PERINATAL ENDOCRINOLOGY AND PREGNANCY-RELATED ENDOCRINE DISORDERS

EDITED BY: Qiongjie Zhou and Ganesh Acharya
PUBLISHED IN: Frontiers in Endocrinology and Frontiers in Physiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88976-203-3
DOI 10.3389/978-2-88976-203-3

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

PERINATAL ENDOCRINOLOGY AND PREGNANCY-RELATED ENDOCRINE DISORDERS

Topic Editors:
Qiongjie Zhou, Fudan University, China
Ganesh Acharya, Department of Clinical Science, Intervention and Technology, Karolinska Institutet (KI), Sweden

Citation: Zhou, Q., Acharya, G., eds. (2022). Perinatal Endocrinology and Pregnancy-Related Endocrine Disorders. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-203-3

Table of Contents

frontiers | Frontiers in Endocrinology

EDITORIAL
published: 28 April 2022
doi: 10.3389/fendo.2022.905829

Editorial: Placental Hormones and Pregnancy-Related Endocrine Disorders

Qiongjie Zhou[1*] *and Ganesh Acharya*[2,3,4]

[1] Department of Obstetrics, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China, [2] Department of Clinical Sciences, Intervention and Technology, Karolinska Institute, Stockholm, Sweden, [3] Center for Fetal Medicine, Karolinska University Hospital, Stockholm, Sweden, [4] Department of Clinical Medicine, Faculty of Health Sciences, UiT - The Arctic University of Norway, Tromsø, Norway

Keywords: Placental Hormones, Pregnanacy, Endocrine disorders, Environmental factor, Preeclampsia, Gestational diabetes, Hypothyroidism

Editorial on the Research Topic

Perinatal Endocrinology and Pregnancy-Related Endocrine Disorders

OPEN ACCESS

Edited and reviewed by:
Richard Ivell,
University of Nottingham,
United Kingdom

***Correspondence:**
Qiongjie Zhou
zhouqiongjie1732@fckyy.org.cn

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *27 March 2022*
Accepted: *04 April 2022*
Published: *28 April 2022*

Citation:
Zhou Q and Acharya G (2022) Editorial: Placental Hormones and Pregnancy-Related Endocrine Disorders. Front. Endocrinol. 13:905829. doi: 10.3389/fendo.2022.905829

Metabolic changes occur continuously in the mother and her fetus during pregnancy, and the role of hormones in maintaining normal fetal growth and development cannot be overemphasized. The scale of endocrine control needed to support physiological functions during pregnancy is clearly demonstrated by the drastic changes in hormone profiles and their fluctuations observed from preconception to the postnatal period. Differential sensitivity to the fluctuation of placental hormones can also play a role in the development of perinatal mental health disorders. Thus, endocrine homeostasis is important for a successful pregnancy and its favorable outcome. Placenta is an important endocrine organ in this regard as it plays a crucial role in mediating maternal physiological adaptation to pregnancy.

Endocrine disorders during pregnancy have become a major concern in recent years due to their rapidly increasing prevalence (1, 2) as well as their adverse impact on maternal and offspring health (3). Placental dysfunction remains a root cause of many pregnancy-associated endocrine disorders. This could be associated with several intrinsic as well as extrinsic factors, including genetic aberrations as well as functional changes related to dietary habits, lifestyle factors and environmental exposures.

With emerging new technologies in molecular biology, together with the real possibility of using mega-data and artificial intelligence, significant progress has been made in our understanding of key regulatory mechanisms involved in the pathophysiology of pregnancy related endocrine disorders, associated risk factors, and their short- and long-term consequences. The topic of "Perinatal Endocrinology and Pregnancy-related Endocrine Disorders" in Frontiers in Endocrinology presents 14 high quality original research and review articles on this theme contributed by researchers from around the world. The focus is both on basic science exploring pathophysiological mechanisms as well as clinical practice of screening, diagnosis and management of pregnancy-associated endocrine disorders.

Regarding physiological endocrine changes in pregnancy, Garces et al. focus on angiopoietin-like protein 3 (ANGPTL3) that plays a significant role in lipoprotein metabolism. They measured its levels in healthy nonpregnant women, pregnant women at different trimesters of gestation and in the postpartum period. They report lower levels of ANGPTL3 during pregnancy which may promote lipid uptake by highly oxidative tissues leading to preservation of glucose for the growth and development of the feto-placental unit during pregnancy.

Three papers address the pathophysiological characteristics of pregnancy-associated endocrine disorders. Cathey et al. have addressed an interesting topic exploring the association between gestational hormones and timing of delivery. In this longitudinal cohort study, higher corticotropin releasing hormone (CRH), estriol, progesterone, total triiodothyronine and free thyroxine were observed among male fetuses with spontaneous preterm birth and higher testosterone in female fetuses, indicating intrauterine sexual dimorphism in the association between hormones and preterm birth. Han et al. have investigated the variations in adiponectin, leptin, insulin and ghrelin levels in the umbilical cord blood of preterm infants and further elucidated their link with fetal growth. A lower concentration of these hormones was found in preterm and small for gestational age infants, suggesting an impaired maturation of adipose tissue and gastrointestinal tract. The authors hypothesize that dysregulation of these hormones may be a risk factor for abnormal fetal growth. Liu et al. have explored the associations between triglycerides, lymphocyte subsets and insulin resistance among patients with polycystic ovarian syndrome. Interestingly, elevated level of triglycerides was significantly associated with higher risk of hyperinsulinemia and impaired immune response among patients with insulin resistance and recurrent pregnancy loss, providing a new insights into the pathophysiology of these conditions.

Two studies have reported on regulatory molecular mechanisms underlying uterine receptivity, decidualization of endometrium, and placentation. Ashour et al. have used a rat model of vitamin D deficiency to investigate the role of HOXA-10/FKBP52 axis in regulating uterine receptivity. Downregulated HOXA-10/FKBP52 was noted in vitamin D-deficient rats, in association with increased amplitude and frequency of uterine contractility. More importantly, they show that vitamin D supplementation might be potentially useful for improving endometrial decidualization, uterine receptivity, and reducing myometrial contractility. On the other hand, Feng et al. demonstrate the role of collagen I in suppressing the proliferation and invasion capacity of trophoblasts by inhibiting ERK phosphorylation and WNT/b-catenin signaling pathways to induce preeclampsia-like symptoms.

Our Research Topic also discusses the new concepts in the pathogenesis and regulatory mechanism of thyroid disease in pregnancy and postpartum period. Synthesizing currently available evidence based on high-quality randomized studies, Girolamo et al. have concluded that levothyroxine (LT4) supplementation is not effective in improving adverse pregnancy outcomes among euthyroid pregnant women with thyroperoxidase (TPO) antibodies. Gao et al. (4), have evaluated maternal postpartum thyroid function within one year after delivery among women with pre-existing or newly diagnosed hypothyroidism in early pregnancy. They found that women diagnosed to have subclinical hypothyroidism in early pregnancy were at higher risk requiring LT4 treatment within one-year postpartum if they stopped LT4 treatment at delivery, had abnormal TSH level before eight gestational weeks and near delivery, or had TPOAb≥300 mIU/mL. Thus, a modified strategy for close monitoring of thyroid function during early pregnancy and postpartum period may be indicated. Additionally, Kankanamalage et al. have written a broad review on the pathogenesis of gestational hypothyroidism. The concept of gestational hypothyroidism as a separate entity of thyroid hormone dysregulation during pregnancy is proposed. Possible mechanisms could be impaired iodine regulation, elevated estrogen levels, malplacentation and dysregulation of placental endocrine function.

Studies on pregnancy-related metabolic disorders, especially gestational diabetes, and their impact on pregnancy outcome have also been included in this themed issue. Wang et al. have explored the association between increased fibroblast growth factor 21 (FGF21) and subsequent development of gestational diabetes mellitus (GDM), utilizing a nested case-control design. The authors report that serum FGF21 level is significantly elevated several weeks before the diagnosis of GDM is made, suggesting a potential value of FGF21 in predicting GDM. Bai et al. have utilized the GDM model to investigate the role of angiopoietin like 8 (ANGPTL8), a secretory protein involved in lipid metabolism, in GDM and insulin resistance. They have demonstrated that the silencing of ANGPTL8 promotes insulin resistance in trophoblast cells by inhibiting JNK signaling. Han Z. et al. assessed the changes of placental volume and vascular indices in pregnant women with GDM using three-dimensional power Doppler, and found them to be significantly lower in the GDM group in the first trimester compared to healthy pregnant women. This noninvasive approach might be useful in assessing the degree of placental vasculopathy in GDM. In addition, Han S. et al. conducted a retrospective cohort study to investigate the risk of adverse pregnancy outcomes among women with diminished ovarian reserve. An increased risk of hypertensive disorders of pregnancy and slightly elevated incidence of preterm birth were observed.

The role of environmental factors on placental development and function was also addressed in this Research Topic. Per- and poly-fluoroalkyl substances (PFAS) are persistent pollutants, which accumulate in humans *via* food, drinking water, outdoor air, etc (5, 6). Marchese et al. assessed the gestational characteristics of placental brain-derived neurotrophic factor (BDNF) signaling and the impact of PFAS exposure on this pathway in trophoblast. They report that BDNF signaling is diverse during placental development and is affected by PFNA exposure. This PFAS potentially interferes with the key signaling pathway of BDNF in fetal neurodevelopment.

We believe that this Research Topic has been enriched by the inclusion of basic and clinical studies and will appeal to both

clinicians and scientists equally. We hope that it will improve our understanding of pregnancy-related endocrine disorders, and motivate further research into perinatal endocrinology and pregnancy associated endocrine disorders.

AUTHOR CONTRIBUTIONS

Both authors conceptualized and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

QZ was funded by the National Key Research and Development Program (2021YFC2701600, 2021YFC2701601), Clinical Research Plan of SHDC (SHDC2020CR1047B), the Shanghai Excellent Young Scholar Plan of Public Health (2020-2022, GWV-10.2-YQ13), Elite Young Scholar 2025 of Fudan University (2020-2023), Shanghai Medical Center of Key Programs for Female Reproductive Diseases (2017ZZ01016), and the Science Foundation of Shanghai (21ZR1410600). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Morton A, Teasdale S. Physiological Changes in Pregnancy and Their Influence on the Endocrine Investigation. *Clin Endocrinol (Oxf)* (2022) 96(1):3–11. doi: 10.1111/cen.14624
2. Gingrich J, Ticiani E, Veiga-Lopez A. Placenta Disrupted: Endocrine Disrupting Chemicals and Pregnancy. *Trends Endocrinol Metab* (2020) 31 (7):508–24. doi: 10.1016/j.tem.2020.03.003
3. Solano ME, Arck PC. Steroids, Pregnancy and Fetal Development. *Front Immunol* (2020) 10:3017. doi: 10.3389/fimmu.2019.03017
4. Shan Z, Teng W. Thyroid Hormone Therapy of Hypothyroidism in Pregnancy. *Endocrine* (2019) 66(1):35–42. doi: 10.1007/s12020-019-02044-2
5. Ding N, Harlow SD, Randolph JFJr, Loch-Caruso R, Park SK. Perfluoroalkyl and Polyfluoroalkyl Substances (PFAS) and Their Effects on the Ovary. *Hum Reprod Update* (2020) 26(5):724–52. doi: 10.1093/humupd/dmaa018
6. Wang B, Fu J, Gao K, Liu Q, Zhuang L, Zhang G, et al. Early Pregnancy Loss: Do Per- and Polyfluoroalkyl Substances Matter? *Environ Int* (2021) 157:106837. doi: 10.1016/j.envint.2021.106837

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 13 April 2021
doi: 10.3389/fendo.2021.670357

Maternal Serum Angiopoietin-Like 3 Levels in Healthy and Mild Preeclamptic Pregnant Women

María Fernanda Garces[1], Haiver Antonio Rodriguez - Navarro[1†], Julieth Daniela Buell - Acosta[1†], Alvaro Javier Burgos - Cardenas[2], Roberto Franco - Vega[2], Luis Miguel Maldonado - Acosta[2], Javier Eslava - Schmalbach[3], Arturo José Parada - Baños[4], Andres Castro - Pinzon[2], Elizabeth Sanchez[1], Edith Angel - Muller[4], Ezequiel Lacunza[5], Justo P. Castaño[6], Carlos Dieguez[7], Rubén Nogueiras[7], Ariel Ivan Ruiz - Parra[4] and Jorge Eduardo Caminos[1]*

[1] Department of Physiology, Universidad Nacional de Colombia, Bogota, Colombia, [2] Department of Internal Medicine Endocrine Unit, Universidad Nacional de Colombia, Bogota, Colombia, [3] Department of Surgery, Universidad Nacional de Colombia, Bogota, Colombia, [4] Department of Obstetrics and Gynecology, Universidad Nacional de Colombia, Bogota, Colombia, [5] Centro de Investigaciones Inmunológicas Básicas y Aplicadas (CINIBA), Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina, [6] Maimonides Institute of Biomedical Research of Cordoba, Department of Cell Biology, University of Córdoba, Córdoba, Spain, [7] CIBER Physiopathology of Obesity and Nutrition (CIBERobn), Instituto de Investigaciones Sanitarias (IDIS), Department of Physiology (CIMUS), Universidad de Santiago de Compostela, Santiago de Compostela, Spain

OPEN ACCESS

Edited by:
Qiongjie Zhou,
Fudan University, China

Reviewed by:
Purnima Dey Sarkar,
Mahatma Gandhi Memorial Medical College, Indore, India
Gendie Lash,
Guangzhou Medical University, China

***Correspondence:**
Jorge Eduardo Caminos
jecaminosp@unal.edu.co

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *21 February 2021*
Accepted: *23 March 2021*
Published: *13 April 2021*

Citation:
Garces MF, Rodriguez - Navarro HA, Buell - Acosta JD, Burgos - Cardenas AJ, Franco - Vega R, Maldonado - Acosta LM, Eslava - Schmalbach J, Parada - Baños AJ, Castro - Pinzon A, Sanchez E, Angel - Muller E, Lacunza E, Castaño JP, Dieguez C, Nogueiras R, Ruiz - Parra AI and Caminos JE (2021) Maternal Serum Angiopoietin-Like 3 Levels in Healthy and Mild Preeclamptic Pregnant Women. Front. Endocrinol. 12:670357. doi: 10.3389/fendo.2021.670357

Objective: Angiopoietin-like protein 3(ANGPTL3) is an important regulator of lipoprotein metabolism in the fed state by inhibiting the enzyme lipoprotein lipase in oxidative tissues. However, the possible role of ANGPTL3 throughout gestation and its relationship with hormonal and biochemical variables are still unknown. The aim of this study was to determinate serum ANGPTL3 level in healthy non-pregnant women, during healthy and preeclamptic pregnancy and postpartum.

Methods: Serum ANGPTL3 was analyzed by enzyme-linked immunosorbent assay (ELISA), in a prospective cohort of healthy pregnant women *(n = 52)* and women with mild preeclampsia *(n = 21)*, and women at three months postpartum *(n = 20)* and healthy non-pregnant women *(n = 20).* The results obtained were correlated with biochemical, hormonal and anthropometric variables and insulin resistance indices.

Results: Levels of ANGPTL3 were not different between the follicular and the luteal phases of the cycle in healthy non-pregnant women. There was a significant reduction in serum ANGPTL3 levels from the first to the third trimester in healthy pregnant women compared with healthy non-pregnant and postpartum women ($p < 0.01$). ANGPTL3 levels do not differ significantly during the three trimesters of pregnancy neither in healthy women nor in preeclamptic women. The serum levels of ANGPTL3 in women who developed preeclampsia are not statistically different from those observed in healthy pregnant women in each trimester of pregnancy. A significant lineal positive correlation was observed between serum ANGPTL3 levels and triglyceride ($P = 0.0186$, $r = 0.52$), very low-density lipoprotein cholesterol ($P = 0.0224$, $r = 0.50$), and total cholesterol levels ($P = 0.0220$,

r =0.50) in healthy non-pregnant women (P 0.05). Besides, there were no significant correlations between serum ANGPTL3 and body mass index (BMI), high-density lipoprotein cholesterol, glucose, insulin, leptin, or HOMA-IR (P >0.05)

Conclusions: We describe for the first time the profile of ANGPTL3 throughout pregnancy and postpartum as well as and discussed about explore their potential contribution interactions with lipoprotein metabolism throughout pregnancy and postpartum. Thus, low levels of ANGPTL3 during pregnancy might favor lipid uptake in oxidative tissues as the main maternal energy source, while may helping to preserve glucose for use by the fetus and placenta.

Keywords: ANGPTL3, pregnancy, preeclampsia, insulin resistance, ANGPTL

INTRODUCTION

It is well known that during pregnancy, a series of profound metabolic adaptations occur to favor and ensure fetal development and maternal survival. As a result, maternal plasma triglyceride (TG) concentrations rise significantly, 2 to 4 fold, in uncomplicated late gestation and revert to pre-pregnancy levels after delivery (1–4). These metabolic changes during pregnancy appear to be attributable to different factors such as lipoprotein lipase (LPL), estrogen, progesterone, cortisol, leptin and prolactin, among others (5). On the other hand, during the third trimester of pregnancy, high levels of human placental lactogen (hPL), decrease insulin sensitivity and increase production of catecholamines, altogether stimulating lipolysis in adipose tissue and increasing free fatty acids as substrate for endogenous synthesis of other lipoproteins in the liver (5–9).

Lipoprotein lipase (LPL) plays a fundamental role in normal lipid metabolism and energy balance, by catalyzing the hydrolysis of TG component of circulating chylomicrons and very low -density lipoprotein (VLDL-c) at the luminal surface of endothelial cells from extrahepatic tissues, to release fatty acids that can be used or stored (10–12). Additionally, it has been shown that during the first trimester of pregnancy LPL activity increases in adipose tissue whereas, in the third trimester of pregnancy this activity decreases significantly (12, 13). This situation coincides with the transformation from anabolic to catabolic condition and the increase in insulin resistance as pregnancy advances (13–16). In contrast, LPL expression and activity increase significantly in placental syncytiotrophoblast during the third trimester of pregnancy and correlate with increase of placental capacity to transport lipid for fetal growth (17, 18).

Moreover, different proteins participate in the regulation of LPL activity through posttranslational mechanisms, including the inhibitory proteins ANGPTL3, ANGPTL4 and ANGPTL8 (19, 20). ANGPTL3 is expressed mainly in the liver and its inhibitory -and irreversible- activity on LPL is developed through the ANGPTL3/ANGPTL8 complex (21–24) and is exerted mostly in the fed state in oxidative tissues, such as heart, skeletal muscle, brown adipose tissue and liver, accordingly storage of TG in the white adipose tissue (WAT) is favored (20). It is now generally accepted that the relative activity of LPL in WAT and oxidative tissues reflects a balance between the systemic effects of circulating, liver-derived ANGPTL3 and ANGPTL8 and the local effects of ANGPTL4 expression in WAT (20).

Different studies have shown that women who develop preeclampsia have high TG levels throughout pregnancy (25). Data gleaned recently have exposed the important role of ANGPTL3 in lipid metabolism by inhibiting LPL activity and therefore increasing triglycerides and other lipids. Furthermore, it was recently shown that the monoclonal antibody, Evinacumab, an ANGPTL3 inhibitor, reduced triglycerides in healthy human volunteers and in homozygous familial hypercholesterolemic individuals (26, 27). Since nothing is known about ANGPTL3 role in normal pregnancy and preeclampsia, we hypothesized that the pregnancy-induced increased in TG levels could be, at least in part, mediated by ANGPTL3. Thus, in this study we determined the profile of its serum levels in a cohort of healthy pregnant women, during the three trimesters of pregnancy and three months after delivery, and in pregnant women who developed mild preeclampsia. The correlation between serum ANGPTL3 levels and biochemical, hormonal variables, anthropometric and insulin resistance indices was investigated as well.

MATERIALS AND METHODS

Ethical Considerations

The study was approved by the Institutional Review Board at the School of Medicine of the Universidad Nacional de Colombia and all participants signed informed consent forms. Also, the study was conducted by healthcare personnel working at the Department of Obstetrics and Gynecology of the School of Medicine of the Universidad Nacional de Colombia and Hospital de Engativá in Bogotá Colombia, between May 2012 and November 2015. Additionally, all clinical care was performed following relevant institutional, national, and international guidelines.

Study Population

This is a case-control study nested in a prospective cohort study (n=465) that analyzed ANGPTL3 serum levels during three periods of pregnancy in healthy pregnant women (n=52),

women with mild preeclampsia (n=21), postpartum women (n=20), and healthy non-pregnant women at reproductive age (n=20). Healthy and preeclamptic women were randomly selected from the original prospective cohort study taken into account the pregnancy outcome. Additionally, normal pregnant women and those who developed mild preeclampsia belong to the full original cohort of pregnant women.

During their first visit, maternal demographics and baseline characteristics were collected and gestational ages were determined based on last menstrual period and ultrasound during the first trimester. Additionally, maternal anthropometric, biochemical and clinical parameters were determined at the time of each routine prenatal visit scheduled. Healthy women were studied at three-time points during pregnancy: 12.14 (11.3–13.6) (1st trimester), 24.60 (23.4–27.3) (2nd trimester) and 34.90 (33.5–38.6) (3rd trimester) weeks of gestation and three months postpartum. The inclusion criteria for healthy pregnant women were: women without pathologic antecedents, with a normal pregnancy course and outcome, that is, women with full-term delivery or cesarean section, with babies with normal Apgar score and birth weight, who did not present fetal abnormalities or malformations and women who did not develop pathologies associated with pregnancy or adverse maternal-perinatal outcomes (28).

Additionally, women with mild preeclampsia were studied in the same cohort study during 12.22 (11.2–13.1) (1st trimester), 24.43 (24.0–26.0) (2nd trimester) and 34.95 (34.0–37.0) (3rd trimester) weeks of gestation. Mild to moderate preeclampsia may be asymptomatic and was diagnosed as preeclampsia with systolic blood pressure < 160 mmHg, or diastolic blood pressure < 110 mmHg, normal platelet count, on-elevated liver enzymes, absence of renal insufficiency, pulmonary edema, cyanosis, new-onset headaches or visual disturbances and/or right upper quadrant or epigastric pain (29). Age matched healthy non–pregnant women (BMI 18.0 – 25.0 kg/m^2) with regular menstrual cycles, normotensive, euglycemic and with triglycerides and cholesterol levels within the normal range were included in the study (30).

Women who met any of the following criteria were excluded from the study: current smoking, alcoholism, mental illness, pre-existing heart disease, pre-existing chronic hypertension, pre-existing diabetes mellitus, gestational diabetes, autoimmune, thyroid dysfunction, chronic kidney disease, cardiac failure, hepatic failure, thyroid diseases, renal or hepatic disease, multiple pregnancy, preterm delivery (before the 34th week), preterm premature rupture of membranes, ongoing infection and miscarriage, use of approved weight lowering pharmacotherapy or patients with a history of gastric bypass and other bariatric surgery.

