 100–300 μg/day. In 13 out of 62 (21%) cases, iodine was not supplemented throughout the entire pregnancy, 4/62 (6.5%) of patients administered it only in the 1st and 2nd trimester, and 9/62 (14.5%) only in the 3rd trimester of pregnancy. The dose and formula of the dietary supplements are presented in Table 3.

In the subsequent stages of the analysis, women who did not supplement iodine in the 3rd trimester and patients who presented abnormal TSH for this period of pregnancy were excluded.

No significant differences in UIC (median, Q1–Q3) were found in the group which declared iodine supplementation (105 μg/liter, 69–170, $n = 50$) in comparison with the women who denied iodine supplementation (99 μg/liter, 84–130, $n = 27$) during pregnancy ($p = 0.55$); see Figure 1.

There was no significant difference in UIC between the iodine-supplemented and non-supplemented groups, also in the subgroups of patients not treated with LT4 ($p = 0.47$) as well as those receiving the medication ($p = 0.15$).

No difference was found between the group of iodine-supplementing and non-supplementing mothers and those showing UIC below and above 150 μg/liter in maternal and neonatal thyroid hormone indices (TSH, ft3, ft4, and TSHs), even after excluding patients receiving LT4. There were also no differences in maternal and child antibodies (a-TPO, a-Tg, and TRAb) with regards to iodine supplementation, tested in both AIT(+) and AIT (−) subgroups.

All thyroid antibodies of mothers and their children correlated positively (a-TPO with $p < 0.001$, $R = 0.59$; a-Tg with $p < 0.001$, $R = 0.52$; TRAb with $p < 0.001$, $R = 0.49$). Additionally, the children's ft4 correlated with maternal TRAb level ($p = 0.03$, $R = -0.26$), and cord blood TSH correlated with the mothers' UIC ($p = 0.03$, $R = 0.25$).

A comparison of the three groups of women receiving external iodine source, that is, iodine without LT4, LT4 without iodine and the combined iodine and LT4 intake, revealed no significant differences regarding UIC ($p = 0.18$) or UIC/crea ($p = 0.09$). However, the highest ioduria was reported in the group with the combined LT4 and iodine intake; see Figure 2.

TABLE 1 Clinical and biochemical characteristics of the recruited women and newborns.

Clinical parameter (median, IQR)	Mothers [*n* = 91]	Newborns [*n* = 101] [b]
Age, years	33 (31–35)	
Body weight before delivery, kg	62 (56–70)	
Pregnancy week at delivery	39 (38–40)	
Body weight at delivery, g		3415 (3100–3650)
5th minute Apgar score		10 (10–10)
LT4 treated/non-treated, number of patients	50/41	
LT4 dose in 3rd trimester, µg/24h	75 (50–91)	
AIT [+]/AIT [−], number of patients	18/71	
Biochemical parameter (median, IQR)	**Mothers [*n* = 89]**	**Newborns [*n* = 94]**
TSH from venous/cord blood, µU/ml	1.99 (1.32–2.81)	8.53 (5.91–11.2)
TSHs, mIU/liter [*n* = 101]		1.88 (0.63–2.78)
ft3, pmol/liter	4.05 (3.64–4.38)	2.05 (1.78–2.35)
ft4, pmol/liter	13.7 (12.2–15.5)	16.4 (15.1–17.6)
a-TPO, IU/ml	12 (10–20)	9 (9–12)
a-Tg, IU/ml	13 (11–17)	13 (10–15)

LT4, levothyroxine; AIT, autoimmune thyroiditis: present [+], absent [−]; IQR, interquartile range; TSH, thyrotropin; TSHs, TSH at 3rd–4th day of life; ft3, free triiodothyronine; ft4, free thyroxine; a-TPO, anti-thyroid peroxidase; a-Tg, anti-thyroglobulin; [b] data comprises 10 twin pregnancies.

Women with UIC/crea within 150–249 µg/g presented the lowest values of TSH (in the subgroup after excluding LT4-treated patients) and a-TPO (in the subgroup with the negative antithyroid antibodies) in comparison with those with a lower or higher ioduria (Table 4).

Additionally, TSHs was above 5 mIU/liter in 6% of newborns. All newborns showed TSH < 12 mIU/liter (one had > 10 mIU/liter); thus, none of the children required further investigation for congenital HT.

4 Discussion

Our study aimed to characterize the iodine status of pregnant women in the leading obstetric center in western Poland, as well as to analyze whether supplementation involving regular nutritional supplement preparations was effective in correcting iodine deficiency. The data indicate that the presented group of pregnant women residing in the area of western Poland is iodine deficient, despite the fact that the entire country is considered to be optimally iodine-supplied at the population level. Approximately one-third of pregnant women denied supplementing iodine in the course of pregnancy, and many supplemented the microelement inappropriately with regard to the onset and duration of the supplementation, disregarding both Polish and international guidelines.

TABLE 2 Median ioduria of pregnant women according to the WHO classification.

Category of iodine supply in population of pregnant women	WHO criteria for ioduria, expressed by a median UIC, µg/liter or UIC/crea, µg/g
Severe deficiency	< 50
Insufficient	< 150
Sufficient	150–249
Above requirements	250–499
Excessive	> 500

WHO, World Health Organization; UIC, urinary iodine concentration; UIC/crea, iodine/creatinine ratio.

Despite the differences in UIC among the supplemented and non-supplemented subgroups of women found in the literature, no study has shown sufficient iodine concentrations in pregnant Polish women with regard to the UIC reference ranges established by the WHO (see Table 5), with the exception of the data provided in one interventional study by Jastrzębska et al. (22). In the presented study, the supplemented group did not show a significantly higher UIC, although the subjects declared taking a proper iodine dose. The abovementioned notion is in line with the observation that the supplementation rate was one of the highest in comparison with the previous Polish reports, yet it still did not significantly affect the overall ioduria. This may be attributed to inappropriate formulas used by the patients, whereby in our study only one participant received KI in the form of the prescription drug, and the rest used a multi-nutrient over-the-counter pregnancy dietary supplements. In addition, iodine supplementation was frequently initiated too late or discontinued too early (21% of subjects in our cohort received iodine only through a short period). This unfortunate inconsequence of supplementation, was already observed in the previous analyses (23). The only report where borderline sufficient

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TABLE 3 Iodine supplements, doses, and formulas declared by pregnant women.

Percentage of women on iodine supplements, number from total [$n = 62$]	Formula (multivitamin supplements of diet)	Dose, µg/per day
63% (39)	KIO_3, KI	200
31% (19)	KI	150
3% (2)	KI	220/300
1.5% (1)	KI	100
1.5% (1)	Not known	Not known

KIO_3, potassium iodate; KI, potassium iodide.

ioduria was achieved, was an interventional study involving the administration of 150 µg of KI per day in a separate tablet (22). In terms of dietary supplements, KI is the most recommended form, whereas Kelp should be avoided, due to high-dose variation (24). Interestingly, the U.S. market analysis revealed that 24% of prenatal multivitamin supplements did not contain iodine at all, and in those containing iodine, the doses varied considerably (25–290 µg), with still others contained Kelp (25).

Furthermore, the study group had already been evaluated with regard to the selenium intake, where the authors found both poor selenium status and a similar ineffectiveness of self-administered micronutrient supplementation (26). A similar observation was made in Latvia where, despite a wide supplement usage (70%), pregnant women failed to achieve optimal selenium and iodine status. However, the baseline concentrations of both trace elements were higher than in Poland (26). Therefore, local guidelines concerning microelement and vitamin supplementation are necessary to provide the evidence-based medicine background for physicians and self-reported users, with particular emphasis on the most beneficial groups, such as pregnant women and autoimmune thyroiditis patients (27, 28). In other European countries, where salt iodization had been introduced, the optimal ioduria was achieved in school-aged children, although not in pregnant women (29, 30). Similar results were obtained in Portuguese pregnant women in the 1st trimester, where median UIC was 104 µg/liter, 19% had UIC < 50 µg/liter, and the supplementation rate was 57%, despite a decade long official recommendations regarding iodine supplementation during pregnancy, thus, showing poor adherence to the guidelines (31). The low iodine status was associated with poorer knowledge in terms of iodine significance and its sources (32). This may also potentially account for the results obtained in the presented study.

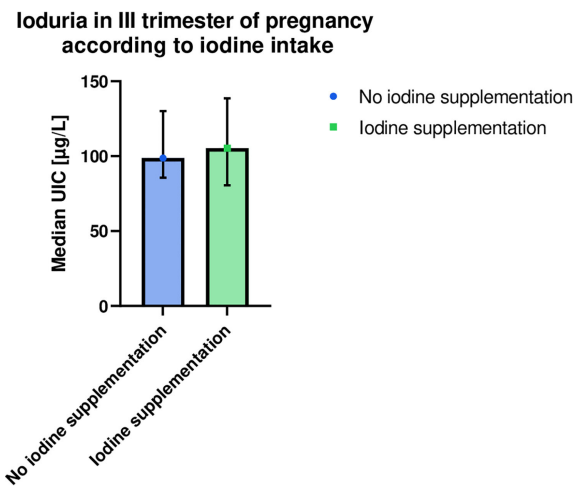

FIGURE 1
Comparison of ioduria in the last trimester of pregnancy in women who confirmed or denied iodine supplementation. Higher median UIC and wider confidence interval (95% CI) were observed in the iodine supplementing group, although the result was not significant ($p = 0.55$). Both intervals are below the normal range established by the World Health Organization (< 150 µg/liter). Medians (symbols), 95% CI (error bars). UIC, urinary iodine concentration.

Notably, our cohort included also hypothyroid patients, some of whom were treated with LT4, which releases 64% of iodine itself. Possibly, the local population, or individuals with a disturbed thyroid function, experienced excessive local tissue iodine deficits in the thyroid gland, or the need for this micronutrient is too great to be effectively supplemented with the recommended dose. Nevertheless, in our further calculations, we excluded the hypothyroid patients with abnormal TSH, based on the population-adjusted referenced values, established by the Polish Society of Endocrinology for the 3rd trimester of pregnancy (TSH 0.11–3.53). Additionally, bearing in mind the effect of iodine supplementation on thyroid function, in calculations comprising thyroid parameters, only women not receiving LT4 therapy were included, and accounted for the presence or absence of AIT.

Only women in the 3rd trimester of pregnancy were enrolled in this study (at one time point—a few days before the delivery, following hospital admission) in order to avoid inconsistency due to changes in UIC during pregnancy, where increased urine volume may lead to a decrease in iodine concentration in comparison with the 1st trimester (33). This may account for the lower value of UIC in this study in comparison with research including also the 1st and 2nd trimesters of pregnancy. Nevertheless, it should be emphasized that the second half of the 1st trimester is the most crucial time for the newborn's maternal iodine intake, as the placenta is the only source of supplying a sufficient amount of T3 for the development of the fetal central nervous system until midgestation (2).

Conversely, elevated population ioduria may be harmful when median UIC exceeds 500 µg/liter, or the daily intake increases above 500 µg (twofolds higher than recommended). Elevated iodine may serve as an endocrine disruptor, causing oxidative damage to lipid membranes, disturbing thyroid hormones metabolism, or enhancing autoimmune processes, particularly in vulnerable individuals with preexisting iodine deficiencies (34). It is vital to note that, according to one report, iodine and selenium content in multivitamin supplements exceeds the amount stated on the label by the manufacturer, by up to 25% (35). Therefore, it is essential to avoid overdosing, or ingesting unknown microelements dosages, as well as poorly characterized supplement mixtures.

According to the WHO, iodine sufficiency at pregnancy, expressed by ioduria, can be estimated only on the population level (due to the significant intra- and interindividual differences). It reflects the total iodine consumption within the past few days. The

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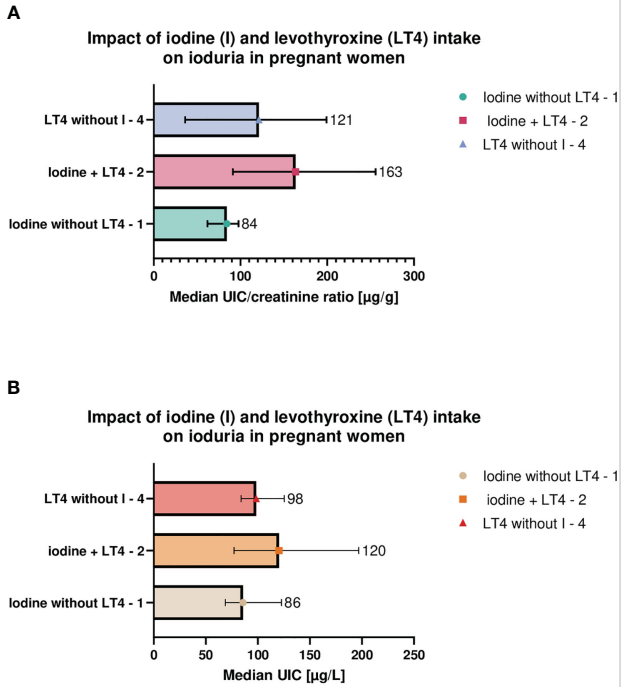

FIGURE 2
Maternal urinary iodine concentration **(A)** and iodine/creatinine ratio **(B)** in relation to levothyroxine and/or iodine supplements intake. Pregnant women differed with respect to iodine **(I)** and levothyroxine (LT4) intake and were divided into three groups; I only (group 1), LT4 only (group 2), or the combined I and LT4 intake (group 3). No significant differences in urinary iodine concentration (UIC, p = 0.18, KW-H = 3.46) and iodine/creatinine ratio (UIC/crea, p = 0.09, KW-H = 5.84) between the groups were observed. However, the highest UIC or UIC/crea were observed in the group with the combined LT4 therapy and I supplementation. Medians (symbols and numbers), 95% CI (error bars) are shown. UIC/crea, urine/creatinine ratio; LT4, levothyroxine; I, iodine; KW-H, the result of Kruskal–Wallis test.

median UIC values between 150 and 249 µg/liter are considered an optimal supply. As iodine is excreted in > 90% by kidneys, UIC in a spot urine sample is a biomarker of iodine status during gestation in epidemiological studies, recommended by the WHO (36). Nonetheless, in the course of pregnancy, physiological increase in renal filtration and urine iodine dilution may result in underestimating iodine concentration level. Moreover, hydration status and day-to-day iodine concentration differences may also impact the results. Hence, a reasonable additional indicator seems to be UIC/crea ratio, which reduces the impact of changes in urine volume. In pregnant women, this biomarker correlates with 24h urine iodine collection, and is consistent with serum concentration during pregnancy (33). Bearing in mind the aforementioned, in this study, both biomarkers were assessed. Additionally, for the purpose of the presented study, a new inexpensive and selective ion-pair HPLC-UV technique for iodine assessment in human urine was developed and validated. In comparison with the former method, inductively coupled plasma mass spectrometry (ICP–MS), HPLC-UV is less complicated and does not require any advanced equipment. Compared with the spectrophotometric method, using the Sandell–Kolthoff reaction (S–K), HPLC-UV does not involve the ingestion of the initial material, or the use of noxious substances (arsenic and cerium) (19, 37). Moreover, HPLC-UV provides better selectivity of the analytes to be determined in such a complex biological matrix as urine.

The impact of iodine intake on the thyroid may be reflected by the lowest TSH and a-TPO of mothers in the subgroup where ioduria was within the recommended ranges. Analogously, the Chinese cohort of more than a thousand pregnant women revealed lower TSH in the group with UIC 150–249 µg/liter, than in those with UIC 250 µg/liter or above, and estimated the 2.5 higher risk for developing subclinical HT in late pregnancy for women with UIC lower than 100 µg/liter in the 1st trimester (38). Two randomized controlled trials demonstrated a lower increase in TSH throughout pregnancy when supplemented with 200–225 µg iodine/day and in one study, and a lower maternal TSH was found at the 1st trimester in the supplemented group (39, 40). Additionally, in our study, a weak-positive correlation was observed between maternal UIC and neonatal TSH from the cord blood. However, according to meta-analyses, in the majority of studies, iodine supplementation itself did not impact maternal TSH, ft4, a-TPO and neonatal TSH (41). Low UIC (< 100 µg/liter) was found as an independent risk factor for positive a-Tg among iodine-sufficient population (42). Our research indicated that the lowest a-TPO was seen in the subgroup with the optimal ioduria. In terms of the reluctance to supplement iodine in pregnant women suffering from AIT, due to the fact that excessive iodine is considered a trigger of antithyroid autoimmunity, no significant increase in a-TPO was found after administrating a dose of 100 or 150 µg daily (43), and the prevalence of a-TPO positivity among Iranian pregnant women did not increase 2 years following introduction of a national iodine supplementation (44). Notably, in our study, a

TABLE 4 The impact of maternal ioduria (according to the WHO criteria) on thyroid parameters.

	UIC/crea, µg/g			H	P
	< 150	150–249	> 249		
M UIC/crea, µg/g [Me, Q1–Q3]	64.9 ^{c,d} [35.7–91.2]	182.8 ^{c} [164.3–203.5]	348.2 ^{d} [271–457.1]	60.4	< 0.01
M TSH, µU/ml [Mean, SD]*	2.06 ± 0.82 ^{b}	1.42 ± 0.69 ^{b}	1.97 ± 0.73	7.9	0.02
M a-TPO, IU/ml [Me, Q1–Q3]**	11^{e} [10–13.5]	10 ^{a,e} [9–11]	13 ^{a} [10.5–16]	7.96	0.02

UIC/crea, iodine/creatinine ratio; Me, median; H, the result of Kruskal–Wallis test; P, level of significance for Kruskal–Wallis test; Q1, first quartile; Q3, third quartile; M, mothers; TSH, thyrotropin; a-TPO, anti-thyroid peroxidase; post-hoc analysis revealed significant differences between the labeled subgroups with ^{a}p = 0.025, ^{b}p = 0.014, ^{c}p < 0.001, ^{d}p < 0.001, ^{e}p = 0.063; *analysis among patients not treated with levothyroxine; **analysis in the group of patients with excluded elevated antithyroid antibodies levels.

TABLE 5 A comparison of the Polish studies with regard to pregnancy ioduria in the past 14 years.

Author and type of the study	Year	Region of Poland	Number of women	Trimester	Median UIC, µg/liter	Amount of women with UIC ≥ 150 µg/liter	Neonatal TSH > 5 mIU/liter on 3rd day of life	Method of UIC measurement	Iodine supplemented (supplementation rate) *vs.* non-supplemented
D Filipowicz, (2022) [cross-sectional]	2019–2021	Western (Poznań)	91	3rd	106	28.5%	6%	HPLC–UV (spot urine sample)	105 (68%) *vs.* 99, $p = 0.55$
M Trofimiuk-Müldner, (2020) (13) [cross-sectional]	2017	Northern, southern, central, north-eastern, south-eastern	300	1st (14.7%) 2nd (21%) 3rd (64.7%)	112	–	–	Sandell–Kolthoff reaction (spot urine sample)	–
H Jastrzębska, (2016) (22) [prospective]	2008–2013	Central (Warsaw)	92	1st	1st - 83 3rd - 101	–	8.77%	Sandell–Kolthoff reaction (spot urine sample)	151.5 (100% in 2nd and 3rd) *vs.* 101, $p < 0.01$
M Krasnodebska-Kiljańska, (2013) (17) [prospective]	–	Central (Warsaw)	62	1st	1st - 96 2nd - 122 3rd - 129	14%	4.41%	–	(100% in 2nd and 3rd)
A Zygmunt, (2015) (18) [cross-sectional]	2010	Central (Łódź)	115	1st (6%) 2nd (53%) 3rd (41%)	1st - 80.1 2nd - 81.3 3rd - 78.4	–	2%	Sandell–Kolthoff reaction (spot urine sample)	129.4 (45%, 1st - 0, 2nd - 24.6%, 3rd - 78.7%) *vs.* 73.0, $p < 0.001$
M Gietka-Czernel, (2010) (12) **[cross-sectional]**	2007-2008	Central (Warsaw)	100 (72)	1st (32%) 2nd (36%) 3rd (32%)	112.6	28%	2.9%	Sandell–Kolthoff reaction (24h urine collection)	146.9 (35%, 1st - 31%, 2nd - 39%, 3rd - 34%) *vs.* 97.3, $p < 0.001$
T Milewicz, (2011) (16) [cross-sectional, survey]	–	Southern (Kraków)	500	–	–	–	–	–	(59%)

UIC, urinary iodine concentration; TSH, thyrotropin; HPLC-UV, high-performance liquid chromatography with ultraviolet detection.

positive correlation was found between all maternal and newborns' thyroid autoantibodies, which proved maternofetal transplacental transfer of IgG antibodies, since a neonate is unable to produce antibodies during the first months of life. Moreover, higher maternal TRAb concentration was related to a lower fetal ft4 level, which may be attributed to the presence of TSH receptor blocking autoantibodies fraction, detected by TRAb assessment, which reduce fetal thyroid hormones production.

Neonatal TSHs evaluated in a dried whole blood sample (heel prick) 3 to 4 days after birth may serve as a sensitive indicator of population iodine deficiency. The latter is recognized when TSH is above 5 mIU/liter in more than 3% of neonates, as presented in this study (20, 45). Newborns poorly supplied in iodine showed an elevated thyroidal iodine turnover. As a consequence, TSH increases in the first weeks of life, causing neonatal transient hyperthyrotropinemia. This condition resolves spontaneously after 2 weeks and should be distinguished from the physiological TSH elevation in the first 36h of life due to perinatal stress. Nevertheless, neonatal transient hyperthyrotropinemia increases the risk of developing persistent hyperthyrotropinemia in childhood (46). In the presented study, TSH elevation in the first minutes of life was verified after 3 days, and achieved physiological concentrations in all newborns, showing no correlations with former TSH results. Therefore, it should be emphasized that only a delayed TSH assessment should be taken into account.

The American Thyroid Association guidelines recommend supplementation of 150-µg iodine daily during pregnancy and lactation, although the optimal ioduria was demonstrated in this geographical area (47). In contrast to the Polish guidelines in hypothyroid women treated with LT4, they discourage additional supplementation of iodine. In the present study, among patients treated with LT4, supplementing only iodine or receiving both LT4 and iodine, no significant difference was found. However, it is worth bearing in mind that the addition of iodine to LT4 in hypothyroid

TABLE 6 Summary of the recommendations for iodine supplementation in pre-conception, pregnancy and the lactation period.

Author and publication year	Guideline title	Iodine dose per day and the formulation	Time of supplementation initiation and duration	The recommendation in case of thyroid disturbances
A Hubalewska-Dydejczyk, (2021) (14)	Thyroid diseases in pregnancy: Guidelines of the Polish Society of Endocrinology	150–200 µg of iodine or 400 mg of iodized oil per year	Pregnancy planning (50 µg), pregnant and breastfeeding	150 µg/day in mild hypothyroidism treated with a low dose of LT4 [b], lower iodine dose in high dose of LT4
M Zimmer, (2020) (48)	Polish Society of Gynecologists and Obstetricians recommendations on supplementation during pregnancy	150–200 µg	Pregnancy	Supplementation under control of thyroid hormones and antithyroid antibodies level
EK Alexander, (2017) (24)	2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum	150 µg of potassium iodide or 400 mg of iodized oil per year	3 months before pregnancy, pregnancy and lactation	No supplementation in hyperthyroidism and in hypothyroidism treated with LT4 [a]
L De Groot, (2012) (49)	Management of Thyroid Dysfunction during Pregnancy and Postpartum: An Endocrine Society Clinical Practice Guideline	150–200 µg of potassium iodide or iodate	Before conception, pregnancy and lactation	–
J H Lazarus (2014) (50)	2014 European thyroid association guidelines for the management of subclinical hypothyroidism in pregnancy and in children	150 µg of potassium iodide	Before conception, pregnancy and lactation	Need for studies on the efficacy and side effects of combined LT4 and iodine or iodine alone in subclinical hypothyroidism

LT4, levothyroxine; [a]weak recommendation, low-quality evidence; [b]strong recommendation, low-quality evidence.

patients may improve the iodine status to a greater extent than iodine or LT4 alone, which would favor local recommendations (see Table 6). This is supported by another Polish study, where optimal UIC was achieved after the addition of 150 µg of KI to the standard LT4 dose (22). Nevertheless, more studies are necessary to prove the currently observed tendency.

The conducted study also has a few limitations. Median ioduria was assessed in the heterogenous group in terms of the thyroid status (including euthyroid, hypothyroid women, with and without AIT, patients receiving LT4). The data concerning supplement intake were interview-based; thus, it is impossible to exclude the bias of underreporting. Additionally, no detailed daily food questionnaire was performed; hence, the impact of individual diets, possibly rich in iodine, was also not addressed. However, any specific diet followers and individuals residing abroad for longer periods were excluded, and due to obligatory salt iodization, the population baseline iodine status was assumed to be nearly equal. Iodine was assessed only in the 3rd trimester of pregnancy, where the most complete iodine status would have been documented, with the assessments performed at least once per trimester, including the most crucial for the neonatal development —the 1st trimester.