Biochemical and Hormonal Analysis

Blood samples were collected and processed to yield serum on the same protocol. Thus, overnight fasting venous blood samples were drawn from all women after an overnight fast of 10 - 12 h (0700 – 0800 h). BD Vacutainer dry tubes (5 mL) were used to draw blood, and serum aliquots were stored at -80°C until assays. All the participants underwent a serum biochemical analysis of fasting insulin, blood glucose, total cholesterol, triglyceride, HDL-c and High-sensitivity C-reactive protein (hs-CRP) as described elsewhere (28). HOMA-IR index was calculated as described by Matthews et al. (31).

$$HOMA\ \ IR = \left(\frac{Fasting\ \ glucose\ \ (mg/dL) \times\ \ Fasting\ \ insulin\ \ (\mu UI/L)}{405} \right)$$

Serum specimen samples from each women participating in the cohort study have been stored appropriately (-80˚C) and thawed immediately before used in the recent ELISA assay reported here for the analysis of circulating levels of ANGPTL3. Additionally, human serum ANGPTL3 levels were measured using a commercially available ELISA kit (Catalog Number DANL30 - R&D Systems, Inc. USA) (32). The intra and inter-assay coefficients of variation (CV) were <4.1% and <8.5%, respectively. All samples were analyzed in duplicate and the mean value of the two measurements was reported. Serum levels of leptin were analyzed as described elsewhere (28).

Statistical Analysis

Descriptive statistical analysis was performed using statistical software R (version 3.5.1). Data with normal distribution were reported as mean (±) and standard deviation (SD), while data with non-normal distribution were reported as median and interquartile range (IQ 25 - 75), but statistical testing was conducted after logarithmic transformation (33, 34). Variables with normal distribution were compared by unpaired Student's t-test and one-way ANOVA and repeated-measured ANOVA. Additionally, a *post hoc* analysis was made among the groups. Mann-Whitney U test, Kruskal-Wallis one-way analysis of variance and Friedman test were performed for non-normally distributed variables.

Additionally, after log transformation, continuous variables not normally distributed were normally distributed and these log-transformed variables were used for correlations and linear regression analyses using Pearson or Spearman analysis to assess the association (33, 34). Using a single regression analysis, we evaluated the potential association of serum levels of ANGPTL3 with BMI, fasting glucose, triglycerides, VLDL-c, total cholesterol, HDL-c, fasting insulin, HOMA-IR and leptin levels, during each trimester of pregnancy in healthy pregnant women and healthy non–pregnant women. A p-$value$ < 0.05 was considered statistically significant

RESULTS

Characteristics for healthy pregnant, preeclamptic, postpartum and healthy non-pregnant women are described in **Table 1**.

Serum levels of ANGPTL3 were not different between the follicular and the luteal phases of the menstrual cycle in healthy non-pregnant women (**Table 1**). Additionally, **Figure 1** shows a significant reduction in serum ANGPTL3 levels from the first to the third trimester of pregnancy in healthy pregnant women compared with healthy non-pregnant and postpartum women (P <0.01).

Serum ANGPTL3 levels did not differ significantly during the three trimesters of pregnancy in healthy and preeclamptic women (**Figure 1** and **Supplementary Table 1**). In addition, serum levels of ANGPTL3 in women who developed

TABLE 1 | Characteristics of healthy non-pregnant women, healthy pregnant women and preeclamptic pregnant women.

	Healthy non-pregnant women	Healthy pregnant (trimester)			Healthy women at delivery	Preeclamptic pregnant (trimester)		
		1st	2nd	3rd		1st	2nd	3rd
N	20	52	52	52	20	21	21	21
Age (years)	20 (19-23.5)	24 (19.5-31)	–	–	23 (19-27)	20.5 (18.5-28)	–	–
Gestational age (weeks)	–	12.14 (11.3 - 13.6)	24.60 (23.4–27.3)	34.90 (33.5–38.6)	–	12.22 (11.2–13.1)	24.43 (24.0–26.0)	34.95 (34.0–37.0)
BMI (Kg/m^2)	21.45 (19.78-23.68)	22.33 (20.71-23.66)	24.28 (22.51-25.76)	26.32 (24.42-27.7)	23.19 (21.1-25.43)	23.5 (22.2-25.5)	26.1 (24.45-28.75)	29.70 (27.4-31.6)
Fasting glucose (mg/dL)	84.2 (80-90.5)	78 (73-83)	73.5 (69-78)	75 (68.5-79.5)	79 (77-83)	80.5 (76.8-84)	77.5 (70-81)	73 (70-78)
Insulin (µU/dL)	1.90 ± 1.03	9.48 ± 4.17	10.69 ± 4.51	12.94 ± 5.64	6.60 ± 3.60	12.01 ± 4.35	15.58 ± 5.42	15.08 ± 6.32
HOMA – IR index	0.40 ± 0.22	1.84 ± 0.87	1.96 ± 0.86	2.42 ± 1.13	1.32 ± 0.74	2.32 ± 0.89	2.97 ± 1.07	2.81 ± 1.31
Total cholesterol (mg/dL)	157.86 ± 24.18	167.95 ± 31.47	219.43 ± 40.92	241.26 ± 54.74	159.67 ± 30.6	170.03 ± 30.56	219.76 ± 41.95	235.98 ± 45.70
HDL cholesterol (mg/dL)	52.13 ± 8.14	57.85 ± 11.49	67.74 ± 12.41	65.68 ± 11.36	48.00 ± 6.80	53.54 ± 11.72	64.54 ± 13.81	59.55 ± 17.45
LDL cholesterol, mg/dL	114.18 ± 26.38	113.27 ± 30.30	145.76 ± 465.85	157.65 ± 48.72	95.90 ± 29.35	117.27 ± 34.94	146.65 ± 55.48	150.43 ± 55.58
VLDL cholesterol (mg/dL)	14.11 (12.2-18.35)	21.66 (17.89-25.64)	34.09 (28.58-44.85)	50.65 (40.77-59.59)	14.08 (10.22-23)	24.04 (18.31-33.41)	33.3 (26.57-41.72)	46.86 (35.4364.13)
Triglycerides (mg/dL)	10.55 (61-91.75)	108.3 (89.45-128.2)	170.45 (143.15-224.25)	253.25 (203.85-297.95)	70.4 (51.5-115.7)	120.4 (91.5-135.9)	166.5 (132.85-208.6)	243,7 (187.25-321.15)
hs-CRP (mg/L)	1.65 ± 1.05	5.46 ± 3.10	4.70 ± 2.29	5.50 ± 3.20	3.62 ± 3.68	5.62 ± 3.44	7.43 ± 2.86	6.79 ± 3.38
Leptin (ng/mL)	Follicular: 16.32 ± 2.25 Luteal: 23.02 ± 4.60	20.54 ± 5.17	26.80 ± 9.66	36.96 ± 11.09	–	35.15 ± 12.54	68.98 ± 32.47	91.64 ± 41.50
Fasting ANGPTL3 (ng/mL)	Follicular 113.59 ± 18.68 Luteal 115.83 ± 22.36	78.54 ± 41.34	81.96 ± 38.44	94.10 ± 41.81	124.30 ± 36.11	79.71 ± 34.55	67.36 ± 31.62	85.31 ± 23.48

Data with normal distribution were reported as mean +/- standard deviation (SD) and data with non-normal distribution were reported as median and interquartile range (IQR).

BMI, Body mass index; HDL-C, High-Density Lipoprotein Cholesterol; VLDL, Very Low-Density Lipoprotein; LDL-C, low-density lipoprotein-cholesterol; hs-CRP, High-Sensitivity C-Reactive Protein.

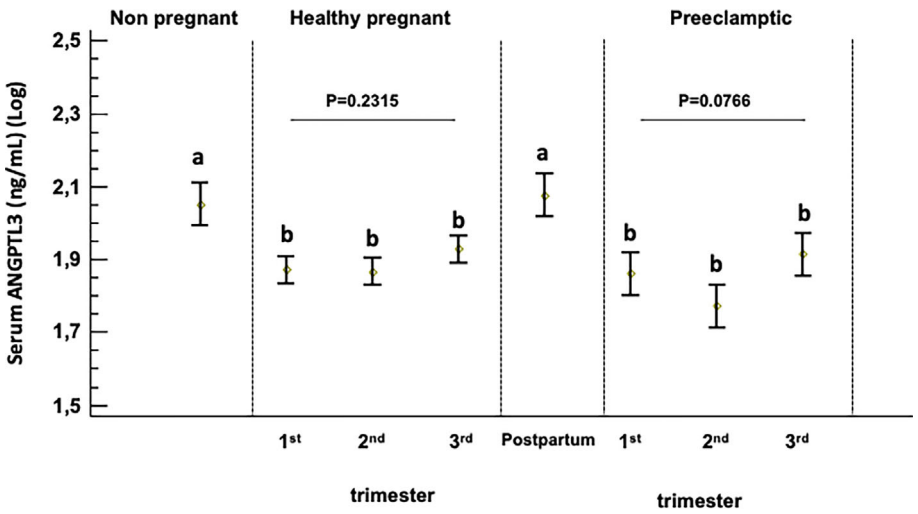


FIGURE 1 | Serum ANGPTL-3 levels in healthy non-pregnant women, healthy pregnant women and women at three months postpartum and preeclamptic women. Box plot showing serum ANGPTL3 levels across pregnancy and compared with non-pregnant controls. A p-value < 0.05 was considered statistically significant. Different superscript letters (a and b) indicate which means differ significantly from which other (p<0.05) and described in statically method section. Log-transformed (log10) values were used.

preeclampsia are not statistically different from the levels observed in healthy pregnant women throughout the three trimesters of pregnancy studied (**Supplementary Table 2**).

On the other hand, correlation coefficients between ANGPTL3 serum levels and different anthropometric and metabolic parameters in healthy pregnant and healthy non-pregnant women are described in **Table 2**, **Figures 2A, B** and **Supplementary Tables 1–3**. Serum ANGPTL3 levels were positively correlated with triglycerides, VLDL-c and total cholesterol in healthy non-pregnant women (**Table 2** and **Figures 2A, B**). Conversely, serum ANGPTL3 levels were not significantly correlated with any of the biochemical variables previously described at each trimester of pregnancy in healthy pregnant women (**Supplementary Tables 1–3**).

Finally, there was not a significant relationship between serum ANGPTL3 levels and BMI, HDL-c, glucose, insulin,

TABLE 2 | Pearson's correlation coefficient between serum ANGPTL3 levels and study variables in healthy non – pregnant women.

Variable	Valor R	*Valor P*
BMI	-0.0825	0.7294
Triglycerides	0.5206	0.0186*
VLDL –c	0.5205	0.0186*
HDL –c	-0.0545	0.8195
Total cholesterol	0.5088	0.0220*
Glucose	-0.0874	0.7139
Insulin	0.0144	0.9518
Leptin	-0.0199	0.9336
HOMA - IR	-0.0001	0.9996

*BMI, Body mass index; HDL-C, High-Density Lipoprotein Cholesterol; VLDL, Very Low-Density Lipoprotein. *P<0.05 (two-tailed significance). Log-transformed (log10) values were used.*

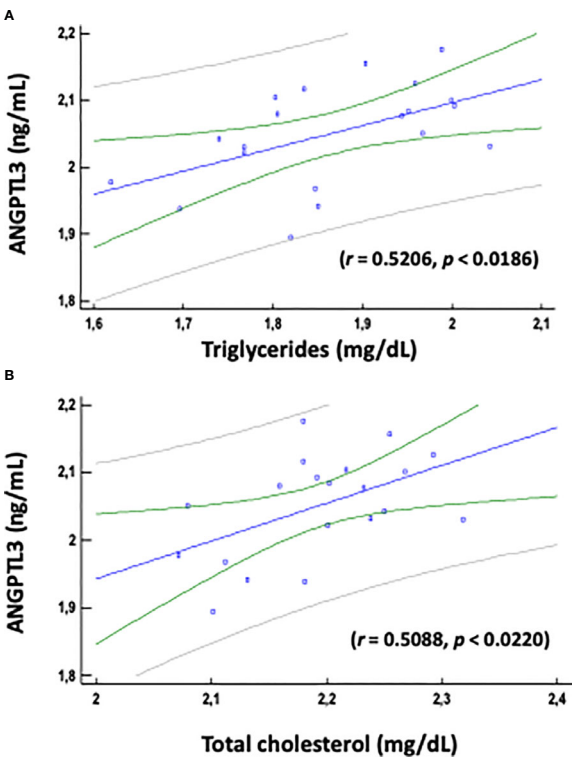


FIGURE 2 | Pearson's correlation coefficient between serum ANGPTL3 levels and triglycerides **(A)** and total cholesterol **(B)** variables was determined for healthy non – pregnant women. P<0.05 (two-tailed significance). Log-transformed (log10) values were used. Linear regression presenting the associations of ANGPTL3 with triglycerides and total cholesterol.

leptin, and HOMA-IR index in the different groups of women studied (**Table 2** and **Supplementary Tables 1-3**).

DISCUSSION

The present study describes for the first time, the serum profile of ANGPTL3 during the three trimesters of gestation in healthy pregnant and preeclamptic women, as well as in postpartum. Our results reveal that serum ANGPTL3 levels decreased significantly from the first to the third trimester in both healthy and preeclamptic pregnant women when compared with healthy non-pregnant and postpartum women. Additionally, there were no significant differences in serum ANGPTL3 levels between normal and preeclamptic women during each trimester of pregnancy examined in this study. Furthermore, results showed a significantly positive correlation between serum ANGPTL3 levels with triglyceride, VLDL-c and total cholesterol levels in healthy non-pregnant; however, we did not observe any significant relationship between serum ANGPTL3 and those lipids in healthy and preeclamptic pregnant women, across all trimesters of pregnancy.

Maternal accumulation of fat depots occurs in the first two trimesters of pregnancy to supply energy requirements during late pregnancy and lactation, and are attributed to different factors that can act synergistically, including increasing lipogenesis and LPL activity of adipose tissue (12–16). Furthermore, hormonal related changes during pregnancy, such as the increases of estrogen, progesterone and cortisol levels, favors endogenous *de novo* lipogenesis and fat deposition in adipose tissue (35). Previous studies have shown that adipose tissue LPL activity increases in early pregnancy or anabolic state, contributing to the accumulation of maternal fat depots during early pregnancy, and decreases as gestation progresses, favoring the adipose tissue lipolytic activity during the last third of gestation or catabolic state (35). Additionally, it has been shown that hormone-sensitive lipase (HSL) expression and activity in white adipose tissue are increased in the third trimester of pregnancy, accelerating breakdown of fat deposits and enhanced adipose tissue lipolytic activity (35). In this way, at the end of pregnancy the increased concentration of plasma non-esterified fatty acids (NEFA) and glycerol are re-esterified to triglycerides in the liver and subsequently released into the maternal circulation as native VLDL particles (12–14). As pregnancy advances, plasma triglyceride levels may raise 200 – 400% and this increase is primarily due to VLDL–TG particles (10). These VLDL-TG particles are transported to peripheral tissues, such as muscle and heart, where the triglycerides are hydrolyzed into fatty acid by action of LPL and are uptake into tissues as an important source of energy, favoring placental transfer and fetal uptake of glucose as the main energy source for the growth of the fetoplacental unit (36). In this way, it is possible that low serum ANGPTL3 concentrations profile might play a critical role during the different maternal metabolic adaptations that occur throughout pregnancy.

The results of the present study show that the serum levels of ANGPTL3 decrease significantly during the three trimesters of gestation, a condition that could contribute to the change in lipid metabolism during the anabolic and catabolic phases of pregnancy. Therefore, low levels of ANGPTL3 during the anabolic phase of pregnancy would favor the increase in *de novo* lipogenesis and the deposition of lipids due to the high activity of LPL in adipose tissue, while in oxidative tissues it would favor the activity of LPL and, therefore, the uptake of lipids as an energy source. Alternatively, during the catabolic phase of gestation, the low levels of ANGPTL3 would favor the hydrolysis of VLDL-TG and fatty acids as the main sources of energy in oxidative tissues, favoring glucose to be transported to the fetoplacental unit. In this sense, early pregnancy is an anabolic phase characterized by fat accumulation in maternal depots (2, 4, 12), while the third trimester is a catabolic condition with a breakdown of fat deposits and hyperlipidemia, and a decrease in removal the TG-rich lipoproteins from the circulation by LPL, favoring the placental lipid transport to the fetus (2, 37, 38). In this regard, previous studies have shown that ANGPTL3 is a key regulator of circulating TG levels due to its inhibitory action on the activity of LPL in the lipolysis of TG of VLDL and chylomicrons. Thus, the significant reduction in maternal circulating levels of ANGPTL3 throughout gestation could contribute that placental hormones would be the principal driving for the regulation of maternal lipids metabolism during pregnancy and these findings suggest that ANGPTL3 might be playing a reduced role in the inhibitory LPL activity during this transitory stage. Thus, the sharp decline in maternal ANGPTL3 levels throughout pregnancy might play a key role in maternal-fetal lipid metabolism adaptations.

On the other hand, serum levels of ANGPTL3 were positively and significantly associated with triglycerides, VLDL-c and total cholesterol levels in healthy non - pregnant women, which agree with previously reported results (39, 40). However, we could not find the same correlation in healthy women in any trimester of pregnancy. These data might provide potentially valuable information to further understanding of some of the features related to lipid metabolism during this critically dynamic period. In this way, these controversial relationships between ANGPTL3 levels and cholesterol and triglycerides suggest that there are other maternal factors during pregnancy that may affect the relationship between ANGPTL3 levels and other metabolic, hormonal and anthropometric factor. In addition, in controversy with previously reported results, we did not find any significant correlation between ANGPTL3 with other variables such as BMI, glucose, HDL–c, insulin, leptin and HOMA – IR index, in healthy non - pregnant and healthy pregnant women (39, 41, 42). Therefore, it is evident that assessing ANGPTL3 during pregnancy requires further investigation, in particular conducting other large prospective cohort studies associated with metabolic diseases

In conclusion, we show that in both healthy pregnant and preeclamptic women, serum ANGPTL3 levels are significantly decreased during all trimesters of pregnancy and might play a fundamental role in lipid maternal metabolism during gestation, favoring the transfer of glucose to the fetus and placenta as the main source of energy.

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 study was approved by the Institutional Review Board at the School of Medicine of the Universidad Nacional de Colombia and all participants signed informed consent forms. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by Government grants of The Universidad Nacional de Colombia (Dirección de investigaciones de Sede Bogotá and School of Medicine - código Hermes: 41802) and Colciencias (Departamento Administrativo de Ciencia, Tecnología e Innovación - Cod. 110154531660).

ACKNOWLEDGMENTS

The authors thank the women whose participation made this study possible. We are also grateful to the Promotion and Prevention Care Program, nurses and staff of Hospital Engativá of Bogotá, Colombia. The authors thank the women whose participation made this study possible. We are also grateful to the Promotion and Prevention Care Program, nurses and staff of Hospital Engativá of Bogotá, Colombia.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.670357/full#supplementary-material

REFERENCES

1. Geraghty AA, Alberdi G, O'Sullivan EJ, O'Brien EC, Crosbie B, Twomey PJ, et al. Maternal and fetal blood lipid concentrations during pregnancy differ by maternal body mass index: findings from the ROLO study. *BMC Pregnancy Childbirth* (2017) 17:360. doi: 10.1186/s12884-017-1543-x
2. Grimes SB, Wild R. Effect of Pregnancy on Lipid Metabolism and Lipoprotein Levels, in: *Endotext*. South Dartmouth (MA: MDText.com, Inc. Available at: http://www.ncbi.nlm.nih.gov/books/NBK498654/ (Accessed January 26, 2021).
3. Sattar N, Greer IA, Louden J, Lindsay G, McConnell M, Shepherd J, et al. Lipoprotein Subfraction Changes in Normal Pregnancy: Threshold Effect of Plasma Triglyceride on Appearance of Small, Dense Low Density Lipoprotein1. *J Clin Endocrinol Metab* (1997) 82:2483–91. doi: 10.1210/jcem.82.8.4126
4. Goldberg AS, Hegele RA. Severe Hypertriglyceridemia in Pregnancy. *J Clin Endocrinol Metab* (2012) 97:2589–96. doi: 10.1210/jc.2012-1250
5. Pecks U, Rath W, Kleine-Eggebrecht N, Maass N, Voigt F, Goecke TW, et al. Maternal Serum Lipid, Estradiol, and Progesterone Levels in Pregnancy, and the Impact of Placental and Hepatic Pathologies. *Geburtshilfe Frauenheilkd* (2016) 76:799–808. doi: 10.1055/s-0042-107078
6. Desoye G, Schweditsch MO, Pfeiffer KP, Zechner R, Kostner GM. Correlation of Hormones with Lipid and Lipoprotein Levels During Normal Pregnancy and Postpartum*. *J Clin Endocrinol Metab* (1987) 64:704–12. doi: 10.1210/jcem-64-4-704
7. Ursell W, Brudenell M, Chard T. Placental Lactogen Levels in Diabetic Pregnancy. *Br Med J* (1973) 2:80–2. doi: 10.1136/bmj.2.5858.80
8. Herrera E, Ortega-Senovilla H. Implications of Lipids in Neonatal Body Weight and Fat Mass in Gestational Diabetic Mothers and Non-Diabetic Controls. *Curr Diabetes Rep* (2018) 18:7. doi: 10.1007/s11892-018-0978-4
9. Herrera E, Desoye G. Maternal and fetal lipid metabolism under normal and gestational diabetic conditions. *Hormone Mol Biol Clin Invest* (2016) 26:109–27. doi: 10.1515/hmbci-2015-0025
10. Serrano NC, Guio-Mahecha E, Quintero-Lesmes DC, Bayona S–, Paez MC, Beltran M, et al. Lipid profile, plasma apolipoproteins, and pre-eclampsia risk in the GenPE case-control study. *Atherosclerosis* (2018) 276:189–94. doi: 10.1016/j.atherosclerosis.2018.05.051
11. Olivecrona G. Role of lipoprotein lipase in lipid metabolism. *Curr Opin Lipidol* (2016) 27:233–41. doi: 10.1097/MOL.0000000000000297
12. Herrera E, Ortega-Senovilla H. Lipid metabolism during pregnancy and its implications for fetal growth. *Curr Pharm Biotechnol* (2014) 15:24–31. doi: 10.2174/1389201015666140330192345
13. Herrera E, Lasunción MA, Gomez-Coronado D, Aranda P, López-Luna P, Maier I. Role of lipoprotein lipase activity on lipoprotein metabolism and the fate of circulating triglycerides in pregnancy. *Am J Obstetrics Gynecology* (1988) 158:1575–83. doi: 10.1016/0002-9378(88)90193-7
14. Knopp RH, Boroush MA, O'Sullivan JB. Lipid metabolism in pregnancy. II. Postheparin lipolytic activity and hypertriglyceridemia in the pregnant rat. *Metabolism* (1975) 24:481–93. doi: 10.1016/0026-0495(75)90073-6
15. Alvarez JJ, Montelongo A, Iglesias A, Lasunción MA, Herrera E. Longitudinal study on lipoprotein profile, high density lipoprotein subclass, and postheparin lipases during gestation in women. *J Lipid Res* (1996) 37:299–308. doi: 10.1016/S0022-2275(20)37617-3
16. Knopp RH, Herrera E, Freinkel N. Carbohydrate metabolism in pregnancy: *VIII. Metabolism of adipose tissue isolated from fed and fasted pregnant rats during late gestation*. *J Clin Invest* (1970) 49:1438–46. doi: 10.1172/JCI106361
17. Lindegaard MLS, Olivecrona G, Christoffersen C, Kratky D, Hannibal J, Petersen BL, et al. Endothelial and lipoprotein lipases in human and mouse placenta. *J Lipid Res* (2005) 46:2339–46. doi: 10.1194/jlr.M500277-JLR200
18. Bonet B, Brunzell JD, Gown AM, Knopp RH. Metabolism of very-low-density lipoprotein triglyceride by human placental cells: the role of lipoprotein lipase. *Metabolism* (1992) 41:596–603. doi: 10.1016/0026-0495(92)90051-b
19. Zhang R. The ANGPTL3-4-8 model, a molecular mechanism for triglyceride trafficking. *Open Biol* (2016) 6:150272. doi: 10.1098/rsob.150272
20. Wang Y, McNutt MC, Banfi S, Levin MG, Holland WL, Gusarova V, et al. Hepatic ANGPTL3 regulates adipose tissue energy homeostasis. *PNAS* (2015) 112(37):11630–5. doi: 10.1073/pnas.1515374112
21. Kovrov O, Kristensen KK, Larsson E, Ploug M, Olivecrona G. On the mechanism of angiopoietin-like protein 8 for control of lipoprotein lipase activity. *J Lipid Res* (2019) 60:783–93. doi: 10.1194/jlr.M088807
22. Haller JF, Mintah IJ, Shihanian LM, Stevis P, Buckler D, Alexa-Braun CA, et al. ANGPTL8 requires ANGPTL3 to inhibit lipoprotein lipase and plasma triglyceride clearance [S]. *J Lipid Res* (2017) 58:1166–73. doi: 10.1194/jlr.M075689