In most countries, universal salt iodization programs are ineffective in restoring adequate maternal ioduria with regard to the ranges established by the WHO. Despite the recommendation to additionally supplement iodine during pregnancy, the real-life assessment of this trace element supplementation revealed the ineffectiveness of the current model. An interventional study would presumably need to be conducted in order to verify the effectiveness of the dose and avoid a possible bias due to self-reporting supplementation, irregularity in the supplement intake (particularly in the end of pregnancy), HT, or LT4 impact, as well as diversity of the preparations declared. Physicians should consider prescribing KI as a medication (verified composition), and it should possibly come as a separate formulation. It is essential to increase the awareness of endocrinologists, gynecologists, general practitioners, and the society, especially pregnant women, regarding the significance and the benefits of a proper iodine supplementation during pregnancy for mothers and their newborns to restore appropriate levels of this element.

In conclusion, despite the relevant guidelines, the analyzed group of pregnant women from the western Poland demonstrated an insufficient iodine status, which may present potential negative implications for pregnancy and child development. Considering that this issue is preventable, additional measures are essential in order to provide a more comprehensive information to attending physicians and medical caregivers, as well as to the general public, including young women, and to improve the iodine intake in pregnancy to the level which safely allows to avoid iodine deficiency.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Local Bioethics Committee of Poznan University of Medical Sciences (protocol no. 104/19, date of approval: 10 January 2019, annexed 4 February 2021, protocol no. 132/21). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, MR, DF, ES-P. Methodology, DF, FG, MK-Ł, AM-S, KS. Statistical analysis, DF. Investigation, DF, MR, ES-P. Data collection, DF, KS, MO. Writing - original draft preparation, DF. Writing - review and editing, MR, ES-P, LS, KS, FG, MK-Ł, AM-S, MO. Supervision, MR, ES-P. Funding acquisition, DF, MR. 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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The effect of peak serum estradiol level during ovarian stimulation on cumulative live birth and obstetric outcomes in freeze-all cycles

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Objective: To determine whether the peak serum estradiol (E2) level during ovarian stimulation affects the cumulative live birth rate (CLBR) and obstetric outcomes in freeze-all cycles.

Methods: This retrospective cohort study involved patients who underwent their first cycle of *in vitro* fertilization followed by a freeze-all strategy and frozen embryo transfer cycles between January 2014 and June 2019 at a tertiary care center. Patients were categorized into four groups according to quartiles of peak serum E2 levels during ovarian stimulation (Q1-Q4). The primary outcome was CLBR. Secondary outcomes included obstetric and neonatal outcomes of singleton and twin pregnancies. Poisson or logistic regression was applied to control for potential confounders for outcome measures, as appropriate. Generalized estimating equations were used to account for multiple cycles from the same patient for the outcome of CLBR.

Result(s): A total of 11237 patients were included in the analysis. Cumulatively, live births occurred in 8410 women (74.8%). The live birth rate (LBR) and CLBR improved as quartiles of peak E2 levels increased (49.7%, 52.1%, 54.9%, and 56.4% for LBR; 65.1%, 74.3%, 78.4%, and 81.6% for CLBR, from the lowest to the highest quartile of estradiol levels, respectively, $P<0.001$). Such association remained significant for CLBR after accounting for potential confounders in multivariable regression models, whereas the relationship between LBR and peak E2 levels did not reach statistical significance. In addition, no significant differences were noticed in adverse obstetric and neonatal outcomes (gestational diabetes mellitus, pregnancy-induced hypertension, preeclampsia, placental disorders, preterm birth, low birthweight, and small

for gestational age) amongst E2 quartiles for either singleton or twin live births, both before and after adjustment.

Conclusion: In freeze-all cycles, higher peak serum E2 levels during ovarian stimulation were associated with increased CLBR, without increasing the risks of adverse obstetric and neonatal outcomes.

KEYWORDS

estradiol, cumulative live birth, freeze-all, embryo transfer, obstetric outcome

1 Introduction

Controlled ovarian stimulation (COS) is undoubtedly one of the milestones in assisted reproductive treatments (1), which has resulted in a significant increase in pregnancy rates as compared with unstimulated *in vitro* fertilization (IVF) cycles (2, 3). However, COS, by stimulating multi-follicular growth, often increases serum estradiol (E2) to supraphysiologic levels, and the question of whether high E2 levels during COS may influence reproductive outcomes has been a matter of debate over the past few decades (4, 5). Existing data have reported that there may be a detrimental effect of high E2 level, which could lead to impaired endometrial receptivity (6–8). In addition, the increased incidence of ovarian hyperstimulation syndrome (OHSS) with high E2 exposure cannot be neglected (9). Studies have also suggested that a high response to ovarian stimulation may affect the quality of oocytes or embryos by altering the epigenetic programming of oocytes including DNA methylation, histone acetylation and epigenetic modifier expression (10–13), and potentially resulting in higher risks of implantation failure and pregnancy loss (14–16). Another concern is that the effect of supraphysiologic E2 level may further extend into placentation and subsequent fetal development, leading to higher risks of preeclampsia, low birthweight, and small for gestational age (SGA) (17–19).

Yet, published studies addressing the association between peak E2 level and pregnancy-related outcomes have focused mainly on fresh IVF cycles (4, 5), where top-quality embryos of the cohort were transferred into a suboptimal peri-implantation environment. In addition, very few data have reported the outcome of CLBR following multiple embryo transfer cycles after COS, which is of utmost importance to understand whether supraphysiologic E2 level during COS could affect the entire cohort of embryos. Taking into account the advances in cryopreservation technique, frozen embryo transfer (FET) has become an alternative to fresh embryo transfer (20), and FET cycles have contributed to an increased chance of live birth and better perinatal outcomes in clinical practice (21–23). Thus, it is vital to evaluate whether the high E2 levels during COS have any effects on CLBR and placentation following FET.

Given the increased utilization of the freeze-all strategy (24), which provides a novel model to assess separately the impact of ovarian stimulation on oocyte and embryo quality to that on the endometrium, we conducted the present study to investigate the association between peak serum E2 level during COS and CLBR, as well as obstetric and neonatal outcomes in freeze-all cycles.

2 Materials and methods

2.1 Study design

This retrospective study was conducted at the Reproductive Center of Ren Ji Hospital of Shanghai Jiao Tong University School of Medicine. All patients aged 20-40 years old, undergoing their first autologous cycle of IVF or intracytoplasmic sperm injection (ICSI) treatment followed by a freeze-all strategy between January 2014 and June 2019 were reviewed for eligibility (Figure 1). Women who utilized gonadotropin-releasing hormone (GnRH) agonist or antagonist protocol for COS were included. Excluded were individuals diagnosed with congenital uterine malformation, or with untreated diabetes and hypertension, those with no viable embryos for transfer, and those undergoing preimplantation genetic testing or freezing of oocytes. Cycles with remaining frozen embryos that have not yet achieved a live delivery and those without available information on peak serum E2 level during COS and pregnancy outcomes were also excluded. The study protocol was approved by the Institutional Review Board of the hospital.

2.2 Ovarian stimulation protocols

Protocols for ovarian stimulation were determined at the discretion of patients' preference and physicians' recommendation. COS was performed with injections of 150-300 IU/day recombinant follicle-stimulating hormone (rFSH, Merck Serono) and/or urinary human menopausal gonadotropin (uHMG, Ferring). The starting dose was individualized based on the patient's age, body mass index (BMI), and ovarian reserve makers. For patients using GnRH-agonist long protocol, Triptorelin (0.05 mg daily, Ferring) was administered on day 7 after ovulation and lasted for 10-14 days. For those using GnRH-agonist short protocol, Triptorelin (0.1 mg daily, Ferring) was injected starting on day 2 or 3 of their menstrual cycle and continued until the trigger day. In participants using the GnRH-antagonist protocol, the antagonist (0.25 mg by daily subcutaneous injection,

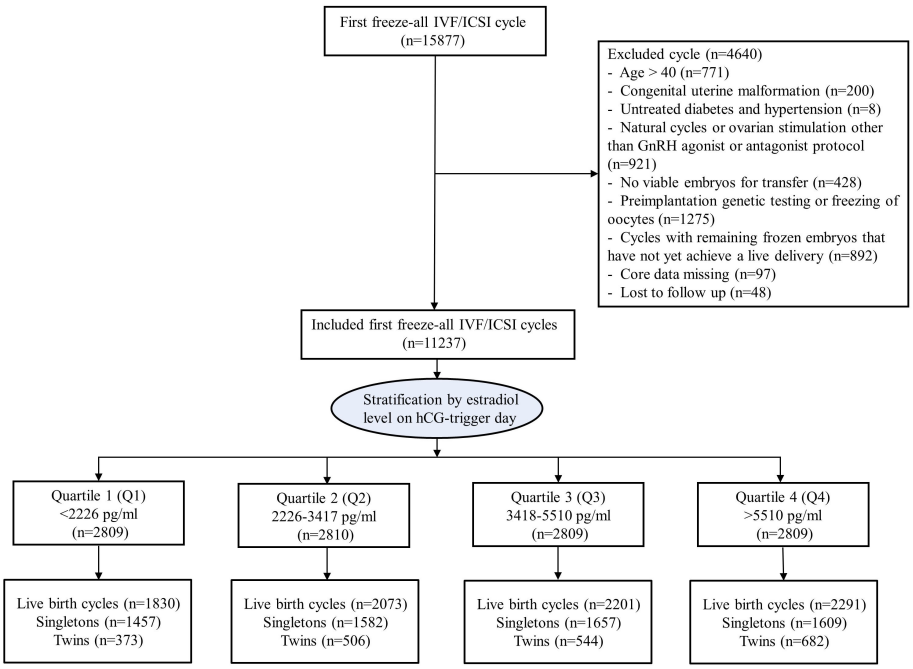

FIGURE 1
Flow chart.

Vetter Pharma-Fertigung GmbH & Co. KG or Merck Serono) was introduced when the leading follicle reached 12mm in average diameter.

Follicle development during COS was monitored by serial transvaginal ultrasound and serum E2, luteinizing hormone (LH), and progesterone (P) levels starting from day 4-5 of stimulation. Monitoring frequency was individualized, and the dose of gonadotropin (Gn) was adjusted accordingly. Final oocyte maturation was induced by administering 250 μg of recombinant human chorionic gonadotropin (hCG, Merck Serono) when at least one lead follicle reached 18 mm in mean diameter. Oocyte retrieval was conducted by vaginal ultrasound-guided puncture 36 hours later.

2.3 IVF, endometrial preparation, and embryo transfer

Retrieved oocytes were fertilized either via conventional IVF or ICSI based on serum analysis. Fertilization was examined 16-18 hours post insemination or microinjection by the presence of two pronuclei. Then the embryos were placed into individual droplets of cleavage culture medium (G1.5, Vitrolife, Gothenburg, Sweden) for three consecutive days and in the sequential culture medium (G2.5, Vitrolife, Gothenburg, Sweden) thereafter. Cleavage embryos with ≥6 blastomeres and <20% fragmentation on day 3 were defined as good quality and were frozen by vitrification. Embryos that did not meet the criteria were extendedly cultured for blastocyst, and those scored ≥4BC were eligible for vitrification on day 5 or 6 according to the Gardner criteria (25). Blastocysts scored ≥3BB were defined as good-quality embryos. Culture media, laboratory conditions, and procedures remain unchanged during the study period.

Endometrial preparation was performed in an artificial cycle, a modified natural cycle or a stimulated cycle. The endometrial preparation regimen was based on the physicians' discretion. For the artificial cycle, oral administration of estrogen valerate (4-6 mg daily, Bayer Vital GmbH) was started on day 2-5 of the menstrual cycle, vaginal progesterone gel (90 mg daily; Merck Serono) and oral dydrogesterone (10 mg, 2-3 times daily; Abbott) were added when endometrial thickness reached 7 mm. For the modified natural cycle, ovulation was determined by serum hormone levels and ultrasound monitoring. For the stimulation cycle, letrozole (2.5 mg daily, Hengrui Pharma) was orally administered on cycle day 3 for 5 days, and follicle growth was monitored from cycle day 10. If the diameter of the dominant follicle was <14 mm, an additional 75 IU of uHMG was supplemented until the diameter ≥17 mm. If the diameter of the dominant follicle was ≥14 mm on cycle day 10, no more uHMG was given. In both the modified natural and stimulation cycles, ovulation was triggered by hCG either when the mean diameter of the dominant follicle was ≥17 mm or when the serum luteinizing hormone (LH) surge was detected, and oral dydrogesterone (10 mg, 2-3 times daily) was started 2 days after triggering for luteal phase support. In all FET cycles, no more than two embryos were transferred. Cleavage-stage embryos were transferred three days after progesterone administration and blastocysts were transferred five days after progesterone administration. If pregnancy was achieved, luteal support was continued to 10-12 weeks of gestation.

2.4 Outcome measures

The primary outcome of the study was CLBR. The secondary outcomes included obstetric and neonatal outcomes of live births,

as well as pregnancy outcomes of the first FET cycle, namely the implantation rate, clinical pregnancy rate, early miscarriage rate, and live birth rate (LBR).

The implantation rate was defined as the number of fetal heartbeats observed per number of embryos transferred. Clinical pregnancy was defined as the observation of at least one gestational sac at 6-8 weeks of gestation. Early miscarriage rate was defined as a loss of clinical pregnancy before the 12th gestational week. Live birth was defined as the delivery of at least one living child (≥28 weeks of gestation). CLBR was calculated by including only the first live birth born after all FET cycles resulting from the associated ovarian stimulation.

Obstetric outcomes included gestational diabetes mellitus (GDM, 10th revision of the International Statistical Classification of Diseases and Related Health Problems [ICD-10] code O24.4), pregnancy-induced hypertension (PIH, ICD-10 code O13), preeclampsia (ICD-10 code O14-O15), and placental disorders (placenta previa [ICD-10 code O44], placental abruption [ICD-10 code O45], placenta accreta, placenta increta, or placenta percreta [ICD-10 code O43.21, O43.23]). Neonatal outcomes included gestational age, birthweight, preterm birth, low birthweight, SGA, and birth defects (ICD-10 codes Q00-Q99). Preterm birth was defined as delivery before 37 complete weeks of gestation. Low birthweight was defined as birthweight <2500g. SGA was defined as birthweight <10th percentile of gender-specific birthweight reference at the same gestational week (26). The dataset collected maternal and neonatal conditions from electronic medical records of neonates born in our university hospital. While for neonates delivered elsewhere, the information was obtained from responsible obstetricians and/or pediatricians at the local hospitals.

2.5 Statistical analysis

Patients were categorized into groups according to quartiles (Q1-Q4) of peak serum E2 levels on hCG-trigger day: Q1 (<2226 pg/ml), Q2 (2226-3417 pg/ml), Q3 (3418-5510 pg/ml) and Q4 (>5510 pg/ml). Descriptive statistics were presented as mean (standard deviation [SD]) or numbers and percentages according to the nature of the variables. The distribution of normality was tested by the Kolmogorov–Smirnov test, and nonparametric tests were preferred according to the results. The Mann-Whitney test was applied to analyze the between-group differences of continuous variables, while comparisons of categorical variables were performed by Pearson's chi-squared test. Poisson regression was performed to investigate the effect of peak E2 level on implantation, and logistic regression was used to evaluate the impact of peak E2 level on clinical pregnancy, early miscarriage, live birth, and obstetric and neonatal outcomes. Multivariable generalized estimating equations (GEE) analysis was applied to fit the logistic regression models and further explored the possible relationship between E_2 level and CLBR by accounting for the clustering of FET cycles within individuals. For pregnancy outcomes, confounding factors adjusted in the multivariable models included: maternal age, maternal BMI, primary or secondary infertility, parity, basal FSH, infertility diagnosis, protocol for stimulation, P level on hCG-trigger day, IVF or ICSI, endometrial preparation regimen, embryo developmental stage, embryo quality, and number of embryos transferred. For obstetric and neonatal outcomes, factors including maternal age, maternal BMI, primary or secondary infertility, parity, infertility diagnosis, protocol for stimulation, endometrial preparation regimen, embryo developmental stage, embryo quality, and number of embryos transferred were adjusted. The group of Q1 was taken as the reference group.

Additionally, the predictive probability of cumulative live birth according to E2 levels on hCG-trigger day and maternal age was evaluated using the generalized additive model (GAM). All statistical analyses were performed using R statistical programming language (version 4.2.1; R Foundation for Statistical Computing, Vienna, Austria). Two-tailed *P*-value <0.05 was considered statistically significant.

3 Results

3.1 Patient demographic and cycle characteristics

A total of 11237 women were included in the analysis, with an average age of 29.7 ± 3.8 years and a BMI of 21.8 ± 3.2 kg/m^2. The mean ± SD peak serum E2 level on hCG-trigger day in the study cohort was 4065.7 ± 2456.6 pg/ml. The patients' baseline and cycle characteristics are presented in Table 1. Women with peak E2 levels in the highest quartile (Q4) were younger, with lower BMI, and were more likely to be diagnosed with primary infertility and polycystic

TABLE 1 Patient demographic and cycle characteristics.

Variable	Overall	Q1 (<2226 pg/ml)	Q2 (2226-3417 pg/ml)	Q3 (3418-5510 pg/ml)	Q4 (>5510 pg/ml)	*P* value
No. of patients	11237	2809	2810	2809	2809	
Maternal age (y)	29.7 ± 4.0	30.6 ± 4.2	29.8 ± 3.9	29.4 ± 3.8	29.0 ± 3.8	<0.001
Maternal BMI (kg/m^2)	21.8 ± 3.2	22.6 ± 3.4	22.0 ± 3.1	21.6 ± 3.1	21.2 ± 2.8	<0.001
Infertility duration (y)	3.2 ± 2.3	3.2 ± 2.3	3.2 ± 2.3	3.3 ± 2.3	3.3 ± 2.2	0.035
Primary infertility	7629(67.9)	1811(64.5)	1867(66.4)	1956(69.6)	1995(71.0)	<0.001

(Continued)

TABLE 1 Continued

Variable	Overall	Q1 (<2226 pg/ml)	Q2 (2226-3417 pg/ml)	Q3 (3418-5510 pg/ml)	Q4 (>5510 pg/ml)	*P* value
Parity						<0.001
0	10407(92.6)	2560(91.1)	2574(91.6)	2623(93.4)	2650(94.3)	
≥1	830(7.4)	249(8.9)	236(8.4)	186(6.6)	159(5.7)	
Basal FSH (IU//L)	6.6 ± 1.7	6.9 ± 2.0	6.5 ± 1.7	6.5 ± 1.6	6.4 ± 1.6	<0.001
Infertility diagnosis						<0.001
Tubal	6103(54.3)	1541(54.9)	1526(54.3)	1536(54.7)	1500(53.4)	0.697
Diminished ovarian reserve	439(3.9)	336(12.0)	100(3.6)	3(0.1)	0(0)	<0.001
PCOS	2777(24.7)	546(19.4)	708(25.2)	726(25.8)	797(28.4)	<0.001
Endometriosis	874(7.8)	273(9.7)	244(8.7)	195(6.9)	162(5.8)	<0.001
Male factors	4021(35.8)	1036(36.9)	1015(36.1)	967(34.4)	1003(35.7)	0.275
Other	419(3.7)	103(3.7)	126(4.5)	117(4.2)	73(2.6)	0.001
Protocol for ovarian stimulation						<0.001
GnRH-agonist long	3887(34.6)	475 (16.9)	845(30.1)	1136(40.4)	1431(50.9)	
GnRH-agonist short	2802(24.9)	1047(37.3)	679(24.2)	561(20.0)	515(18.3)	
GnRH-antagonist	4548(40.5)	1287(45.8)	1286(45.8)	1112(39.6)	863(30.7)	
Total Gn dose (IU)	1453.8 ± 487.0	1494.7 ± 564.0	1457.7 ± 506.4	1457.4 ± 461.5	1405.3 ± 396.7	<0.001
Progesterone level on hCG-trigger day (ng/mL)	1.0 ± 0.7	0.7 ± 0.7	0.8 ± 0.5	1.0 ± 0.9	1.3 ± 0.7	<0.001
Cycles with ICSI	3199(28.5)	770(27.4)	813(28.9)	800(28.5)	816(29.0)	0.512
No. of oocytes retrieved	15.5 ± 7.7	9.4 ± 5.1	14.3 ± 5.7	17.4 ± 6.5	21.1 ± 8.1	<0.001
Fertilization rate	80.9 ± 16.0	80.6 ± 18.8	80.7 ± 16.6	80.9 ± 15.7	81.2 ± 15.5	0.458
No. of viable embryos	5.7 ± 3.6	3.7 ± 2.3	5.2 ± 2.9	6.3 ± 3.5	7.6 ± 4.1	<0.001
No. of good quality embryos	3.4 ± 2.5	2.1 ± 1.2	3.2 ± 2.3	3.6 ± 2.9	4.5 ± 3.4	<0.001
No. of FET cycles	1.5 ± 0.8	1.4 ± 0.6	1.5 ± 0.7	1.5 ± 0.8	1.6 ± 0.9	<0.001
No. of total embryos transferred	2.4 ± 1.3	2.1 ± 1.1	2.3 ± 1.3	2.5 ± 1.4	2.7 ± 1.5	<0.001
No. of cleavage-stage embryos transferred	1.7 ± 1.3	1.4 ± 1.1	1.6 ± 1.3	1.8 ± 1.3	2.0 ± 1.4	<0.001
No. of blastocysts transferred	0.7 ± 1.0	0.7 ± 0.9	0.8 ± 1.0	0.8 ± 1.1	0.7 ± 1.1	<0.001
FET endometrial preparation						<0.001
Artificial cycle	14006(84.0)	3294(85.5)	3507(83.5)	3616(84.2)	3589(82.5)	
Modified natural cycle	1398(8.4)	291(7.6)	332(7.9)	367(8.5)	408(9.4)	
Stimulated cycle	1227(7.7)	253(6.6)	362(8.6)	310(7.2)	352(8.1)	
Moderate or severe OHSS	59(0.5)	3(0.1)	7(0.2)	15(0.5)	34(1.2)	<0.001

Data are presented as mean ± standard deviation or number (%).
BMI, body mass index; FSH, follicle-stimulating hormone; PCOS, polycystic ovarian syndrome; GnRH, gonadotropin-releasing hormone; Gn, gonadotropin; IU, in units; hCG, human chorionic gonadotropin; ICSI, intracytoplasmic sperm injection; FET, Frozen embryo transfer; OHSS, ovarian hyperstimulation syndrome.

ovarian syndrome (PCOS). Regarding the outcomes of ovarian stimulation, women with higher E2 quartiles resulted in increased number of retrieved oocytes and good-quality embryos, while fertilization rates remained similar across groups. Furthermore, more subsequent FET cycles were observed in higher E2 quartiles, where more embryos were transferred cumulatively.

Moderate and severe OHSS occurred in 59 patients (0.5%) (27). The rates of OHSS were 0.1%, 0.2%, 0.5%, and 1.2% for Q1, Q2, Q3

and Q4 respectively, which increased significantly across groups (P<0.001).

3.2 Live birth rate and cumulative live birth rate

A total of 8410 (74.8%) women achieved live births following their FET cycles. Pregnancy outcomes of the first FET cycle and CLBR by quartiles of peak E2 levels are shown in Table 2. The clinical pregnancy rate, LBR, and CLBR improved as peak E2 quartiles increased (P<0.01), while the rate of implantation remained similar across different quartiles. The early miscarriage rate was lower in Q4 group compared with Q1 group in univariate analysis. After adjusting for potential confounders in multivariate regression models, results showed no statistically significant between peak E2 level and the rates of implantation, clinical pregnancy, early miscarriage, and live birth following the first FET. However, a positive association was detected between peak E2 level and CLBR after adjustment in multivariate regression and GEE models. The results of each FET cycle for the cumulative live birth are shown in Supplemental Table 1.

Analysis by age strata (<31, 31-34, 35-37, 38-40 years) showed a steady increase in CLBR with the peak E2 levels on hCG-trigger day (Supplemental Figure 1). However, for a given E2 level, CLBR decreased with increasing age, with the most prominent decline observed at 38-40 years old.

3.3 Maternal and neonatal outcomes

There were 6305 singletons (75.0%) and 2105 twins (25.0%) born during the study period (Table 3). No differences were noticed amongst peak E2 quartiles in terms of obstetric complications including GDM, PIH, preeclampsia, and placental disorders for both singleton and twin live births. Birthweights were similar amongst different quartiles. The incidence of preterm birth, low birthweight, SGA, and birth defect were also comparable across

TABLE 2 Pregnancy outcomes and its association with peak serum estradiol levels.

Outcomes	Q1 (<2226 pg/ml)	Q2 (2226-3417 pg/ml)	Q3 (3418-5510 pg/ml)	Q4 (>5510 pg/ml)
Implantation rate [a]				
n, (%)	2012/4352 (46.2)	2202/4594 (47.9)	2307/4787(48.2)	2425/5074(47.8)
Crude OR(95%CI) [c]	Ref.	1.06(0.98-1.16)	1.04(0.95-1.14)	1.02(0.93-1.11)
Adjusted OR(95%CI) [b,c]	Ref.	1.04(0.95-1.13)	1.04(0.94-1.14)	1.08(0.98-1.20)
Clinical pregnancy rate [a]				
n, (%)	1685/2809(60.0)	1742/2810(62.0)	1796/2809(63.9)	1811/2809(64.5)
Crude OR(95%CI) [d]	Ref.	1.09(0.98-1.22)	1.19(1.07-1.32) *	1.21(1.09-1.35) *
Adjusted OR(95%CI) [b,d]	Ref.	0.98(0.88-1.10)	1.03(0.92-1.16)	1.08(0.95-1.22)
Early miscarriage rate [a]				
n, (%)	216/1685(12.8)	211/1742(12.1)	183/1796(10.2)	174/1811(9.6)
Crude OR(95%CI) [d]	Ref.	0.98(0.80-1.19)	0.84(0.68-1.03)	0.79(0.65-0.98) *
Adjusted OR(95%CI) [b,d]	Ref.	1.09(0.89-1.34)	1.01(0.81-1.27)	1.06(0.84-1.35)
LBR [a]				
n, (%)	1397/2809(49.7)	1465/2810(52.1)	1541/2809(54.9)	1583/2809(56.4)
Crude OR(95%CI) [d]	Ref.	1.10(0.99-1.22)	1.23(1.11-1.36) *	1.31(1.18-1.45) *
Adjusted OR(95%CI) [b,d]	Ref.	0.97(0.87-1.08)	1.03(0.92-1.15)	1.08(0.96-1.22)
CLBR				
n, (%)	1830/2809(65.1)	2088/2810(74.3)	2201/2809(78.4)	2291/2809(81.6)
Crude OR(95%CI) [d]	Ref.	1.55(1.38-1.74) *	1.94(1.72-2.18) *	2.37(2.09-2.68) *
Adjusted OR(95%CI) [b,e]	Ref.	1.06(0.96-1.16)	1.12(1.02-1.24) *	1.21(1.09-1.35) *

OR, odds ratio; CI, confidence interval; LBR, live birth rate; CLBR, cumulative live birth rate.
[a]Results of the first frozen embryo transfer cycle;
[b]Models were adjusted for maternal age, maternal BMI, primary or secondary infertility, parity, basal FSH, infertility diagnosis, protocol for stimulation, progesterone level on hCG day, IVF or ICSI, endometrial preparation regimen, embryo developmental stage, embryo quality, and number of embryos transferred; [c]Results of poisson regression analysis; [d]Results of logistic regression analysis; [e]Results of generalized estimating equations regression analysis. *P value <0.05.

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TABLE 3 Maternal complications and neonatal outcomes, stratified by estradiol levels on hCG-trigger day.

Outcome	Overall	Q1 (<2226 pg/ml)	Q2 (2226-3417 pg/ml)	Q3 (3418-5510 pg/ml)	Q4 (>5510 pg/ml)	*P* value
No. of live birth	8410	1830	2088	2201	2291	
Singleton	6305(75.0)	1457(79.6)	1582(75.8)	1657(75.3)	1609(70.2)	
Twins	2105(25.0)	373(20.4)	506(24.2)	544(24.7)	682(29.8)	
Gestational diabetes mellitus						
Singleton	754(12.0)	188(12.9)	202(12.8)	197(11.9)	167(10.4)	0.110
Twins	210(10.0)	43(11.5)	57(11.3)	55(10.1)	55(8.1)	0.193
Pregnancy-induced hypertension						
Singleton	262(4.2)	68(4.7)	71(4.5)	58(3.5)	65(4.0)	0.357
Twins	177(8.4)	35(9.4)	43 (8.5)	40(7.4)	59(8.7)	0.728
Preeclampsia						
Singleton	194(3.1)	52(3.6)	58(3.7)	44(2.7)	40(2.5)	0.117
Twins	136(6.5)	26(7.0)	37(7.3)	34(6.3)	39(5.7)	0.699
Placental disorders						
Singleton	146(2.3)	35(2.4)	41(2.6)	43(2.6)	27(1.7)	0.257
Twins	44(2.1)	8(2.1)	15(3.0)	11(2.0)	10(1.5)	0.361
Male gender						
Singleton	3354(53.2)	790(54.2)	822(52.0)	896(54.1)	846(52.6)	0.504
Twins	2188(52.0)	382(51.2)	537(53.1)	565(51.9)	704(51.6)	0.866
Gestational age (weeks)						
Singleton	38.7 ± 1.7	38.7 ± 1.7	38.7 ± 1.7	38.8 ± 1.6	38.8 ± 1.8	0.134
Twins	36.0 ± 1.9	35.9 ± 2.1	35.9 ± 1.0	36.2 ± 1.8	36.1 ± 1.9	0.083
Preterm birth						
Singleton	422(6.7)	96(6.6)	126(8.0)	94(5.7)	106(6.6)	0.075
Twins	1021(48.5)	182(48.8)	258(51.0)	251(46.1)	330(48.4)	0.478
Birthweight (g)						
Singleton	3388.0 ± 513.8	3406.0 ± 527.3	3374.4 ± 520.2	3404.7 ± 493.3	3367.7 ± 515.3	0.066
Twins	2552.4 ± 426.2	2539.3 ± 466.5	2538.9 ± 434.1	2571.6 ± 398.9	2554.3 ± 430.4	0.578
Low birthweight						
Singleton	264(4.2)	61(4.2)	69(4.4)	64(3.9)	70(4.4)	0.882
Twins	1593(37.8)	282(37.8)	388(38.3)	392(36.0)	531(38.9)	0.510
SGA						
Singleton	286(4.5)	65(4.5)	74(4.7)	66(4.0)	81(5.0)	0.535
Twins	149(3.5)	34(4.6)	34(3.4)	31(2.8)	50(3.7)	0.268
Birth defect						
Singleton	175(1.3)	17(1.2)	17(1.1)	22(1.3)	24(1.5)	0.734
Twins	66(1.6)	11(1.5)	18(1.8)	15(1.4)	22(1.6)	0.896

Data are presented as mean ± standard deviation or number (percentage).
SGA, small for gestational age.

groups. Details of birth defects that occurred in all live-born babies were presented in Supplemental Table 2.

Results of multivariable logistic regression adjusting for potential confounders revealed no associations between peak E2 level and adverse obstetric and neonatal outcomes (Figure 2).

4 Discussion

Results of this large cohort study demonstrated that peak serum E2 level during COS was positively associated with CLBR in freeze-all cycles, while no association was found between E2 level and adverse obstetric and neonatal outcomes.

In fresh embryo transfer cycles, evidence about whether the peak serum E2 level during COS affects pregnancy outcomes remains conflicting (4, 5). The heterogeneity of the population sampled, study sizes and E2 cut-off levels in these studies may account for their discrepancies. For instance, Moralog˘lu et al. involved 106 patients with ≥ 5 oocytes retrieved and suggested that peak E2 levels >2500 pg/ml were negatively associated with implantation rates (16). However, a large cohort study by Mustafa et al. included 6478 ICSI cycles and found that E2 levels over the 90th percentile (>4200 pg/ml) had increased clinical pregnancy rate, while the implantation rate was similar across the E2 percentile groups (28). Bianco et al. using a threshold of 2000 pg/ml, reviewed 58 oocyte donation cycles and reported that E2 concentration did not affect clinical pregnancy rate and LBR (29). In addition, other researchers showed no influence of peak E2 levels during COS on IVF success rates in autologous cycles (30–33). Among the existing studies, Yu Ng et al. and Chen et al. have further explored the subsequent FET cycles after the initial fresh cycles and reported similar pregnancy outcomes across different E2 concentrations, but they mainly looked at the rates of implantation and clinical pregnancy, without evaluating the CLBR (31, 32).

The impact of peak E2 levels during COS on the CLBR can be interpreted as a useful indicator of its effect on embryo development and implantation potential. A retrospective study included 1141 non-PCOS patients and assessed the outcomes of fresh and frozen cycles (34). They reported that the peak E2 level had a concentration-dependent effect on CLBR, with the optimal CLBR achieved between the E2 range of 2185-6361 pg/ml and a remarkable decrease afterward. However, the results could have been confounded since the CLBR in this study was calculated based on pooled data of both fresh and frozen cycles in the first embryo transfer attempt. Besides, the authors acknowledged that the sample size was relatively small in high E2 levels. The present study, conducted in a large general population, has estimated both reproductive outcomes after the first FET cycle and CLBR to explore the applicability of the freeze-all strategy in IVF patients with different peak E2 levels. Our results found that a higher E2 level not only does not decrease the implantation rate and LBR but, on the contrary, increases CLBR following the use of frozen-thawed embryos. Furthermore, the benefit from high ovarian response is limited for patients with advanced age as the CLBR reaches a plateau in extremely elevated E2 levels. These results, based on the freeze-all setting, added information on the association between peak E2 levels during COS and CLBR.

Concerning obstetric and neonatal outcomes, the maternal hyperestrogenic milieu has been reported to has an adverse effect on placentation and subsequent fetal growth (17–19). In comparison with previous studies presenting increased risks of preeclampsia, low birthweight and SGA with elevating E2 levels in fresh embryo transfer cycles, the present study found no association between E2 levels and adverse maternal and neonatal outcomes. Cai et al. and Zhang et al. conducted their studies in FET cycles and suggested that singleton birthweight was negatively influenced by increasing peak E2 levels during COS (35, 36). However, both studies were limited by the main FET indication of failed fresh transfer and OHSS risk, which would

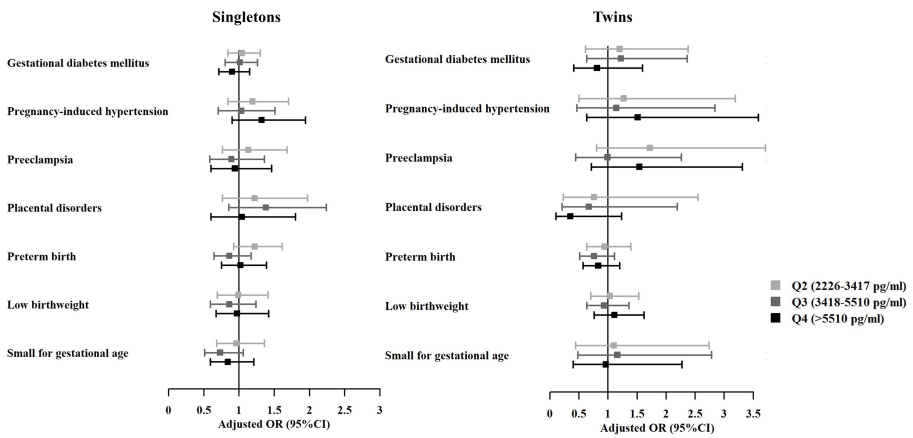

FIGURE 2
Adjusted odds ratios of adverse maternal and neonatal outcomes among live births with different estradiol levels on hCG-trigger day. OR, odds ratio; CI, confidence interval. The analyses were adjusted for maternal age, maternal BMI, primary or secondary infertility, parity, infertility diagnosis, protocol for stimulation, endometrial preparation regimen, embryo developmental stage, embryo quality, and number of embryos transferred (The group of Q1 was taken as the reference group).

lead to confounding outcomes as the patients included were generally with worse prognoses. Consistent with our findings, a more recent large cohort study reported that peak E2 level during COS was not related to increased risks of low birthweight and SGA in freeze-all cycles, although patients with maternal complications including GDM and hypertensive disorders were excluded in the study (37).

A significant body of evidence has demonstrated that the supraphysiologic E2 level in IVF treatments may impair endometrial receptivity and adversely affect trophoblastic invasion or placentation, which may explain the unfavorable results associated with high E2 levels in fresh cycles (6–8, 17–19). However, it is also of concern whether there is a negative effect on the quality of oocytes or embryos attributable to high E2 levels (10–13). Many studies indicated that the embryonic viability decreased and chromosomal abnormalities increased after superovulation in animal experiments (10, 11). In addition, hormonal stimulation has been hypothesized to induce epigenetic alterations in both human and murine oocytes or embryos derived from assisted reproduction treatment (12, 13). However, contemporary studies have reported that ovarian stimulation was not related to the chromosomal status of embryos (38, 39) and no drastic epigenetic changes were found in placental tissues with or without superovulation (40).

Furthermore, it is difficult to distinguish the effects of supraphysiologic E2 level on oocytes or embryos from those on the endometrium in fresh cycles, whereas a freeze-all strategy provides a novel model to assess the sole impact of ovarian stimulation on oocyte and embryo quality after ruling out the potential deleterious influences on endometrium caused by a hyperestrogenic milieu (41). Our study, focusing on pregnancy and obstetric outcomes, adds further to the currently existing evidence by suggesting that the high E2 levels do not appear to pose adverse effects on oocyte or embryo quality, and the detrimental effect of intrauterine high E2 exposure could be avoided by transferring embryos into a more physiologic uterine environment.

This is the first study to evaluate the impact of peak E2 level during COS in freeze-all cycles. The strength of our study is the large cohort size with an organized dataset that offered all the relevant parameters in the analysis. The primary outcome of this study, CLBR, allows us to capture all live births after one ovarian stimulation cycle and the corresponding obstetric and fetal outcomes, which also provides new insight into the relationship between E2 level and the success of an IVF program.

We acknowledged that there are limitations in this study. The retrospective nature of the analysis may increase the chance of selection bias regarding the population characteristics as well as cycle parameters (e.g. basal ovarian reserve, fertilization method, embryo stage at transfer). In this regard, we utilized multivariable regression models to adjust for potential confounders, and the result of CLBR was reinforced by the GEE analysis. In addition, the policy of transferring two cleavage-stage embryos was taken as a priority in our IVF centers before 2019 and single blastocyst transfer was encouraged afterward given the advantages of reduced multiple pregnancies and improved pregnancy rates (42, 43). Thus, the results of our analysis may not be generalizable to other populations where blastocysts were cultured and transferred primarily. Further investigations on this subject are still needed to evaluate the effect of high E2 levels on the oocyte competence and embryo developmental potential, as well as the long-term health of IVF offspring.

5 Conclusion

Our study demonstrated that, in freeze-all cycles, the CLBR progressively increased with the higher levels of peak serum E2 after COS, while the risks of adverse obstetric and neonatal outcomes were not increased, suggesting that high E2 levels may actually have a very limited or no adverse effect on oocyte or embryo quality. Our results provide reassuring findings for patients with high E2 levels during COS and suggest that they may benefit from freeze-all cycles. Nevertheless, given that the extremely elevated E2 levels would pose additional risks such as OHSS and thromboembolic complications after oocyte retrieval, COS for freeze-all cycles should be rational to avoid aggressive stimulation and focus on the balance between treatment efficiency and patients' safety.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University. This study did not require informed consent for participation following the national legislation and the institutional requirements.

Author contributions

JH and YL: study design, analysis and interpretation of data, and drafting of the manuscript. YW, QZ, JQ, YD and HZ: data collection. YH and ZD: assessed the article. YS: study concept and revise of article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1130211/full#supplementary-material

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Association of gestational hypertriglyceridemia, diabetes with serum ferritin levels in early pregnancy: a retrospective cohort study

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Aims: Previous studies showed conflicting results linking body iron stores to the risk of gestational diabetes mellitus (GDM) and dyslipidemia. We aim to investigate the relationship between serum ferritin, and the prevalence of GDM, insulin resistance (IR) and hypertriglyceridemia.

Methods: A total of 781 singleton pregnant women of gestation in Shanghai General Hospital took part in the retrospective cohort study conducted. The participants were divided into four groups by quartiles of serum ferritin levels (Q1–4). Binary logistic regressions were used to examine the strength of association between the different traits and the serum ferritin (sFer) quartiles separately, where Q1 (lowest ferritin quartile) was taken as the base reference. One-way ANOVA was adopted to compare the averages of the different variables across Sfer quartiles.

Results: Compared with the lowest serum ferritin quartile (Q1), the ORs for Q3, and Q4 in our population were 1.79 (1.01–2.646), and 2.07 (1.089-2.562) respectively and this trend persisted even after adjusted for age and pre-BMI. Women with higher serum ferritin quartile including Q3 (OR=2.182, 95%CI=1.729-5.527, P=0.003) and Q4(OR=3.137, 95%CI=3.137-8.523, P<0.01)are prone to develop insulin resistance disorders. No significant difference was observed between sFer concentrations and gestational hypertriglyceridemia(GTG) in the comparison among these 4 groups across logistic regressions but TG was found positively correlated with increased ferritin values in the second trimester.

Conclusions: Increased concentrations of plasma ferritin in early pregnancy are significantly and positively associated with insulin resistance and incidence of GDM but not gestational dyslipidemia. Further clinical studies are warranted to determine whether it is necessary to encourage pregnant women to take iron supplement as a part of routine antenatal care.

KEYWORDS

gestational diabetes, serum ferritin levels, insulin resistance, hypertriglyceridemia, metabolism & endocrinology, gestational diabetes

1 Introduction

The morbid effects of gestational iron deficiency on both maternal and fetal outcomes remains a global health problem affecting 10–90% of pregnant women (1) as iron is a detrimental supplement. According to the World Health Organization, daily oral iron supplementation (30-60 mg of elemental iron daily intake) should be a part of routine antenatal care with a purpose of avoiding poor maternal-fetal outcomes including intrauterine growth restriction, premature delivery, and neonatal and perinatal death (1) (2). However, when pregnant women sunk in excessive amounts of iron, it is prone to cause potential damage to newborns and mothers since emerging researches suggested that exposures to high iron during hematopoiesis in early life might induce anemia with profound developmental effects and probably worse erythropoietin sensitivity to limit erythropoiesis (3) (4) (5).

Serum ferritin is a major iron storage protein, a widely used marker for total body Fe stores, with a Nano-sized core of hydrated iron oxide and a cage-shaped protein shell, containing 20% iron. Recently increasing studies have discovered the phenomenon that higher serum ferritin concentrations are also related with metabolic disorders of pregnancy such as gestational diabetes (GDM), serum dyslipidemia, insulin resistance (IR) calculated by indexes such as homeostasis model assessment-insulin resistance (HOMA-IR), homeostasis model assessment-insulin secretion (HOMA-IS), and homeostasis model assessment- β cell function (HOMA-β) (6) (7) (8) (9). On the contrary, there are still other conflicting researches pronounced that iron supplementation does not increase the risk of GDM but benefit a lot for mothers and fetus in terms of pregnancy outcomes (10) (11).

Considering the paucity of researches but with conflicting findings, evaluating the relationship between serum ferritin and metabolic disorders in the Chinese pregnant population, we utilized epidemiological data of pregnant women from Shanghai General Hospital, China to explore the association between serum ferritin levels and the prevalence of metabolic disorders including GDM, serum dyslipidemia, and IR.

2 Materials and methods

2.1 Study population

This retrospective study used data from department of obstetrics in Shanghai General Hospital on 781 singleton pregnant women aged 21-44 years hospitalized for delivery during January 2021 to Oct 2021 after approval of Ethical Review Board Committee of our institution. Informed patient consent was not required as this was a retrospective research, and this study was conducted in accordance with the Declaration of Helsinki.

Inclusion criteria of the study population were listed as:1)live-birth singleton pregnancy; 2)women's age ≥18 years old; 3)had their first antenatal care visit between 10 and 20 weeks of gestation. Patients were excluded if they were complicated by 1) stillbirths 2) malformation congenital of the fetus 3)chronic diseases including hypertension, lupus, dyslipidemia, diabetes mellitus, acute or chronic liver diseases through medical history taking at the beginning of enrolment 4) multiple gestations, 5) incomplete medical records.

Among 800 women who met these criteria, 5 refused to participate, 6 had abortions before 24 weeks of gestation, and 4 did not undergo an assessment for GDM diagnosis and 4 for not performing serum lipids tests. Ultimately a total of 781 pregnant women were included in this analysis.

2.2 Clinical measurements

All measurements and the sample collections were performed in the morning after an overnight fast. Blood samples was extracted in gestation 10-14 weeks for early pregnancy and 24-28 weeks for middle pregnancy. Pre-pregnancy body mass index (P-BMI) (kg/m (2)) was calculated through weight(kg)/height (2)(m (2)). Enzymatic colorimetric GPO-PAP method (Siemens Healthcare diagnostics Inc) was used to measure serum TGs while serum cholesterol level was calculated by the enzymatic endpoint (CHOD-PAP) method on an automatic analyzer (ADVIA Chemistry XPT). Routine immuno-turbidimetry methods on an automatic analyzer (ADVIA Chemistry XPT) was adopted to quantify ApoA1 and ApoB. Serum low-density lipoprotein-cholesterol (LDL-C) and Serum high-density lipoprotein-cholesterol (HDL-C) levels were directly measured through homogeneous enzymatic colorimetric assay (Siemens Healthcare diagnostics Inc) on an automatic analyzer (ADVIA Chemistry XPT). The quantitative determination of glycosylated hemoglobin (HbA1c) was observed with cyanmethemoglobin. Serum glucose level including 0h, 1h and 2h glucose level was determined by glucose oxidase method. Direct chemiluminescence technology with bright spot sandwich method was used to measure insulin level by a fully automatic biochemical analyzer. HOMA-β HOME-IS and HOMA-IR were calculated using the following formulas. HOMA-β was calculated through the formula: HOMA-β= (20 × insulin)/(glucose − 23·5). HOMA-IR was calculated by the standard formula: HOMA-IR= (glucose × insulin)/22·5. HOME– IS was evaluated using the formula: HOME–IS=1/(FINS×FPG). Insulin resistance is defined as the value of HOMA-IR above or equal 2.69 according to a published paper (12). OGTT was performed in the gestation age between 24 and 28 weeks. GDM was diagnosed if one of the points exceeded the below values: 5.1mmol/L (FBG), 10mmol/l (1h), and 8.5mmol/L(2h). There has not been a consensus about gestational hypertriglyceridemia. We regarded those with TG ≥ 3.4mmol/L(reference cut-off for general population is 1.7mmol/L) as patients diagnosed with gestational hypertriglyceridemia since there is a growing trend of TG in this period.

2.3 Statistical analysis

The participants were stratified by sFer levels. One-way ANOVA was adopted to compare the averages of the different variables across sFer quartiles. Chi-square tests were used to analyze statistical differences among the study participant's characteristics

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in relation to serum ferritin quartile (Q1-4) groups. Binary logistic regressions were used to examine the strength of association between the different traits and the sFer quartiles separately, where Q1 (lowest ferritin quartile) was taken as the base reference.

3 Results

3.1 Characteristics of the study population according to the ferritin tertials of early gestation

The baseline characteristics of the 781 subjects and metabolism values are detailed in Table 1. To assess the relationship between ferritin levels and abnormal plasma glucose in pregnant women, we compared HbA1c and FBG among these 4 groups. The mean HbA1c of Q4 (5.15 ± 0.35) was significantly higher than the other groups (5.14 ± 0.38, 5.10 ± 0.33, 5.11 ± 0.34). Likewise, it can be observed that the mean value of FBG of Q4 (4.90 ± 0.55)was statistically significantly higher than Q1 (4.75 ± 0.48), Q2 (4.72 ± 0.42) and Q3 (4.79 ± 0.44). However, we didn't notice any obvious correlations between lipid profiles including TC, TG, H-LDL, L-LDL, ApoA1, ApoB, ApoE and Lp(a), and values of ferritin in early pregnancy.

3.2 Relationship between ferritin of early gestation and metabolism indexes in middle pregnancy

Association between ferritin and metabolism disorders across the tertials of serum ferritin concentrations was presented in Table 2. There were no statistically significant differences in 2-h OGTT level and insulin index, whereas the difference was statistically significant for fasting plasma glucose, 1-h OGTT level, GA, HbA1 c, HOMA-β, HOMA-IS and HOMA- IR, which may be correlated to the incidence of GDM. Unlike lipid levels in early pregnancy, however, TG was found positively correlated with mounting ferritin values in the second trimester.

3.3 The association of serum ferritin in early gestation with GDM and insulin resistance by serum ferritin quartile

To further clarify the correlation between sFer levels and Carbohydrate metabolism, we analyzed Odds ratios and adjusted odds ratios for the association of serum ferritin with insulin resistance by serum ferritin quartile (Table 3). Here, IR was

TABLE 1 Characteristics of the study population & relationship between ferritin of early pregnancy and metabolism in early pregnancy.