23. Chi X, Britt EC, Shows HW, Hjelmaas AJ, Shetty SK, Cushing EM, et al. ANGPTL8 promotes the ability of ANGPTL3 to bind and inhibit lipoprotein lipase. *Mol Metab* (2017) 6:1137–49. doi: 10.1016/j.molmet.2017.06.014
24. Kersten S. Angiopoietin-like 3 in lipoprotein metabolism. *Nat Rev Endocrinol* (2017) 13:731–9. doi: 10.1038/nrendo.2017.119
25. Spracklen CN, Smith CJ, Saftlas AF, Robinson JG, Ryckman KK. Maternal Hyperlipidemia and the Risk of Preeclampsia: a Meta-Analysis. *Am J Epidemiol* (2014) 180:346–58. doi: 10.1093/aje/kwu145
26. Ahmad Z, Banerjee P, Hamon S, Chan K-C, Bouzelmat A, Sasiela William J, et al. Inhibition of Angiopoietin-Like Protein 3 With a Monoclonal Antibody Reduces Triglycerides in Hypertriglyceridemia. *Circulation* (2019) 140:470–86. doi: 10.1161/CIRCULATIONAHA.118.039107
27. Gaudet D, Gipe DA, Pordy R, Ahmad Z, Cuchel M, Shah PK, et al. ANGPTL3 Inhibition in Homozygous Familial Hypercholesterolemia. *N Engl J Med* (2017) 377:296–7. doi: 10.1056/NEJMc1705994
28. Poveda NE, Garcés MF, Ruiz-Linares CE, Varón D, Valderrama S, Sanchez E, et al. Serum Adipsin Levels throughout Normal Pregnancy and Preeclampsia. *Sci Rep* (2016) 6:20073. doi: 10.1038/srep20073
29. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet Gynecol* (2013) 122:1122–31. doi: 10.1097/01.AOG.0000437382.03963.88
30. Alberti KGMM, Zimmet P, Shaw J. IDF Epidemiology Task Force Consensus Group. The metabolic syndrome–a new worldwide definition. *Lancet* (2005) 366:1059–62. doi: 10.1016/S0140-6736(05)67402-8
31. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* (1985) 28:412–9. doi: 10.1007/BF00280883
32. Chung HS, Lee MJ, Hwang SY, Lee HJ, Yoo HJ, Seo J-A, et al. Circulating angiopoietin-like protein 8 (ANGPTL8) and ANGPTL3 concentrations in relation to anthropometric and metabolic profiles in Korean children: a prospective cohort study. *Cardiovasc Diabetol* (2016) 15:1. doi: 10.1186/s12933-015-0324-y
33. Lacroix M, Battista M-C, Doyon M, Ménard J, Ardilouze J-L, Perron P, et al. Lower Adiponectin Levels at First Trimester of Pregnancy Are Associated With Increased Insulin Resistance and Higher Risk of Developing Gestational Diabetes Mellitus. *Diabetes Care* (2013) 36:1577–83. doi: 10.2337/dc12-1731
34. Lacroix M, Battista M-C, Doyon M, Moreau J, Patenaude J, Guillemette L, et al. Higher maternal leptin levels at second trimester are associated with subsequent greater gestational weight gain in late pregnancy. *BMC Pregnancy Childbirth* (2016) 16:62. doi: 10.1186/s12884-016-0842-y
35. Zeng Z, Liu F, Li S. Metabolic Adaptations in Pregnancy: A Review. *ANM* (2017) 70:59–65. doi: 10.1159/000459633
36. Goldberg IJ, Eckel RH, Abumrad NA. Regulation of fatty acid uptake into tissues: lipoprotein lipase- and CD36-mediated pathways. *J Lipid Res* (2009) 50 Suppl:S86–90. doi: 10.1194/jlr.R800085-JLR200
37. Hodson K, Man CD, Smith FE, Thelwall PE, Cobelli C, Robson SC, et al. Mechanism of Insulin Resistance in Normal Pregnancy. *Horm Metab Res* (2013) 45:567–71. doi: 10.1055/s-0033-1337988
38. Dubé E, Desparois G, Lafond J. Placental Lipid Transport. *Methods Mol Biol* (2018) 1710:305–16. doi: 10.1007/978-1-4939-7498-6_24
39. Zhao D, Yang L-Y, Wang X-H, Yuan S-S, Yu C-G, Wang Z-W, et al. Different relationship between ANGPTL3 and HDL components in female non-diabetic subjects and type-2 diabetic patients. *Cardiovasc Diabetol* (2016) 15:132. doi: 10.1186/s12933-016-0450-1
40. Yu C-G, Yuan S-S, Yang L-Y, Ke J, Zhang L-J, Lang J-N, et al. Angiopoietin-like 3 Is a Potential Biomarker for Retinopathy in Type 2 Diabetic Patients. *Am J Ophthalmol* (2018) 191:34–41. doi: 10.1016/j.ajo.2018.03.040
41. Yilmaz Y, Ulukaya E, Atug O, Dolar E. Serum concentrations of human angiopoietin-like protein 3 in patients with nonalcoholic fatty liver disease: association with insulin resistance. *Eur J Gastroenterol Hepatol* (2009) 21:1247–51. doi: 10.1097/MEG.0b013e32832b77ae
42. Cinkajzlová A, Mráz M, Lacinová Z, Kloučková J, Kaválková P, Kratochvílová H, et al. Angiopoietin-like protein 3 and 4 in obesity, type 2 diabetes mellitus, and malnutrition: the effect of weight reduction and realimentation. *Nutr Diabetes* (2018) 8:1–11. doi: 10.1038/s41387-018-0032-2

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

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 15 April 2021
doi: 10.3389/fendo.2021.621845

Triglyceride Induced Metabolic Inflammation: Potential Connection of Insulin Resistance and Recurrent Pregnancy Loss

Yongjie Liu[1], Mengyang Du[2], Yuexin Gan[1], Shihua Bao[2], Liping Feng[1,3]* and Jun Zhang[1]**

[1] Ministry of Education and Shanghai Key Laboratory of Children's Environmental Health, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] Department of Reproductive Immunology, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, China, [3] Department of Obstetrics and Gynecology, Duke University School of Medicine, Durham, NC, United States

OPEN ACCESS

Edited by:
Qiongjie Zhou,
Fudan University, China

Reviewed by:
Kok-Min Seow,
Shin Kong Wu Ho-Su Memorial Hospital, Taiwan
Yimin Zhu,
Zhejiang University, China

***Correspondence:**
Liping Feng
liping.feng@duke.edu
Shihua Bao
baoshihua@tongji.edu.cn
Jun Zhang
junjimzhang@sina.com

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *27 October 2020*
Accepted: *22 March 2021*
Published: *15 April 2021*

Citation:
Liu Y, Du M, Gan Y, Bao S, Feng L and Zhang J (2021) Triglyceride Induced Metabolic Inflammation: Potential Connection of Insulin Resistance and Recurrent Pregnancy Loss. Front. Endocrinol. 12:621845. doi: 10.3389/fendo.2021.621845

The underlying correlative mechanisms between Insulin resistance (IR) and recurrent pregnancy loss (RPL) in patients without polycystic ovarian syndrome (PCOS) remain inconclusive. To investigate the association between triglyceride (TG) levels, lymphocyte subsets, and IR in RPL patients without PCOS and obesity. Eighty-nine subjects with an unexplained RPL, independent of PCOS/obesity were enrolled in this study. A 75-g oral glucose tolerance test was performed on each subject with plasma tested for glucose and insulin. The fasting venous blood of all subjects was collected for routine clinical chemistry analysis. Lymphocyte subsets were analyzed by four-color flow cytometry. As a result, TG levels were significantly elevated in RPL patients with IR compared to those without IR. Pearson linear correlation model and receiver operating characteristic (ROC) curve analyses revealed a significant positive association between TG and HOMA-IR index value. In multiple logistic regression analysis, TG was significantly associated with the risk of hyperinsulinemia and increased $CD3^+CD4^+/CD3^+CD8^+$ ratio which was significantly negatively correlated with disposition index (DI30) and DI120, indicators for insulin sensitivity. In addition, DI30 and DI120 were significantly decreased in the higher $CD3^+CD4^+/CD3^+CD8^+$ group. Our findings showed that the elevated TG and altered immune responses in RPL patients with IR are independent of PCOS and obesity, and could be used as an indicator of IR in RPL patients. These results contribute to the understanding of the pathophysiology of IR in RPL for potential prevention and therapeutic targets.

Keywords: triglyceride, $CD3^+CD4^+/CD3^+CD8^+$ ratio, insulin resistance, recurrent pregnancy loss, insulin sensitivity

INTRODUCTION

Miscarriage, the most common complication of pregnancy, is the spontaneous loss of a conceptus before 20 weeks' gestation, and it affects approximately 15% of reproductive-age women recognized clinically (1). There are two types of miscarriage: sporadic and recurrent. Recurrent pregnancy loss (RPL), defined as three or more consecutive pregnancy losses, occurs in approximately 1% of childbearing couples (2). Actually, many clinicians define RPL as two or more losses, due to the recurrence rate is similar to that after three losses, and it affects around 1% to 5% of couples (2–4). Historically, RPL has been attributed to either genetic, structural, infective, endocrine, immune, or unexplained causes (2, 5–8). Endocrine disturbances have been postulated as an important cause of RPL, which widens the scope of investigation.

In recent years, researchers have focused on the connection between polycystic ovarian syndrome (PCOS), insulin resistance (IR), and RPL (9, 10). About 70% of women with PCOS have IR (11), and spontaneous pregnancy loss occurs in 40% women with PCOS (12). Data from clinical trials also showed that IR is common in women with RPL (13–16), and has been associated with an increased rate of pregnancy loss in PCOS patients (17). In fact, there are many RPL subjects with IR who do not have PCOS and/or obesity. It has been reported that non-obese Asians (BMI < 25 kg/m^2) can readily develop metabolic abnormalities (18, 19). Tamura et al., also reported that impaired insulin clearance and hyperinsulinemia could occur in apparently healthy subjects (20). Studies on the underlying correlative mechanisms between IR and RPL in patients without PCOS remain inconclusive. Guidelines from the Endocrine Society commonly recommend using metformin for women with IR and glucose intolerance due to its functional role on activating the insulin receptor and increasing glucose uptake. However, a randomized double-blind study also indicated that metformin has no major effects on glucose homeostasis in pregnant women with PCOS (21, 22), which indicates there are other underlying causes of IR in RPL patients beyond abnormal glucose metabolism. Therefore, it is imperative to understand the pathophysiology of IR in RPL for potential prevention and therapeutic targets.

Insulin resistance is characterized by a decreased responsiveness of insulin sensitivity and supraphysiological levels of insulin. It has been observed that a chronic inflammatory state may contribute to the development of IR (23, 24). Dysregulation of fat metabolism, characterized by significantly increased lipid accumulations, and triglycerides (TG) level, are known to induce low grade inflammation (25, 26). It was reported that the level of LDL cholesterol increases, whereas that of HDL cholesterol decreases in PCOS (27). Furthermore, elevated TG/HDL cholesterol ratio was significantly associated with IR and could be used as a predictor of IR in women with PCOS (28). However, the link between TG levels, inflammation, and IR in women without PCOS is unclear and need further study. It's worth noting that the T cell lymphocytes play a pivotal role in regulating the effector stage of the immune response, and increased lymphocyte proliferation is associated with an inflammatory response (29–31). An increased ratio of $CD4^+/CD8^+$ lymphocytes was found in lipopolysaccharide (LPS) challenged pigs (32). IR was reported to be related to the abnormal percentage of lymphocyte subsets in RPL (33, 34). A recent study revealed that a decrease of regulatory B cells ($CD19^+CD24^{hi}CD38^{hi}$) in peripheral blood is associated with IR, and can increase the risk of postprandial hyperinsulinemia (35). Yan et al. also demonstrated that IR was associated with cellular immune abnormalities in RPL patients (34).

Taken together, we hypothesized that cellular immune abnormalities and/or abnormal fat metabolism might contribute to the pathophysiology of IR in RPL subjects without PCOS. To test our hypothesis, we investigated the relationship between fat metabolism and immune cell status in peripheral blood and homeostasis model assessment for insulin resistance (HOMA-IR) parameters in an RPL cohort with/without IR, independent of PCOS/obesity.

MATERIALS AND METHODS

Participant Enrolment

Women were recruited at the first visit and were eligible for enrollment in this study if: (i) they were age less than 35 years with a body-mass index (BMI) no more than 27.9 kg/m^2 (non-obesity reference value standards of BMI of Chinese), and (ii) had regular ovulatory menstrual cycles and were actively trying to conceive with a history of unexplained RPL (two or more consecutive pregnancy losses before 20 weeks of gestation). The diagnosis of pregnancy loss was confirmed by ultrasound. (iii) The current male partner had normal semen testing by computer-assisted analysis. Age criterion was applied because the likelihood of pregnancy loss, due to chromosomal abnormalities is higher in older women. Subjects with a verifiable cause of miscarriage were excluded (**Figure 1**). The exclusion criteria were: (i) abnormality of the uterus such as uterine septae and bicornuate uterus; (ii) karyotype abnormality in either the parents or the embryo. Karyotyping abnormity was defined as abnormal chromosome number (such as trisomy, polyploidy, and monosomy X) and abnormal chromosome structure (either a balanced reciprocal translocation or a Robertsonian translocation). (iii) luteal phase defect (diagnosed by combining basal body temperature and serum progesterone levels < 10 ng/mL) (36, 37), hyperprolactinemia (non-pregnant serum prolactin levels > 30 ng/mL), or hyperandrogenemia (serum testosterone levels > 0.7 ng/mL); (iv) with PCOS which was diagnosed by the presence of all following criteria: (menstrual irregularities, polycystic ovarian morphology (PCOM), and/or hyperandrogenemia). Moreover, women diagnosed with non-PCOS with hyperandrogenemia, ovulatory dysfunction, or PCOM are excluded for further analyses; (v) presence of autoantibodies including antinuclear antibodies (ANA), anticardiolipin antibodies (ACL), anti-Beta 2 glycoprotein and extractable nuclear antigens antibodies associated with systemic lupus erythematosus (SLE). The study

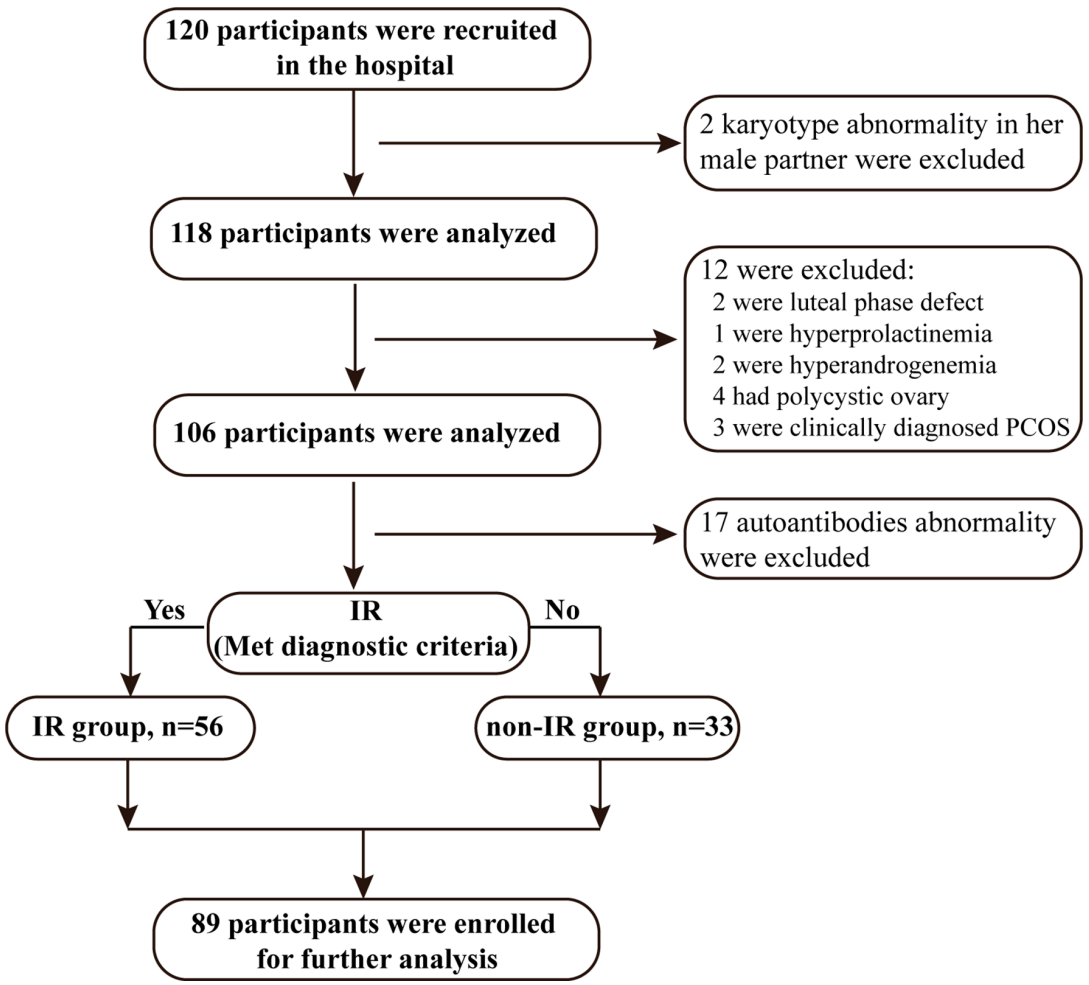


FIGURE 1 | Flow chart for the exclusion in this study.

protocol was approved by the Ethics Committees of Shanghai First Maternity and Infant Hospital (KS1812). Eighty-nine participants were recruited at the Department of Reproductive Immunology, Shanghai First Maternity and Infant Hospital between April 2018 and March 2019. Informed consent was obtained from all participants.

Oral Glucose Tolerance Test and Insulin Releasing Test

A 75 g oral glucose tolerance test (OGTT) was performed on each subject with plasma tested for glucose and insulin in a fasting state. Glucose levels were measured by a glucose oxidase-based assay (Roche Diagnostics GmbH, Mannheim, Germany). Insulin levels were measured by enzyme immunoassay (Roche Diagnostics GmbH, Mannheim, Germany). The plasma glucose levels at time points 0, 30, 60, 120, and 180 min were designated as Glu0, Glu30, Glu60, Glu120, and Glu180, and the insulin level at these time points were designated as Ins0, Ins30, Ins60, Ins120, and Ins180, respectively. In addition, the mean glucose and insulin levels during OGTT were designated as Glu_{mean} and Ins_{mean}, respectively.

Diagnosis

In this study, IR was diagnosed by the following indicators: 1) impaired glucose tolerance (IGT) or diabetic (38); 2) fasting insulin levels (FINS) ≥ 15 mIU/L (39); 3) HOMA-IR = Ins0 (μIU/ml) × Glu0 (mmol/L)/22.5 > 2.5 (40); and 4) normal FINS, but Ins180 level cannot be back to the normal level (41). The area under the curve (AUC) for glucose and insulin (GluAUC and InsAUC) was determined using the insulin secretion curve adjusted for glucose. The islet function and sensitivity index included the following: (i) a homeostasis model assessment of β cell function (HOMA-β) = [20 × Ins0 (μIU/ml)]/[Glu0 (mmol/L) − 3.5]; (ii) the early insulin secretion index (ΔI30/ΔG30) = [(Ins30 − Ins0) (μIU/ml)]/[(Glu30 − Glu0) (mmol/L)]; (iii) AUC for early-phase insulin secretion (InsAUC30/GluAUC30) = [(Ins0 + Ins30) (pmol/L)]/[(Glu0 + Glu30) (mmol/L)]; (iv) AUC for total insulin secretion (InsAUC120/GluAUC120) = [(Ins0+4×Ins30+3×Ins120) (pmol/L)]/[(Glu0+4×Glu30+ 3×Glu120) (mmol/L)]; (v) the Matsuda insulin sensitivity index (ISI_M) = 10,000/[(Glu0 (mg/dl) × Ins0 (μIU/ml) × Glu_{mean} (mg/dl) × Ins_{mean} (uIU/ml)]$^{0.5}$; (vi) the

disposition index (DI) = HOMA–β/HOMA-IR, representing an adjusted insulin sensitivity according to HOMA-IR; vii) the early-phase disposition index (DI30) = InsAUC30/GluAUC30 × ISI_M and the total disposition index (DI120) = InsAUC120/GluAUC120 × ISI_M.

Blood Chemistry Assay

The morning fasting venous blood of all subjects was collected for routine clinical chemistry analysis. Serum lipid content, including total cholesterol (TCH), triglyceride (TG), and homocysteine (HCY) was measured using an enzymatic colorimetric assay (Beckman Coulter Inc., Brea, USA). Serum levels of insulin, in addition to thyroid function, including thyrotropin (TSH), free triiodothyronin (FT3), and free thyroxine (FT4) was measured by enzyme immunoassay (Roche Diagnostics GmbH, Mannheim, Germany).