Items/Groups	Q1 (196)	Q2 (195)	Q3 (195)	Q4 (195)	All	*p*
Age	30.61 ± 3.82	29.75 ± 3.98	29.47 ± 4.09	29.31 ± 4.18	29.79 ± 4.04	0.007*
Height	160.80 ± 4.78	160.77 ± 6.70	160.02 ± 4.62	160.29 ± 5.00	160.47 ± 5.34	0.4
Weight	56.53 ± 10.04	56.88 ± 10.33	56.90 ± 10.33	57.03 ± 10.33	56.84 ± 10.33	0.97
PreBMI	21.86 ± 3.78	22.00 ± 3.79	22.22 ± 4.10	22.21 ± 4.12	22.07 ± 3.95	0.778
HbA1c	5.14 ± 0.38	5.10 ± 0.33	5.11 ± 0.34	5.24 ± 0.32	5.15 ± 0.35	<0.01*
FBG	4.75 ± 0.48	4.72 ± 0.42	4.79 ± 0.44	4.90 ± 0.55	4.79 ± 0.48	<0.01*
GA	14.54 ± 1.56	12.00 ± 0.00	12.80 ± 0.29	11.25 ± 0.00	12.99 ± 1.51	0.314
TC	5.28 ± 0.85	5.05 ± 0.87	5.13 ± 1.13	5.14 ± 0.87	5.15 ± 0.94	0.122
TG	1.57 ± 0.59	1.50 ± 0.54	1.75 ± 2.31	1.78 ± 0.73	1.65 ± 1.28	0.097
H-LDL	1.75 ± 0.36	1.68 ± 0.38	1.68 ± 0.37	1.66 ± 0.38	1.69 ± 0.37	0.078
L-LDL	2.55 ± 0.65	2.46 ± 0.70	2.45 ± 0.66	2.49 ± 0.64	2.49 ± 0.66	0.498
ApoA1	1.64 ± 0.22	1.60 ± 0.21	1.62 ± 0.22	1.60 ± 0.22	1.61 ± 0.22	0.181
ApoB	0.78 ± 0.17	0.75 ± 0.19	0.75 ± 0.17	0.77 ± 0.17	0.76 ± 0.18	0.143
ApoE	25.89 ± 10.19	25.19 ± 10.04	29.52 ± 63.96	25.38 ± 9.74	26.50 ± 33.17	0.532
Lp(a)	296.33 ± 385.08	253.10 ± 332.46	230.51 ± 289.11	297.31 ± 418.34	269.28 ± 360.07	0.177
Hb	120.68 ± 15.33	129.01 ± 9.50	130.67 ± 13.83	129.22 ± 14.35	127.39 ± 13.98	<0.01*
Ferritin	13.98 ± 5.78	31.40 ± 5.01	53.58 ± 8.18	116.61 ± 59.33	53.84 ± 49.16	<0.01*
Ferritin	3-23.1	23.2-41	41.1-69.6	69.7-560.6	3-560.6	

Age in years, kg, cm, kg/m2, g, and days are the units of age, weight, height, BMI, review intervals, and mmol/for TC, TG, LDL, HDL, and baby weight, while g/l is for ApoA1 and ApoB. TC, TG, HDL, LDL, Hb represents Glycosylated hemoglobin, fasting plasma glucose glycated albumin, triglycerides, cholesterol, High density lipoprotein Low density lipoprotein and hemoglobin respectively.

The participants were classified into four groups based on their serum ferritin quartile (Q1: 13.98 ± 5.78μg/L, N=196; Q2: 31.40 ± 5.01μg/L, N= 195; Q3: 53.58 ± 8.18μg/L, N= 195; and Q4: 116.61 ± 59.33μg/L, N= 195) at the beginning of the study, and their characteristics are presented in Table 1.

* is displayed when p value is no more than 0.05.

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TABLE 2 Relationship between ferritin of early gestation and metabolism indexes in middle pregnancy.

items/groups	Q1 (196)	Q2 (195)	Q3 (195)	Q4 (195)	All	*p*
HbA1c	5.03 ± 0.36	4.95 ± 0.33	5.42 ± 0.65	5.92 ± 0.78	5.33 ± 0.68	0.00*
GA	12.01 ± 1.19	12.12 ± 1.24	11.99 ± 1.17	11.69 ± 0.87	11.9 ± 1.14	0.00*
OGTT0	4.46 ± 0.44	4.41 ± 0.43	4.53 ± 0.49	4.56 ± 0.49	4.49 ± 0.47	0.01*
OGTT1	7.64 ± 1.78	8.02 ± 1.74	9.74 ± 1.96	10.63 ± 1.81	9.00 ± 2.19	0.00*
OGTT2	6.52 ± 1.39	6.56 ± 1.43	6.78 ± 1.51	6.75 ± 1.31	6.65 ± 1.42	0.15
INS0	55.65 ± 30.14	55.76 ± 34.63	63.48 ± 38.50	66.71 ± 37.26	60.41 ± 35.53	0.00*
INS1	448.76 ± 290.57	465.60 ± 301.25	493.70 ± 299.65	491.48 ± 308.81	474.88 ± 300.13	0.39
INS2	385.2 ± 268.57	394.75 ± 248.44	419.90 ± 301.01	445.44 ± 296.19	411.32 ± 279.86	0.15
HOMA-β	353.92 ± 365.65	301.24 ± 312.08	221.64 ± 194.50	191.94 ± 116.47	267.24 ± 273.03	0.00*
HOMA-IS	0.71 ± 0.22	0.68 ± 0.20	0.55 ± 0.17	0.49 ± 0.11	0.61 ± 0.20	0.00*
HOMA-IR	1.53 ± 0.45	1.59 ± 0.45	1.98 ± 0.54	2.15 ± 0.55	1.81 ± 0.56	0.00*
TG	2.315 ± 0.76	2.342 ± 0.89	2.539 ± 1.947	2.675 ± 0.968	2.46 ± 1.24	0.01*
TC	6.393 ± 1.09	6.207 ± 1.128	38.81 ± 453.85	6.576 ± 1.290	14.56 ± 227.80	0.4
LDL	1.941 ± 0.3772	1.885 ± 0.449	1.887 ± 0.409	1.919 ± 0.447	1.90 ± 0.42	0.44
HDL	3.351 ± 0.8988	3.296 ± 0.921	3.185 ± 0.917	3.249 ± 0.919	3.27 ± 0.91	0.28
ApoA1	1.855 ± 0.22	1.831 ± 0.292	1.856 ± 0.302	1.871 ± 0.252	1.85 ± 0.271	0.49
ApoB	1.004 ± 0.14	0.956 ± 0.291	0.953 ± 0.245	1.183 ± 2.689	1.02 ± 1.358	0.28
ApoE	39.15 ± 31.04	38.81 ± 19.08	40.78 ± 50.77	38.72 ± 17.99	39.3 ± 32.48	0.93
LP (a)	305.74 ± 343.73	241.70 ± 240.96	251.72 ± 308.86	306.27 ± 360.99	276. ± 317.88	0.07
Hb	111.24 ± 14.87	114.62 ± 12.14	115.19 ± 12.77	114.67 ± 12.83	113.92 ± 13.27	0.01*
Ferritin	9.52 ± 6.39	10.30 ± 6.22	12.85 ± 8.00	29.93 ± 59.21	15.63 ± 31.26	0.00*

* is displayed when p value is no more than 0.05.

measured by HOMA-IR and HOMA-%B of the participants. A positive relation was observed as showed in Table 2. Compared with people with Q1, the lowest serum ferritin quartile, we found women with higher serum ferritin quartile including Q3(OR=2.182, 95% CI=1.729-5.527, P=0.003) and Q4(OR=3.137, 95%CI=3.137-8.523, P<0.01)are prone to develop insulin resistance disorders (Table 4). This association persisted after adjusting for potential confounders factors.

3.4 Association of serum ferritin in early gestation with GTG in pregnancy by serum ferritin

To assess the relationship between ferritin levels and dyslipidemia, OR for the occurrence of hypertriglyceridemia was estimated for participants across the SFer quartiles (Tables 5, 6). However, no significant difference was observed in the comparison among these different groups.

4 Discussion

We investigated the relationship between sFer levels in early pregnancy and metabolic disorders including GDM, insulin resistance, hypertriglyceridemia in early and middle pregnancy, and SCH in early and middle pregnancy. The results of our study revealed that elevated serum ferritin levels are positively independently associated with GDM. Furthermore, an increased β-cell function associated with higher ferritin level was observed in pregnancy period. Nevertheless, no obvious correlation between ferritin levels and hypertriglyceridemia in early and middle pregnancy was found.

Our findings correspond well with the previous study by Cheng et al. (13). Cheng et al. conducted a prospective observational study of 851 Chinese pregnant women, and they concluded that increased serum ferritin concentrations of first trimester are involved with an elevated risk of GDM. A longitudinal study of iron status during pregnancy and the risk of GDM from a prospective multiracial cohort (14) suggested that higher iron stores may be related with the

TABLE 3 Odds ratios and adjusted odds ratios for the association of serum ferritin in early gestation with GDM by serum ferritin quartile (reference group = Q1).

GDM		Q1	Q2	Q3	Q4
model1	*p*	Ref	0.91	0.00*	0.00
	OR		0.97	1.79*	2.07*
	95% CI		0.569-1.652	1.01-2.646	1.089-2.562
model2	*P*	Ref	0.91	0.00*	0.00*
	OR		0.97	1.80*	2.07*
	95% CI		0.568-1.65	1.002-2.678	1.998-2.6

Q1-4 were grouped according to serum ferritin quartiles. Q, quartile; OR, odds ratio; CI, confidence interval.
Model 1 shows ORs and their 95% CIs calculated from a logistic regression model. Model 2 shows adjusted ORs and their 95% CIs calculated from a logistic regression model adjusted for age and pre-BMI. Compared with the lowest serum ferritin quartile (Q1), the ORs for Q3, and Q4 in our population were 1.79 (1.01–2.646), and 2.07 (1.089-2.562) respectively and this trend remained almost same even after adjusted for age and pre-BMI.
* is displayed when p value is no more than 0.05.

development of GDM from early pregnancy, a >2-fold increased odds of GDM with highest quintiles of ferritin compared with lowest ones, which are also in line with our result. The majority of other previous studies are retrospective researches (15) and they also demonstrated that elevated sFer concentrations is positively related with incidence of GDM. Furthermore, our findings are also in accordance with results from previously published prospective studies in non-pregnant individuals on iron store and type 2 diabetes (16) (17) (18). However, 1 large randomized controlled trial conducted in Hong Kong found no association between iron supplementation and GDM (19). The divergence was potentially owing to various body iron stores, and different dosage and time length of iron supplementation in multitudinous pregnant populations.

In terms of insulin resistance, the findings of our research partially concurs with previous work (20) (21). These studies stated that higher content of ferritin and transferrin at baseline were correlated with HOMA-IR, and low HOMA-%, hyperinsulinemia and the metabolic syndrome anomalies (20).

Based on our current study and previous researches, though WHO suggests daily oral iron supplementation (30-60 mg of elemental iron daily intake) should become a part of routine antenatal care, women with lower serum ferritin levels might benefit from iron supplementation but it is likely to contribute some side effects to those with normal plasma ferritin concentrations. Our research suggests that routine iron supplementation for pregnant women may not that suitable considering risk of GDM. Thus, it is necessary for clinicians to assess iron status of pregnant women in early pregnancy to offer individualized iron supplementation recommendations to reduce the incidence of GDM.

Biologically speaking, the observed positive correlation between sFer and glucose homoeostasis is plausible. The underline mechanism by which elevated fasting SF levels pronounce diabetes, damage beta-cell function and decrease insulin sensitivity have not been fully illuminated. However, it has been noted that heme iron shoulder the responsibility of increasing the body's iron store and thus leading to oxidative injury to pancreatic cells. Furthermore, growing insulin resistance and elevated insulin secretion from the pancreas could cause pancreatic beta-cell exhaustion (22). Some other researches have revealed that high plasma ferritin mirrors increased iron stores of the body and might be taken as an acute phase inflammatory reactant (23) (24). Besides, increased plasma ferritin levels motivate the inflammatory process inducing elevated insulin resistance,decreased insulin secretion by the pancreas, and hepatic dysfunction (25) Ultimately it follows reduced glucose uptake by muscles and mounting gluconeogenesis, hence spelling the development of GDM (26).

In the year of 2017, Li et al. found that growing serum ferritin levels are significantly related to higher risk of of dyslipidemia, independent of other confounding factors such as age and BMI etc. (8) Nevertheless, our findings did not come in accordance with theirs, since our data showed no statistical significance.

TABLE 4 Odds ratios and adjusted odds ratios for the association of serum ferritin in early gestation with insulin resistance by serum ferritin quartile (reference group = Q1).

Insulin resistance		Q1	Q2	Q3	Q4
	p		0.519	0.003*	0*
model1	OR	Ref	1.524	2.182*	3.137*
	95% CI		0.423-3.486	1.729-5.527	3.137-8.523
	P		0.508	0.003*	0*
model2	OR	Ref	1.542	2.718*	3.242*
	95% CI		0.428-3.557	1.769-5.985	3.555-9.506

Q1-4 were grouped according to serum ferritin quartiles. Q, quartile; OR, odds ratio; CI, confidence interval.
* Model 1 shows ORs and their 95% CIs calculated from a logistic regression model. Model 2 shows adjusted ORs and their 95% CIs calculated from a logistic regression model adjusted for age and pre-BMI. * is displayed when p value is no more than 0.05.

TABLE 5 Odds ratios and adjusted odds ratios for the association of serum ferritin in early gestation with GTG in middle pregnancy by serum ferritin quartile (reference group = Q1).

GTG in middle pregnancy		Q1	Q2	Q3	Q4
model1	*p*	Ref	0.258	0.258	0.056
	OR		0.719	0.719	0.553
	95% CI		0.406	0.406	0.302-1.230
model2	*p*	Ref	0.229	0.298	0.066
	OR		0.703	0.737	0.565
	95% CI		0.396	0.415	0.307-1.233

Q1-4 were grouped according to serum ferritin quartiles. Q, quartile; OR, odds ratio; CI, confidence interval.
* Model 1 shows ORs and their 95% CIs calculated from a logistic regression model. Model 2 shows adjusted ORs and their 95% CIs calculated from a logistic regression model adjusted for age and pre-BMI.

TABLE 6 Odds ratios and adjusted odds ratios for the association of serum ferritin with GTG in early pregnancy by serum ferritin quartile (reference group = Q1).

GTG in early pregnancy		Q1	Q2	Q3	Q4
model1	*P*	Ref	0.243	0.988	0.213
	OR		2.065	1.011	0.247
	95% CI		0.611	0.249	0.027-2.210
model2	*P*	Ref	0.266	0.94	0.216
	OR		2.015	1.057	0.246
	95% CI		0.586-6.925	0.255-4.382	0.027-2.269

Q1-4 were grouped according to serum ferritin quartiles. Q, quartile; OR, odds ratio; CI, confidence interval.
* Model 1 shows ORs and their 95% CIs calculated from a logistic regression model. Model 2 shows adjusted ORs and their 95% CIs calculated from a logistic regression model adjusted for age and pre-BMI.

The present study has several advantages. Firstly, we evaluated the relationship between serum ferritin and metabolic disorders in Chinese pregnant population as there is a paucity of studies among Chinese pregnant women. Secondly, we analyzed serum ferritin concentrations in early pregnancy, prior to the diagnosis of GDM, which indicates that plasma ferritin would less be influenced by progress of

GDM. Thirdly, the current study suggests that routine iron supplementation for pregnant women may not that suitable considering risk of GDM, which is innovative. Nevertheless, some limitations are also needed to be noticed:Firstly, this study was conducted in a retrospective cohort with a relatively small sample. Secondly, the information on iron supplement use was not provided, so further investigations on the relation of iron supplement with fetal iron status and GDM are needed. Thirdly, we did not assess concentrations of biomarkers of inflammation, but previous studies indicated that plasma ferritin levels can be slightly influenced by inflammation.

5 Conclusions

In summary, findings from our present study suggest that increased concentrations of plasma ferritin in early pregnancy are significantly and positively associated with insulin resistance and incidence of GDM but not gestational dyslipidemia. Similar studies, especially well-designed cohort studies or randomized trials, should be performed in different types of populations, since plasma ferritin concentrations may play an important role for predication and prevention of GDM, IR and their complications. Further clinical studies are warranted to determine whether it is necessary to encourage pregnant women to take iron supplement as a part of antenatal care. In addition, further researches are warranted to clarify the underlying mechanisms. Anyway, in order to prevent excessive iron intake, a lower-dose or intermittent iron supplements instead of regular daily supplementation of iron possibly is more suitable for women without iron deficiency.

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

Author contributions

All the authors participated in designing this study. ZZ and XL collected data. XZ and XG undertook the statistical analyses and interpreted the data. ZZ and YZ wrote the first draft of the manuscript, which was reviewed by all the other authors, especially HW and XX, who also offered further contributions as well as advice.

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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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The duration of estrogen treatment before progesterone application does not affect neonatal and perinatal outcomes in frozen embryo transfer cycles

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Objective: To explore whether the duration of estrogen treatment before progesterone application affects neonatal and perinatal outcomes in artificial frozen embryo transfer (FET) cycles.

Methods: This was a retrospective cohort study. Patients who underwent FET via artificial cycles and delivered a singleton live birth between January 2015 and August 2019 were included in the analysis. According to the duration of estrogen treatment before progesterone application, we divided the cycles into four groups: ①≤12 days, ②13-15 days, ③16-19 days, and ④≥20 days. The '≤12 days group' was considered the reference group. The main outcome measures were preterm birth (PTB), small-for-gestational age (SGA), low birth weight (LBW), macrosomia, large-for-gestational age (LGA), gestational diabetes mellitus (GDM), gestational hypertension, premature rupture and placenta previa.

Results: Overall, 2010 FET cycles with singleton live births were included for analysis. Cycles were allocated to four groups according to the duration of estrogen treatment before progesterone application: ①≤12 days (n=372), ②13-15 days (n=745), ③16-19 days (n=654), ④≥20 days (n=239). The neonatal outcomes, including PTB, SGA, LBW, macrosomia and LGA, were comparable among the groups (P=0.328, P=0.390, P=0.551, P=0.565, P=0.358). The rates of gestational hypertension, premature rupture and placenta previa (P=0.676, P=0.662, P=0.211) were similar among the groups. The rates of GDM among the four groups were 4.0% (15/372), 6.7% (50/745), 6.4% (42/654), and 11.3% (27/239), with statistical significance (P=0.006). After multiple logistic regression analysis, the duration of estrogen treatment did not affect the rate of GDM or other outcomes.

Conclusion: The estrogen treatment duration before progesterone application does not affect neonatal and perinatal outcomes in single frozen blastocyst transfer cycles.

KEYWORDS

frozen embryo transfer, artificial cycle, duration of estrogen, gestational diabetes mellitus, neonatal outcomes

Introduction

In the past decade, the number of frozen embryo transfer (FET) cycles has substantially increased. Currently, up to half of embryos are cryopreserved, and FET is performed (1). This trend may be related to the improvement and development of vitrification technology and a rapid rise in single embryo transfer (ET), combined with the development of preimplantation genetic tests (PGTs) (2–4). In addition, current studies have shown that FET can reduce *in vitro* fertilization (IVF) complications, such as ovarian hyperstimulation syndrome, increase the cumulative live birth rate, and improve perinatal and offspring outcomes (5–7). How to improve the live birth rate of FET cycles and ensure the safety of mothers and offspring is the focus of our research. Ensuring the synchronization of embryo and endometrial development is a key step in obtaining pregnancy. However, the optimal endometrial preparation protocols for FET are still a topic of constant debate (8–10). The artificial cycle is mainly suitable for patients with abnormal ovulation or nonovulation. The application of exogenous estrogen and progesterone can promote the development of the endometrium and promote the synchronization of the development of the endometrium and the embryo. Preparing the endometrium in artificial cycles, which can flexibly arrange the transplantation time and reduce the number of monitoring procedures, is widely used in clinical practice (1). Therefore, how to optimize this protocol, improve the clinical outcome and ensure the safety of the perinatal period is the focus of our attention. At present, there are studies exploring the optimal duration of estrogen treatment before progesterone application; however, most studies focus on the pregnancy rate or live birth rate (11–15). To the best of our knowledge, only two studies have analyzed the relationship between the duration of estrogen treatment and offspring and perinatal safety (13, 15). However, the conclusions of the two studies are not consistent. Due to the differences in the included population and groupings, it is necessary to further analyze the effect of estrogen use time on neonatal and perinatal outcomes. Therefore, the purpose of this study was to analyze whether the duration of estrogen treatment before progesterone application affects neonatal and perinatal outcomes in artificial FET cycles.

Materials and methods

Study design and population

This was a retrospective cohort study conducted in the reproductive center of the Third Affiliated Hospital of Zhengzhou University. Permission to conduct this study was obtained from the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University. The outcomes of patients who underwent the first FET of whole embryo freezing and delivered a singleton live birth between January 2015 and August 2019 were included for potential analysis. All included patients had the endometrium prepared for ET through artificial cycles. All included patients followed the oral glucose tolerance test and insulin release test, and patients with abnormal blood glucose or insulin were excluded. We excluded cycles with oocyte donation(n=92), PGT(n=176) and incomplete data(n=45). In addition, studies have shown that thin endometrium thickness (EMT) may have an impact on offspring weight and perinatal outcomes (16–19). To reduce the influence of thin EMT, cycles with EMT ≤7 mm within 10 days of the initiation of estrogen administration were excluded from the analysis(n=81).

Endometrial preparation and ET

The controlled ovarian stimulation protocols and IVF/ICSI process have been described in our previous studies (5, 20, 21). In the present study, all patients underwent artificial cycles for FET. Vaginal ultrasound examination was performed on the 2nd/3rd day of the menstrual cycle, 2-3 mg of estradiol valerate was taken orally three times daily (Bayer Co. Germany), and vaginal ultrasound examination was performed 7 days later. The drug dose was adjusted according to the thickness of the endometrium (up to 9 mg per day). The duration of estrogen treatment continued for at least 10 days. If conditions were appropriate (EMT>7 mm, serum estrogen level ≥150 pg/ml and serum progesterone < 1.5 ng/ml) and the patient's schedule was feasible, the progesterone exposure day was formulated. Routine corpus luteum support, namely, oral dydrogesterone (2 times daily, 10 mg once) (Abbott Co. America) and intravaginal administration of 90 mg of a progesterone sustained-release vaginal gel (Merck Co. Germany), was given. Transfer 1-2 cleavage-stage embryos 3 days after endometrial progesterone conversion or transfer 1 blastocyst 5 days after endometrial transformation. Vitrified frozen embryos were thawed according to our reproductive standard procedure (22). Specifically, embryos were quickly released and immersed in thawing solution for 1 minute at 37°C. Then, embryos were moved into dilution solution for 3 minutes, followed by two steps in washing solution for 3 min each at room temperature. Thawed embryos were incubated for 2 h before transfer. Embryo transplantation was carried out by abdominal ultrasound. Corpus luteum support was performed 55 days after transplantation if pregnancy occurred.

According to the duration of estrogen treatment before progesterone application, we divided the cycles into four groups: ①≤12 days, ②13-15 days, ③16-19 days, and ④≥20 days. The '≤12 days group' was considered the reference group. The grouping was mainly based on the interquartile range of estrogen treatment.

Outcome measures and definition

Outcome measures of this study included neonatal and perinatal outcomes. Small-for-gestational age (SGA) was defined as a birth weight less than the 10th centile for gestational age (23). Large-for-gestational age [LGA, defined as a birth weight greater than the 90th centile of the sex-specific birth weight (23)]. The weight criteria refer to the weight of Chinese newborns (24). Other neonatal outcomes included preterm birth (PTB) (defined as a birth that takes place after 28 weeks and before 37 completed weeks of

gestational age), low birth weight (LBW, defined as a neonatal birth weight less than 2500 g) (23) and macrosomia (defined as a neonatal birth weight more than 4000 g).

We also analyzed the rate of pregnancy-related complications among the following groups: ①Hypertensive disorders of pregnancy, including chronic hypertension, preeclampsia-eclampsia, preeclampsia superimposed on chronic hypertension, and gestational hypertension. ②Gestational diabetes mellitus: GDM was defined as carbohydrate intolerance of variable severity with onset or first recognition during pregnancy as determined from the diagnosis in the obstetrical medical record. ③ Premature rupture of membrane (PROM): PROM was defined as rupture of the amniotic membranes before the onset of labor, including PROM of term and preterm PROM. ④ Placenta previa: Placenta previa was used to describe a placenta that is implanted over or very near the internal cervical os (7).

Statistical analysis

All the data in this article were obtained from the electronic medical record system of the reproductive center of the Third Affiliated Hospital of Zhengzhou University.

The one-sample Kolmogorov–Smirnov test was used to check for normality of continuous variables. Continuous variables with abnormal distributions are expressed as the median (P25, P75), and the between-group differences were assessed by the Wilcoxon rank sum test. Categorical variables are represented as the number of cases (n) and percentage (%). The means from chi-square analyses were used to assess the differences between groups with Fisher's exact test when necessary. For the neonatal and perinatal outcomes of this study, multiple logistic regression was used to adjust for the baseline characteristics. Adjusted odds ratios (AORs) with 95% confidence intervals (CIs) were calculated. $P<0.05$ was considered to be statistically significant. Statistical management and analyses were performed usingSPSS software, version 22.0.

Results

Study population

Overall, 2010 FET cycles with singleton live births from January 2015 to August 2019 were included for analysis. Cycles were allocated to four groups according to the duration of estrogen treatment before progesterone application,

①≤12 days (n=372), ②13-15 days (n=745), ③16-19 days (n=654), ④≥20 days (n=239).

Baseline characteristics

The details of the baseline and cycle characteristics among the four groups are described in Table 1. The endometrial thickness was different among groups on the first day of progesterone administration (≤12 days, 9.5 (8.7, 10.5); 13-15 days, 9.0 (8.3, 10.0); 16-19 days, 8.5 (8.0, 9.3); ≥20 days, 8.0 (7.4, 8.7, P<0.001). Apart from this, there were significant differences in maternal age at oocyte retrieval (P=0.017), paternal age (P=0.024), duration of infertility (P=0.002), body mass index (BMI) (P=0.011), gravidity (P=0.023), parity (P=0.019), number of previous miscarriages (P=0.008), infertility diagnosis (P=0.014) and AMH (P<0.001). Other basic characteristics, including type of infertility (P=0.062), PCOS ratio (P<0.001), basal serum FSH (P=0.384), basal antral follicle count (P=0.059), method of ART (P=0.648), number of transferred embryos (P=0.838) and type of transferred embryos (P=0.191), were similar among groups.

Neonatal and perinatal outcomes

The specific rates of neonatal and perinatal outcomes are described in Table 2. The mean neonatal birth weight was comparable among groups (≤12 days, 3500 (3150, 3800); 13-15 days, 3500 (3150, 3800); 16-19 days, 3405 (3100, 3800); ≥20 days, 3490 (3100, 3700), P=0.168). The neonatal sex ratio (P=0.693) and gestational weeks at delivery were similar between the groups (P=0.940). The rates of PTB, LBW, SGA, macrosomia, LGA, gestational hypertension, premature rupture and placenta previa were comparable among the groups (P=0.328, P=0.551, P=0.390, P=0.565, P=0.358, P=0.676, P=0.662, P=0.211). The rate of gestational diabetes mellitus was different among groups, which was 4.0% (15/372), 6.7% (50/745), 6.4% (42/654), and 11.3% (27/239), P=0.006.

To adjust for the influence of confounding factors, we conducted multiple logistic regression analysis. The included factors were maternal age (continuous variable), paternal age (continuous variable), BMI (continuous variable), gravidity (continuous variable), duration of infertility (continuous variable), type of infertility (primary/secondary infertility), infertility diagnosis (tubal/male/both/others), PCOS (yes/no), basal antral follicle count, basal serum FSH level, fertilization method (IVF/ICSI), endometrial thickness on the first day of progesterone administration (continuous variable) and type of transferred embryos (cleavage embryo/blastocyst). After adjustments for confounding factors, taking the '≤12 days group' as the reference group, the duration of estrogen treatment did not affect the rate of GDM and other neonatal and perinatal outcomes. The specific AOR values with their 95% CIs are presented in Table 3.

Discussion

The purpose of this study was to analyze whether the duration of estrogen treatment before progesterone application affects neonatal and perinatal outcomes of singleton live births after FET via artificial cycles. Through our single-center retrospective cohort study, our results show that the duration of estrogen treatment does not affect offspring and perinatal outcomes.

Globally, the incidence of infertility is as high as 10%, and IVF is an important method of infertility treatment (25, 26). However, adverse pregnancy and perinatal outcomes, such as SGA, LBW, preterm birth,

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TABLE 1 Comparison of demographic characteristics among the four groups.

Item	Duration of estrogen treatment before progesterone administration (days)				*P* value
	≤12	13-15	16-19	≥20	
No. of cases	372	745	654	239	
Maternal age (year)	31.0 (28.0,34.0)	30.0 (28.0,34.0)	30.0 (28.0,33.0)	30.0 (27.0,33.0)	0.017
Paternal age (year)	31.0 (29.0,35.0)	31.0 (28.0,35.0)	31.0 (28.0,35.0)	30.0 (28.0,34.0)	0.024
Body mass index (kg/m2)	24.1 (22.0,26.4)	23.7 (21.5,26.0)	23.6 (21.5,25.6)	23.4 (21.0,25.5)	0.011
Gravidity	0 (0,1)	1 (0,2)	1 (0,2)	1 (0,2)	0.023
Parity	0 (0,0)	0 (0,1)	0 (0,0)	0 (0,0)	0.019
Number of previous miscarriages	0 (0,0)	0 (0,0)	0 (0,0)	0 (0,1)	0.008
Duration of infertility (year)	3.0 (2.0,6.0)	3.0 (1.0,5.0)	3.0 (1.0,4.0)	3.0 (1.0,5.0)	0.002
Type of infertility (%)					0.062
Primary infertility	50.3 (187/372)	45.2 (337/745)	46.8 (306/654)	39.3 (94/239)	
Secondary infertility	49.7 (185/372)	54.8 (408/745)	53.2 (348/654)	60.7 (145/239)	
Infertility diagnosis (%)					0.014
Tubal factor	29.0 (108/372)	33.0 (246/745)	33.0 (216/654)	41.4 (99/239)	
Male factor	16.7 (62/372)	20.8 (155/745)	23.2 (152/654)	17.6 (42/239)	
Male+female factors	21.0 (78/372)	18.0 (134/745)	17.7 (116/654)	15.5 (37/239)	
Others	33.3 (124/372)	28.2 (210/745)	26.0 (170/654)	25.5 (61/239)	
PCOS ratio	15.9 (59/372)	20.0 (149/745)	15.1 (99/654)	32.6 (78/239)	<0.001
Basal FSH (IU/L)	6.5 (5.0,8.2)	6.2 (5.0,7.7)	6.2 (4.8,7.6)	6.4 (5.1,7.7)	0.384
AMH (ng/ml)	3.5 (1.6,6.7)	4.1 (2.1,7.1)	4.6 (2.5,7.5)	5.2 (2.9,7.9)	<0.001
Basal antral follicle count	17.0 (11.0,24.0)	19.0 (12.5,24.0)	20.0 (13.0,24.0)	20.0 (15.0,24.0)	0.059
Method of ART (%)					0.648
IVF	76.9 (286/372)	74.1 (552/745)	73.4 (480/654)	73.6 (176/239)	
ICSI	23.1 (86/372)	25.9 (193/745)	26.6 (174/654)	26.4 (63/239)	
Endometrial thickness on the first day of progesterone administration (mm)	9.5 (8.7,10.5)	9.0 (8.3,10.0)	8.5 (8.0,9.3)	8.0 (7.4,8.7)	<0.001
No. of transferred embryos					0.838
1	52.7 (196/372)	54.1 (403/745)	54.1 (354/654)	56.5 (135/239)	
2	47.3 (176/372)	45.9 (342/745)	45.9 (300/654)	43.5 (104/239)	
Type of transferred embryos					0.191
Cleavage embryo	33.1 (123/372)	33.4 (249/745)	28.9 (189/654)	28.5 (68/239)	
Blastocyst	66.9 (249/372)	66.6 (496/745)	71.1 (465/654)	71.5 (171/239)	

Data are presented as median (P25, P75) for continuous variable and %(n/N) for categorical variable.

gestational hypertension and gestational diabetes, were increased in IVF/ICSI cycles, even for singleton births (27, 28). However, the exact biological mechanism leading to adverse perinatal outcomes is unclear. Some studies have shown that infertility disease itself is the main reason for the poor perinatal outcome of assisted reproductive technology (ART) singleton offspring (29). However, an increasing number of studies have shown that the process of ART, such as exposure to superphysiological doses of estrogen, may have an adverse effect on perinatal outcomes (20, 30, 31). The mechanism may be that superphysiological doses of estrogen may affect the development of the endometrium rather than the development and quality of the embryo (32, 33). In the artificial cycle of FET, exogenous estrogen is supplemented to cause endometrial hyperplasia while inhibiting the development of dominant follicles. When the endometrium is suitable, exogenous progesterone is supplemented to promote the synchronous development of the endometrium and the embryo. It is not clear

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TABLE 2 Comparison of neonatal and perinatal outcomes among groups.

	Duration of estrogen treatment before progesterone administration (days)				*P value*
	≤12	13-15	16-19	≥20	
No. of cases	372	745	654	239	
Neonatal outcomes					
Neonatal birth weight(g)	3500 (3150,3800)	3500 (3150,3800)	3405 (3100,3800)	3490 (3150,3700)	0.168
Neonatal sex (%)					0.693
Male	56.2 (209/372)	54.2 (404/745)	53.4 (349/654)	51.5 (123/239)	
Female	43.8 (163/372)	45.8 (341/745)	46.6 (305/654)	48.5 (116/239)	
Gestational weeks at delivery(week)	39 (38,40)	39 (38,40)	39 (38,40)	39 (38,40)	0.940
Preterm birth (%)	6.7 (25/372)	7.5 (56/745)	9.2 (60/654)	10.0 (24/239)	0.328
Low birth weight (%)	5.6 (21/372)	4.6 (34/745)	6.3 (41/654)	5.0 (12/239)	0.551
Small-for-gestational age (%)	4.3 (16/372)	4.4 (33/745)	5.2 (34/654)	2.5 (6/239)	0.390
Macrosomia (%)	13.4 (50/372)	16.0 (119/745)	15.1 (99/654)	13.0 (31/239)	0.565
Large-for-gestational age (%)	28.0 (104/372)	26.6 (198/745)	25.4 (166/654)	21.8 (52/239)	0.358
Pregnancy-related complications					
Gestational diabetes mellitus (%)	4.0 (15/372)	6.7 (50/745)	6.4 (42/654)	11.3 (27/239)	0.006
Gestational hypertension (%)	7.3 (27/372)	7.4 (55/745)	8.9 (58/654)	7.1 (17/239)	0.676
Premature rupture (%)	3.0 (11/372)	4.2 (31/745)	4.4 (29/654)	4.6 (11/239)	0.662
Placenta previa (%)	2.4 (9/372)	0.9 (7/745)	2.1 (14/654)	1.7 (4/239)	0.211

Data are presented as median (P25, P75) for continuous variable and %(n/N) for categorical variable.

TABLE 3 Multiple logistic regression analysis to account for confounding variables for neonatal and perinatal outcomes.

	Duration of estrogen treatment before progesterone administration (days)			
	≤12 (Reference)	13-15	16-19	≥20
Preterm birth				
% (n/N)	6.7 (25/372)	7.5 (56/745)	9.2 (60/654)	10.0 (24/239)
AOR (95% CI)	Ref	1.13 (0.69-1.86)	1.40 (0.84-2.33)	1.52 (0.82-2.86)
Low birth weight				
% (n/N)	5.6 (21/372)	4.6 (34/745)	6.3 (41/654)	5.0 (12/239)
AOR (95% CI)	Ref	0.75 (0.42-1.33)	1.01 (0.57-1.79)	0.71 (0.33-1.56)
Small-for-gestational age				
% (n/N)	4.3 (16/372)	4.4 (33/745)	5.2 (34/654)	2.5 (6/239)
AOR (95% CI)	Ref	0.96 (0.51-1.79)	1.05 (0.56-2.00)	0.47 (0.17-1.29)
Macrosomia				
% (n/N)	13.4 (50/372)	16.0 (119/745)	15.1 (99/654)	13.0 (31/239)
AOR (95% CI)	Ref	1.29 (0.90-1.86)	1.27 (0.86-1.86)	1.05 (0.63-1.75)
Large-for-gestational age				
% (n/N)	28.0 (104/372)	26.6 (198/745)	25.4 (166/654)	21.8 (52/239)
AOR (95% CI)	Ref	1.01 (0.75-1.34)	1.01 (0.74-1.36)	0.84 (0.56-1.27)

(Continued)

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TABLE 3 Continued

	Duration of estrogen treatment before progesterone administration (days)			
	≤12 (Reference)	13-15	16-19	≥20
Gestational hypertension				
% (n/N)	7.3 (27/372)	7.4 (55/745)	8.9 (58/654)	7.1 (17/239)
AOR (95% CI)	Ref	0.96 (0.59-1.57)	1.09 (0.66-1.79)	0.75 (0.38-1.48)
Gestational diabetes mellitus				
% (n/N)	4.0 (15/372)	6.7 (50/745)	6.4 (42/654)	11.3 (27/239)
AOR (95% CI)	Ref	1.48 (0.77-2.82)	1.87 (0.95-3.66)	1.43 (0.64-3.16)
Premature rupture				
% (n/N)	3.0 (11/372)	4.2 (31/745)	4.4 (29/654)	4.6 (11/239)
AOR (95% CI)	Ref	1.46 (0.72-2.97)	1.65 (0.79-3.42)	1.81 (0.74-4.45)
Placenta previa				
% (n/N)	2.4 (9/372)	0.9 (7/745)	2.1 (14/654)	1.7 (4/239)
AOR (95% CI)	Ref	0.49 (0.18-1.33)	0.64 (0.26-1.57)	0.39 (0.10-1.46)

AOR, adjusted odds ratio; CI, confidence interval. Multiple logistic regression model included maternal age, paternal age, BMI, gravidity, duration of infertility, type of infertility (primary/secondary infertility), infertility diagnosis(tubal/male/both/others), PCOS(yes/no), basal antral follicle count, basal serum FSH level, fertilization method (IVF/ICSI), endometrial thickness on the first day of progesterone administration, type of transferred embryos (cleavage embryo/blastocyst) and duration of estrogen treatment.

whether prolonging the use of estrogen would affect offspring and perinatal outcomes.

To the best of our knowledge, only two studies have analyzed the relationship between the duration of estrogen treatment and neonatal and perinatal outcomes. Bourdon M et al. (13) reported that the mean birth weight and Z scores were significantly lower for the '36–48 days' group than for the '≤21 days' group (3042 ± 801.2 g and −0.44 ± 1.50 vs. 3362 ± 602.9 g and 0.10 ± 0.94, respectively). Another study included only single, vitrified-warmed, euploid blastocysts, and the results showed that variation in the duration of estradiol supplementation before progesterone initiation did not impact frozen, euploid blastocyst transfer outcomes. The duration of estrogen administration was inversely correlated with gestational age at delivery, but this did not translate into an increase in preterm delivery (15). To date, our research is the largest retrospective cohort study to analyze the relationship between the duration of estrogen treatment before progesterone application and neonatal outcomes. This is the first study to analyze the relationship of this duration to pregnancy complications, including hypertensive states of pregnancy, GDM, placenta previa, placental abruption and premature rupture. Through analysis, different estrogen use times had no significant effect on offspring or perinatal outcomes. However, this study also has certain limitations. First, it suffers from the limitation of a retrospective cohort, although we used strict inclusion and exclusion criteria and multiple logistic regression to correct for the influence of confounding factors. Second, regarding the duration of estrogen use, the time range of hormone use at our center is 10-30 days, most of the treatment durations are 13-18 days (25%-75%), and only 275 cases have been treated for more than 19 days; this estrogen use time is shorter than that in Bourdon M's (13) study, and his research shows that a duration of estrogen treatment greater than 35 days may have an impact on the outcome of offspring mean birth weight. However, in our center, there are almost no transplantation cycles with an estrogen application time greater than 35 days. According to our current data in our reproductive center, the duration of estrogen therapy does not affect offspring and perinatal outcomes, but it is uncertain whether extending the estrogen application time will affect the outcome. Consequently, further analysis of the safety of the estrogen treatment duration before progesterone application is needed for a well-designed, prospective, and large-scale cohort study.

Conclusion

In conclusion, the duration of estrogen treatment before progesterone application does not affect neonatal and perinatal outcomes in FET cycles. For patients and clinicians, the transplant time can be flexibly arranged for FET in artificial cycles. However, due to the retrospective cohort study limitation, further randomized controlled studies with large samples are needed.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of The Third Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JZ and LS designed the study and selected the populations to be included and excluded. JZ and MD were involved in the data extraction and analysis. YG reviewed the data. ZW and SW participated in the revision of the manuscript. JZ and MD were involved in drafting this article. 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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Effects of body mass index and insulin resistance on first-time assisted conception and perinatal outcomes in young polycystic ovary syndrome patients

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Objective: The objective of the study was to explore the effect of body mass index (BMI) and insulin resistance (IR) levels on first-time assisted conception results and perinatal outcomes in young polycystic ovary syndrome (PCOS) patients.

Design: This was a single-center, retrospective, observational cohort study.

Patients: Young women with PCOS undergoing their first embryo transfer were included in the study.

Main outcome measure: Early pregnancy loss rate was the main outcome measure.

Results: The early pregnancy loss rate in the overweight + insulin resistance group (OW+IR group) was significantly higher than that in the non-overweight + non-insulin resistance group (NOW+NIR group) (18.16% *vs.* 9.02%, Bonferroni correction, P = 0.012). The early pregnancy loss rate in the non-overweight + insulin resistance group (NOW+IR group) and overweight + non-insulin resistance group (OW+NIR group) (18.18% and 17.14%, respectively) were also higher than that in the NOW+NIR group (6.07%), but the difference was not statistically significant (Bonferroni correction, all P > 0.05). No significant difference was found in clinical pregnancy rate, live birth rate, and macrosomia rate (all P > 0.05). After adjusting for confounding factors, BMI and IR levels were identified as independent risk factors for early pregnancy loss rate.

Conclusion: BMI and IR levels are independent risk factors for early pregnancy loss in young PCOS patients during the first embryo transfer cycle. Multiple indicators should be considered when assessing pregnancy outcomes, which will promote individualized pregnancy guidance and treatment procedures for PCOS patients.

KEYWORDS

insulin resistance, polycystic ovary syndrome, overweight, early pregnancy loss rate, multivariate logistic regression analysis

1 Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder in women of reproductive age, accounting for approximately 80% of anovulatory infertility (1). The clinical manifestations of PCOS include sporadic ovulation or anovulation, polycystic ovarian changes, hyperandrogenemia, obesity, and insulin resistance (IR). Many women with PCOS are overweight (OW) or obese (2). Obesity raises the risk of infertility and is a major contributor to the metabolic syndrome. This risk is mostly attributed to the defective hypothalamic-pituitary-ovarian (HPO) axis, low oocyte quality, and decreased endometrial tolerance (3). Obesity is typically accompanied by elevated levels of circulating insulin and a concurrent rise in ovarian androgen production. Extra adipose tissue causes these androgens to aromatize into estrogens, which exerts a negative feedback on the HPO axis and affects gonadotropin (Gn) production. These changes lead to ovulatory dysfunction and menstrual abnormalities. Hyperinsulinemia plays a fundamental role in the pathogenesis of PCOS and is characterized by hypomenorrhea and hyperandrogenemia (4). The symptoms of PCOS are exacerbated by the concurrent presence of obesity, which further raises IR (5). In addition, the increased testosterone (T) production in PCOS causes visceral fat accumulation, which in turn raises IR and hyperinsulinemia, aggravating the vicious cycle (3).

IR is generally considered to be closely associated with obesity. Other studies have found that PCOS patients with IR have a significantly higher incidence of ovulation disorders, anovulation miscarriage, and complications such as gestational diabetes mellitus and gestational hypertension. PCOS patients with IR also exhibit significantly lower conception rates (6–8). However, not much research focused on how IR and BMI levels affect first-time assisted conception results and perinatal outcomes in people with PCOS. Therefore, this study aimed to compare the effects of various levels of IR and OW on first-time assisted conception outcomes and perinatal outcomes in patients with PCOS. Furthermore, the effects of various levels of IR and OW on early pregnancy loss rate were investigated during the first embryo transfer in PCOS patients.

2 Materials and methods

2.1 Study population

This single-center retrospective cohort study was approved by the Ethics Committee of the People's Hospital of Zhengzhou University. Retrospective data analysis was performed on PCOS women who underwent their first *in vitro* fertilization (IVF) or intracytoplasmic sperm microinjection (ICSI) procedures at the Reproductive Medicine Center of Henan Provincial People's Hospital between January 2016 and December 2021. The inclusion criteria were as follows: 1) age ≤35 years; 2) normal ovarian reserve with anti-Mullerian hormone (AMH) >1.1 ng/ml; 3) patients in their first IVF/ICSI-ET assisted conception cycle. The exclusion criteria were as follows: 1) chromosome abnormalities detected on preimplantation genetic screening of preimplantation genetic diagnosis; 2) recurrent spontaneous abortion; 3) uterine cavity abnormalities (uterine adhesions, endometrial polyps, endometritis, unicornuate/bicornuate uterus, etc.); 4) incomplete data cycles, no embryo transfer cycles; 5) thyroid disease, diabetes mellitus, and other systemic endocrine diseases; and 6) uterine fibroids, adenomyosis, and endometriosis. All couples included in the study signed a consent for assisted reproduction treatment (ART) therapy. The study adhered to the fundamental tenets of the Helsinki Declaration.

2.2 Diagnostic criteria and formulas

The diagnosis of PCOS was based on the Rotterdam criteria (2003), which requires at least two of the following three criteria: oligomenorrhea and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries on ultrasound scanning (9). IR was assessed using the homeostasis model assessment insulin resistance index (HOMA-IR), HOMA-IR = fasting blood glucose (FPG) (mmol/L)/22.5 × fasting insulin (FINS) (μU/ml). HOMA-IR ≥2.69 was considered IR. According to the criteria of the Chinese Working Group on Obesity, body mass index (BMI) ≥24 kg/m^2 was considered OW and ≥28 kg/m^2 was considered obese.

2.3 Grouping

PCOS patients were divided into four groups according to HOMA-IR and BMI: Group 1: non-overweight + non-insulin resistance group (NOW+NIR group), Group 2: non-overweight + insulin resistance group (NOW+IR group), Group 3: overweight + non-insulin resistance group (OW+NIR group), and Group 4: overweight + insulin resistance group (OW+IR group) according to IR index and BMI.

2.4 Ovulation promotion protocol

The controlled ovulation induction protocol in this study was carried out by the same team based on the patients' conditions. All of the women underwent either gonadotropin-releasing hormone (GnRH) agonist or flexible GnRH antagonist protocol. After ovulation induction at the appropriate time, oocytes were retrieved by transvaginal ultrasound 36–37 h later according to the fertility center's protocol, and IVF/ICSI insemination was performed based on the male partner's condition.

2.4.1 GnRH agonist protocol

For the GnRH agonist protocol, the long-acting GnRH agonist (Diphereline, Ipsen, Tianjin) was injected once at a total of 3.75 mg on the second or third day of the menstrual cycle, 30–35 days after which the serum hormone levels were monitored and the ultrasound was undertaken. On top of that, the same examinations could be accomplished after a short-acting GnRH agonist (Decapeptyl, 0.1 mg/day, Germany Ferring) was injected each day for 14–18 days starting from the middle luteal phase of the

previous menstrual cycle. When the requirements for downregulation were met, a dose of 75–300 IU Gn was administered based on age, ovarian reserve, and AMH levels. Depending on the ovarian response and hormone levels, the Gn dosage was modified after 4–5 days. Subcutaneous injection of urinary human chorionic gonadotropin (hCG) was administered when at least two follicles measured ≥18 mm or three follicles measured ≥17 mm. A dose of 4,000–10,000 IU of hCG (Lizhu Pharmaceutical Trading, China) was given to induce ovulation according to the peak estradiol level and age. Extraction of the oocytes was guided by vaginal ultrasonography 36–37 h later.

2.4.2 GnRH antagonist protocol

Ultrasound and basal sex hormone measurements were performed on the second or third day of the menstrual cycle. If no dominant follicles and functional ovarian cysts were observed, Gn was injected to trigger ovulation, starting at the previously mentioned dosage. The size of the follicle and hormone levels were monitored for 4–5 days following Gn treatment. A daily dose of 0.25 mg GnRH antagonist was initiated when dominant follicles showed a mean diameter of 12 mm or estrogen levels ≥200 ng/L or blood luteinizing hormone (LH) levels significantly increased. The process was continued until the hCG dosing day. Subcutaneous injection of urinary hCG was administered when at least two follicles measured ≥18 mm or three follicles measured ≥17 mm. A dose of 4,000–10,000 IU of hCG was given to induce ovulation depending on the peak estradiol level and age. Extraction of the oocytes was guided by vaginal ultrasonography 36–37 h later.