Flow Cytometry Analysis for Lymphocyte Subsets

The lymphocyte subsets, including $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD19^+$, and $CD16^+CD56^+$ (NK) cells were analyzed by four-color flow cytometry. Venous blood was drawn into EDTA anticoagulant tubes and then 200 μL of the fresh whole blood samples were stained with monoclonal antibodies conjugated with fluorochromes fluorescein isothiocyanate [FITC], phycoerythrin [PE], PE cyanine 5 [PE-Cy 5], and allophycocyanin [APC] and incubated for 20 min in the dark at room temperature. Erythrocytes were lysed by FACS lysing solution (BD Biosciences) and then the blood sample was washed with PBS twice prior to flow cytometric analysis. A FACSCalibur flow cytometer with multiset software (BD Biosciences) was used to analyze the levels of subpopulations.

Statistical Analysis

All data are shown as means ± standard deviation (SD), and statistically analyzed using the SPSS 22.0 software package. Comparisons of continuous variables with normal distribution between the groups were performed using Student's t-test, and Chi-square test was performed for categorical data analysis. Pearson's correlation test was used to analyze the correlation between lipid profiles, the lymphocyte subsets, and insulin resistance by regarding as continuous variables. Multiple-factor regression analysis was performed for assessing the association between TG levels, hyperinsulinemia, and $CD3^+CD4^+$/$CD3^+CD8^+$ ratio. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of Participants.

According to the standard of IR, of the 89 RPL women, 56 participants were diagnosed with insulin resistance and defined as the IR group, and the remaining 33 were the non-IR group. The general characteristics of the study participants are presented in **Table 1**, which include the following epidemiological factors: age, BMI, education level, smoking status, alcohol-drinking status, reproductive tract virus infection, antibodies screen and medical history. The RPL women with IR exhibited significantly higher BMI compared with the non-IR group although all subjects' BMI were in a healthy weight range. No significant differences were observed regarding other characteristics.

Comparison of β-Cell Function and Insulin Sensitivity Between IR and Non-IR Group

As shown in **Table 2**, HOMA-β was significantly higher in the IR group than the non-IR group. Patients with IR exhibited higher values of InsAUC30/GluAUC30 and InsAUC120/GluAUC120, which reflected higher early-phase insulin release and total insulin release, respectively, as opposed to the controls. In addition, RPL women with IR showed a significant decrease in Matsuda index, DI, DI30, and DI120 during OGTT, which indicates the reduced insulin sensitivity. Moreover, patients with IR manifested significantly higher glucose levels at 0, 60, 120, 180 min during OGTT in comparison with the controls, while the insulin levels were markedly higher during the entire period of the OGTT compared to the non-IR group (**Figure 2**).

TABLE 1 | Characteristics of participants in this study.

Variables	Non-IR (n = 33)	IR (n = 56)	*P-value*
Age (years)[a]	30.5 ± 4.8	30.6 ± 3.4	0.95
Body mass index (BMI, kg/m^2)	20.9 ± 2.0	22.2 ± 2.6	0.009*
<18.5	6 (18) [b]	5 (9)	0.34
18.5 ≤ BMI < 24	24 (73)	37 (66)	0.68
24 ≤ BMI < 28	3 (9)	14 (25)	0.12
Waist circumstance (cm)	66.0 ± 2.2	65.9 ± 2.6	0.92
Education			
Illiterate	0 (0)	0 (0)	NE[c]
High school or lower	7 (21)	9 (16)	0.75
College	23 (70)	45 (80)	0.38
Postgraduate or higher	3 (9)	2 (4)	0.54
History of smoking	0 (0)	0 (0)	NE
History of drinking	0 (0)	0 (0)	NE
History of diabetes	0 (0)	2 (4)	0.72
History of hypertension	0 (0)	3 (5)	0.46
Virus			
HIV+	0 (0)	0 (0)	NE
HPV+	1 (3)	0 (0)	0.79
Syphilis	0 (0)	0 (0)	NE
Antibody			
Anticardiolipin antibody (IgA, IgM, IgG)	0 (0)	0 (0)	NE
Anti DNA antibody (single- and double-stranded)	0 (0)	0 (0)	NE
Anti ENA (extractable nuclear antigen, 7 subtypes) antibody	0 (0)	0 (0)	NE
Anti-beta 2 glycoprotein antibody (Ig, IgG)	0 (0)	0 (0)	NE
Irregular antibody	0 (0)	0 (0)	NE
Medical history			
Endometriosis	0 (0)	0 (0)	NE
Uterine fibroids	2 (6)	0 (0)	0.26
Endometrial polyps	0 (0)	0 (0)	NE
Intrauterine adhesion	2 (6)	2 (4)	0.99
Ovarian cysts	0 (0)	0(0)	NE
Pelvic inflammation	0 (0)	0 (0)	NE

[a] Data are presented as mean ± SD. [b] Data are presented as n (%). [c] NE, not estimable (due to nullity of category in both groups). Pelvic inflammation means Pelvic Inflammatory Diseases, including endometritis, salpingitis, and chronic pelvic inflammation.

TABLE 2 | Comparison of β-cell function and insulin sensitivity, lipid profiles, and lymphocyte subsets of RPL patient with IR and non-IR.

		Non-IR (n = 33)	IR (n = 56)	*P*-value
β-cell function and insulin sensitivity	HOMA-IR	1.3 ± 0.5	2.6 ± 1.5	<0.0001*
	HOMA-β	92.5 ± 33.4	144.2 ± 69.2	0.0002*
	ΔI30/ΔG30	20.7 ± 18.1	21.6 ± 10.7	0.78
	InsAUC30/GluAUC30	36.2 ± 16.8	46.6 ± 21.1	0.03*
	InsAUC120/GluAUC120	49.8 ± 21.0	67.4 ± 30.0	0.007*
	Matsuda index (ISI_M)	8.0 ± 3.0	4.9 ± 2.3	<0.0001*
	DI	75.7 ± 20.4	62.0 ± 20.1	0.005*
	DI30	261.0 ± 96.1	194.7 ± 72.7	0.0009*
	DI120	358.1 ± 117.2	275.8 ± 81.9	0.0005*
Lipid profiles	TCH (mmol/L)	4.4 ± 0.7	4.4 ± 0.9	0.97
	TG (mmol/L)	0.8 ± 0.4	1.1 ± 0.6	0.025*
	LDL-C (mmol/L)	2.8 ± 0.3	2.9 ± 0.4	0.35
	HDL-C (mmol/L)	1.3 ± 0.3	1.4 ± 0.4	0.17
	HCY (μmol/L)	9.5 ± 2.8	8.6 ± 2.3	0.20
	TSH (mIU/L)	1.8 ± 0.9	2.2 ± 1.0	0.11
	FT3 (pmol/L)	5.0 ± 0.4	5.0 ± 0.6	0.97
	FT4 (pmol/L)	16.2 ± 2.1	17.2 ± 2.3	0.12
Lymphocyte subsets	$CD3^+$ cells (%)	70.1 ± 9.7	67.6 ± 8.4	0.22
	$CD3^+CD4^+$ cells (%)	36.1 ± 6.9	34.9 ± 6.4	0.43
	$CD3^+CD8^+$ cells (%)	28.1 ± 7.6	26.9 ± 6.0	0.39
	$CD3^+CD4^+/CD3^+CD8^+$ cell Ratio	1.4 ± 0.5	1.4 ± 0.4	0.89
	$CD16^+CD56^+$ cells (%)	16.6 ± 9.1	18.7 ± 8.5	0.27
	$CD19^+$ cells (%)	11.2 ± 3.0	11.9 ± 3.8	0.76

*IR, insulin resistance; non-IR, none insulin resistance; HOMA-IR, homeostasis model assessment for insulin resistance; HOMA-β, homeostasis model assessment of β cell function; DI, disposition index, representing an adjusted insulin sensitivity; TCH, total cholesterol, TG, triglyceride; LDL, low density lipoprotein-cholesterol; HDL, high density lipoprotein-cholesterol; HCY, homocysteine; TSH, thyrotropin; FT3, free triiodothyronin; and FT4, free thyroxine. *Significant difference between the two groups.*

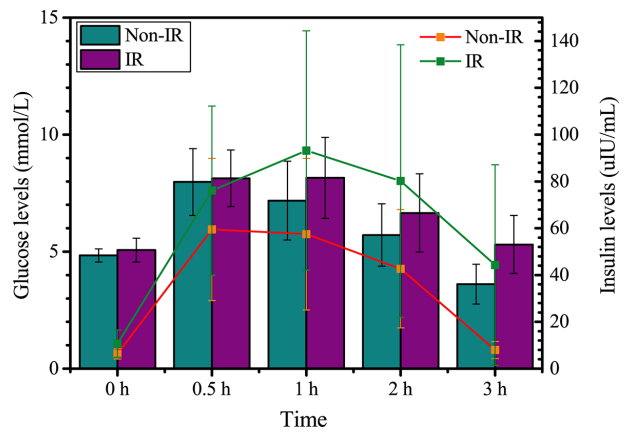


FIGURE 2 | Glucose and insulin levels between insulin resistance group (IR) and non-insulin resistance group (Non-IR).

Association Between TG and Insulin Resistance or Insulin Sensitivity

In the measures of lipid profile, TG levels were significantly elevated in patients with IR (**Table 2**). Pearson linear correlation model revealed a significant positive association between TG and HOMA-IR, suggesting an increased possibility of insulin resistance as TG levels increased (**Figure 3A**). A significant negative association was also identified between TG and DI (**Figure 3B**), DI30 (**Figure 3C**), DI120 (**Figure 3D**), indicating that a high TG level was closely associated with a decrease in the insulin sensitivity. Furthermore, a receiver operating characteristic (ROC) curve analysis indicated that TGs were associated with HOMA-IR (**Figure 3E**), DI (**Figure 3F**), and DI120 (**Figure 3G**). Moreover, a significant negative association was identified between TSH and HOMA-IR, however, no significant association was found between TG and TSH (**Supplementary Figure 1**).

An Increase in TG Levels Was Positively Associated With Hyperinsulinemia.

Hyperinsulinemia was defined as a fasting insulin ≥15 mU/L, and/or insulin levels at 2 h ≥80 mU/L during OGTT. In total, 25 enrollments were defined as hyperinsulinemia. Multivariate logistic regression analysis was performed to assess the association between TG and hyperinsulinemia in RPL patients. The results demonstrated that increase in TG was positively associated with hyperinsulinemia ($p = 0.02$). After adjusting for confounding factors including age, and BMI, there was a 2.77-fold higher risk of developing hyperinsulinemia with increasing TG levels ($p = 0.03$) (**Table 3**).

INCREASED TG LEVEL WAS POSITIVELY ASSOCIATED WITH THE RATIO OF $CD3^+CD4^+$ TO $CD3^+CD8^+$ LYMPHOCYTES

The ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ lymphocytes ($CD3^+CD4^+/CD3^+CD8^+$) was calculated by using the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$lymphocyte cells. The ratio of < 1.0 was set as decreased group (N = 22) and those with a ratio ≥ 1.0 were normal group (N = 67) (42). Multivariate logistic regression

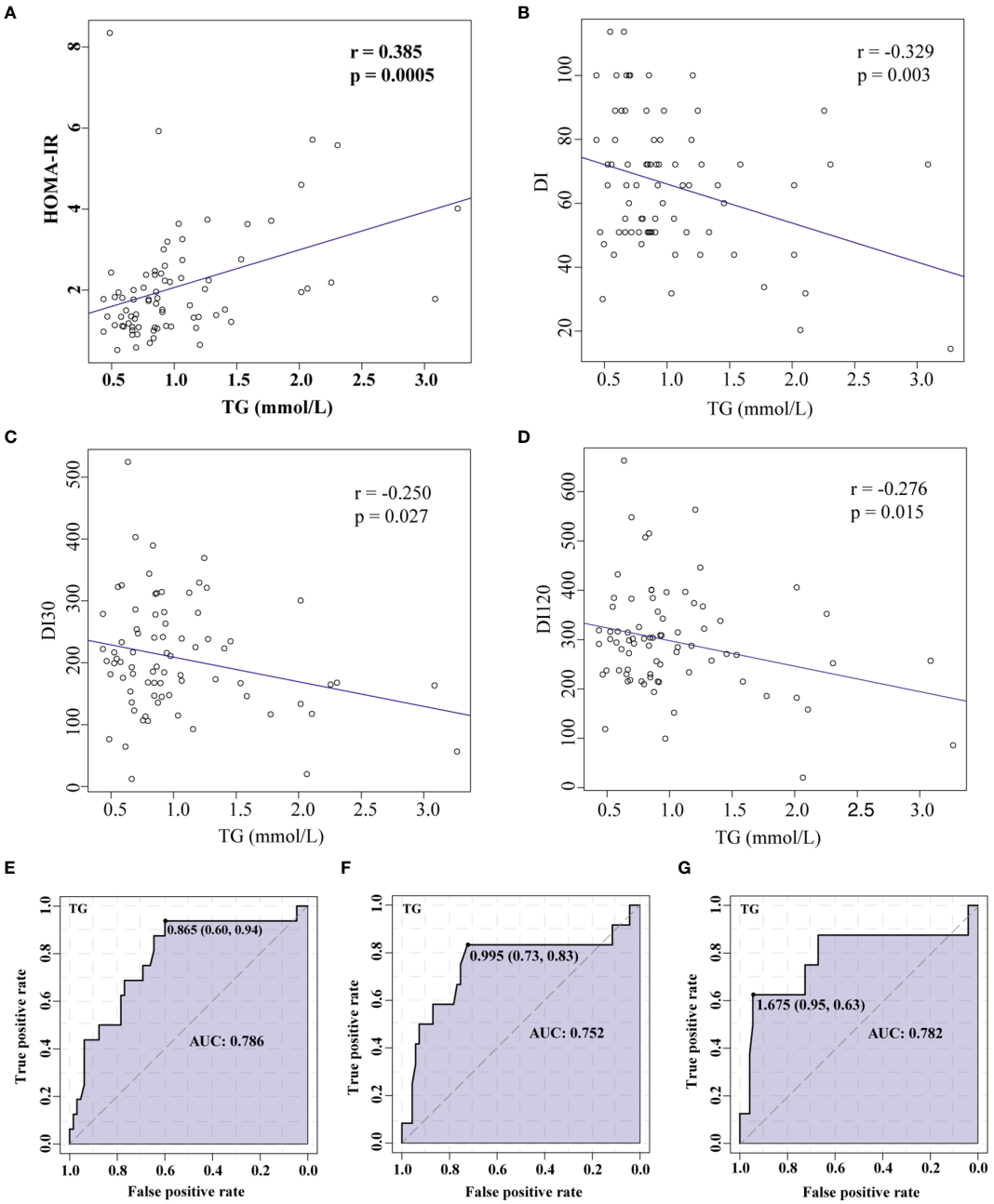


FIGURE 3 | Correlation between TG levels and HOMA-IR or insulin sensitivity demonstrated using Pearson's linear correlation model and ROC curve analysis. Association between TG and HOMA-IR **(A)**, TG and DI **(B)**, TG and DI30 **(C)**, and TG and DI120 **(D)**. ROC curve analysis for TGs and HOMA-IR **(E)**, DI **(F)**, and DI120 **(G)**.TG, Triglyceride; HOMA-IR, homeostasis model assessment for insulin resistance; DI, disposition index, representing adjusted insulin sensitivity according to HOMA-IR.

analysis was performed to assess the association between increased levels of TG and $CD3^+CD4^+/CD3^+CD8^+$ ratio in RPL patients. The results showed that increase in TG was positively associated with an increased $CD3^+CD4^+/CD3^+CD8^+$ ratio (p = 0.02). After adjusting for confounding factors including age, and BMI, there was a 2.85-fold increase in the ratio with an increasing TG levels (p = 0.04) (**Table 3**).

Increased Ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ Lymphocytes Was Negatively Associated With DI30 and DI120

The RPL women with a lower $CD3^+CD4^+/CD3^+CD8^+$ ratio of < 1.0 were set as decreased group and those with a ratio ≥ 1.0 were normal group. As presented in **Figure 4A**, DI30 and DI120 were significantly decreased in the high $CD3^+CD4^+/CD3^+CD8^+$

TABLE 3 | Logistic regression analysis for hyperinsulinemia and $CD3^+CD4^+/CD3^+CD8^+$ ratio.

	Models	B	SE	*P*-value	OR (95% CI)
Hyperinsulinemia	Model I				
	TG levels	1.02	0.46	0.02	2.78 (1.14 - 6.78)
	Model II				
	TG levels	1.02	0.46	0.03	2.77 (1.12 - 6.84)
CD3+CD4+/CD3+CD8+	Model I				
	TG levels	1.13	0.47	0.02	3.10 (1.24–7.72)
	Model II				
	TG levels	1.05	0.63	0.04	2.85 (0.83–9.77)

Model I, variables were introduced into the logistic regression model; Model II, adjustment for confounding factors including age, and BMI. BMI, Body mass index; TG, triglyceride; B, regression coefficient; SE, standard error; OR, odds ratio; CI, confidence interval.

group, and the ratio of $CD3^+CD4^+/CD3^+CD8^+$ was significantly negatively correlated with both DI30 (**Figure 4B**) and DI120 (**Figure 4C**). Moreover, ROC analysis indicated that $CD3^+CD4^+/CD3^+CD8^+$ were associated with DI30 (**Figure 4D**), and DI120 (**Figure 4E**), suggesting that the increased ratio of $CD3^+CD4^+/CD3^+CD8^+$ is accompanied by the reduced insulin sensitivity.

DISCUSSION

RPL has complex pathogenic mechanisms. Using a unique cohort of RPL patients without PCOS and obesity, we found increased TG levels in subjects with IR compared to those without IR. Moreover, the increased TG was positively associated with the ratio of $CD3^+CD4^+/CD3^+CD8^+$, and was accompanied by reduced insulin sensitivity and hyperinsulinemia. Thus, our study suggests that elevated TG and altered immune response are associated with insulin resistance in this cohort of RPL patients. Strategies to reduce the elevated TG and/or modulate immune system could be considered for treatments of these patients.

Insulin resistance is a condition in which the body fails to properly use insulin to regulate glucose metabolism. The accumulation of excessive insulin in the blood, described as hyperinsulinemia, with normal blood glucose levels indicates the presence of IR (43–45). Patients with PCOS and/or obese appear to have a higher prevalence of IR, and increased rate of pregnancy loss. Recently, it also has been reported that impaired insulin clearance and hyperinsulinemia could occur in non-obese Asians (20). However, the underlying correlative mechanisms between IR and RPL in patients without PCOS/obese remain inconclusive. To investigate the underlying mechanisms, we recruited a unique cohort of RPL patients without PCOS, suspected PCOS, and obese. The current study revealed that the glucose remained normal levels, however, the insulin levels at corresponding time points were significantly higher during OGTT in the IR group. Additionally, increased early-phase insulin release, total insulin release and lower insulin sensitivity indicated the occurrence of IR. Notably, the diagnose of IR in this study was determined based on a Chinese clinical expert consensus (46), which might be better representation for RPL patients in China. These appearance makes it more consistence to investigate the underlying mechanism between IR and RPL patients without PCOS and obese.

There is evidence that the higher fasting levels of TG are associated with IR (47, 48). These findings are consistent with the results of the present study. In this study, we found that higher TG levels were positively correlated with the risk of hyperinsulinemia in RPL women with and without adjustment of age and BMI. However, the underlying connection between higher TG levels and RPL patients with IR remained elusive. It has been demonstrated in mice that lymphocytes participate in the pathogenesis of IR (49). Yan et al. found that a far greater proportion of newly diagnosed RPL patients with IR had an aberrant $CD3^+CD4^+$, and $CD56^+CD16^+$ cell population compared to RPL patients without IR (34). Our study found that an increase in TG levels positively associated with an increased $CD3^+CD4^+/CD3^+CD8^+$ ratio. Furthermore, an inverse correlation between the $CD3^+CD4^+/CD3^+CD8^+$ ratio and DI values, reflecting insulin sensitivity were found, and increased $CD3^+CD4^+/CD3^+CD8^+$ is an indicator for hyperinsulinemia, revealing the involvement of $CD3^+CD4^+/CD3^+CD8^+$ in RPL patients with IR.

Previous studies indicated that an increased ratio of $CD4^+/CD8^+$ was positively correlated with tumor necrosis factor-α (TNF-α) level (50, 51). Notably, TG levels induced a high levels of plasma TNF-α by the increased production of very low density lipoprotein (VLDL) particles (52). And increased TNF-α expression contribute to decreased adiponectin, which plays as an important modulator of insulin sensitivity *via* the downregulation of the peroxisome proliferator activated receptor-α (PPAR-α) (53, 54). From our observations, we postulate that high TG levels induce IR in RPL patients *via* the elevated $CD3^+CD4^+/CD3^+CD8^+$ ratio. One possible hypothesis is elevated TG induce aberrant lymphocyte subsets, including increased $CD3^+CD4^+/CD3^+CD8^+$, which serves a proinflammatory role by producing TNF-α, leading to reduced adiponectin production and blocking activation of PPAR-α, thereby resulting in decreased insulin sensitivity. All these findings indicate that higher TG levels are associated with the development of IR.