2.5 Fresh embryo transfer and luteal support

IVF/ICSI fertilization was performed depending on male semen parameters. After 3–6 days of *in vitro* culture, the best 1 or 2 cleavage embryos or blastocysts were selected according to the routine protocol of our center and then transferred into the uterus under abdominal ultrasound guidance. The embryos that were not transferred were frozen by vitrification with the informed consent of the couple. Fresh cycle transplant patients started receiving luteal support on ovulation day with a progesterone vaginal gel (Crinone, 90 mg/tablet, Merck Serono, Germany) 90 mg/day vaginal medication and dydrogesterone (Duphaston, 10 mg/tablet, Abbott, Netherlands) 10 mg orally bid.

2.6 First freeze-thaw embryo transfer and luteal support after whole embryo freezing

The endometrium was prepared by choosing a natural cycle or artificial replacement cycle according to the patient's condition. In the natural cycle, follicle development and endometrial thickness were monitored by vaginal ultrasound from the 9th or 10th day of the menstrual cycle, and luteal support was given after ovulation (medication as above). One or two cleavage embryos or blastocysts with the best score were transferred at the appropriate time. During the artificial cycle, estradiol valerate tablets (Progynova, 1 mg/tablet, Bayer Pharmaceutical Company, Germany) were taken orally 4–8 mg/day from the second to fourth day of the menstrual cycle, and the endometrial thickness was monitored after 7–8 days. The original dosage was kept the same or increased based on the endometrial thickness. Progesterone was used to transform the endometrium when the medication's duration was ≥11 days or the endometrial thickness was ≥8 mm. Transforming endometrial medication and embryo transfer strategies are basically consistent with the natural cycle.

2.7 Outcomes

Comparison of the general conditions of patients in the four groups: Age, BMI, HOMA, LH, FSH, AMH, years of infertility, and type of infertility

Comparison of assisted conception outcomes in the four groups: duration of Gn, total dosage of Gn, number of oocytes retrieved, number of mature oocytes, number of normal fertilization oocytes, number of available embryos, and blastocyst formation rate

Comparison of perinatal outcomes in the four groups: early miscarriage rate, early pregnancy loss rate, live birth rate, and macrosomia birth rate

The primary outcome of this study was the early pregnancy loss rate. Peripheral blood hCG >50 mIU/ml at 14 days after embryo transfer was used to define pregnancy, and clinical pregnancy was defined as the presence of at least one intrauterine gestational sac 4–5 weeks after transfer (ectopic pregnancy was not included in this study). Biochemical pregnancy was characterized by hCG-positive results in the absence of an intrauterine gestational sac *in utero*. Early miscarriage was defined as a miscarriage occurring within the first 12 weeks of pregnancy. Early pregnancy loss included biochemical pregnancy and early miscarriage. When an early pregnancy loss was determined, all luteal support medications were discontinued. In pregnant patients, luteal support medications were discontinued, and luteal support medications were maintained until 8–10 weeks of gestation. Early pregnancy loss rate = (biochemical pregnancy cycles + early miscarriage cycles)/hCG positive cycles × 100%.

2.8 Statistical analysis

All statistical management and analyses were performed using SPSS 24.0 software. The measurement data conforming to normal distribution were expressed as mean ± SD, and one-way ANOVA was used for comparison between groups. All counting data were expressed by percentage (%), and the chi-square test was used to compare the count data between groups. The Bonferroni method was used to compare multiple groups by pairwise comparison. Univariate logistic analysis was conducted to determine the factors affecting clinical outcomes. After adjusting for confounding variables, a logistic regression model was used to investigate the effects of different BMI and IR levels on the outcome indicators. A two-sided P-value <0.05 was considered statistically significant.

3 Results

The study initially screened 2,876 patients with PCOS who underwent a first-time embryo transfer cycle, and 1,240 eligible patients were finally enrolled based on the inclusion and exclusion criteria.

3.1 Patient demographic and clinical characteristics

Table 1 shows the demographic and clinical characteristics of the four groups of patients. IR and NIR accounted for 55.65% (690/1,240) and 44.35% (550/1,240) of the total PCOS cases, respectively. OW patients accounted for 54.44% (675/1,240) of all PCOS patients, and 510 cases (75.56%, 510/675) also had IR. NOW patients accounted for 45.56% (565/1,240) of all PCOS patients, and 385 cases (68.14%, 385/565) were NIR. The differences in HOMA-IR (Group 1, Group 2, Group 3, Group 4: 1.73 ± 0.53, 4.00 ± 1.55, 2.02 ± 0.48, 5.11 ± 2.26, respectively, $P < 0.001$) and BMI (Group 1, Group 2, Group 3, Group 4: 21.06 ± 1.67, 22.29 ± 1.43, 26.54 ± 2.27, 28.27 ± 2.85, respectively, $P < 0.001$) were statistically significant. AMH, basal follicle-stimulating hormone (FSH), basal LH, and T levels were significantly lower in the OW+IR group compared to those in the NOW+NIR group, whereas the number of years of infertility was significantly higher. No statistically significant differences were observed in age, infertility type, and fertilization method ($P > 0.05$).

3.2 Ovarian stimulation and first embryo transfer results

As shown in Table 2, the starting dosage of Gn, the total dosage of Gn, and the duration of Gn became higher in the OW+IR and OW+NIR groups compared to those in the NOW+NIR and NOW+IR groups, while the number of oocytes retrieved, the number of mature oocytes, the number of normally fertilized oocytes, and the number of normal cleavage embryos decreased. No statistically significant difference was observed in the type of protocol, number of available embryos, number of good quality embryos, and blastocyst formation rate.

Furthermore, the early miscarriage rate in the OW+IR group (13.41%) was significantly higher than that in the NOW+NIR group (6.07%) (Bonferroni correction, $P = 0.024$). The early miscarriage rate in the NOW+IR group and OW+NIR group (13.6% and 12.07%, respectively) was higher than that in the NOW+NIR group (6.07%), but the differences were not statistically significant, and there was no difference in the other pairwise comparisons (Bonferroni correction, all $P > 0.05$). Similar results were observed in the early pregnancy loss rate: the early pregnancy loss rate in the OW+IR group (18.16%) was higher than that in the NOW+NIR group (9.02%), and the difference was statistically significant (Bonferroni correction, $P = 0.012$). The early pregnancy loss rate in the NOW+IR group and OW+NIR group (18.18% and 17.14%, respectively) was higher than that in the NOW+NIR group (6.07%), but the difference was not statistically significant (Bonferroni correction, all $P > 0.05$). There were no significant differences in the other pairwise comparisons. The differences in endometrial thickness, clinical pregnancy rate, and live birth rate were not statistically significant when compared after the first embryo transfer ($P > 0.05$) (Table 3).

TABLE 1 Comparison of demographic and clinical characteristics of the four groups.

GROUP	Group 1	Group 2	Group 3	Group 4	P
No. of cases	385	180	165	510	
Age (years)	28.91 ± 3.38	28.27 ± 3.55	29.12 ± 3.47	28.51 ± 3.56	0.051
BMI (kg/m^2)	21.06 ± 1.67	22.29 ± 1.43^{a}	26.54 ± 2.27^{ab}	28.27 ± 2.85^{abc}	<0.001
HOMA-IR	1.73 ± 0.53	4.00 ± 1.55^{a}	2.02 ± 0.48^{ab}	5.11 ± 2.26^{abc}	<0.001
LH (IU/L)	10.85 ± 6.42	9.63 ± 5.47	9.52 ± 5.17	8.51 ± 4.62^{a}	<0.001
FSH (IU/L)	6.13 ± 1.44	5.81 ± 1.45	5.90 ± 1.56	5.82 ± 1.55^{a}	0.016
T(ng/m)	0.39 ± 0.20	0.43 ± 0.22	0.41 ± 0.18	0.45 ± 0.22^{a}	<0.001
AMH (ng/ml)	9.18 ± 4.70	8.87 ± 4.51	8.93 ± 4.92	7.80 ± 4.33^{ab}	<0.001
Duration of infertility (years)	3.34 ± 2.07	3.66 ± 2.18	3.56 ± 2.13	4.15 ± 2.41^{ac}	<0.001
Type of infertility (%)					0.639
Primary	69.09 (266/385)	66.11 (119/180)	63.64 (105/165)	66.67 (340/510)	
Secondary	30.91 (119/385)	33.89 (61/180)	36.36 (60/165)	33.33 (170/510)	
Methods of ART (%)					0.061
IVF	82.34 (317/385)	84.44 (152/180)	90.30 (149/149)	87.06 (444/510)	
ICSI	17.66 (68/385)	15.56 (28/180)	9.70 (16/149)	12.94 (66/510)	

[1a,] Significantly different to Group 1; [b,] Significantly different to Group 2; [c,] Significantly different to Group 3.
Group 1, NOW-NIR; Group 2, NOW-IR; Group 3, OW-NIR; Group 4, OW-IR.

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TABLE 2 Ovarian stimulation characteristics among the four groups.

GROUP	Group 1	Group 2	Group 3	Group 4	P
No. of cases	385	180	165	510	
Protocol (%)					0.190
GnRH agonist protocol	83.64 (322/385)	85.56 (154/180)	86.67 (143/165)	88.63 (452/510)	
GnRH antagonist protocol	16.36 (63/385)	14.44 (26/180)	13.33 (22/165)	11.37 (58/510)	
No. of basal antral follicles	22.77 ± 4.15	23.18 ± 3.64	22.48 ± 4.61	23.50 ± 3.99[ac]	0.010
Starting dosage of Gn (IU)	121.04 ± 27.62	123.12 ± 25.19	135.45 ± 28.73[ab]	144.44 ± 34.65[abc]	<0.001
Total dosage of Gn (IU)	1631.67 ± 692.83	1696.81 ± 761.10	2245.70 ± 1060.56[ab]	2644.74 ± 1163.04[abc]	<0.001
Duration of Gn (d)	10.98 ± 2.68	10.95 ± 2.74	12.29 ± 3.32[ab]	13.04 ± 3.49[ab]	<0.001
No. of oocytes retrieved	14.87 ± 7.80	14.89 ± 8.61	13.30 ± 7.33	12.85 ± 7.21[ab]	<0.001
No. of mature oocytes	12.68 ± 6.90	12.72 ± 7.43	11.25 ± 6.62	11.08 ± 6.46[a]	<0.001
No. of normal fertilization oocytes	9.08 ± 5.60	9.04 ± 6.01	7.72 ± 4.63[a]	7.83 ± 5.15[a]	<0.001
No. of normal cleavage embryos	8.84 ± 5.49	8.80 ± 5.87	7.50 ± 4.52[a]	7.58 ± 5.08[a]	<0.001
No. of available embryos	7.47 ± 4.97	7.57 ± 5.50	6.89 ± 4.43	6.85 ± 4.77	0.219
No. of good embryos	3.04 ± 3.56	3.46 ± 3.94	2.99 ± 3.23	3.08 ± 3.58	0.633
Blastocyst formation rate(%)	69.98 (1902/2718)	70.99 (893/1258)	68.00 (622/916)	71.98 (2168/3012)	0.087

2 [a:] Significantly different to Group 1 [b:] Significantly different to Group 2 [c:] Significantly different to Group 3.

TABLE 3 Outcomes of first embryo transfer cycle.

Group	Group 1	Group 2	Group 3	Group 4	P
No. of cases	385	180	165	510	
Type of transfer (%)					0.026
Fresh cycle	43.64 (168/385)	42.78 (77/180)	51.52 (85/165)	52.16 (266/510)	
Frozen cycle	56.36 (217/385)	57.22 (103/180)	48.48 (80/165)	47.84 (244/510)	
No of embryo transferred	1.39 ± 0.49	1.40 ± 0.49	1.49 ± 0.50	1.48 ± 0.50	0.028
Type of transfer embryos (%)					0.008
cleavage	52.73 (203/385)	55.56 (100/180)	65.45 (108/165)[a]	61.96 (316/510)[a]	
blastocyst	47.27 (182/385)	44.44 (80/180)	34.55 (57/165)[a]	38.04 (194/510)[a]	
Endometrium (mm)	10.10 ± 1.93	10.22 ± 2.23	9.87 ± 1.96	10.10 ± 2.12	0.443
Clinical pregnancy rate (%)	64.16 (247/385)	69.44 (125/180)	70.30 (116/165)	64.31 (328/510)	0.190
Early miscarriage rate (%)	6.07 (15/247)	13.60 (17/125)	12.07 (14/116)	13.41 (44/328)[a]	0.028
Late miscarriage rate (%)	4.45 (11/247)	1.60 (2/125)	6.90 (8/116)	8.84 (29/328)[ab]	0.019
Early pregnancy loss rate	9.02 (23/255)	18.18 (24/132)	17.14 (22/124)	18.16 (63/347)[a]	0.010
Live birth rate (%)	55.32 (213/385)	53.33 (96/180)	55.15 (91/165)	47.06 (240/510)	0.060
Single live birth rate (%)	45.45 (175/385)	44.44 (80/180)	43.03 (71/165)	37.65 (192/510)	0.096
Low birth weight rate (%)	6.29 (11/175)	6.25 (5/80)	1.41 (1/71)	6.77 (13/192)	0.400
Macrosomia rate (%)	9.14 (16/175)	11.25 (9/80)	11.27 (8/71)	13.02 (25/192)	0.709
Twin live birth rate (%)	9.87 (38/385)	8.89 (16/180)	12.12 (20/165)	9.41 (48/510)	0.740
Low birth weight rate (%)	52.63 (20/38)	81.25 (13/16)	50.00 (10/20)	56.25 (27/48)	0.208
Macrosomia rate (%)	0	0	0	0	

2 [a,] Significantly different to Group 1; [b,] Significantly different to Group 2.

Birth weight is an important predictor of neonatal and infant survival and a crucial indicator of pregnancy outcome. Low birth weight was defined as fetal birth weight <2,500 g. Macrosomia was defined as birth weight ≥4,000 g. The results of this study indicated that the other three groups may increase the macrosomia rate compared with the NOW+NIR group, but the differences were not significant (Table 3).

3.3 Univariate logistic regression analysis

The univariate logistic regression analysis revealed that the endometrial thickness on the day of transfer, type of transfer, and groups had statistically significant effects on the early pregnancy loss rate (P < 0.05). In contrast, AMH, age, T, type of infertility, duration of infertility, methods of ART, protocol, type of transfer embryos, and number of embryos transferred showed no statistical significance (P > 0.05). The details are shown in Table 4.

3.4 Multivariate logistic regression analysis

Multivariate logistic regression analysis was performed to explore the risk factors of early pregnancy loss rate. After adjusting for confounding factors such as age, AMH, T, protocol, methods of ART, endometrial thickness on the day of transfer, type of transfer, and the number of embryos transferred, the results showed that the group was an independent risk factor of early pregnancy loss rate. Compared with group 1, groups 2–4 had significantly higher early pregnancy loss rates (group 2, aOR = 2.377, 95% CI: 1.261, 4.480, P = 0.007; group 3, aOR = 2.304, 95% CI: 1.185, 4.477, P = 0.014; group 4, aOR = 2.229, 95% CI: 1.295, 3.835, P = 0.004). The details are shown in Table 5.

TABLE 4 Univariate analysis of effects on early pregnancy loss rate.

	Early pregnancy loss rate		
	B	OR (95% confidence interval)	P
Group1		1	0.012
Group2	0.807	2.242(1.211,4.149)	0.010
Group3	0.777	2.176(1.160,4.082)	0.015
Group4	0.805	2.238(1.346,3.716)	0.002
AGE(year)	0.004	1.004(0.952,1.060)	0.871
AMH(ng/ml)	-0.033	0.968(0.926,1.011)	0.141
T(ng/ml)	-0.184	0.832(0.325,2.131)	0.701
Endometrium(mm)	-0.109	0.897(0.811,0.992)	0.035
Duration of infertility (year)	0.023	1.024(0.945,1.109)	0.568
No. of embryo transferred	-0.252	0.777(0.534,1.133)	0.190
Type of transfer			
Fresh cycle		1	
Frozen cycle	0.471	1.602(1.092,2.351)	0.016
Type of infertility			
Primary		1	
Secondary	-0.013	0.987(0.663,1.470)	0.950
Protocol			
GnRH agonist protocol		1	
GnRH antagonist protocol	0.123	1.130(0.664,1.925)	0.651
Methods of ART			
IVF		1	
ICSI	0.247	1.281(0.731,2.244)	0.387
Type of transfer embryos			
cleavage		1	
blastocyst	0.031	1.031(0.710,1.498)	0.892

TABLE 5 Multivariate logistic regression on the early pregnancy loss rate according to BMI and IR level.

	Early pregnancy loss rate		
	B	aOR (95% confidence interval)	P
Group1		1	0.015
Group2	0.866	2.377(1.261,4.480)	0.007
Group3	0.834	2.304(1.185,4.477)	0.014
Group4	0.801	2.229(1.295,3.835)	0.004
AGE(year)	-0.010	0.990(0.934,1.048)	0.723
AMH(ng/ml)	-0.039	0.962(0.917,1.009)	0.115
T(ng/ml)	-0.582	0.559(0.195,1.599)	0.278
Endometrium(mm)	-0.075	0.928(0.831,1.036)	0.182
No. of embryo transferred	-0.232	0.793(0.528,1.191)	0.263
Type of transfer			
Fresh cycle		1	
Frozen cycle	0.602	1.825(1.166,2.856)	0.008
Protocol			
GnRH agonist protocol		1	
GnRH antagonist protocol	0.109	1.115(0.637,1.953)	0.703
Methods of ART			
IVF		1	
ICSI	-0.180	0.835(0.466,1.495)	0.544

4 Discussion

According to epidemiological studies, approximately 50% of PCOS patients are obese, and the prevalence of PCOS is gradually rising among OW or obese people (10). In the present study, IR and OW in PCOS patients accounted for 55.65% and 54.44% of the included cases, respectively, which is similar to the previously reported incidence of IR in Asian PCOS patients (11). Studies have shown that body mass reduction had a corrective effect on reproductive outcomes in obese infertile patients and increased spontaneous fertility in patients with anovulatory PCOS (12). Increased abdominal or visceral fat is associated with IR, which plays a central role in the pathophysiology of PCOS (13), while IR also leads to metabolic abnormalities in women with PCOS (14).

BMI is the most widely used clinical indicator to evaluate OW/obesity. Studies have shown that elevated BMI may lead to alterations in preovulatory follicular fluid metabolites, including elevated levels of insulin, triglycerides, and androgens and decreased hCG levels (15–17). Hassani et al. (18) demonstrated that IR decreases the number of mature oocytes in patients with PCOS by impeding oocyte meiosis and delaying oocyte maturation. In the current study, the duration of Gn and total dosage of Gn were higher in the overweight PCOS group compared with the normal body mass PCOS group, but the number of oocytes retrieved, mature oocytes, normal fertilization oocytes, and normal cleavage embryos were reduced. The OW combined with IR group had the lowest number of these indicators, but the number of available embryos and the number of good embryos were not significantly different.

Earlier investigations indicated that different BMI and IR levels did not impact the outcome of pregnancies. However, the current study confirmed that different BMI and IR levels may affect oocyte maturation, although no discernible effect was found on the number of available embryos, the number of good embryos, the clinical pregnancy rate, and the live birth rate. The differences may be observed in the cumulative pregnancy rate if follow-up statistics are continued.

Similar to previous studies, the present study showed that different BMI and IR levels were associated with early pregnancy loss rates, which were higher in the remaining three groups compared to the NOW+NIR group. The influences persisted after adjusting for factors such as age, AMH, number of embryos transferred, type of transfer embryo, and endometrial thickness. In addition, Li et al. (19) showed that the early spontaneous miscarriage rate was significantly higher and the live birth rate was lower in patients with PCOS with central obesity. Wu et al. (20) showed that weight loss of more than 5 kg may regulate the neuroreproductive endocrine hormone secretion, IR, and gene expression profiles of ovarian granulosa cells. These

changes resulted in improved ovarian responsiveness to Gn, the embryo quality, embryo implantation rate, clinical pregnancy rate, and live birth rate and reduced the spontaneous abortion rate in obese infertile PCOS patients undergoing IVF-ET. Chen et al. (8) investigated the effects of IR on the outcome of the first embryo transfer cycle in PCOS patients. The findings of this study revealed that higher IR levels were associated with significantly lower numbers of oocytes retrieved, fewer good embryos, poorer clinical pregnancy rate, and lower live birth rate. In contrast, the rate of early miscarriage and macrosomia increased significantly, and IR was an independent risk factor for early miscarriage and macrosomia in PCOS patients. The hyperinsulinemic state in PCOS patients can stimulate the secretion of androgens from ovaries and adrenal glands, and in the presence of androgen excess, PCOS patients are prone to visceral fat hypertrophy. Moreover, androgens can act directly on oocytes, leading to impaired follicular development and affecting the quality and quantity of follicular cells, ultimately leading to an increased rate of early pregnancy loss in patients with OW+IR. An altered uterine environment may be another factor contributing to the increased early pregnancy loss rate in OW and IR patients (21). Furthermore, because obesity alters Gn pharmacokinetics, obese patients require more Gn to stimulate the ovaries, and higher Gn doses may contribute to their poor pregnancy outcome.

5 Conclusion

In conclusion, this study showed heterogeneity in the clinical presentation of basal hormone levels in patients with different levels of BMI and IR, with hyperandrogenemia being more prominent in OW PCOS patients with IR. In terms of first-time assisted conception outcomes, the number of oocytes retrieved, mature oocytes, and normal fertilization oocytes were also significantly lower in patients with OW and IR, while no significant difference was found in the number of available embryos and clinical pregnancy rate, which may be related to the limitations of the retrospective analysis. However, the early pregnancy loss rate was significantly higher in patients with OW combined with IR, and different levels of BMI and IR were independent risk factors for early pregnancy loss rate. Therefore, in clinical practice, multiple indicators should be considered when assessing the pregnancy outcomes of infertile women, in order to promote individualized tailored pregnancy guidance and treatment procedures for young PCOS patients.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Reproductive Medicine Ethics Committee of Henan Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JG designed and organized the study. YC and YJ were involved in the data extraction and analysis. CZ was responsible for providing data and guiding research. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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

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The association between umbilical cord blood fat-soluble vitamin concentrations and infant birth weight

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Background: Fat-soluble vitamins, including vitamins A, D and E, play an important role in the regulation of glucose and lipid metabolism, and may affect infant birth weight. Evidence on the association of birthweight with fat-soluble vitamins is controversial. Therefore, this study aims is to determine the associations of birthweight with vitamin A, D, and E concentrations in cord blood.

Methods: A total of 199 mother–infant pairs were enrolled in the study. According to gestational age and birth weight, the mother–infant pairs were divided into small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA). The Vitamin A, D, and E concentrations in serum were measured by high-performance liquid chromatography tandem-mass spectrometry.

Results: The concentrations of vitamin A in the SGA group were significantly lower than those in the AGA and LGA groups. The concentrations of vitamin E in the SGA group were significantly higher than those in the AGA and LGA groups. However, no significant differences were observed in vitamin D among the three groups. Being male ($\beta = 0.317$, $p < 0.001$) and birth weight ($\beta = 0.229$, $p = 0.014$) were positively correlated with the levels of vitamin A. Birth weight ($\beta = -0.213$, $p= 0.026$) was correlated with lower levels of vitamin E. No correlation was found between influencing Factors and the levels of vitamin D ($p> 0.05$). After adjusting for gestational age, sex, mother's age, delivery mode, pre-pregnancy BMI, and weight gain during pregnancy, the levels of cord blood vitamin A were positively correlated with birth weight ($p=0.012$).

Conclusion: The infant's birth weight is associated with the levels of cord blood vitamins A and E. The dysregulation of vitamins A and E in infants may be a risk factor for fetal growth and future metabolic diseases.

KEYWORDS

vitamin A, vitamin D, vitamin E, fat-soluble vitamin, umbilical cord blood, birth weight

1 Introduction

Birth weight (BW) is important to the health of the fetus, and it also predicts the subsequent development of the child. There are two types of extreme birth weight according to the gestational age and sex-specific (1): large for gestational age (LGA) and small for gestational age (SGA), and both of them increase the risk of cardiovascular disease, diabetes, and obesity later in life (2, 3). However, the mechanisms underlying the extreme birth weight and subsequent metabolic dysfunction are not well understood.

Maternal nutrition is important for fetal development and maternal health. Fat-soluble vitamins, including vitamins A, D and E, play an essential role in the regulation of glucose and lipid metabolism. Vitamin A and Vitamin D can affect obesity, cardiovascular disease, insulin resistance and type 2 diabetes (4, 5). While recent literature has found new mechanistic insights into the vitamin E derivatives in cardiovascular disease regulation (6). Vitamin E improves lipid metabolism in mice with nonalcoholic fatty liver through Nrf2/CES1 signaling pathway (7). Optimal maternal nutrition is a major factor in regulating fetal development and its lifelong consequences (8). The cord blood concentration of fat-soluble vitamins may influence the intrauterine environment, and may be associated with birth weight and lifelong metabolic dysfunction.