The strengths of this study include: (i) thoroughly consideration of the exclusion criteria for common causes of pregnancy loss such as uterine abnormalities, karyotype abnormality, luteal phase defect, and abnormal antibodies; (ii) an RPL cohort with and without IR independent of PCOS and abnormal BMI. Several limitations were associated with our study: (i) some information related to IR was not obtained,

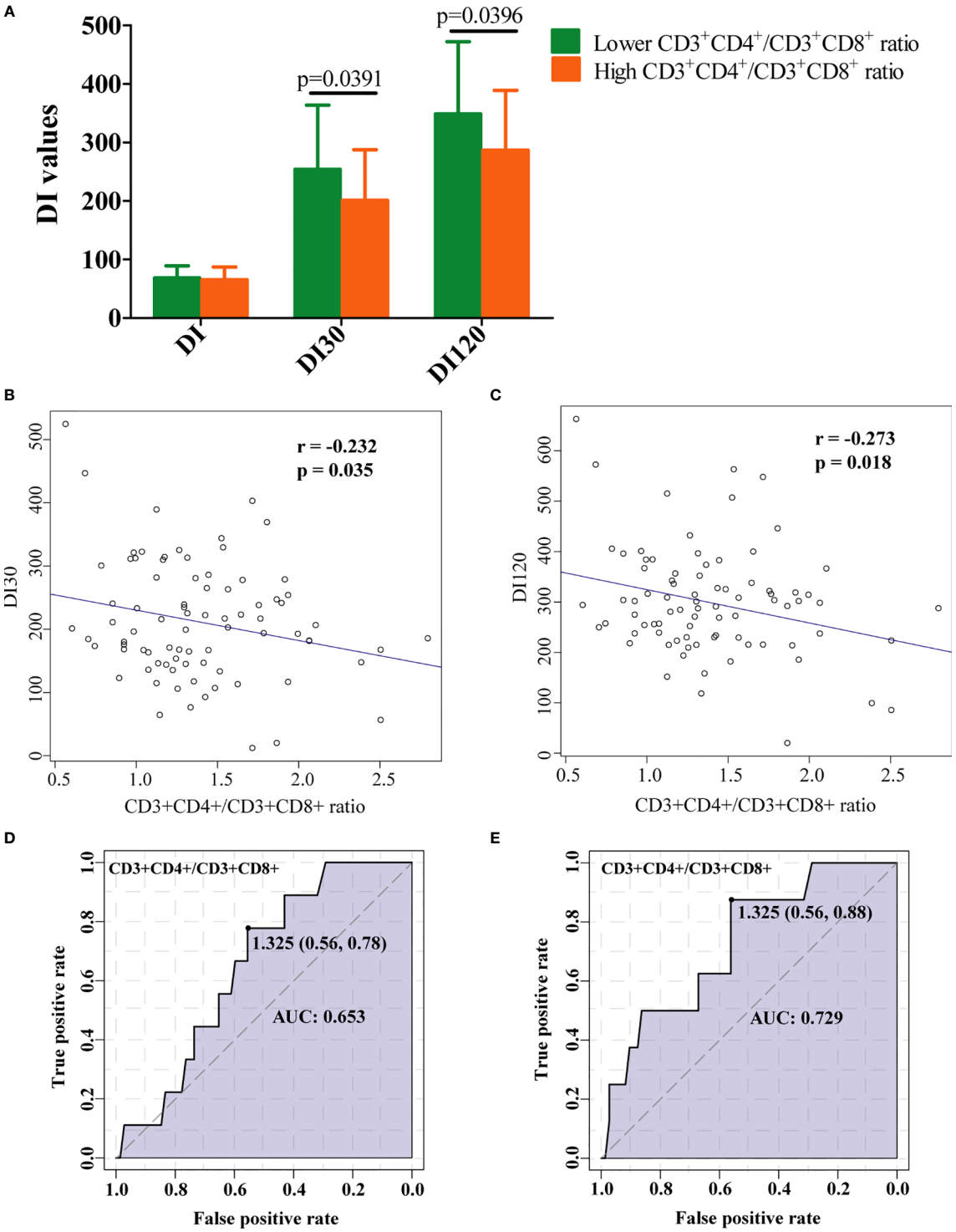


FIGURE 4 | Clinical implication of $CD3^+CD4^+/CD3^+CD8^+$ ratio in RPL patients with IR. **(A)** RPL women with decreased $CD3^+CD4^+/CD3^+CD8^+$ ratio had significantly higher levels of DI30 and DI120. The increase in the ratio of $CD3^+CD4^+/CD3^+CD8^+$ was marked associated with low **(B)** DI30 and **(C)** DI120 levels. ROC curve analysis for $CD3^+CD4^+/CD3^+CD8^+$ ratio and DI30 **(D)**, and DI120 **(E)**. Data is presented as mean ± standard deviation. DI, disposition index, representing adjusted insulin sensitivity according to HOMA-IR.

such as the free fatty acid levels, the detail of regular physical exercise, and family history of diabetes; (ii) this study was a cross-sectional design that made it difficult to explore the causal relationship between TG, $CD3^+CD4^+/CD3^+CD8^+$ ratio and IR. (iii) there is lack of racial diversity in the studied cohort. It was reported that ethnic differences in TG levels should be considered for identifying insulin resistance (55). Tamura et al., reported that impaired insulin clearance and hyperinsulinemia could occur in non-obese Asians and decreased insulin sensitivity in muscle was well correlated with elevated TG levels in non-obese Asians (20). It is possible that TG can be used as an indicator of IR in RPL patients with racial and ethnic disparities. Finally, future studies with diverse populations and large survey samples are warranted.

In conclusion, the results of the present study demonstrated significant increases in the early phase insulin secretion and total insulin secretion during OGTT and an increased TG levels in RPL patients with IR. The increased serum level of TG was positively associated with postprandial hyperinsulinemia. The role of $CD3^+CD4^+/CD3^+CD8^+$ ratio in insulin resistance contributes to the associated interference with islet compensation function. Further investigation is required to determine if $CD3^+CD4^+/CD3^+CD8^+$ ratio is able to act as immune indicators of insulin resistance and islet β-cell function in RPL 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 Ethics Committees of Shanghai First Maternity and Infant Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LF and YL conceived and designed the experiments. MD, YG, and YL performed sample collection. YL performed experimental processing of the samples and analyzed the data. YL wrote the manuscript, and LF revised the manuscript. SB, LF, and JZ contributed to discussions and reviewed the paper, and gave their approval to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (81903325), and the China Postdoctoral Science Foundation (2018M632138).

ACKNOWLEDGMENTS

We thank obstetrician Mengyang Du, Guohua Li, and Na Yu (Department of Reproductive Immunology, Shanghai First Maternity and Infant Hospital, Shanghai, China) for helpful sampling assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.621845/full#supplementary-material

Supplementary Figure 1 | Correlation analyses between lipid profiles, the lymphocyte subsets and insulin resistance. HOMA-IR, homeostasis model assessment for insulin resistance; HOMA-β, homeostasis model assessment of β cell function; ISI_M, Matsuda insulin sensitivity index; DI, disposition index, representing an adjusted insulin sensitivity; TCH, total cholesterol; TG, triglyceride; TSH, thyrotropin; * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.0001$.

REFERENCES

1. Hertz-Picciotto I, Samuels S. Incidence of early loss of pregnancy. *N Engl J Med* (1988) 319(22):1483–4. doi: 10.1056/NEJM198812013192214
2. Rai R, Regan L. Recurrent miscarriage. *Lancet* (2006) 368(9535):601–11. doi: 10.1016/S0140-6736(06)69204-0
3. Branch D, Gibson M, Silver R. Recurrent Miscarriage. *N Engl J Med* (2010) 363:1740–7. doi: 10.1056/NEJMcp1005330
4. Bender Atik R, Christiansen OB, Elson J, Kolte AM, Lewis S, Middeldorp S, et al. ESHRE guideline: recurrent pregnancy loss. *Hum Reprod Open* (2018) 2018(2):hoy004. doi: 10.1093/hropen/hoy004
5. Kaur R, Gupta K. Endocrine dysfunction and recurrent spontaneous abortion: An overview. *Int J Appl Basic Med Res* (2016) 6(2):79–83. doi: 10.4103/2229-516X.179024
6. Medicine PCotASfR. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. *Fertil Steril* (2012) 98(1556-5653(1556-5653 (Electronic):1103–11. doi: 10.1016/j.fertnstert.2012.06.048
7. Pereza N, Ostojić S, Kapović M, Peterlin B. Systematic review and meta-analysis of genetic association studies in idiopathic recurrent spontaneous abortion. *Fertil Steril* (2016) 107(1):S0015028216629242. doi: 10.1016/j.fertnstert.2016.10.007
8. Murugappan G, Vyas NM, Lathi RB. Karyotype of first miscarriage is prognostic of subsequent pregnancy outcome. *Fertil Steril* (2019) 111(4, Supplement):e11–2. doi: 10.1016/j.fertnstert.2019.02.048
9. Rajani S, Mukherjee S, Sharma S, Sarkar HS, Goswami SK, Chakravarty B. Insulin resistance, homocysteine & pregnancy loss in polycystic ovarian syndrome (PCOS): are they related? *Fertil Steril* (2010) 94(4):S194–4. doi: 10.1016/j.fertnstert.2010.07.754
10. Chen L, Xu WM, Zhang D. Association of abdominal obesity, insulin resistance, and oxidative stress in adipose tissue in women with polycystic ovary syndrome. *Fertil Steril* (2014) 102(4):1167–74.e1164. doi: 10.1016/j.fertnstert.2014.06.027
11. Goodarzi MO, Korenman SG. The importance of insulin resistance in polycystic ovary syndrome. *Fertil Steril* (2003) 80(0015-0282(0015-0282 (Print):255–8. doi: 10.1016/S0015-0282(03)00734-9
12. Rai R, Backos M, Rushworth F, Regan L. Polycystic ovaries and recurrent miscarriage–a reappraisal. *Hum Reprod* (2000) 15(3):612–5. doi: 10.1093/humrep/15.3.612

13. Hong Y, Xie QX, Chen CY, Yang C, Li YZ, Chen DM, et al. Insulin resistance in first-trimester pregnant women with pre-pregnant glucose tolerance and history of recurrent spontaneous abortion. *J Biol Regul Homeost Agents* (2013) 27(1):225–31. doi: 10.7868/S004446771301005X
14. Banu J, Fatima P, Sultana P, Chowdhury MA, Begum N, Anwary SA, et al. Association of infertile patients having polycystic ovarian syndrome with recurrent miscarriage. *Mymen Med J* (2014) 23(4):770–3.
15. Ghaffarzad A, Amani R, Darabi M, Cheraghian B. Correlation of Serum Lipoprotein Ratios with Insulin Resistance in Infertile Women with Polycystic Ovarian Syndrome: A Case Control Study. *Int J Fertil Steril* (2016) 10(1):29–35. doi: 10.22074/ijfs.2016.4765
16. Craig LB, Ke RW, Kutteh WH. Increased prevalence of insulin resistance in women with a history of recurrent pregnancy loss. *Fertil Steril* (2002) 78 (3):487–90. doi: 10.1016/S0015-0282(02)03247-8
17. Ispasoiu CA, Chicea R, Stamatian FV, Ispasoiu F. High fasting insulin levels and insulin resistance may be linked to idiopathic recurrent pregnancy loss: a case-control study. *Int J Endocrinol* (2013) 2013(2013):576926. doi: 10.1155/2013/576926
18. Chan J, Malik V, Jia W, Kadowaki T, Yajnik C, Yoon K, et al. Diabetes in Asia: epidemiology, risk factors, and pathophysiology. *JAMA* (2009) 301(20):2129–40. doi: 10.1001/jama.2009.726
19. Pan W, Yeh W, Weng L. Epidemiology of metabolic syndrome in Asia. *Asia Pac J Clin Nutr* (2008) 17(Suppl 1):37–42.
20. Tamura Y. Ectopic fat, insulin resistance and metabolic disease in non-obese Asians: investigating metabolic gradation. *Endocr J* (2019) 66(1):1–9. doi: 10.1507/endocrj.EJ18-0435
21. Fougner KJ, Vanky E, Carlsen SM. Metformin has no major effects on glucose homeostasis in pregnant women with PCOS: results of a randomized double-blind study. *Scand J Clin Lab Inv* (2008) 68(8):771–6. doi: 10.1080/00365510802254620
22. Song J, Ruan X, Gu M, Wang L, Wang H, Mueck AO. Effect of orlistat or metformin in overweight and obese polycystic ovary syndrome patients with insulin resistance. *Gynecol Endocrinol* (2018) 34(5):413–7. doi: 10.1080/09513590.2017.1407752
23. Xu H, Barnes GT, Yang Q, Tan G, Daseng Y, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* (2003) 112(12):1821–30. doi: 10.1172/JCI200319451
24. Sáinz N, González-Navarro CJ, Martínez JA, Moreno-Aliaga MJ. Leptin signaling as a therapeutic target of obesity. *Expert Opin Ther Tar* (2015) 19 (7):1–17. doi: 10.1517/14728222.2015.1018824
25. Jin Y, Wu Y, Zeng Z, Jin C, Wu S, Wang Y, et al. From the Cover: Exposure to Oral Antibiotics Induces Gut Microbiota Dysbiosis Associated with Lipid Metabolism Dysfunction and Low-Grade Inflammation in Mice. *Toxicol Sci* (2016) 154(1):140. doi: 10.1093/toxsci/kfw150
26. Elhady M, Elazab AAAM, Bahagat KA, Abdallah NA, Ibrahim ES. Fatty pancreas in relation to insulin resistance and metabolic syndrome in children with obesity. *J Pediatr Endocr Met* (2019) 32:19–26. doi: 10.1515/jpem-2018-0315
27. Patel S. Polycystic ovary syndrome (PCOS), an inflammatory, systemic, lifestyle endocrinopathy. *J Steroid Biochem* (2018) 182:27–36. doi: 10.1016/j.jsbmb.2018.04.008
28. Ketefian A, Amin M, Manning D, Azziz R, Pisarska M, Alexander C. Triglyceride to high-density lipoprotein cholesterol ratio as a predictor of insulin resistance in women with polycystic ovary syndrome (PCOS). *Fertil Steril* (2011) 96(3):S129. doi: 10.1016/j.fertnstert.2011.07.503
29. Liu YL, Li DF, Gong LM, Yi GF, Gaines AM, Carroll JA. Effects of fish oil supplementation on the performance and the immunological, adrenal, and somatotropic responses of weaned pigs after an lipopolysaccharide challenge. *J Anim Sci* (2003) 81(11):2758–65. doi: 10.2527/2003.81112758x
30. Romualdo MCDS, Nóbrega FJD. Insulin resistance in obese children and adolescents. *J Pediat Brazil* (2014) 90(6):600–7. doi: 10.1016/j.jped.2014.03.005
31. Zhu LY, Chen X, Xu ZZ, Xu L, Mao T, Zhang H. Changes and clinical significance of peripheral blood helper T lymphocyte and natural killer (NK) cells in unexplained recurrent spontaneous abortion (URSA) patients after abortion and successful pregnancy. *Clin Exp Obstet Gyn* (2015) 42(1):62–6.
32. Lai C JY, Li D, Zhao L, Chen X. Effects of dietary conjugated linoleic acid supplementation on performance and immune function of weaned pigs. *Arch Anim Nutr* (2005) 59(1745-039X(1745-039X (Print):41–51. doi: 10.1080/17450390512331342377
33. Kanninen TT, Bruna Ribeiro DAR, Shirlee J, Ann Marie B, Iara Moreno L, Renzo GCD, et al. Inhibition of autophagy by sera from pregnant women. *Reprod Sci* (2013) 20(11):1327–31. doi: 10.1177/1933719113485301
34. Yan Y, Bao S, Sheng S, Wang L, Tu W. Insulin resistance in patients with recurrent pregnancy loss is associated with lymphocyte population aberration. *Syst Biol Reprod Med* (2017) 63(6):397–404. doi: 10.1080/19396368.2017.1378936
35. Yang M, Du C, Wang Y, Liu J. CD19(+)CD24(hi)CD38(hi) regulatory B cells are associated with insulin resistance in type I Hashimoto's thyroiditis in Chinese females. *Exp Ther Med* (2017) 14(4):3887–93. doi: 10.3892/etm.2017.4925
36. Jordan J, Craig K, Clifton D, Soules M. Luteal phase defect: the sensitivity and specificity of diagnostic methods in common clinical use. *Fertil Steril* (1994) 62(1):54–62. doi: 10.1016/S0015-0282(16)56815-0
37. Czyzyk A, Podfigurna A, Genazzani A, Meczekalski B. The role of progesterone therapy in early pregnancy: from physiological role to therapeutic utility. *Gynecol Endocrinol* (2017) 33(6):421–4. doi: 10.1080/09513590.2017.1291615
38. Kim J, Bacha F, Tfayli H, Michaliszyn S, Yousuf S, Arslanian S. Adipose Tissue Insulin Resistance in Youth on the Spectrum From Normal Weight to Obese and From Normal Glucose Tolerance to Impaired Glucose Tolerance to Type 2 Diabetes. *Diabetes Care* (2019) 42(2):265–72. doi: 10.2337/dc18-1178
39. Tohidi M, Ghasemi A, Hadaegh F, Derakhshan A, Chary A, Azizi F. Age- and sex-specific reference values for fasting serum insulin levels and insulin resistance/sensitivity indices in healthy Iranian adults: Tehran Lipid and Glucose Study. *Clin Biochem* (2014) 47(6):432–8. doi: 10.1016/j.clinbiochem.2014.02.007
40. DeUgarte C, Bartolucci A, Azziz R. Prevalence of insulin resistance in the polycystic ovary syndrome using the homeostasis model assessment. *Fertil Steril* (2005) 83(5):1454–60. doi: 10.1016/j.fertnstert.2004.11.070
41. Jinfang L, Xin L, Su C. Diagnosis and treatment of insulin resistance in patients with polycystic ovary syndrome. *Chin J Pract Gynecolo Obstet* (2007) 09):663–7. doi: 1005-2216(2007)09-0667-03
42. Chen X, Jl Y, Ye J. Analysis of peripheral blood lymphocyte subsets and prognosis in patients with septic shock. *Microbiol Immunol* (2011) 55(1348-0421(1348-0421 (Electronic):736–42. doi: 10.1111/j.1348-0421.2011.00373.x
43. Yang X, Quan X, Lan Y, Wei Q, Ye J, Yin X, et al. Serum chemerin level in women with PCOS and its relation with the risk of spontaneous abortion. *Gynecol Endocrinol* (2018) 34(10):864–7. doi: 10.1080/09513590.2018.1462316
44. Cefalu WT. Insulin resistance: cellular and clinical concepts. *Exp Biol Med* (2001) 226(1):13–26. doi: 10.1177/153537020122600103
45. Reaven GM. Insulin resistance, cardiovascular disease, and the metabolic syndrome: how well do the emperor's clothes fit? *Diabetes Care* (2004) 27 (4):1011–2. doi: 10.2337/diacare.27.4.1011
46. Association EamboCmd. Consensus of endocrinologists on diagnosis and treatment of polycystic ovary syndrome. *Chin J Endocrinol Metab* (2018) 34 (1):1–7. doi: 10.3760/cma.j.Łissn.1000-6699.2018.01.001
47. Yeh WC, Tsao YC, Li WC, Tzeng IS, Chen LS, Chen JY. Elevated triglyceride-to-HDL cholesterol ratio is an indicator for insulin resistance in middle-aged and elderly Taiwanese population: a cross-sectional study. *Lipids Health Dis* (2019) 18(1):176. doi: 10.1186/s12944-019-1123-3
48. Martins LM, Oliveira ARS, Cruz KJC, Torres-Leal FL, Marreiro D. Obesity, inflammation, and insulin resistance. *Braz J Pharm Sci* (2014) 50(4):677–92. doi: 10.1590/S1984-82502014000400003
49. Solano ME, Sander VA, Ho H, Motta AB, Arck PC. Systemic inflammation, cellular influx and up-regulation of ovarian VCAM-1 expression in a mouse model of polycystic ovary syndrome (PCOS). *J Reprod Immunol* (2011) 92 (1):33–44. doi: 10.1016/j.jri.2011.09.003
50. Moodley Y, Dorasamy T, Venketasamy S, Naicker V, Lalloo U. Correlation of CD4:CD8 ratio and tumour necrosis factor (TNF)alpha levels in induced sputum with bronchoalveolar lavage fluid in pulmonary sarcoidosis. *Thorax* (2000) 55(8):696–9. doi: 10.1136/thorax.55.8.696
51. Wang K, Wang DC, Feng YQ, Xiang-Feng L. Changes in cytokine levels and CD4+/CD8+ T cells ratio in draining lymph node of burn wound. *J Burn Care Res* (2007) 28(5):747–53. doi: 10.1097/BCR.0B013E318148C95A
52. Deng Y, Scherer P. Adipokines as novel biomarkers and regulators of the metabolic syndrome. *Ann NY Acad Sci* (2010) 1212:E1–19. doi: 10.1111/j.1749-6632.2010.05875.x

53. Hajri T, Tao H, Wattacheril J, Marks-Shulman P, Abumrad N. Regulation of adiponectin production by insulin: interactions with tumor necrosis factor-α and interleukin-6. *Am J Physiol Endoc M* (2011) 300(2):E350–60. doi: 10.1152/ajpendo.00307.2010
54. Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* (2002) 8(11):1288–95. doi: 10.1038/nm788
55. Sumner A, Cowie C. Ethnic differences in the ability of triglyceride levels to identify insulin resistance. *Atherosclerosis* (2008) 196(2):696–703. doi: 10.1016/j.atherosclerosis.2006.12.018

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 28 April 2021
doi: 10.3389/fendo.2021.630287

FGF21 Serum Levels in the Early Second Trimester Are Positively Correlated With the Risk of Subsequent Gestational Diabetes Mellitus: A Propensity-Matched Nested Case-Control Study

Zhiheng Wang[1†‡], Min Yuan[2‡], Chengjie Xu[3], Yang Zhang[2], Chunmei Ying[1] and Xirong Xiao[2*]*

[1] Clinical Laboratory, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China, [2] Department of Obstetrics, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China, [3] Information Section, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China

Background: As an important endocrine hormone regulating glucose metabolism, fibroblast growth factor 21 (FGF21) is increased in individuals with gestational diabetes mellitus (GDM) after 24 gestational weeks. However, it is unknown whether the increase in FGF21 precedes the diagnosis of GDM.

Methods: In this nested case-control study, 133 pregnant women with GDM and 133 pregnant women with normal glucose tolerance (NGT) were identified through propensity score matching, and serum FGF21 levels were measured at 14 to 21 gestational weeks, before GDM is routinely identified. The differences in FGF21 levels were compared. The association between FGF21 and the occurrence of GDM was evaluated using logistic regression models with adjustment for confounders.

Results: The serum FGF21 levels of the GDM group at 14 to 21 gestational weeks were significantly higher than those of the NGT group overall ($P < 0.001$), with similar results observed between the corresponding BMI subgroups ($P < 0.05$). The 2nd (OR 1.224, 95% CI 0.603–2.485), 3rd (OR 2.478, 1.229–5.000), and 4th (OR 3.419, 95% CI 1.626–7.188) FGF21 quartiles were associated with greater odds of GDM occurrence than the 1st quartile after multivariable adjustments.

Conclusions: The serum FGF21 levels in GDM groups increased in the early second trimester, regardless of whether participants were stratified according to BMI. After adjusting for confounding factors, the FGF21 levels in the highest quartile were associated with more than three times higher probability of the diagnosis of GDM in the pregnancy as compared to levels in the first quartile.

Keywords: FGF21, gestational diabetes mellitus (GDM), nested case-control study, propensity score matching (PSM), early second trimester

OPEN ACCESS

Edited by:
Ganesh Acharya,
Karolinska Institute, Sweden

Reviewed by:
Kok Hian Tan,
KK Women's and Children's Hospital, Singapore
Amarnath Bhide,
St George's University Hospitals NHS Foundation Trust, United Kingdom

***Correspondence:**
Chunmei Ying
ycmzh2012@163.com
Xirong Xiao
xiaoxirong@fudan.edu.cn

†ORCID:
Zhiheng Wang
orcid.org/0000-0003-4186-5564

‡These authors have contributed equally to this work

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *17 November 2020*
Accepted: *08 April 2021*
Published: *28 April 2021*

Citation:
Wang Z, Yuan M, Xu C, Zhang Y, Ying C and Xiao X (2021) FGF21 Serum Levels in the Early Second Trimester Are Positively Correlated With the Risk of Subsequent Gestational Diabetes Mellitus: A Propensity-Matched Nested Case-Control Study. Front. Endocrinol. 12:630287. doi: 10.3389/fendo.2021.630287

INTRODUCTION

Gestational diabetes mellitus (GDM), defined as glucose intolerance during pregnancy, has an incidence of 7% to 17.5% among pregnant women in China (1–4). GDM can adversely affect mothers and fetuses, resulting in an increased risk of delivery-related complications and type 2 diabetes mellitus (T2DM) (3, 5, 6). Appropriate intervention in pregnant women with GDM can reduce the risk of adverse pregnancy outcomes (7–10). Early diagnosis of GDM before 24 weeks of gestation may provide more time for pregnant women with high risk of GDM for appropriate intervention. However, early identification of GDM is very difficult (2, 11), as GDM is a complex metabolic process of insulin resistance and islet β cell proliferation disorders (2, 12), with many changes in metabolic factors preceding hyperglycemia (13, 14).

Fibroblast growth factor 21 (FGF21), an important endocrine factor regulating glucose and lipid metabolism (15–17), may be a potential factor associated with GDM prediction, as it was recently found that serum FGF21 increased after 24 gestational weeks in GDM patients (18–20). Studies showed that FGF21 reduces blood glucose and regulates blood lipids without causing hypoglycemia in obese or type 2 diabetes patients (21–23), and FGF21 increases insulin sensitivity and improves islet β cell secretion and proliferation (15, 22, 23). As a metabolic factor, FGF21 increases in type 2 diabetes and obesity patients (23, 24), which might be a kind of compensation for insulin deficiency (25) and one study confirmed that serum FGF21 increased before type 2 diabetes was diagnosed in women (26). Due to FGF21 increasing in both GDM and T2DM and the similarity of the pathogenesis of these two diseases (2), we hypothesized that increases in FGF21 compensate for GDM. FGF21 may increase earlier than we are generally aware, and this increase may precede the hyperglycemia of GDM. However, to the best of our knowledge, no study has focused on the changes in FGF21 before the diagnosis of GDM.

Therefore, we intend to analyze the difference in serum levels of FGF21 between individuals with GDM and those without GDM early in the second trimester and analyze the relationship between FGF21 and GDM through a nested case-control study by propensity score matching to eliminate confounding factors.

MATERIALS AND METHODS

Study Population

This study was a nested case-control design carried out by screening the Down's syndrome screening cohort (14–21 gestational weeks) between January 2019 and October 2019 and was approved by the Ethics Committee of Obstetrics and Gynecology Hospital of Fudan University. All participants were informed of the purpose of the study and signed informed consent forms. Among the cohort of 2,540 participants, 1,368 pregnant women signed informed consent forms for our study.

The inclusion criteria: women with a singleton pregnancy in the Down's syndrome screening cohort (14–21 gestational weeks) and records of oral glucose test (OGTT) at 24 to 28 weeks of pregnancy. A total of 1247 pregnant women who met the inclusion criteria were included in the study. Individuals with any of the following were excluded: no GDM diagnostic information; pregnancy with twins or triplets; diabetes history; malignant tumors; serious metabolic diseases including Cushing's syndrome, hyperthyroidism and hypothyroidism; or severe hypertension.

One hundred forty subjects were diagnosed with GDM by a 75-g oral glucose test (OGTT) at 24 to 28 weeks of pregnancy according to the Diagnostic Criteria for Gestational Diabetes issued by the International Association of Diabetes and Pregnancy Study Groups (27, 28). GDM was diagnosed if any one of the following was met: (1) fasting plasma glucose ≥5.1 mmol/L; (2) 1-h plasma glucose ≥10 mmol/L; or (3) 2-h plasma glucose ≥8.5 mmol/L.

Finally, 133 individuals with GDM and 133 individuals with normal glucose tolerance (NGT) were identified as our research subjects through propensity score matching. In the process of object matching, the following multiple covariates and potential confounding factors associated with GDM were considered: age, body mass index (BMI) early in the second trimester, ethnicity, and the number of parities. Since all GDM subjects were Han Chinese and the number of parities is similar (zero or one) in Chinese women, age and BMI were finally determined as matching variables. The GDM and NGT pregnant women were matched in a 1:1 ratio using the nearest neighbor algorithm (caliper width 0.04 of the SD for the logit propensity score) without replacement. The flowchart of the selection of the analyzed GDM population and the matched control population is shown in **Figure 1**.

Considering that BMI is an important factor that affects FGF21 levels and the occurrence of GDM (29–32), comparative analyses of the BMI subgroups were performed in this study. Participants were grouped on the basis of the Chinese BMI classification standard into underweight (BMI < 18.5 kg/m^2), normal weight (18.5 ≤ BMI < 24 kg/m^2), overweight (24 ≤ BMI < 28 kg/m^2) and obese (BMI ≥ 28 kg/m^2) subgroups (33).

Detection of Serum FGF21

Serum was collected during Down's syndrome screening (14–21 gestational weeks) and stored at −20°C. The serum levels of FGF21 were detected by an Abcam ELISA kit (ab222506, Cambridge, UK).

Statistical Analysis

All statistics were performed by SPSS software. Psmatch 3.04 of the R language extender in SPSS software (Version 25.0; IBM, NY, US) was used for propensity score matching. The results are expressed as the mean ± standard deviation if the data followed a normal distribution, while abnormally distributed measurements are reported as the median (interquartile range). The t-test, Mann-Whitney U test, Kruskal-Wallis one-way ANOVA test, chi-square test, or Fisher's exact test was used to compare the differences in variables among groups as required. All reported P values were two-tailed. Binary logistic regression analysis models were used to estimate the associations of FGF21 with GDM.

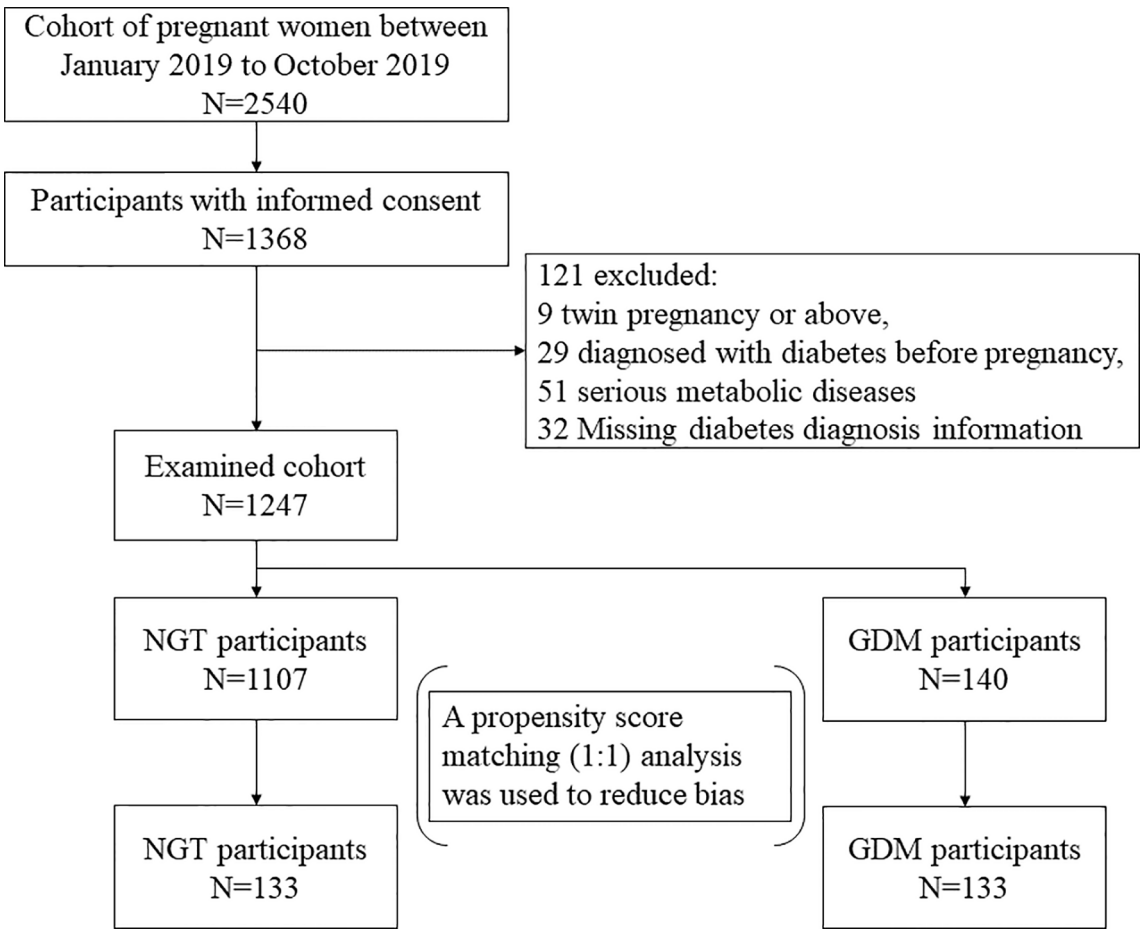


FIGURE 1 | The flowchart of the selection of the analyzed GDM population and the matched control population.

Model 1 was adjusted for the known risk factors for GDM, including age, BMI, family history of metabolic diseases and parity. $P < 0.05$ was considered statistically significant.

RESULTS

To verify the effect of eliminating confounding factors after sample matching, the age, BMI, gestational weeks at blood sampling, gestational weeks at OGTT, family history of metabolic diseases and parity in the overall GDM and NGT groups and each corresponding subgroup were compared, and the results are reported in **Table 1**. It should be noted that statistical analysis between underweight subgroups was not performed due to the small number of cases (2 of GDM, 1 of NGT). As expected, there was no significant difference in age, BMI, gestational age, family history of metabolic diseases or parity between the GDM and NGT groups, regardless of whether analyzed overall or by corresponding subgroup (P>0.05, **Table 1**), which suggested that the effect of confounding factors was sufficiently reduced after matching.

Then, the differences in serum levels of FGF21 among the corresponding groups were analyzed (**Table 1**). The FGF21 levels of the GDM group [58.59 (33.34–105.03) pg/ml] were significantly higher than those of the NGT group [24.20 (23.91–67.89) pg/ml] overall (P<0.001), with similar results observed between the corresponding BMI subgroups (all P<0.05). In GDM group, the FGF21 levels in the normal BMI subgroup [51.67 (28.86–87.49) pg/ml] were the lowest, followed by the overweight group [75.43 (43.52–159.17) pg/ml], and the levels were highest in the obese group [177.42 (88.89–307.22) pg/ml], with a significant difference between the obese group and the normal BMI group (P = 0.002, Bonferroni corrected). However, there was no significant difference among the different BMI NGT subgroups (Bonferroni, P = 0.154).

Subsequently, the relationship of FGF21 and GDM was explored using logistic regression analysis by stratifying study subjects into quartiles for FGF21 (Q1 ≤ 27.83 pg/ml, Q2 28.00 to 45.82 pg/ml, Q3 46.81 to 83.87 pg/ml, Q4 ≥84.09 pg/ml; **Table 2**). Compared with Q1, we found that Q3 was associated with a high risk of GDM (adjusted for age, BMI, family history of metabolic diseases and parity) (OR 2.478, 95% CI 1.229–5.000; P = 0.011). Moreover, Q4 was related to a higher risk of GDM

TABLE 1 | Baseline characteristics of the study population and the serum levels of FGF21 in different groups.

		NGT	GDM	*P* value
Total				
	N	133	133	NA
	Age (y)	29.54 ± 2.62	29.53 ± 2.27	0.972
	BMI (kg/m^2)	22.50 (20.85–24.21)	22.80 (20.70–24.50)	0.704
	Gestational weeks at blood sampling (w)	16.39 ± 0.92	16.48 ± 0.79	0.393
	Gestational weeks at OGTT (w)	25.93 ± 1.36	25.63 ± 1.25	0.339
	Family history of metabolic diseases (N)	22	30	0.216
	Parity			0.360
	0	109(82.0)	103(77.4)	
	1	24(18.0)	30(22.6)	
	FGF21 (pg/ml)	24.20 (23.91–67.89)	58.59 (33.34–105.03)**	<0.001
BMI (kg/m^2) ≥28				
	N	9	9	NA
	Age (y)	28.67 ± 1.73	28.70 ± 1.65	0.967
	BMI (kg/m2)	29.73 (28.68–33.59)	30.70 (29.60–32.65)	0.730
	Gestational weeks at blood sampling (w)	16.00 ± 1.12	16.44 ± 0.73	0.332
	Family history of metabolic diseases (N)	4	2	0.620
	Parity			1.000
	0	8(88.9)	7(77.8)	
	1	1(11.1)	2(22.2)	
	FGF21 (pg/ml)	33.35 (30.60–103.90)	177.42 (88.89–307.22)*	0.011
24≤BMI (kg/m^2) <28				
	N	28	32	NA
	Age (y)	29.75 ± 2.96	29.42 ± 2.19	0.625
	BMI (kg/m^2)	25.48 (24.50–26.40)	25.40 (24.50–26.38)	0.935
	Gestational weeks at blood sampling (w)	16.57 ± 1.03	16.63 ± 0.83	0.825
	Family history of metabolic diseases (N)	5	13	0.102
	Parity			0.165
	0	21(75)	29(90.6)	
	1	7(25)	3(9.4)	
	FGF21 (pg/ml)	44.20 (27.38–109.83)	75.43 (43.52–159.17)*	0.042
18.5≤BMI (kg/m^2) <24				
	N	95	90	NA
	Age (y)	29.59 ± 2.59	29.70 ± 2.35	0.804
	BMI (kg/m^2)	21.58 (20.39–22.86)	21.55 (20.30–23.03)	0.981
	Gestational weeks at blood sampling (w)	16.37 ± 0.86	16.43 ± 0.79	0.621
	Family history of metabolic diseases (N)	13	15	0.572
	Parity			0.073
	0	79(83.2)	65(72.2)	
	1	16(16.8)	25(27.8)	
	FGF21 (pg/ml)	33.36 (21.74–66.78)	51.67 (28.86–87.49)**	0.005

*Data are presented as the mean ± standard deviation, median (interquartile range) or N (%). Data on age and gestational weeks at blood sampling were compared using the t-test; BMI and FGF21 were compared using the Mann-Whitney U test; family history of metabolic diseases and parity were compared using the chi-square test and Fisher's exact test. *P<0.05 and **P<0.01 compared with the control groups. NGT, normal glucose tolerance group; GDM, gestational diabetes mellitus group; N, number; BMI, body mass index; FGF21, fibroblast growth factor 21, NA, not applicable.*

TABLE 2 | Associations of serum FGF21 concentrations (pg/ml) early in the second trimester of pregnancy with the risk of gestational diabetes mellitus.

FGF21 (pg/ml) (quartiles)	NGT (N=133)	GDM (N=133)	Crude OR	adjusted OR[a]
1st Quartile (≤27.83)	43 (32.3%)	24 (18.0%)	1.000 (referent)	1.000 (referent)
2nd Quartile (28.00–45.82)	39 (29.3%)	27 (20.3%)	1.240 (0.616–2.498)	1.224 (0.603–2.485)
3rd Quartile (46.81–83.87)	28 (21.1%)	39 (29.3%)	2.496 (1.244–5.008)	2.478 (1.229–5.000)
4th Quartile (≥84.09)	23 (17.3%)	43 (32.3%)	3.350 (1.645–6.821)	3.419 (1.626–7.188)
P for trend	NA	NA	0.002	0.002

Values are given as OR (95% CI).

[a]Adjusted for age, BMI, family history of metabolic diseases and parity.

OR, odds ratio; CI, confidence interval; NGT, normal glucose tolerance group; GDM, gestational diabetes mellitus group; N, number; BMI, body mass index; FGF21, fibroblast growth factor 21; NA, not applicable.

(OR 3.419, 95% CI 1.626–7.188; P = 0.001). This demonstrated that FGF21 levels significantly increased early in the second trimester, which was an independent risk factor for GDM.

DISCUSSION

To the best of our knowledge, this is the first study to evaluate the association between the serum levels of FGF21 and GDM before recommended routine screening period. Through a propensity-matched nested case-control study, we found that the FGF21 levels at 14 to 21 gestational weeks in the GDM groups were significantly higher than those in the NGT groups, regardless of whether analyzed overall or by corresponding BMI subgroup. And higher levels of FGF21 were significantly associated with a higher risk of GDM. Of note, these relationships were independent of known risk factors for GDM, including age, BMI, family history of metabolic diseases and parity.

Due to the important role of FGF21 in regulating glucose metabolism in the body, its potential role in GDM has gradually received increasing attention in recent years. Most of the studies found that the serum levels of FGF21 in GDM women were all elevated after 24 gestational weeks. Li et al. and Bonakdaran et al. found that GDM women had higher levels of FGF21 than NGT pregnant women at 24 to 28 gestational weeks (18, 34). ŠIMJÁK et al. found that FGF21 in GDM women was higher than that in NGT pregnant women at 28 to 32 gestational weeks (35). In addition, four studies showed that FGF21 was also higher in the GDM group than in the NGT group in the third trimester and prenatally (20, 35–37).

Notably, we discovered that FGF21 increased significantly at 14 to 21 gestational weeks before the routine GDM diagnosis time (24–28 gestational weeks). The impaired glucose tolerance on pregnancy is a continuum from normal to established GDM (38). It is possible that glucose tolerance of GDM women is impaired right at the outset (even outside of pregnancy) than that of non-GDM women. It may be why FGF21 levels in women who later were diagnosed with GDM were higher than those with normal OGTT. Our findings might make it possible for considering FGF21 as a predictor of GDM. Moreover, GDM women with normal BMI account for more than half of all GDM women in China (1, 2, 30, 39), and the possibility of GDM onset in women with normal BMI is more likely to be ignored and difficult to predict. Importantly, we found that the FGF21 levels of the normal BMI GDM subgroup were significantly higher than that of the normal BMI NGT subgroup, which provided important theoretical evidence for the early prediction of GDM in normal BMI pregnant individuals. Prospective studies are needed to confirm the predictive value of FGF21 for identifying the women at high risk of GDM.

In addition, our study showed that higher levels of FGF21 were associated with a higher risk of GDM. Our findings raise an important question regarding the potential mechanism or causality for the development of GDM with elevation of FGF21. As studies have found that FGF21 can improve tissue insulin sensitivity, promote the proliferation of pancreatic β-cells, and increase insulin secretion (15, 22, 23, 25), we suspected that there was a compensatory effect of FGF21 in pregnant women before GDM was identified (25). In addition, studies found that there were some differences in pathogenesis between obese and nonobese GDM women (2, 33, 40), and in our study, the FGF21 levels of the obese GDM subgroup were significantly higher than those of normal BMI GDM subgroup, which suggested that the mechanism of FGF21 resistance might be different in GDM women with different BMIs. Therefore, interventions that influence FGF21 levels based on different BMIs should be focused on for future research to prevent subsequent GDM.

Though high-quality results were demonstrated in our study by eliminating the effects of BMI and age through propensity score matching in a large cohort, there were still limitations. This was a retrospective study carried out by a Down's syndrome screening cohort, which lacked some important information, such as real-time blood glucose and glycosylated hemoglobin levels; therefore, the hyperglycemia and insulin resistance status of the individuals included were unknown. Therefore, it was not known whether some individuals already had hyperglycemia at 14 to 21 weeks of gestation, which brought a certain degree of uncertainty in the clinical value of the availability of high FGF21 levels. Nevertheless, our results still showed that higher levels of FGF21 were significantly associated with higher detection rate of GDM, which could provide evidence for the earlier identification of women at high risk of GDM before the recommended GDM screening period. Prospective studies are needed to confirm the clinical value of FGF21 for identifying the women at high risk of GDM. Another limitation was that the pregnant women in this study cohort were generally younger than 35 years old; therefore, research on FGF21 in GDM women over 35 years old still needs to be performed. However, studies have shown that pregnant women younger than 35 years old account for more than 85% of the total number of pregnant women in China (41). Therefore, the findings of this study are still very instructive and interesting.

In summary, the serum FGF21 levels in GDM women increased early in the second trimester, regardless of whether participants were stratified according to BMI. After adjusting for confounding factors, the FGF21 levels in the highest quartile were associated with more than three times higher probability of the diagnosis of GDM in the pregnancy as compared to levels in the first quartile. In addition to the currently established clinical and biochemical risk factors, the circulating concentration of FGF21 represents a potentially useful new biomarker that can identify pregnant women at risk for GDM.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions. Requests to access these data sets should be directed to ZW, wzh.0409@163.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Obstetrics and Gynecology Hospital of Fudan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XX, CY, and ZW contributed to the conception of the study. ZW, CX, and YZ contributed significantly to analysis and manuscript preparation. ZW and MY performed the data analyses and wrote the manuscript. XX and ZW helped perform the analysis with constructive discussions. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partly supported by the Shanghai Municipal Health Bureau (grant 20204Y0404) and National Natural Science Foundation of China (81871183), and Shanghai Committee of Science and Technology (18411963400).