Evidence on the association of birthweight with fat-soluble vitamins is controversial. Recent studies have shown that premature low birth weight (LBW) infants have significantly lower cord blood vitamin A levels, and vitamin A levels in LBW infants correlate with their birth weight (9). However, Enfu Tao et al. found that cord blood vitamin A concentration in late preterm infants was not associated with birth weight (10). Some meta-analysis relating to vitamin revealed that maternal vitamin D deficiency increased the risk of SGA (OR=1.588, 95% CI 1.138-2.216) (11) and LBW (OR=2.39, 95% CI 1.25-4.57) (12). However, in the current literature, the difference in neonatal anthropometric measurements was not found between infants born to normal and vitamin D deficient mothers (13). Vitamin E was found to be positively associated with birth weight and reduced the risk of SGA (14). And one finding had been reported that high levels of vitamin E were associated with macrosomia (15).

Understanding fat-soluble vitamins in low and high birthweight infants may provide new insights into metabolic mechanisms in newborns and help prevent subsequent metabolic diseases. This study aims to determine associations between birthweight and vitamin A, E, and D concentrations in cord blood.

Abbreviations: SGA, small for gestational age; LGA, large for gestational age; AGA, appropriate for gestational age group; LBW, low birth weight; GA, gestational age; CS, cesarean section; BMI, Body Mass Index; BW, Birth weight; SD, standard deviation.

2 Methods

2.1 Study population

Cord blood samples were collected from 199 newborns in the Department of Obstetrics of Chongqing Maternal and Child Health Hospital between June 2021 and December 2021. Inclusion criteria were singleton pregnancy, live, term birth (37–42 weeks), aged 18–42 years, no smoking and drinking history. Women with pregnancy complications, such as gestational hypertension, preeclampsia, cancer, or asthma were excluded. According to birth weight and gestational age (GA), a total of 199 mother–infant pairs were divided into SGA group (63), AGA (appropriate for gestational age) group (87), LGA group (49). Informed written consent was obtained. This study was approved by the Ethics Committee of our hospital.

2.2 Data collection

Data were collected from our hospital's electronic information system, including pre-pregnancy weight, maternal height, gestational weight gain, sociodemographic information, delivery and neonatal outcomes (GA, newborn sex, birth weight, birth length). Baby weight and birth length were measured immediately after birth. Neonatal cord blood was collected during delivery. As soon as the samples were collected, they were sent without delay to the hospital laboratory, centrifuged, and frozen at -80°C until fat-soluble vitamin levels were analyzed.

2.3 Vitamin A, D and E measurement

The samples were analyzed at the Neonatal Screening Center, Chongqing Health Center for Women and Children. The Vitamin A, D and E concentrations in serum were measured by high-performance liquid chromatography tandem-mass spectrometry (HPLCMS/MS) using a Waters UPLC Xevo TQS (Waters Corporation, Milford, MA, USA) described by Hao Liu et al. (16). Briefly, QCs, standards, and plasma samples were added into the corresponding wells of 96-wells plates. Protein was precipitated by adding isotopic IS in a 35:1 (v/v) mixture of methanol: isopropanol, then hexane was added to each well for extraction. The Vitamin A, D and E in serum was separated by HPLC on a Shimadzu Waters BEH C18μm 1.7 2.1*50mm column and quantitated by MS.

2.4 Variables and definitions

Birth weight was measured immediately after delivery by midwives using electronic scales to the nearest 10 g. According to the birth weight curve of newborns in China, infants with birth weight less than the 10th percentile and greater than the 90th percentile for gestational age and sex were divided into SGA and LGA (17). Prepregnancy BMI was calculated by dividing self-reported weight in kilograms by height in meters squared.

Gestational weight gain was calculated from prenatal weight and pre-pregnancy weight. Babies born between 37-42 weeks of pregnancy were defined as term birth. The GA was confirmed through the use of ultrasound before week 20 of gestation.

2.5 Statistical analysis

All analyses were done with SPSS version 25.0. Data are presented as mean ± standard deviation (SD) for continuous variables, medians and interquartile range for skewed distributions, and counts (percentages) for categorical variables. Comparisons among subjects in the SGA, AGA, and LGA groups were made using One-way ANOVA. Multivariate linear regression models were used to estimate the influencing factors on vitamins A, D, and E in cord blood and birth weight after adjusting for confounders. Associations between fat-soluble vitamins and neonatal birth weight adjusted for confounders were evaluated by partial correlation analysis. The confounders considered were mode of delivery, GA, maternal age, pre-pregnancy BMI, and sex of the infants. A p-value <0.05 was considered statistically significant.

3 Results

3.1 Baseline characteristics of the mother–infant pairs

Table 1 shows baseline characteristics of mothers and birth outcomes. This study enrolled a total of 199 mother-infant pairs, including 63 in the SGA group, 87 in the AGA group and 49 in the LGA group. Statistically significant differences were found in maternal age (P=0.005), delivery mode (P<0.001), gestational age (P=0.005), birth weight (P<0.001), birth length (P<0.001), and weight gain (P<0.001) among the three groups. Women with LGA babies were less likely to deliver spontaneously, and have higher weight gain during pregnancy and prepregnancy BMI than SGA and AGA groups.

3.2 Comparisons of fat-soluble vitamins

Statistically significant differences were found in cord blood vitamin A and E concentrations among the groups; No significant differences were observed in vitamin D. The concentrations of vitamin A in the SGA group were significantly lower than those in the AGA and LGA group; however, the concentrations of vitamin E in the SGA group were significantly higher than those in the AGA and LGA group (Table 1).

3.3 Analysis of the influencing factors on birth weight

GA, spontaneous vaginal delivery (SVD), weight gain during pregnancy, pre-pregnancy BMI, Vitamin A, and Vitamin E were associated with birth weight. GA (β = 0.480, p<0.001), Pre-pregnancy BMI (β = 0.260, p<0.001), Weight gain during pregnancy (β = 0.248, p<0.001), Vitamin A (β = 0.146, p=0.009) and being SVD (β = 0.277, p<0.001) were positively correlated with the birth weight, while Vitamin E (β = −0.135, p = 0.015) negatively correlated with the birth weight (Table 2).

TABLE 1 Baseline characteristics of the mother–infant pairs in the three groups.

	Total(n=199)	SGA(n=63)	AGA(n=87)	LGA(n=49)	*P*-value
Maternal information					
Maternal age (years)	29.9 ± 3.8	28.9 ± 3.8	30.6 ± 3.8	29.4 ± 3.8	**0.005***
Delivery mode (SVD/CS), n (%)	100(50.2%)/99(49.8%)	48(76.2%)/15(23.8%)	37(42.5%)/50(57.5%)	15(30.6%)/34(69.4%)	**<0.001*****
Prepregnancy maternal body mass index, mean ± SD	21.2 ± 2.9	20.3 ± 2.5	21.0 ± 2.8	22.6 ± 3.3	**<0.001*****
Weight gain during pregnancy	13.2 ± 4.4	12.3 ± 4.3	12.7 ± 4.4	15.5 ± 3.9	**<0.001*****
Neonatal information					
Gestational age (weeks)	38.8 ± 1.06	38.7 ± 1.1	38.7 ± 1.0	39.2 ± 0.9	**0.005***
Gender (male/female), n (%)	96(48.2%)/103(51.8%)	34(54%)/29(46%)	35(40%)/52(60%)	27(55%)/22(45%)	0.136
Birth weight (g)	3120(2750,3760)	2650(2480,2790)	3200(2930,3420)	4010(3870,4265)	**<0.001*****
Birth length (cm)	49.4 ± 2.2	47.4 ± 1.6	49.4 ± 1.3	51.9 ± 1.3	**<0.001*****
Cord blood metabolic measures					
vitamin A (ng/ml)	229.6 ± 67.4	214.3 ± 58.1	226.0 ± 65.5	255.5 ± 75.3	**0.004***
vitamin D (ng/ml)	19.7 ± 8.0	19.9 ± 8.8	19.2 ± 8.0	20.3 ± 7.2	0.711
vitamin E (ng/ml)	2196.0 ± 600.6	2388.0 ± 649.9	2069.6 ± 516.2	2176.2 ± 622.6	**0.005***

Data presented are medians, mean ± SD for continuous variables, n (%) for categorical variables, and interquartile range for skewed distributions. The p-values are from One-way ANOVA for differences in means for continuous variables or chi-square tests for differences in proportions for categorical variables groups. Values in bold are significant at p < 0.05.SVD, spontaneous vaginal delivery; CS, cesarean section. *p < 0.05, ***p < 0.001.

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TABLE 2 Multivariate linear regression analysis of the influencing factors on birth weight.

	β	95%IC		p-value
Maternal age	0.058	0.285	-7.394	0.285
Gestational age	0.480	207.516	323.76	**<0.001*****
Gender (male = 1)	-0.092	-236.166	19.283	0.096
SVD (yes = 1)	0.277	193.034	455.842	**<0.001*****
Pre-pregnancy BMI (kg/m2)	0.260	29.676	73.567	**<0.001*****
Weight gain during pregnancy	0.258	19.567	49.513	**<0.001*****
Vitamin A	0.146	0.320	2.230	**0.009***
Vitamin D	0.011	-7.117	8.711	0.843
Vitamin E	-0.135	-0.283	-0.125	**0.015***

Multivariate linear regression analysis was used with adjustments for maternal age, mode of delivery, gestational age, sex, pre-pregnancy BMI, weight gain during pregnancy and fat-soluble vitamins. Values in bold are significant at p <0.05.SVD, spontaneous vaginal delivery; BMI, body mass index. *p < 0.05, ***p < 0.001.

3.4 Analysis of factors of fat-soluble vitamins

Sex and birth weight were associated with the levels of vitamin A in umbilical cord blood. Being male (β = 0.317, $p < 0.001$) and birth weight (β = 0.229, $p = 0.014$) were positively correlated with the levels of vitamin A. Birth weight (β = -0.213, $p= 0.026$) was correlated with lower levels of vitamin E. No correlation was found between influencing Factors and the levels of vitamin D ($p> 0.05$) (Table 3).

3.5 Partial correlations between fat-soluble vitamins and newborn birth weight

The partial correlation coefficients between fat-soluble vitamins and newborn birth weight are shown in Table 4. After adjusting for delivery mode, GA, mother's age, sex, pre-pregnancy BMI, pregnancy weight gain and other factors, the cord blood vitamin A level i was positively correlated with body weight (p=0.012). However, no statistically significant association was found between vitamin D or vitamin E level and birth weight.

4 Discussion

Our study demonstrates the relationship between the levels of cord blood fat-soluble vitamins and the BW. We found that the cord blood vitamin A level was positively correlated with the infant BW, while the vitamin E level was negatively correlated with BW. When compared with those of AGA and LGA, the vitamin A level was substantially lower in SGA, whereas the vitamin E level was significantly higher. However, we found no significant association between cord blood vitamin D serum level with birth weight.

Vitamin A is critical to ensure proper embryonic development and is involved in several metabolic pathways (18). Observational studies have found that cord blood and maternal vitamin A levels were significantly correlated with birth weight and length (19–22). The largest study investigating untargeted metabolic profiles showed that cord blood vitamin A levels were associated with birthweight (23), after controlling for hereditary factors such as parental size. Indeed, the vitamin A supplementation study in Human Immunodeficiency Virus–Infected Women have indicated that maternal vitamin A supplementation increase birth weight and neonatal growth, and decreases anemia (24). In our study, we found that the cord blood vitamin A concentrations were associated with birth weight, which is consistent with previous research findings. This finding suggests that there might be a role of vitamin A in determining birth weight and underscores the importance of vitamin A nutrition in fetal growth. However, some studies have shown that maternal vitamin A concentrations were not associated with birth weight (10, 25). The reasons behind these discrepancies might be partly due to different study participants. In our study, the Mother–Infant Pairs were all healthy and born full term, therefore, the effects of premature birth, infection, and maternal disease on vitamin A were excluded.

Here, we also discovered that SGA infants exhibit lower concentrations of cord blood vitamin A compared to AGA infants. As some studies have found that umbilical cord blood vitamin A serum concentration is significantly correlated with maternal vitamin A status (21, 26), SGA infants may be born from vitamin A deficient mothers. Unfortunately, we did not measure serum vitamin A levels in pregnant mothers. We further observed that LGA infants had higher concentrations of cord blood vitamin A compared with AGA infants. As maternal nutritional status has a key role on fetal growth (27), this result suggested that maintaining a high concentration of vitamin A during pregnancy may not be good for fetal growth.

In this study, we found no significant association between cord blood vitamin D serum levels with birth weight, which does not agree with the findings of previous studies (11, 12, 28). A meta-analysis of observational studies has shown that vitamin D deficiency during pregnancy is associated with a higher risk of low birth weight (12). While a Brazilian cohort study found that

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TABLE 3 Multivariate linear regression analysis of the influencing factors for the levels vitamin A, vitamin D, and vitamin E in cord blood.

	β	95%IC		p-value
Vitamin A				
Maternal age	-0.50	-3.304	1.543	0.474
Gestational age	-0.86	-15.712	4.834	0.298
Gender (male=1)	0.317	24.416	60.894	**<0.001*****
SVD (yes = 1)	-0.148	-40.421	0.520	0.056
Pre-pregnancy BMI (kg/m2)	0.048	-2.362	4.535	0.535
Weight gain during pregnancy	0.03	-1.875	2.785	0.701
Birth weight	0.229	0.005	0.047	**0.014***
Vitamin D				
Maternal age	0.004	-0.300	0.315	0.961
Gestational age	-0.009	-1.372	1.236	0.918
Gender (male = 1)	0.077	-1.086	3.544	0.296
SVD (yes = 1)	0.050	-1.799	3.397	0.545
Pre-pregnancy BMI (kg/m2)	-0.077	-0.645	0.23	0.950
Weight gain during pregnancy	-0.068	-0.42	0.172	0.409
Birth weight	<0.001	-0.003	0.003	1
Vitamin E				
Maternal age	-0.034	-27.557	17.037	0.643
Gestational age	0.144	-13.074	176.178	0.091
Gender (male = 1)	0.002	-165.384	170.621	0.976
SVD (yes = 1)	-0.118	-330.224	44.896	0.140
Pre-pregnancy BMI (kg/m2)	0.021	27.428	360.097	0.788
Weight gain during pregnancy	0.133	-3.277	39.649	0.096
Birth weight	-0.213	-0.409	-0.026	**0.026***

Multivariate linear regression analysis was used with adjustments for maternal age, mode of delivery, gestational age, sex, pre-pregnancy BMI, birth weight and weight gain during pregnancy. Values in bold are significant at p <0.05.SVD, spontaneous vaginal delivery; BMI, body mass index. *p < 0.05, ***p < 0.001.

higher concentrations of vitamin D during the first, second, and third trimesters increase the risk of preterm birth (28). A meta-analysis has suggested that vitamin D deficiency is associated with an increased risk of SGA (11). However, in our study, we found no association between cord blood vitamin D levels and SGA. Three limitations may have biased the result, particularly the fact that our study subjects were full-term infants, excluding preterm infants. The second limitation the plasma we tested came from cord blood, which does not signify the entire course of pregnancy. The third limitation was the relatively small sample size. In light of the conflicting data from studies, larger-sample studies are required to further confirm the effects of vitamin D on birth weight.

Vitamin E is a regulator of glucose and lipid metabolism and vitamin E deficiency causes metabolic dysregulation in the early stages of development. Therefore, cord blood vitamin E serum levels may influence infant birth weight. Several studies have demonstrated that vitamin E deficiency was found in VLBW infants (29, 30). While a Chinese observational study has indicated that vitamin E

TABLE 4 Relationship between the levels of vitamins A, D and E and infant birth weight.

	vitamins A		vitamins D		vitamins E	
	r	*p-value*	*r*	*p-value*	*r*	*p-value*
Birth weight (g)	0.181	0.012	0.016	0.829	-0.140	0.053

Partial correlation analysis was used with adjustments for maternal age, mode of delivery, gestational age, gender, pre-pregnancy BMI, and weight gain during pregnancy. r, partial correlation coefficient; BMI, body mass index.

concentrations were positively related to increased fetal growth, and a positive association between vitamin E and macrosomia (15). In our study, we found that vitamin E was inversely related to birth weight. The results of this study indicate that the findings are different from what has been previously reported by other researchers. The possible influence factors were the different gestational ages of study participants and the source of the blood. And in another study, vitamin E concentrations were related to decreased risk of SGA births, and an increased risk of LGA (14). Here, we found that cord blood vitamin E concentrations were significantly higher in SGA than those in AGA and LGA. These findings suggest that vitamin E levels may affect on birth weight, but it is still controversial. Further studies are needed to investigate the relationship and mechanism between vitamin E and birth weight.

The strengths of this research include not only comparing the levels of fat-soluble vitamins in umbilical cord blood, but also dividing infants into subgroups based on their GA and BW. One limitation of the present study is the small number of participants from only one hospital in China, thus, it may not be sufficient in power to be generalizable. In the future, a large number of multicenter studies are needed.

In conclusion, this study found that a lower concentration of vitamin A in cord blood was associated with a higher risk of SGA and a lower risk of LGA. Additionally, excessive concentration of vitamin E in cord blood was found to be associated with SGA. Since the vitamin status of newborns is mostly dependent on that of their mothers, women are recommended to avoid having high concentrations of vitamin A and E during pregnancy. Nonetheless, further prospective studies are needed to explore these associations in different populations and to understand the mechanism linking vitamin A and E to infant birth weight.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of Chongqing Health Center for Women and Children. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YZ and GY conceived the study. GY performed the data analysis and wrote the manuscript. HL contributed to manuscript preparation. NW and LS helped with data analysis. All the authors contributed to the manuscript 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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The effect of an extended culture period on birth weight among singletons born after single or double vitrified embryo transfer

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Aim: To evaluate the effect of an extended culture period on birth weight among singletons born after vitrified-warmed embryo transfer

Methods: A retrospective cohort study was performed among 12400 women who gave birth to 1015, 1027, 687, and 9671 singletons after single blastocyst transfer, single cleavage-stage embryo transfer, double blastocyst transfer, and double cleavage-stage embryo transfer, respectively.

Results: The unadjusted birth weight of singletons born after vitrified blastocyst transfer were heavier than those born after cleavage-stage transfer (β=30.28, SE=13.17, P=0.022), as were the adjusted birth weights (β=0.09, SE=0.03, P=0.007). In addition, there was a 37% increased odd of having an infant with high birth weight after vitrified blastocyst transfer compared with vitrified cleavage stage transfer (OR=1.37, 95% CI:1.07-1.77).

Conclusion: The unadjusted and adjusted birth weight and odds of having an infant with high birth weight significantly increased after blastocyst transfer compared with cleavage-stage embryo transfer in vitrified-warmed cycles.

KEYWORDS

birth weight, blastocyst transfer, neonatal outcome, vitrification, culture period

Introduction

Forty years after the birth of the first *in vitro* fertilization (IVF) baby in 1978, many new assisted reproductive technologies (ART) have been widely applied to clinical practice in the past decades. Along with the increasing development and maturity of cryopreservation techniques, frozen embryo transfer (FET) has been a routine procedure that increases the

cumulative pregnancy rate and live birth rate per ovarian stimulation cycle and decreases the risk of ovarian hyperstimulation syndrome (OHSS) (1, 2). Blastocyst transfer has been popular worldwide, as it improves the implantation rate and live birth rate by providing better embryo-endometrium synchrony, more closely mimicking the sequence of events in natural conception, and increasing the chance of transferring a good-quality embryo (3). There is a global trend to reduce the number of transferred embryos aiming to minimize the multiple pregnancy rate and its associated complications (4). Single embryo transfer (SET) as a strategy has been advocated in clinically appropriate patients for years, and even mandated in many countries. Considering that the ultimate outcome of ART therapy should be a healthy singleton baby, not pregnancy or live birth, evaluating the safety of these important technologies regarding neonatal outcomes is necessary.

Birth weight is related to infants' short and long-term health. Previous studies have reported that infants with low birth weight (LBW) have a higher risk of cardiovascular diseases in adulthood, and large-for-gestational-age (LGA) infants have an increased risk of obesity and autism in life (5–7). Therefore, exploring the effect of these clinical techniques on birth weight is important to ensure the healthy growth of ART offspring. Several studies have shown an increased risk of LGA and high birth weight (HBW) using frozen/thawed embryo transfer compared to fresh transfer (8, 9). Although the effect of an extended embryo culture on birth weight has received much attention in recent years, the results are inconsistent. For fresh cycles, some studies have found significantly higher birth weight and an increase in the number of LGA babies after blastocyst transfer compared with those with cleavage-stage embryo transfer (10, 11), while another two studies did not detect significant differences in birth weight between blastocyst transfer and cleavage-stage embryo transfer in fresh cycles (12, 13). For the effect of culture period on birth weight in frozen embryo transfer cycles, two recent studies with small sample sizes also pointed to the opposite conclusions. The study by Zhang et al. reported that the proportion of LGA infants was higher in singletons born after blastocyst transfer than after transfer of cleavage-stage embryos in vitrified-warmed transfer cycles, but Anick et al. found that the transfer of vitrified blastocysts was associated with a lower birth weight compared with the transfer of vitrified cleavage-stage embryos (14, 15). Although the study performed by Holden et al. explored the different obstetrical and perinatal outcomes using the national database, it included embryos cryopreserved by both the conventional slow freeze technique and vitrification (16). Therefore, with the wide application of vitrification, it is important to evaluate the effect of an extended culture period on birth weight in vitrified-warmed transfer cycles.

Considering the popularity of FET, blastocyst transfer, and SET in clinical applications, and the open question with regard to the effect of an extended culture period on birth weight, we conducted this retrospective cohort study to evaluate the effect of an extended culture period on the birth weight of singletons born after single or double blastocyst transfer compared to cleavage-stage embryo transfer in vitrified-warmed cycles.

Materials and methods

Study design and population

This is a retrospective cohort study, conducted in the Shanghai Ninth People's Hospital affiliated with JiaoTong University School of Medicine (a large hospital-based tertiary care reproductive center in Shanghai, China). Women conceived after the transfer of one or two vitrified-warmed embryos and delivered a singleton baby after at least 20 weeks of gestation from January 2013 to December 2018. Patients were excluded if they had mixed cleavage-stage embryo-blastocyst transfer or if they underwent preimplantation genetic testing (PGT). Women were excluded if more than one gestational sac was detected by transvaginal ultrasound in the first trimester of pregnancy. Furthermore, women were excluded who had missing data on infant sex, birth weight, gestational age, number of transferred embryos and stage of embryo development. For women having more than one singleton birth, only the first birth was included. Additionally, patients with pregnancy-associated syndromes, including pregnancy-related hypertension and diabetes, were excluded, because of their influences on neonatal outcomes. The participant selection procedure is presented in Supplemetary Figure S1.

Patients were categorized into four groups: single cleavage-stage embryo transfer (SET-C), single blastocyst transfer (SET-B), double cleavage-stage embryo transfer (DET-C), and double blastocyst transfer (DET-B). This study protocol was approved by the ethics committee of the hospital and was carried out in accordance with the Helsinki Declaration.

Procedures

Our previous studies have described the procedures in detail for the ovulation induction, IVF/ICSI procedure, and embryo culture, freezing, thawing, and transfer (17–19). IVF or ICSI was performed depending on the semen quality. Normal fertilization was assessed 16-18 hours after insemination/injection. Then the embryos were subsequently cultured and embryo morphology was graded on Day 3 or Day 5/6.

Cleavage-stage embryos were classified as high-quality embryos if they had six to eight blastomeres on Day 3, with fragmentation < 20% according to Cummin's criteria. Blastocysts scored according to the Gardner and Schoolcraft grading system were recorded as high quality if they reached at least an expansion stage 3 with A or B for inner cell mass and trophectoderm (3BB). After embryo grading, all cleavage-stage embryos and blastocysts were frozen using the vitrification method. In brief, the cryotop carrier system (Kitazato Biopharma Co. Ltd, Japan) was used for vitrification, and 15% (v/v) ethylene glycol, 15% (v/v) dimethylsulfoxide and 0.5 mol/l sucrose were used as the cryoprotectant. For warming, 1.0 mol/l, 0.5 mol/l and 0.0 mol/l sucrose solutions were used for stepwise cryoprotectant dilution. All vitrification and warming steps were carried out at room temperature except for the first warming step, which was at 37 °C. The same continuous single culture media

(Irvine Scientific) and vitrification method was employed throughout the whole study period.