REFERENCES

1. Gao S, Leng J, Liu H, Wang S, Li W, Wang Y, et al. Development and Validation of an Early Pregnancy Risk Score for the Prediction of Gestational Diabetes Mellitus in Chinese Pregnant Women. *BMJ Open Diabetes Res Care* (2020) 8(1):e000909. doi: 10.1136/bmjdrc-2019-000909
2. McIntyre HD, Catalano P, Zhang C, Desoye G, Mathiesen ER, Damm P. Gestational Diabetes Mellitus. *Nat Rev Dis Primers* (2019) 5(1):47. doi: 10.1038/s41572-019-0098-8
3. Yan J, Yang H. Gestational Diabetes in China: Challenges and Coping Strategies. *Lancet Diabetes Endocrinol* (2014) 2(12):930–1. doi: 10.1016/S2213-8587(14)70154-8
4. Zhu WW, Fan L, Yang HX, Kong LY, Su SP, Wang ZL, et al. Fasting Plasma Glucose At 24-28 Weeks to Screen for Gestational Diabetes Mellitus: New Evidence From China. *Diabetes Care* (2013) 36(7):2038–40. doi: 10.2337/dc12-2465
5. Vounzoulaki E, Khunti K, Abner SC, Tan BK, Davies MJ, Gillies CL. Progression to Type 2 Diabetes in Women With a Known History of Gestational Diabetes: Systematic Review and Meta-Analysis. *BMJ* (2020) 369:m1361. doi: 10.1136/bmj.m1361
6. Yu Y, Arah OA, Liew Z, Cnattingius S, Olsen J, Sorensen HT, et al. Maternal Diabetes During Pregnancy and Early Onset of Cardiovascular Disease in Offspring: Population Based Cohort Study With 40 Years of Follow-Up. *Bmj* (2019) 367:l6398. doi: 10.1136/bmj.l6398
7. Alwan N, Tuffnell DJ, West J. Treatments for Gestational Diabetes. *Cochrane Database Syst Rev* (2009) 3):CD003395. doi: 10.1002/14651858.CD003395.pub2
8. Yew TW, Chi C, Chan SY, van Dam RM, Whitton C, Lim CS, et al. A Randomized Controlled Trial to Evaluate the Effects of a Smartphone Application-Based Lifestyle Coaching Program on Gestational Weight Gain, Glycemic Control, and Maternal and Neonatal Outcomes in Women With Gestational Diabetes Mellitus: The SMART-GDM Study. *Diabetes Care* (2021) 44(2):456–63. doi: 10.2337/figshare.13073030
9. Brown J, Alwan NA, West J, Brown S, McKinlay CJ, Farrar D, et al. Lifestyle Interventions for the Treatment of Women With Gestational Diabetes. *Cochrane Database Syst Rev* (2017) 5:CD011970. doi: 10.1002/14651858.CD011970.pub2
10. Yamamoto JM, Kellett JE, Balsells M, Garcia-Patterson A, Hadar E, Sola I, et al. Gestational Diabetes Mellitus and Diet: A Systematic Review and Meta-analysis of Randomized Controlled Trials Examining the Impact of Modified Dietary Interventions on Maternal Glucose Control and Neonatal Birth Weight. *Diabetes Care* (2018) 41(7):1346–61. doi: 10.2337/dc18-0102
11. Plows JF, Stanley JL, Baker PN, Reynolds CM, Vickers MH. The Pathophysiology of Gestational Diabetes Mellitus. *Int J Mol Sci* (2018) 19(11):3342. doi: 10.3390/ijms19113342
12. Petra IL, Martin-Montalvo A, Cobo Vuilleumier N, Gauthier BR. Molecular Modelling of Islet Beta-Cell Adaptation to Inflammation in Pregnancy and Gestational Diabetes Mellitus. *Int J Mol Sci* (2019) 20(24):6171. doi: 10.3390/ijms20246171
13. Ravnsborg T, Svaneklink S, Andersen LLT, Larsen MR, Jensen DM, Overgaard M. First-Trimester Proteomic Profiling Identifies Novel Predictors of Gestational Diabetes Mellitus. *PloS One* (2019) 14(3):e0214457. doi: 10.1371/journal.pone.0214457
14. Zhao H, Li H, Chung ACK, Xiang L, Li X, Zheng Y, et al. Large-Scale Longitudinal Metabolomics Study Reveals Different Trimester-Specific Alterations of Metabolites in Relation to Gestational Diabetes Mellitus. *J Proteome Res* (2019) 18(1):292–300. doi: 10.1021/acs.jproteome.8b00602
15. BonDurant LD, Ameka M, Naber MC, Markan KR, Idiga SO, Acevedo MR, et al. Fgf21 Regulates Metabolism Through Adipose-Dependent and -Independent Mechanisms. *Cell Metab* (2017) 25(4):935–44.e934. doi: 10.1016/j.cmet.2017.03.005
16. Salminen A, Kaarniranta K, Kauppinen A. Regulation of Longevity by FGF21: Interaction Between Energy Metabolism and Stress Responses. *Ageing Res Rev* (2017) 37:79–93. doi: 10.1016/j.arr.2017.05.004
17. Staiger H, Keuper M, Berti L, Hrabe de Angelis M, Haring HU. Fibroblast Growth Factor 21-Metabolic Role in Mice and Men. *Endocr Rev* (2017) 38(5):468–88. doi: 10.1210/er.2017-00016
18. Li SM, Wang WF, Zhou LH, Ma L, An Y, Xu WJ, et al. Fibroblast Growth Factor 21 Expressions in White Blood Cells and Sera of Patients With Gestational Diabetes Mellitus During Gestation and Postpartum. *Endocrine* (2015) 48(2):519–27. doi: 10.1007/s12020-014-0309-8
19. He Q, Zhu S, Lin M, Yang Q, Wei L, Zhang J, et al. Increased GPR120 Level is Associated With Gestational Diabetes Mellitus. *Biochem Biophys Res Commun* (2019) 512(2):196–201. doi: 10.1016/j.bbrc.2019.03.034
20. Dekker Nitert M, Barrett HL, Kubala MH, Scholz Romero K, Denny KJ, Woodruff TM, et al. Increased Placental Expression of Fibroblast Growth Factor 21 in Gestational Diabetes Mellitus. *J Clin Endocrinol Metab* (2014) 99(4):E591–8. doi: 10.1210/jc.2013-2581
21. Watanabe H, Miyahisa M, Chikamatsu M, Nishida K, Minayoshi Y, Takano M, et al. Development of a Long Acting FGF21 Analogue-Albumin Fusion Protein and its Anti-Diabetic Effects. *J Control Release* (2020) 10(324):522–31. doi: 10.1016/j.jconrel.2020.05.036
22. Xie T, So WY, Li XY, Leung PS. Fibroblast Growth Factor 21 Protects Against Lipotoxicity-Induced Pancreatic Beta-Cell Dysfunction Via Regulation of AMPK Signaling and Lipid Metabolism. *Clin Sci (Lond)* (2019) 133(19):2029–44. doi: 10.1042/CS20190093
23. Pan Y, Wang B, Zheng J, Xiong R, Fan Z, Ye Y, et al. Pancreatic Fibroblast Growth Factor 21 Protects Against Type 2 Diabetes in Mice by Promoting Insulin Expression and Secretion in a PI3K/Akt Signaling-Dependent Manner. *J Cell Mol Med* (2019) 23(2):1059–71. doi: 10.1111/jcmm.14007
24. Reinehr T, Woelfle J, Wunsch R, Roth CL. Fibroblast Growth Factor 21 (FGF-21) and its Relation to Obesity, Metabolic Syndrome, and Nonalcoholic Fatty Liver in Children: A Longitudinal Analysis. *J Clin Endocrinol Metab* (2012) 97(6):2143–50. doi: 10.1210/jc.2012-1221
25. Fisher FM, Maratos-Flier E. Understanding the Physiology of FGF21. *Annu Rev Physiol* (2016) 78:223–41. doi: 10.1146/annurev-physiol-021115-105339
26. Wang Y, Koh WP, Yuan JM, Pan A. Sex-Specific Association Between Fibroblast Growth Factor 21 and Type 2 Diabetes: A Nested Case-Control Study in Singapore Chinese Men and Women. *Nutr Metab (Lond)* (2017) 14:63. doi: 10.1186/s12986-017-0216-0
27. Agarwal MM, Dhatt GS, Shah SM. Gestational Diabetes Mellitus: Simplifying the International Association of Diabetes and Pregnancy Diagnostic

Algorithm Using Fasting Plasma Glucose. *Diabetes Care* (2010) 33(9):2018–20. doi: 10.2337/dc10-0572
28. International Association of D and Pregnancy Study Groups Consensus P, Metzger BE, Gabbe SG, Persson B, Buchanan TA, et al. International Association of Diabetes and Pregnancy Study Groups Recommendations on the Diagnosis and Classification of Hyperglycemia in Pregnancy. *Diabetes Care* (2010) 33(3):676–82. doi: 10.2337/dc09-1848
29. Sorbye LM, Skjaerven R, Klungsoyr K, Morken NH. Gestational Diabetes Mellitus and Interpregnancy Weight Change: A Population-Based Cohort Study. *PloS Med* (2017) 14(8):e1002367. doi: 10.1371/journal.pmed.1002367
30. Hedderson M, Ehrlich S, Sridhar S, Darbinian J, Moore S, Ferrara A. Racial/Ethnic Disparities in the Prevalence of Gestational Diabetes Mellitus by BMI. *Diabetes Care* (2012) 35(7):1492–8. doi: 10.2337/dc11-2267
31. Dushay J, Chui PC, Gopalakrishnan GS, Varela-Rey M, Crawley M, Fisher FM, et al. Increased Fibroblast Growth Factor 21 in Obesity and Nonalcoholic Fatty Liver Disease. *Gastroenterology* (2010) 139(2):456–63. doi: 10.1053/j.gastro.2010.04.054
32. Akyildiz ZI, Polat S, Yurekli BS, Kocabas GU, Tuluce K, Tuluce SY, et al. Epicardial Fat, Body Mass Index, and Triglyceride are Independent Contributors of Serum Fibroblast Growth Factor 21 Level in Obese Premenopausal Women. *J Endocrinol Invest* (2015) 38(3):361–6. doi: 10.1007/s40618-014-0185-3
33. Li W, Zhang S, Liu H, Wang L, Zhang C, Leng J, et al. Different Associations of Diabetes With Beta-Cell Dysfunction and Insulin Resistance Among Obese and Nonobese Chinese Women With Prior Gestational Diabetes Mellitus. *Diabetes Care* (2014) 37(9):2533–9. doi: 10.2337/dc14-0573
34. Bonakdaran S, Khorasani ZM, Jafarzadeh F. Increased Serum Level of Fgf21 in Gestational Diabetes Mellitus. *Acta Endocrinol (Buchar)* (2017) 13(3):278–81. doi: 10.4183/aeb.2017.278
35. Simjak P, Cinkajzlova A, Anderlova K, Klouckova J, Kratochvilova H, Lacinova Z, et al. Changes in Plasma Concentrations and mRNA Expression of Hepatokines Fetuin A, Fetuin B and FGF21 in Physiological Pregnancy and Gestational Diabetes Mellitus. *Physiol Res* (2018) 67(Suppl 3): S531–42. doi: 10.33549/physiolres.934017
36. Megia A, Gil-Lluis P, Naf S, Ceperuelo-Mallafre V, Gonzalez-Clemente JM, Llaurado G, et al. Cord Blood FGF21 in Gestational Diabetes and its Relationship With Postnatal Growth. *Acta Diabetol* (2015) 52(4):693–700. doi: 10.1007/s00592-014-0705-9
37. Tan BK, Sivakumar K, Bari MF, Vatish M, Randeva HS. Lower Cerebrospinal Fluid/Plasma Fibroblast Growth Factor 21 (FGF21) Ratios and Placental FGF21 Production in Gestational Diabetes. *PloS One* (2013) 8(6):e65254. doi: 10.1371/journal.pone.0065254
38. Yee LM, Cheng YW, Liddell J, Block-Kurbisch I, Caughey AB. 50-Gram Glucose Challenge Test: Is it Indicative of Outcomes in Women Without Gestational Diabetes Mellitus? *J Matern Fetal Neonatal Med* (2011) 24 (9):1102–6. doi: 10.3109/14767058.2010.546450
39. Wang H, Zhai F. Programme and Policy Options for Preventing Obesity in China. *Obes Rev* (2013) 14(Suppl 2):134–40. doi: 10.1111/obr.12106
40. Fakhrul-Alam M, Sharmin J, Mashfiqul H, Nusrat S, Mohona Z, Rakibul-Hasan M, et al. Insulin Secretory Defect may be the Major Determinant of GDM in Lean Mothers. *J Clin Transl Endocrinol* (2020) 20:100226. doi: 10.1016/j.jcte.2020.100226
41. Xiong T, Chen P, Mu Y, Li X, Di B, Li J, et al. Association Between Ambient Temperature and Hypertensive Disorders in Pregnancy in China. *Nat Commun* (2020) 11(1):2925. doi: 10.1038/s41467-020-16775-8

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 29 April 2021
doi: 10.3389/fendo.2021.648287

Maternal and Neonatal Complications in Patients With Diminished Ovarian Reserve in *In-Vitro* Fertilization/Intracytoplasmic Sperm Injection Cycles

Shuang Han[1,2,3,4,5], *Yiwei Zhai*[1,2,3,4,5], *Qingqing Guo*[1,2,3,4,5], *Yiming Qin*[6] *and Peihao Liu*[1,2,3,4,5]*

[1] Center for Reproductive Medicine, Cheeloo College of Medicine, Shandong University, Jinan, China, [2] Key Laboratory of Reproductive Endocrinology of Ministry of Education, Shandong University, Jinan, China, [3] Shandong Key Laboratory of Reproductive Medicine, Jinan, China, [4] Shandong Provincial Clinical Research Center for Reproductive Health, Jinan, China, [5] National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, Jinan, China, [6] School of Basic Medical Science, Shandong First Medical University, Jinan, China

OPEN ACCESS

Edited by:
Qiongjie Zhou,
Fudan University, China

Reviewed by:
Borut Kovacic,
Maribor University Medical Centre, Slovenia
Rossella Mazzilli,
Sapienza University of Rome, Italy
Dan Zhang,
Zhejiang University, China

***Correspondence:**
Peihao Liu
drpeihao@163.com

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: 31 December 2020
Accepted: 14 April 2021
Published: 29 April 2021

Citation:
Han S, Zhai Y, Guo Q, Qin Y and Liu P (2021) Maternal and Neonatal Complications in Patients With Diminished Ovarian Reserve in In-Vitro Fertilization/Intracytoplasmic Sperm Injection Cycles. Front. Endocrinol. 12:648287. doi: 10.3389/fendo.2021.648287

Background: Diminished ovarian reserve (DOR) is one of the most intractable clinical issues in human reproduction and is reported to be associated with raised risk of recurrent pregnancy loss and aneuploid blastocysts. In this study, we aimed to explore whether DOR was also associated with maternal and neonatal complications in *in-vitro* fertilization/intracytoplasmic sperm injection cycles.

Methods: A retrospective cohort study including women below 40 years of age who achieved singleton live birth after fresh embryo transfer in *in-vitro* fertilization/intracytoplasmic sperm injection cycles in a single center from January 2012 to June 2019 was conducted. Participants with DOR, defined as basal follicle-stimulating hormone (FSH) ≥ 10IU/L and antimullerian hormone (AMH) < 1.2ng/ml, were enrolled as the study group. The controls were 1:2 matched by age and body mass index with FSH < 10IU/L and AMH ≥ 1.2ng/ml. Maternal and neonatal complications were compared between the DOR group and the controls.

Results: A total of 579 women, 193 in the DOR group and 386 matched as controls, were included in this study. Compared to controls, the incidence of hypertensive disorders of pregnancy was significantly increased in the DOR group (5.7% *vs*. 2.1%, $P = 0.021$). DOR patients also presented slightly higher incidences of preterm birth (10.9% *vs*. 7.5%, $P = 0.174$) and low birthweight (6.2% *vs*. 5.4%, $P = 0.704$) yet without statistical significances. The incidences of gestational diabetes mellitus and placenta previa were comparable between the two groups.

Conclusion: Compared to women with normal ovarian reserve, women with diminished ovarian reserve might have elevated incidence of hypertensive disorders of pregnancy. Patients with diminished ovarian reserve might need more strict antenatal care.

Keywords: diminished ovarian reserve, maternal and neonatal complications, *in-vitro* fertilization, hypertensive disorders of pregnancy, ovarian aging

INTRODUCTION

According to the American Society for Reproductive Medicine, diminished ovarian reserve (DOR) is defined as women of reproductive age having impaired ovarian reserve and/or poor ovarian response to gonadotropin stimulation (1), clinically characterized by elevated concentration of basal follicle-stimulating hormone (FSH), reduced antimullerian hormone (AMH) level as well as declined antral follicle count (AFC). Distinct from the low prevalence of premature ovarian insufficiency, the prevalence of DOR was relatively high, which increased from 19% to 26% from 2004 to 2011 according to a survey among the United States assisted reproductive technology population (2). For DOR patients, the impaired ovarian reserve always leads to fewer oocytes retrieved, less embryos or even no good-quality embryos acquired, which results in poor pregnancy outcomes. Additionally, with the irreversibility of reduced ovarian reserve, DOR becomes one of the most intractable situations in the clinical practice of assisted reproductive technology.

As reported, DOR is associated with raised risk of recurrent pregnancy loss and aneuploid blastocysts (3, 4); however, whether DOR is associated with maternal or neonatal complications remains uncertain. Ovarian aging has been correlated with abnormalities in luteal phase function (5), and recent studies demonstrated that luteal phase defect was related to altered maternal vascular health and higher risk of preeclampsia (6, 7). So, it is worth investigating whether the incidences of hypertensive disorders of pregnancy (HDP) and other perinatal complications are elevated among DOR patients.

The aim of the study was to explore the incidences for adverse maternal and neonatal outcomes, assessed as HDP, preterm birth (PTB), low birthweight (LBW), gestational diabetes mellitus (GDM) and placenta previa in women with DOR after *in-vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment.

MATERIALS AND METHODS

Study Design and Participants

This was a retrospective cohort study of women who achieved singleton live birth after fresh embryo transfer in IVF/ICSI cycles in Center for Reproductive Medicine, Shandong University from January 2012 to June 2019. All participants were below 40 years old. Participants with DOR, defined as basal FSH ≥ 10IU/L measured at least twice and AMH < 1.2ng/ml, were enrolled as the study group. The controls were matched by age and body mass index (BMI) with FSH < 10IU/L and AMH ≥ 1.2ng/ml. To be specific, we stratified age by an interval of 5 years and in each age subgroup we stratified BMI by the category according to the World Health Organization criteria (8) (<18.5 kg/m^2; ≥18.5kg/m^2, <23 kg/m^2; ≥23kg/m^2, <27.5 kg/m^2; ≥27.5kg/m^2), then the controls were 1:2 enrolled. Data in this study were collected from the electronic medical record system in our center. Since female age makes huge impact on the adverse maternal and neonatal outcomes as well as the ovarian reserve, the participants were then divided into two subgroups by age with the cutoff value of 35 years old in order to further explore the associations between DOR and various pregnancy complications in different age groups. Patients with diabetes mellitus or preconceptional fasting glucose (PFG) ≥ 7.0 mmol/L, preconceptional hypertension, polycystic ovary syndrome (PCOS), thyroid dysfunction, hyperprolactinemia, endometriosis, chromosomal abnormalities, chemo-/radio-therapy or autoimmune disorders were excluded from the study. The study was approved by the Institutional Review Board (IRB) of the Center for Reproductive Medicine, Shandong University. All the participants enrolled in this study had signed written informed consent.

Assessment of Maternal and Neonatal Complications

Maternal and neonatal complications analyzed included HDP, PTB, LBW, GDM and placenta previa. Hypertensive disorders were diagnosed according to the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (9). HDP in this study included gestational hypertension and preeclampsia and excluded chronic hypertension. PTB was defined as live birth before 37 gestational age. LBW referred to birth weight of full-term delivered newborns below 2500 g. According to WHO criteria, GDM was diagnosed when met one or more of the following criteria: fasting plasma glucose ≥ 7.0 mmol/l; 2-h plasma glucose ≥ 11.1 mmol/l following a 75 g oral glucose load; random plasma glucose ≥ 11.1 mmol/l in the presence of diabetes symptoms (10). Placenta praevia was diagnosed when the placenta lies directly over the internal os by transvaginal ultrasound (11).

Statistical Analysis

The baseline characteristics of patients were summarized using means ± standard deviations for continuous variables and frequencies and percentages for categorical variables. The between-group differences of continuous data were assessed by student's t-test. Categorical data were tested by χ^2 analysis, with Fisher's exact test for expected frequencies less than five. A conditional logistic regression model was also applied to adjust the factors manifesting significant differences between the DOR and control groups including AFC, basal LH concentration, days of ovarian stimulation, total gonadotropin dose and estradiol level on human chorionic gonadotropin (hCG) trigger day. All the data were analyzed by SPSS 21.0 (SPSS Inc, Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

A total of 579 women, 193 with DOR and 386 as controls, were enrolled in this study. Demographic and ovarian stimulation characteristics were presented in **Table 1**. Due to the disease attributes, between-group significant differences were observed as expected among factors including AFC (5.70 *vs.* 11.52, $P <$ 0.001), AMH (0.56 *vs.* 2.92ng/ml, $P < 0.001$), basal FSH (13.77 *vs.*

TABLE 1 | Demographic and ovarian stimulation characteristics of patients and controls during IVF/ICSI treatment.

Variables	DOR (n=193)	CON (n=386)	*P*-value
Age (year)	33.22 ± 3.78	32.71 ± 3.70	0.124
BMI (kg/m^2)	22.83 ± 3.44	23.19 ± 3.87	0.283
Antral follicle count in both ovaries	5.70 ± 3.77	11.52 ± 4.53	<0.001*
AMH (ng/ml)	0.56 ± 0.32	2.92 ± 1.30	<0.001*
TSH (mIU/L)	2.30 ± 0.91	2.15 ± 0.85	0.059
PRL (ng/ml)	15.31 ± 6.59	16.27 ± 9.69	0.226
Baseline sex hormone			
FSH (IU/L)	13.77 ± 4.48	6.86 ± 1.44	<0.001*
LH (IU/L)	6.01 ± 4.01	4.97 ± 2.33	<0.001*
Estrodiol (pg/ml)	41.83 ± 44.18	38.30 ± 19.70	0.313
Progesterone (ng/ml)	0.59 ± 0.97	0.72 ± 2.29	0.542
Total testosterone (nmol/L)	20.33 ± 12.21	22.20 ± 10.12	0.058
Indication for IVF			
Tubal factors	117/193 (60.6%)	240/386 (62.2%)	0.717
Male factors	32/193 (16.6%)	67/386 (17.4%)	0.815
Combined factors	18/193 (9.3%)	28/386 (7.3%)	0.385
Others	26/193 (13.5%)	51/386 (13.2%)	0.931
Blood pressure			
Systolic pressure (mmHg)	117.54 ± 13.27	118.51 ± 11.07	0.353
Diastolic pressure (mmHg)	70.21 ± 9.71	71.48 ± 8.16	0.099
Preconceptional fasting glucose (mmol/L)	5.23 ± 0.55	5.43 ± 2.33	0.377
Days of ovarian stimulation	9.65 ± 3.58	10.99 ± 2.35	<0.001*
Total gonadotropin dose (IU)	2367.68 ± 1310.03	1975.32 ± 851.84	<0.001*
Estradiol level on hCG trigger day (pg/ml)	1468.70 ± 1075.11	2953.50 ± 1585.56	<0.001*
Progesterone level on hCG trigger day (ng/ml)	0.81 ± 1.03	0.95 ± 1.73	0.346

AMH, antimullerian hormone; BMI, body mass index; PRL, prolactin; FSH, follicle-stimulating hormone; LH, luteinizing hormone; IVF, in-vitro fertilization; ICSI, intracytoplasmic sperm injection; hCG, human chorionic gonadotropin.
**indicates statistical significances of P < 0.05.*

6.86IU/L, $P < 0.001$), luteinizing hormone (LH, 6.01 *vs.* 4.97IU/L, $P < 0.001$), days of stimulation (9.65 *vs.* 10.99, $P < 0.001$), total gonadotropin dose (2367.68 *vs.* 1975.32IU, $P < 0.001$) and estradiol level on hCG trigger day (1468.70 *vs.* 2953.50pg/ml, $P < 0.001$).

Maternal and neonatal complications were listed in **Table 2** and presented in **Figure 1**. Compared to controls, the incidence of HDP was significantly elevated in the DOR group (5.7% *vs.* 2.1%, $P = 0.021$). DOR patients also presented slightly increased incidences of PTB (10.9% *vs.* 7.5%, $P = 0.174$) and LBW (6.2% *vs.* 5.4%, $P = 0.704$) yet without statistical significances. The incidences of GDM and placenta previa were comparable between the two groups. After the adjustment, the DOR group still exhibited a raised incidence of HDP compared to the controls (adjusted OR 2.63, 95%CI 1.01-6.84, P=0.045). None of the other maternal or neonatal complications had significantly different incidences between the DOR and non-DOR groups after the adjustment.

TABLE 2 | Comparison of gestational complications between women with DOR and controls.