Endometrial preparation was performed as previously described (20). A natural cycle was used for patients with regular menstrual cycles, and a hormone therapy cycle or stimulation cycle was used for patients with irregular menstrual cycles. In the natural cycle, we monitored follicular growth by means of serum hormones and transvaginal ultrasound from cycle Day 10 onwards. When the diameter of the dominant follicle was >16 mm and the endometrial thickness was >8 mm, a bolus of hCG was injected to trigger ovulation. In the stimulation cycle, letrozole was prescribed orally for 5 days beginning on cycle Day 3 of menses. In the case of a dominant follicle <14 mm on Day 10, a daily dosage of 75 IU hMG (Anhui Fengyuan Pharmaceutical Co.) was added to stimulate follicle growth and the endometrial lining. In the hormone therapy cycle, oral E2 was commenced on the third day of the menstrual cycle, and progesterone exposure was initiated when the endometrial thickness was appropriate (usually >8 mm). The day of embryo transfer was determined based on the length of the culture period being 3 days or 5/6 days. In all FET cycles, one or two embryos were transferred according to the number of embryos and the patient's intention under ultrasound guidance, and serum β-HCG levels were measured 14 days after embryo transfer.

Data collection

The data used in the study were from the ART database of our center, which included all records about the basic demographic characteristics of patients, treatment details and outcomes. Details of treatments with ART and any birth resulting from ART have been recorded in the database, which was required by the Technical Standard for Human Assisted Reproduction issued by the Chinese Ministry of Health (CMOH). Variables extracted for this study included the following: maternal age, paternal age, maternal body mass index (BMI), type of infertility (primary infertility or second infertility), parity (nulliparous, pluriparous), duration of infertility, causes of infertility (female factor, male factor, combined factor, and unexplained infertility), fertilization type (IVF-only, ICSI-only, or IVF/ICSI split), sperm origin (ejaculation or testicular sperm extraction), number of transferred embryos (one or two embryos), stage of embryo development (blastocyst or cleavage-stage embryo) and endometrial preparation program (natural cycle, mild stimulation cycle, or hormonal replacement cycle). Information regarding infant sex, birthweight (unadjusted), and gestational age was also obtained.

Outcomes

Gestational age was calculated by adding 17 days for cleavage-stage embryo transfer and 19 days for blastocyst transfer from the embryo transfer date (14). Outcomes analyzed included very preterm birth (VPTM, <32 weeks of gestation), preterm birth (PTM, <37 weeks of gestation), very low birth weight (VLBW, <1500 g at birth), low birth weight (LBW, <2500 g at birth), high birth weight (HBW, >4000 g at birth), very high birth weight (VHBW, >4500 g at birth), small for gestational age (SGA, defined as birth weight <10th percentile for gestational age using the Chinese reference singleton newborns stratified by gestational age and neonatal sex), very small for gestational age (VSGA, defined as birth weight <3rd percentile), large for gestational age (LGA, defined as birth weight >90th percentile), and very large for gestational age (VLGA, defined as birth weight >97th percentile) (21). The adjusted birth weight (known as gestational age-adjusted and sex-adjusted birth weights) was also calculated, as described in a previous study (14). Birth defects were defined according to the International Classification of Diseases, 10th Revision (ICD-10), and a detailed description can be found in our previously published paper (19, 22).

Statistical analysis

The baseline characteristics and neonatal outcomes are presented as the mean (standard deviation, SD) for continuous variables and percentage for categorical variables. To investigate the influencing factors on gestational age, unadjusted birth weight, and adjusted birth weight, multivariable linear regression models were performed after controlling for other potential confounders. Logistic regression models were used to compute odds ratios (ORs) and 95% confidence intervals (CIs) for estimating the influencing factors on neonatal outcomes including the same covariates as the linear regression models. All statistical analyses were performed using a two-sided 5% level of significance and the statistical package Stata, Version 12 (StataCorp, USA). $P < 0.05$ was considered to be statistically significant.

Ethical approval

This study protocol was approved by the Ethics Committee (Institutional Review Board) of the Shanghai Ninth People's Hospital (reference number 2017-211).

Results

A total of 12400 singleton live-born infants were included in this study. Of these, 1015, 1027, 687, and 9671 singletons were obtained after single blastocyst transfer, single cleavage-stage embryo transfer, double blastocyst transfer, and double cleavage-stage embryo transfer, respectively. The maternal and treatment characteristics of singletons are presented in Table 1. The average maternal age and paternal age were 31.53 years and 33.45 years, respectively. Slightly more than 50% of women had primary infertility, and the proportion of nulliparous women was above 90%. For the infertility causes, female factors (approximately 60%) were the most common, followed by combined factors. IVF was the main fertilization method.

The percentage of birth outcomes among singletons born after single blastocyst transfer, single cleavage-stage embryo transfer,

TABLE 1 Maternal and treatment characteristics of singletons born after single- or double- vitrified cleavage-stage embryo or blastocyst transfer.

Characteristic	SET-B (n=1015)	SET-C (n=1027)	DET-B (n=687)	DET-C (n= 9671)	Total (n=12400)	P_1	P_2
Maternal age, mean ± SD	31.76(4.12)	32.14(4.08)	30.68(3.74)	31.50(4.20)	31.53(4.17)	0.039	<0.001
Paternal age, mean ± SD	33.60(5.07)	34.07(5.29)	32.53(4.76)	33.44(5.30)	33.45(5.26)	0.044	<0.001
Maternal BMI, mean ± SD	21.01(4.09)	21.61(3.87)	20.78(3.70)	21.25(4.11)	21.23(4.07)	<0.001	0.004
Type of infertility						0.878	0.341
Primary infertility	548(53.99)	551(53.65)	358(52.11)	5221(53.99)	6678(53.85)		
Second infertility	467(46.01)	476(46.35)	329(47.89)	4450(46.01)	5722(46.15)		
Parity,n(%)						0.449	0.529
Nulliparous	942(92.81)	944(91.92)	638(92.87)	8917(92.20)	11441(92.27)		
Pluriparous	73(7.19)	83(8.08)	49(7.13)	754(7.80)	959(7.73)		
uration of infertility, mean ± SD	3.78(2.64)	3.95(2.77)	3.61(2.32)	3.83(2.66)	3.82(2.65)	0.193	0.046
Infertility causes, n (%)						0.491	<0.001
Female factor	630(62.07)	603(58.71)	466(67.83)	5763(59.59)	7462(60.18)		
Male factor	103(10.15)	112(10.91)	61(8.88)	1191(12.32)	1467(11.83)		
Combined factor	194(19.11)	214(20.84)	104(15.14)	1906(19.71)	2418(19.50)		
Unexplained	88(8.67)	98(9.54)	56(8.15)	811(8.39)	1053(8.49)		
Fertilization type, n (%)						0.552	0.040
IVF-only	635(62.56)	646(62.90)	462(67.25)	6050(62.56)	7782(62.76)		
ICSI-only	272(26.80)	259(25.22)	167(24.31)	2588(26.76)	3286(26.50)		
IVF/ICSI split	108(10.64)	122(11.88)	58(8.44)	1033(10.68)	1332(10.74)		
Sperm origin						0.222	0.214
Ejaculation	994(97.93)	1013(98.64)	676(98.40)	9445(97.66)	11827(97.75)		
Testicular sperm extraction	21(2.07)	14(1.36)	11(1.60)	226(2.34)	272(2.25)		

SET-C, single cleavage-stage embryo transfer; SET-B, single blastocyst transfer; DET-C, double cleavage-stage embryo transfer; DET-B, double blastocyst transfer. BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; FET, frozen-thawed embryo transfer.
P<0.05 indicates a statistically significant difference; P_1: SET-B vs. SET-C, P_2: DET-B vs. DET-C.

double blastocyst transfer, and double cleavage-stage embryo transfer are presented in Table 2. The results of the multivariate analysis of birth outcomes are shown in Tables 3 and 4 to explore the influencing factors.

As presented in Table 2, the mean gestational age was 38.83 weeks for single blastocyst transfer, 38.77 weeks for single cleavage-stage embryo transfer, 38.90 weeks for double blastocyst transfer, and 38.94 weeks for double cleavage-stage embryo transfer. The developmental stage of transferred embryos was not significantly associated with gestational age by multivariable linear regression analysis, as shown in Table 3. The mean unadjusted birth weights were 3344.31 g, 3308.32 g, 3372.04 g, and 3345.39 g for singletons born after single blastocyst transfer, single cleavage-stage embryo transfer, double blastocyst transfer, and double cleavage-stage embryo transfer, respectively, as shown in Table 2. After adjusting for several important variables, the multivariable linear regression results in Table 3 showed that the unadjusted birth weights of singletons born after vitrified blastocyst transfer were higher than those after cleavage-stage transfer (β=30.28, SE=13.17, P=0.022), as were the adjusted birth weights (β=0.09, SE=0.03, P=0.007). In addition, birth weight was positively related to maternal BMI, and male newborns had higher birth weights than female newborns, as showed in Table 3.

Furthermore, Table 2 shows that the proportions of infants with high birth weight were 7.39%, 5.16%, 8.15%, and 6.63% among singletons born after single blastocyst transfer, single cleavage-stage embryo transfer, double blastocyst transfer, and double cleavage-stage embryo transfer, respectively, and the corresponding proportions for infants with LGA were 17.54%, 14.41%, 17.61%, and 16.06%. After adjusting for the known confounding factors by logistic regression analysis, as shown in Table 4, there was a 37% increased odd of having an infant with high birth weight after vitrified blastocyst transfer compared with vitrified cleavage stage transfer (OR=1.37, 95% CI:1.07-1.77). However, the number of transferred embryos was not observed to be related to HBW or LGA.

Discussion

To the best of our knowledge, this is the largest study to explore the effect of extended embryo culture on the birth weight of

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TABLE 2 Neonatal outcomes of singletons by number of embryos transferred and stage of embryo development.

	SET-B (n=1015)	SET-C (n=1027)	DET-B (n=687)	DET-C (n=9671)	Total (n=12400)	P1	P2
Newborn gender, n (%)						<0.001	0.274
Female	419(41.28)	514(50.05)	317(46.14)	4671(48.30)	6479(52.25)		
Male	596(58.72)	513(49.95)	370(53.86)	5000(51.70)	5921(47.75)		
Gestational age, mean ± SD	38.83(1.73)	38.77(1.65)	38.90(1.56)	38.94(1.60)	38.92(1.61)	0.471	0.458
Very preterm (<32w), n (%)	10(0.99)	11(1.07)	5(0.73)	89(0.92)	115(0.93)	0.848	0.607
Preterm (<37w), n (%)	89(8.77)	78(7.59)	58(8.44)	662(6.85)	887(7.15)	0.333	0.112
Unadjusted birth weight, mean ± SD	3344.31(533.02)	3308.32(508.35)	3372.04(517.85)	3345.39(500.38)	3343.71(504.85)	0.119	0.178
Adjusted birth weight, mean ± SD	0.30(1.07)	0.23(1.05)	0.35(1.10)	0.28(1.06)	0.28(1.06)	0.142	0.072
Very low birth weight (<1500g), n (%)	11(1.08)	8(0.78)	1(0.15)	55(0.57)	75(0.60)	0.473	0.182
Low birth weight (<2500g), n (%)	47(4.63)	40(3.89)	28(4.08)	403(4.17)	518(4.18)	0.410	0.908
High birth weight (>4000g), n (%)	75(7.39)	53(5.16)	56(8.15)	641(6.63)	825(6.65)	0.038	0.124
Very high birth weight (>4500g), n (%)	7(0.69)	9(0.88)	7(1.02)	83(0.86)	106(0.85)	0.632	0.661
Small for gestational age (<10th percentile), n (%)	60(5.91)	69(6.72)	47(6.84)	584(6.04)	760(6.13)	0.453	0.395
Large for gestation age (>90th percentile), n (%)	178(17.54)	148(14.41)	121(17.61)	1553(16.06)	2000(16.13)	0.054	0.285
Very small for gestational age (<3rd percentile), n (%)	21(2.07)	24(2.34)	16(2.33)	201(2.08)	262(2.11)	0.680	0.658
Very large for gestational age (>97th percentile), n (%)	67(6.60)	54(5.26)	55(8.01)	584(6.04)	760(6.13)	0.199	0.038
Major birth defects, n (%)	8(0.79)	10(0.97)	6(0.87)	65(0.67)	89(0.72)	0.654	0.537

P<0.05 indicates a statistically significant difference; P1: SET-B vs. SET-C, P2: DET-B vs. DET-C.

singletons born after single or double vitrified-warmed embryo transfer. This study showed a significant increase in the unadjusted and adjusted birth weight among singletons born after vitrified blastocyst transfer compared with singletons born after vitrified cleavage-stage embryo transfer after controlling for confounders. Similarly, the results suggested an increased odds of having an infant with HBW after vitrified blastocyst transfer. In addition, only pregnancies with a single gestational sac after single or double embryo transfer were included in the study and no significant differences were found in neonatal outcomes between singletons born after single embryo transfer and singletons born after double embryo transfer.

With advances in embryo culture conditions, extending embryo culture to the blastocyst stage is increasingly used clinically as an effective operation method to reduce the multiple pregnancy rate and improve the implantation rate and live birth rate. Considering that ultimate outcome of ART is a healthy singleton baby, the neonatal outcome was more appropriate as a measure to evaluate effectiveness and safety. In recent years, a few studies on the influences of an extended embryo culture on neonatal outcomes have been conducted. However, many existing studies were performed in fresh embryo transfer cycles (10–13). Even though a few studies focused on frozen embryo transfer, their findings were conflicting and limited by the small sample size or the inclusion of both the slow freeze technique and vitrification method (14–16). Therefore, we conducted this larger study focusing on vitrified-warmed cycles to evaluate the neonatal outcomes after blastocyst transfer. In the present study, the unadjusted and adjusted birth weights for singletons born after vitrified blastocyst transfer were significantly heavier than those born after cleavage-stage embryo transfer, and the risk of delivery of an HBW infant after vitrified blastocyst transfer also increased. This result indicated that blastocyst transfer had a significant impact on birth weight, which was in agreement with some previous studies (11, 14). Considerable evidence in animal models also showed that the exposure of ovine and bovine embryos to *in vitro* culture conditions prior to the blastocyst stage could induce large offspring syndrome (23). The epigenetic reprogramming of the embryo genome in the culture period may be a possible mechanism to explain the significant increase in birth weight after blastocyst transfer. Published studies have reported that epigenetic mechanisms affect neonatal weight (24, 25). Exposure to an *in vitro* culture environment as a perturbation for preimplantation embryo development altered the expression of imprinted genes regulating fetal growth and development by changing DNA methylation (26). Li et al. identified 320 genomic loci alterations in DNA methylation in large offspring syndrome produced by assisted reproduction in bovines. Importantly, their study showed that large offspring

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TABLE 3 Multivariable linear regression analysis for unadjusted birth weight, gestational age, and adjusted birth weight.

	Unadjusted birth weight(g)			Gestational age(weeks)			Adjusted birth weight(g)		
	β	Std. Error	P value	β	Std. Error	P value	β	Std. Error	P value
Blastocyst vs. Cleavage embryo	30.28	13.17	0.022	-0.03	0.05	0.599	0.09	0.03	0.007
Double vs. single embryo	13.93	12.25	0.255	0.11	0.05	0.019	0.05	0.03	0.099
Maternal age	-0.53	1.51	0.727	-0.01	0.01	0.013	0.00	0.00	0.596
Paternal age	0.49	1.16	0.674	-0.01	0.00	0.168	0.00	0.00	0.906
Maternal BMI	10.34	0.97	<0.001	-0.02	0.00	<0.001	0.03	0.00	<0.001
Type of infertility									
Second vs. Primary infertility	44.33	8.87	<0.001	0.00	0.03	0.904	0.09	0.02	<0.001
Parity									
Pluriparous vs. Nulliparous	26.06	16.38	0.112	-0.18	0.06	0.005	0.07	0.04	0.090
Duration of infertility	3.21	2.90	0.269	-0.01	0.01	0.205	0.01	0.01	0.294
Infertility causes									
Female factor(reference)									
Male factor	-11.94	13.52	0.377	0.08	0.05	0.125	-0.03	0.03	0.394
Combined factor	-16.64	10.50	0.113	0.06	0.04	0.145	-0.04	0.03	0.152
Unexplained	-19.92	14.80	0.178	0.15	0.06	0.012	-0.05	0.04	0.156
Fertilization type									
IVF-only (reference)									
ICSI-only	15.00	12.68	0.237	0.00	0.05	0.987	0.04	0.03	0.183
IVF/ICSI split	5.48	20.79	0.792	-0.07	0.08	0.388	0.00	0.05	0.986
Sperm origin									
TESE vs. Ejaculation	-25.05	28.52	0.380	0.15	0.11	0.181	-0.06	0.07	0.453
Endometrial preparation program Natural cycle (reference)									
Mild Stimulation cycle	-12.45	10.01	0.214	-0.16	0.04	<0.001	-0.04	0.03	0.092
Hormonal replacement cycle	14.99	10.48	0.153	-0.19	0.04	<0.001	0.05	0.03	0.069
Neonatal gender									
Female vs. male	-137.93	8.07	<0.001	0.14	0.03	<0.001	-0.07	0.02	0.001
Gestational age	179.04	2.53	<0.001	—	—	—	0.10	0.01	<0.001

syndrome in bovines shares phenotypes and epigenotype aberrations with the most common congenital overgrowth syndrome in humans. Therefore, alterations in DNA methylation during embryo culture are potential mechanisms for explaining the phenomenon in this study (27).

In our study, after controlling for other variables, the birth weight, and odds of HBW or LGA infants were not different between singletons born after single embryo transfer and singletons born after double embryo transfer. The above results indicated that the number of transferred embryos was not related to neonatal outcomes among singletons. However, the different birth weights among singletons after single embryo transfer and double embryo transfer have been reported in a previous study (28). Sutter reported that singletons born after double embryo transfer had a significantly lower birth weight than that of singletons born after single embryo transfer (28). Early vanishing twins, as the important factor, could induce a lower birth weight, which may explain the contradictory results between the study by Sutter and this study.

Many factors can affect birth weight. In the multivariate regression analysis, many variables, such as maternal age, paternal age, maternal BMI, type of infertility, parity, duration of infertility, infertility causes, fertilization type, sperm origin, gestational age and neonatal sex, were included to explore their relationships with birth weight. Consistent with previous reports, birth weight significantly increased with maternal BMI and gestational age, and the birth weight of male infants was significantly heavier than that of female infants.

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TABLE 4 Logistic regression analysis for neonatal outcome according to number and stage of embryo transfer among singletons.

	LBW OR (95%CI)	HBW OR (95%CI)	SGA OR (95%CI)	LGA OR (95%CI)
Blastocyst vs. Cleavage embryo	1.01(0.65,1.56)	1.37(1.07,1.77)	0.96(0.73,1.26)	1.15(0.97,1.37)
Double vs. single embryo	1.34(0.89,2.06)	1.22(0.94,1.56)	1.00(0.78,1.29)	1.06(0.90,1.24)
Maternal age	1.01(0.96,1.06)	0.97(0.94,1.00)	1.01(0.98,1.04)	0.98(0.96,1.00)
Paternal age	1.01(0.97,1.05)	1.02(0.99,1.04)	0.99(0.96,1.01)	1.01(0.99,1.02)
Maternal BMI	0.98(0.95,1.01)	1.12(1.10,1.15)	0.98(0.96,1.00)	1.08(1.05,1.09)
Type of infertility				
Second vs. Primary infertility	0.99(0.73,1.34)	1.16(0.98,1.39)	0.88(0.73,1.05)	1.21(1.07,1.36)
Parity				
Pluriparous vs. Nulliparous	0.79(0.46,1.35)	1.33(0.98,1.79)	0.81(0.56,1.17)	1.17(0.95,1.43)
Duration of infertility	1.03(0.93,1.13)	0.98(0.93,1.04)	1.00(0.95,1.06)	1.00(0.96,1.04)
Infertility causes				
Female factor(reference)				
Male factor	1.35(0.85,2.14)	0.83(0.62,1.10)	1.04(0.79,1.37)	1.00(0.83,1.19)
Combined factor	1.24(0.88,1.75)	0.89(0.72,1.10)	1.15(0.93,1.41)	0.95(0.83,1.09)
Unexplained	1.31(0.79,2.18)	0.89(0.66,1.20)	1.13(0.84,1.52)	0.95(0.78,1.16)
Fertilization type				
IVF-only (reference)				
ICSI-only	1.09(0.70,1.68)	0.96(0.75,1.24)	0.99(0.77,1.29)	1.00(0.84,1.18)
IVF/ICSI split	0.61(0.30,1.24)	1.25(0.83,1.89)	0.97(0.64,1.48)	1.10(0.84,1.45)
Sperm origin				
TESE vs. Ejaculation	0.11(0.02,0.60)	1.21(0.69,2.11)	0.70(0.36,1.37)	0.81(0.54,1.22)
Endometrial preparation program Natural cycle (reference)				
Mild Stimulation cycle	1.10(0.77,1.58)	0.83(0.68,1.02)	0.97(0.79,1.19)	0.92(0.80,1.05)
Hormonal replacement cycle	1.10(0.76,1.58)	1.10(0.90,1.34)	0.93(0.75,1.16)	1.13(0.99,1.30)
Neonatal gender				
Female vs. male	1.54(1.16,2.03)	0.56(0.48,0.66)	1.26(1.07,1.49)	0.96(0.87,1.07)
Gestational age	0.31(0.29,0.34)	1.69(1.56,1.83)	0.84(0.80,0.87)	1.14(1.10,1.19)

OR, odds ratio; CI, confidence interval.

The main strength of the present study was that it had the largest sample size to date, which enhanced the findings in the study. The second strength was that the same embryo culture medium was used during the study period, which avoids the adverse influence of different culture media on neonatal birth weight (29). In addition, we included only vitrified-warmed embryo transfer cycles to avoid the adverse effects of the hyperestrogenic milieu on neonatal outcomes. However, there were several limitations in our study. First, a higher birth weight among singletons born after transfer of vitrified-warmed blastocysts compared to vitrified-warmed cleavage stage embryos was demonstrated. However, the size was rather small compared with the higher birth weight of frozen versus fresh blastocyst transfer (9). Further studies are warranted to clarify the cause of the differences. Second, slight difference exists in the vitrification process between cleavage-stage embryos and blastocysts. Cleavage-stage embryos can be frozen directly, while blastocysts need to be manually shrunk prior to vitrification. It remains unclear whether this difference will yield potential consequences for the birth weight of future offspring (30, 31). Therefore, we cannot rule out the possibility that the higher birth weight is due to a greater effect of the vitrification-thawing process itself on the blastocyst compared to the cleavage-stage embryo, and a randomized study of Day 3 versus Day 5/6 using fresh embryo transfer would be necessary.

Conclusion

In conclusion, this study showed that unadjusted and adjusted birth weights significantly increased after blastocyst transfer compared with cleavage-stage embryo transfer in vitrified-warmed cycles. In addition, blastocyst transfer was significantly associated with an increased odds of having an infant with HBW. Considering the increasing trend towards blastocyst transfer and the freeze-all strategy and a healthy singleton birth as the ultimate goal of IVF, this study provided evidence about the potential clinical safety issues for offspring from blastocyst transfer in vitrified-warmed embryo cycles.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee (Institutional Review Board) of the Shanghai Ninth People's Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

NW drafted the manuscript. KL and XZ collected patient data. XZ and PZ conceived and designed 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1184966/full#supplementary-material

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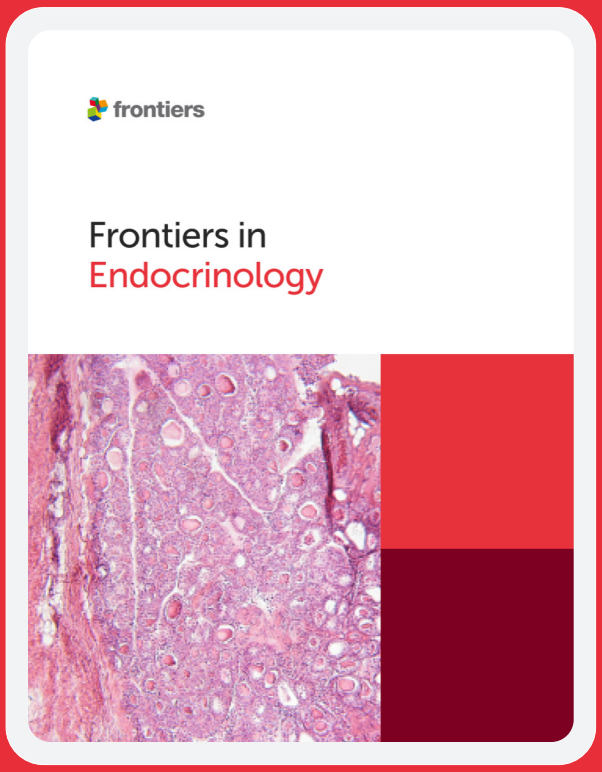

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