Gestational complications	DOR (n=193)	CON (n=386)	*P*-value
Hypertensive disorders of pregnancy	11 (5.7%)	8 (2.1%)	0.021*
Preterm birth	21 (10.9%)	29 (7.5%)	0.174
Low birth weight	12 (6.2%)	21 (5.4%)	0.704
Gestational diabetes mellitus	11 (5.7%)	20 (5.2%)	0.794
Placenta previa	3 (1.6%)	7 (1.8%)	1.000

**indicates statistical significance of P < 0.05.*

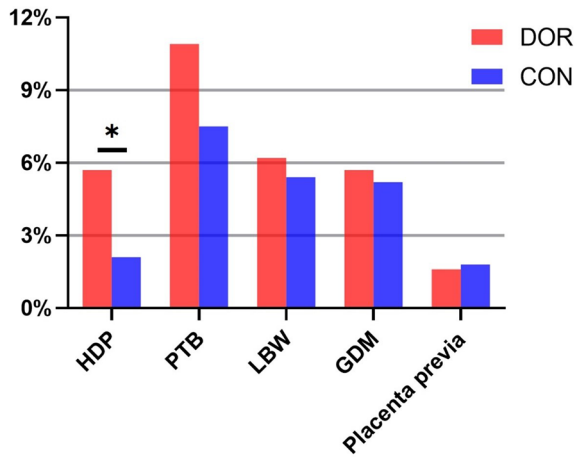


FIGURE 1 | Gestational complications in DOR patients and controls. HDP, hypertensive disorders of pregnancy; PTB, preterm birth; LBW, low birthweight; GDM, gestational diabetes mellitus. *indicates statistical significance of P < 0.05.

Table 3 demonstrated the gestational complications when stratified by age. There were 119 DOR women and 238 controls below 35 years old, leaving 74 DOR patients and 148 age matched controls in the advanced age subgroup. Although lacking of statistical differences, in younger patients with DOR, the incidences of HDP (5.0% *vs.* 1.3%, $P = 0.065$), PTB (11.8% *vs.* 6.3%, $P = 0.075$) and LBW (6.7% *vs.* 5.0%, $P = 0/515$) were increased

TABLE 3 | Gestational complications stratified by age in women with DOR and controls.

Gestational complications with women<35 years old	DOR (n=119)	CON (n=238)	*P*-value
Hypertensive disorders of pregnancy	6 (5.0%)	3 (1.3%)	0.065
Preterm birth	14 (11.8%)	15 (6.3%)	0.075
Low birth weight	8(6.7%)	12 (5.0%)	0.515
Gestational diabetes mellitus	3 (2.5%)	7 (2.9%)	0.821
Placenta previa	1 (0.8%)	4 (1.7%)	0.524
Gestational complications with women ≥ 35 years old	**DOR (n=74)**	**CON (n=148)**	***P*-value**
Hypertensive disorders of pregnancy	5 (6.8%)	5 (3.3%)	0.307
Preterm birth	7 (9.5%)	14 (9.5%)	1.000
Low birth weight	4 (5.4%)	9 (6.1%)	1.000
Gestational diabetes mellitus	8 (10.8%)	13 (8.8%)	0.627
Placenta previa	2 (2.7%)	3 (2.0%)	1.000

compared with controls. However, among women over 35 years of age, only the incidence of HDP represented a rising trend in the DOR group (6.8% *vs.* 3.3%, $P = 0.307$). The incidences of all other complications were similar between the two groups in women of advanced ages.

DISCUSSION

This study analyzed the risk of maternal and neonatal complications, including HDP, PTB, LBW, GDM and placenta previa in women with DOR after IVF/ICSI treatment. The results suggested that women with impaired ovarian function had higher incidence of HDP. And the incidences of PTB and LBW also had an elevated trend. Our study indicates that patients with DOR are supposed to be under more careful antenatal examinations for any signs of pregnancy complications.

With decreased oocyte quantity, DOR is a common status of ovarian aging. According to the committee opinion of the American Society for Reproductive Medicine, ovarian aging is associated with abnormalities of luteal phase function (5). The progesterone and estradiol metabolites production during luteal phase was decreased greatly among advanced aged women (12, 13). It has been reported that vascular problems and hypertensive disorders of pregnancy was related with luteal phase dysfunction and lacking of relaxin (6, 7).

Similar findings were reported that hormone replace therapy cycles with no corpus luteum in frozen embryo transfer had increased incidence of hypertensive disorders of pregnancy (7, 14, 15). And von Versen-Hoynck et al (6, 7) revealed that relaxin concentration in the hormone replace therapy cycles with no corpus luteum was significantly lower than that in the natural cycles, and maternal vascular health and aortic compliance was impaired remarkably in the hormone replace therapy group as well. In our study, patients with DOR, who were supposed to have poor luteal function producing less relaxin, therefore presented with elevated incidence of HDP.

Relaxin is a potent vasodilator produced only by corpus luteum throughout gestation (16). Relaxin has crucial impacts on the vascular health during pregnancy. First, lacking of or low level of relaxin in the DOR patients may results in impaired decidualization, which is the key pathological process of preeclampsia. Second, dramatic remodeling of the uterine artery wall occurs during pregnancy and relaxin is the leading candidate molecule inducing compositional and geometric remodeling of arteries (17, 18). The arterial remodeling fuels the changes of arterial passive mechanical properties which benefits the arterial compliance. Third, relaxin also has vasodilatory effect acting through increased expression of inducible nitric oxide generation (19, 20).

Another explanation for the elevated HDP incidence in women with impaired ovarian reserve could be ascribed to the deteriorative status of oxidative stress. Two cross-sectional studies indicated that serum levels of inducible nitric oxide synthase, total oxidant status, and oxidative stress index in patients with premature ovarian insufficiency were elevated significantly (21, 22). Oxidative stress could decrease the biodisponibility of nitric oxide and prostacyclin and result in increased vasoconstriction and reduced endothelium-dependent vasodilation (23). Excessive oxidative stress is proved to be associated with severe hypertension and target organ damage (24) in human and mice.

Our study also provided the clinicians with more informative clues for antenatal managements of patients with impaired ovarian reserve. HDP is one of the major causes of maternal and neonatal morbidity and mortality (25). Thus, it is crucial to identify the risk factors for HDP and take preventative measures. Since DOR is a risk factor for HDP, obstetricians need to be vigilant on patients' ovarian reserve evaluation and pay special attention to their blood pressure for patients with DOR. For example, frequent home blood pressure monitoring will be helpful, and weight management during pregnancy is recommended as obese is a known risk factor for preeclampsia. Low-dose aspirin for prevention of preeclampsia may also be considered for DOR patients, especially for those who have other risk factors for eclampsia.

Previous studies always focused on the pregnancy outcomes of DOR patients except for one study (26) analyzing the perinatal outcomes as well; however, they only included DOR patients under 35 years old. Calhoun et al' s study (27) was also a retrospective study focusing on the association between FSH and obstetric outcomes including PTB and LBW among women underwent IVF. With a large cohort, their study categorized the participants into three groups (defined by the serum FSH concentration of 1–5, 6–8 and ≥ 9 mIU/ml) and revealed that the incidences of PTB and LBW were not significantly different among the groups. In our study we strictly defined the women with DOR as basal FSH ≥ 10IU/L measured at least twice as well as AMH < 1.2ng/ml, which meant that we homogenized the

patients with impaired ovarian reserve and aimed to figure out the characteristics of this specific population. Both two studies failed to reveal a significant association between ovarian reserve and the incidences of PTB and LBW, which might indicate that poor ovarian reserve did not affect PTB or LBW even taken AMH into consideration or lift the FSH cut-off values. The major limitation of our study was its retrospective design. The levels of relaxin and estrogen concentration during pregnancy were lacking. Information of other potential confounders including second-hand smoking exposure, income levels, nutritional status, physical activity, and sleep during pregnancy could not be collected as well. Prospective studies with larger size of patients are needed in the future.

CONCLUSION

In conclusion, diminished ovarian reserve might be a potential risk factor for hypertensive disorders of pregnancy. Although it still needs further exploration with larger sample size to confirm this conclusion, patients with DOR might be benefit from more careful pregnancy surveillance.

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 (IRB) of the Center for Reproductive Medicine, Shandong University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

In this research, PL and SH contributed to the conception and design. YZ, SH and QG were involved in data collection and analysis. PL, YQ and SH drafted and modified the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by Natural Science Foundation of Shandong Province (ZR2019PH009), Shanghai Commission of Science and Technology (no. 19410760300) and Taishan Scholars Program for Young Experts of Shandong Province (tsqn20161069).

REFERENCES

1. Practice Committee of the American Society for Reproductive M. Testing and Interpreting Measures of Ovarian Reserve: A Committee Opinion. *Fertil Steril* (2015) 103:e9–e17. doi: 10.1016/j.fertnstert.2014.12.093
2. Devine K, Mumford SL, Wu M, DeCherney AH, Hill MJ, Propst A. Diminished Ovarian Reserve in the United States Assisted Reproductive Technology Population: Diagnostic Trends Among 181,536 Cycles From the Society for Assisted Reproductive Technology Clinic Outcomes Reporting System. *Fertil Steril* (2015) 104:612–19 e3. doi: 10.1016/j.fertnstert.2015.05.017
3. Bunnewell SJ, Honess ER, Karia AM, Keay SD, Al Wattar BH, Quenby S. Diminished Ovarian Reserve in Recurrent Pregnancy Loss: A Systematic Review and Meta-Analysis. *Fertil Steril* (2020) 113:818–27 e3. doi: 10.1016/j.fertnstert.2019.11.014
4. Shahine LK, Marshall L, Lamb JD, Hickok LR. Higher Rates of Aneuploidy in Blastocysts and Higher Risk of No Embryo Transfer in Recurrent Pregnancy Loss Patients With Diminished Ovarian Reserve Undergoing in Vitro Fertilization. *Fertil Steril* (2016) 106:1124–8. doi: 10.1016/j.fertnstert.2016.06.016
5. Practice Committee of the American Society for Reproductive M. Current Clinical Irrelevance of Luteal Phase Deficiency: A Committee Opinion. *Fertil Steril* (2015) 103:e27–32. doi: 10.1016/j.fertnstert.2014.12.128
6. von Versen-Hoynck F, Narasimhan P, Selamet Tierney ES, Martinez N, Conrad KP, Baker VL, et al. Absent or Excessive Corpus Luteum Number is Associated With Altered Maternal Vascular Health in Early Pregnancy. *Hypertension* (2019) 73:680–90. doi: 10.1161/HYPERTENSIONAHA.118.12046
7. von Versen-Hoynck F, Schaub AM, Chi YY, Chiu KH, Liu J, Lingis M, et al. Increased Preeclampsia Risk and Reduced Aortic Compliance With in Vitro Fertilization Cycles in the Absence of a Corpus Luteum. *Hypertension* (2019) 73:640–9. doi: 10.1161/HYPERTENSIONAHA.118.12043
8. WHO Expert Consultation. Appropriate Body-Mass Index for Asian Populations and Its Implications for Policy and Intervention Strategies. *Lancet* (2004) 363:157–63. doi: 10.1016/S0140-6736(03)15268-3
9. Tranquilli AL, Dekker G, Magee L, Roberts J, Sibai BM, Steyn W, et al. The Classification, Diagnosis and Management of the Hypertensive Disorders of Pregnancy: A Revised Statement From the ISSHP. *Pregnancy Hypertens* (2014) 4:97–104. doi: 10.1016/j.preghy.2014.02.001
10. World health organization. Diagnostic Criteria and Classification of Hyperglycaemia First Detected in Pregnancy: A World Health Organization Guideline. *Diabetes Res Clin Pract* (2014) 103:341–63. doi: 10.1016/j.diabres.2013.10.012
11. Jauniaux E, Alfirevic Z, Bhide AG, Belfort MA, Burton GJ, Collins SL, et al. Placenta Praevia and Placenta Accreta: Diagnosis and Management: Green-Top Guideline No. *27a. BJOG* (2019) 126:e1–e48. doi: 10.1111/1471-0528.15306
12. Mersereau JE, Evans ML, Moore DH, Liu JH, Thomas MA, Rebar RW, et al. Luteal Phase Estrogen is Decreased in Regularly Menstruating Older Women Compared With a Reference Population of Younger Women. *Menopause* (2008) 15:482–6. doi: 10.1097/gme.0b013e31815982cf
13. Santoro N, Brown JR, Adel T, Skurnick JH. Characterization of Reproductive Hormonal Dynamics in the Perimenopause. *J Clin Endocrinol Metab* (1996) 814:1495–501. doi: 10.1210/jcem.81.4.8636357
14. Ginstrom Ernstad E, Wennerholm UB, Khatibi A, Petzold M, Bergh C. Neonatal and Maternal Outcome After Frozen Embryo Transfer: Increased Risks in Programmed Cycles. *Am J Obstet Gynecol* (2019) 221:126 e1– e18. doi: 10.1016/j.ajog.2019.03.010
15. Zong L, Liu P, Zhou L, Wei D, Ding L, Qin Y. Increased Risk of Maternal and Neonatal Complications in Hormone Replacement Therapy Cycles in Frozen Embryo Transfer. *Reprod Biol Endocrinol* (2020) 18:36. doi: 10.1186/s12958-020-00601-3
16. Singh B, Reschke L, Segars J, Baker VL. Frozen-Thawed Embryo Transfer: The Potential Importance of the Corpus Luteum in Preventing Obstetrical Complications. *Fertil Steril* (2020) 113:252–7. doi: 10.1016/j.fertnstert.2019.12.007
17. Leo CH, Jelinic M, Ng HH, Marshall SA, Novak J, Tare M, et al. Vascular Actions of Relaxin: Nitric Oxide and Beyond. *Br J Pharmacol* (2017) 174:1002–14. doi: 10.1111/bph.13614

18. Osol G, Moore LG. Maternal Uterine Vascular Remodeling During Pregnancy. *Microcirculation* (2014) 21:38–47. doi: 10.1111/micc.12080
19. Nistri S, Bani D. Relaxin Receptors and Nitric Oxide Synthases: Search for the Missing Link. *Reprod Biol Endocrinol* (2003) 1:5. doi: 10.1186/1477-7827-1-5
20. Baccari MC, Bani D. Relaxin and Nitric Oxide Signalling. *Curr Protein Pept Sci* (2008) 96:638–45. doi: 10.2174/138920308786733921
21. Ağaçayak E, Yaman Görük N, Küsen H, Yaman Tunç S, Başaranoğlu S, İçen MS, et al. Role of Inflammation and Oxidative Stress in the Etiology of Primary Ovarian Insufficiency. *Turk J Obstet Gynecol* (2016) 13:109–15. doi: 10.4274/tjod.00334
22. Tokmak A, Yıldırım G, Sarıkaya E, Çınar M, Boğdaycıoğlu N, Yılmaz FM, et al. Increased Oxidative Stress Markers May Be a Promising Indicator of Risk for Primary Ovarian Insufficiency: A Cross-Sectional Case Control Study. *Rev Bras Ginecol Obstet* (2015) 37:411–6. doi: 10.1590/SO100-720320150005397
23. Montezano AC, Dulak-Lis M, Tsiropoulou S, Harvey A, Briones AM, Touyz RM. Oxidative Stress and Human Hypertension: Vascular Mechanisms, Biomarkers, and Novel Therapies. *Can J Cardiol* (2015) 31:631–41. doi: 10.1016/j.cjca.2015.02.008
24. Dikalov SI, Ungvari Z. Role of Mitochondrial Oxidative Stress in Hypertension. *Am J Physiol Heart Circ Physiol* (2013) 305:H1417–27. doi: 10.1152/ajpheart.00089.2013
25. Garovic VD, White WM, Vaughan L, Saiki M, Parashuram S, Garcia-Valencia O, et al. Incidence and Long-Term Outcomes of Hypertensive Disorders of Pregnancy. *J Am Coll Cardiol* (2020) 75:2323–34. doi: 10.1016/j.jacc.2020.03.028
26. Hu S, Xu B, Jin L. Perinatal Outcome in Young Patients With Diminished Ovarian Reserve Undergoing Assisted Reproductive Technology. *Fertil Steril* (2020) 114:118–24 e1. doi: 10.1016/j.fertnstert.2020.02.112
27. Calhoun KC, Fritz MA, Steiner AZ. Examining the Relationship Between Ovarian Reserve, as Measured by Basal FSH Levels, and the Risk of Poor Obstetric Outcome in Singleton IVF Gestations. *Hum Reprod* (2011) 26:3424–30. doi: 10.1093/humrep/der311

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.

frontiers in Endocrinology

REVIEW
published: 25 May 2021
doi: 10.3389/fendo.2021.653407

Understanding the Pathogenesis of Gestational Hypothyroidism

Oshini Mallawa Kankanamalage[1], *Qiongjie Zhou*[2,3]* *and Xiaotian Li*[2,3]*

[1] School of Clinical Medicine, Shanghai Medical College, Fudan University, Shanghai, China, [2] Department of Obstetrics and Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China, [3] Shanghai Key Laboratory of Female Reproductive Endocrine-Related Diseases, Obstetrics and Gynecology Hospital of Fudan University, Shanghai, China

Pregnancy is a complex state with many endocrinological challenges to a woman's physiology. Gestational Hypothyroidism (GHT) is an emerging condition where insufficiency of the thyroid gland has developed during pregnancy in a previously euthyroid woman. It is different to overt hypothyroidism, where marked elevation of thyroid-stimulating hormone with corresponding reduction in free thyroxine levels, is well known to cause detrimental effects to both the mother and the baby. During the past couple of decades, it has been shown that GHT is associated with multiple adverse maternal and fetal outcomes such as miscarriage, pre-eclampsia, placental abruption, fetal loss, premature delivery, neurocognitive and neurobehavioral development. However, three randomized controlled trials and a prospective cohort study performed within the last decade, show that there is no neurodevelopmental improvement in the offspring of mothers who received levothyroxine treatment for GHT. Thus, the benefit of initiating treatment for GHT is highly debated within the clinical community as there may also be risks associated with over-treatment. In addition, regulatory mechanisms that could possibly lead to GHT during pregnancy are not well elucidated. This review aims to unravel pregnancy induced physiological challenges that could provide basis for the development of GHT. During pregnancy, there is increased renal clearance of iodine leading to low iodine state. Also, an elevated estrogen level leading to an increase in circulating thyroglobulin level and a decrease in free thyroxine level. Moreover, placenta secretes compounds such as human chorionic gonadotropin (hCG), placental growth factor (PlGF) and soluble FMS-like tyrosine kinase-1 (s-Flt1) that could affect the thyroid function. In turn, the passage of thyroid hormones and iodine to the fetus is highly regulated within the placental barrier. Together, these mechanisms are hypothesized to contribute to the development of intolerance of thyroid function leading to GHT in a vulnerable individual.

Keywords: gestational hypothyroidism, subclinical hypothyroidism, pregnancy, thyroid disorders, placenta, estrogen, iodine, levothyroxine

OPEN ACCESS

Edited by:
Qi Chen,
The University of Auckland,
New Zealand

Reviewed by:
Yolanda De Rijke,
Erasmus Medical Center,
Netherlands
Stefania Triunfo,
BCNatal, Spain
Weiping Teng,
First Affiliated Hospital of Anhui Medical University, China

***Correspondence:**
Qiongjie Zhou
zhouqiongjie1732@fckyy.org.cn
Xiaotian Li
xiaotianli555@163.com

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *14 January 2021*
Accepted: *01 April 2021*
Published: *25 May 2021*

Citation:
Mallawa Kankanamalage O, Zhou Q and Li X (2021) Understanding the Pathogenesis of Gestational Hypothyroidism. Front. Endocrinol. 12:653407. doi: 10.3389/fendo.2021.653407

INTRODUCTION

Thyroid dysfunction during pregnancy is a well-researched area due to the detrimental effects of either profoundly low or high circulating thyroid hormones. On the lower end of the spectrum where there is an insufficiency in circulating thyroid hormones, exist three distinct conditions, namely; overt hypothyroidism (OH), subclinical hypothyroidism (SCH), and isolated

hypothyroxinemia (IH). All these three conditions can exist with or without the presence of thyroid autoimmunity marked by autoantibodies such as those against thyroid peroxidases (TPOAb +) or thyroglobulin (TgAb +). There is no doubt that overt hypothyroidism should be promptly diagnosed and treated with levothyroxine in order to prevent severe maternal and fetal consequences such as fetal loss, premature birth, neurocognitive impairment of the child (1). However, necessity for screening and treatment of SCH and IH with levothyroxine during pregnancy is still unclear among clinicians. We believe, unraveling pathophysiological changes underlying SCH and IH during pregnancy, will provide foundation for or against the necessity of treatment. This review also attempts to coincide the term 'Gestational Hypothyroidism' (GHT) to identify these conditions as a different entity occurring during pregnancy.

DEFINITIONS – OH, SCH, IH AND GHT

In general terms, OH is defined as elevation of circulating levels of thyroid stimulating hormone (TSH) or thyrotropin with a decrease in free thyroxine (fT4). SCH is defined as elevation of circulating TSH level with normal fT4 levels. On the other hand, IH is defined as reduction of fT4 level with normal TSH level. Among pregnant women, epidemiology studies report 0.3-0.6% prevalence of OH, 18% prevalence of SCH - depending on the TSH cut-off value (2), 1.3-23.9% prevalence of IH - depending on the fT4 cut off value (3).

American Thyroid Association in 2011 and Endocrine Society in 2012, recommended to use a fixed cut off value for TSH above 2.5 uIU/ml during first trimester and 3.0 uIU/ml during second trimester to diagnose women with SCH during pregnancy. However, where available it is highly recommended to use the local TSH range and report the findings as 97.5th percentile or above for diagnosis of SCH (4, 5). While the cut-off value for fT4 could be 5^{th} to 2.5^{th} percentile or low from the locally available range, there has been discrepancies when measuring the circulating fT4 levels during pregnancy. Thus, total T4 or fT4 index is preferred, although many studies to date use the fT4 as a reporting value (6). In this review, we reinforce the term GHT (Gestational Hypothyroidism), for both SCH and IH identified during pregnancy in women who were previously euthyroid. It is to highlight the importance of pregnancy related occurrence of SCH and IH.

GHT IS ASSOCIATED WITH ADVERSE MATERNAL AND FETAL OUTCOMES

Many observational studies have shown significant association between SCH and IH with complications during pregnancy for both the mother and the baby (7). SCH during pregnancy was associated with maternal complications such as pre-eclampsia, miscarriage, placental abruption and fetal complications such as premature delivery, neonatal death (8–10). Some other studies report that SCH is not associated with adverse neurocognitive development of the child (11, 12). Interestingly, many studies have shown that maternal IH was associated with adverse neurocognitive developmental outcomes marked by delays in mental, cognitive, psychomotor assessments and neurobehavioral outcomes including Attention Deficit Hyperactivity Disorder (ADHD), autism spectrum disorder (11–14). IH was also associated with premature delivery (15). These differences of outcomes are pointing towards altered pathophysiology between SCH and IH during pregnancy. Upon meta-analysis evaluating negative outcomes of SCH, Taylor and colleagues (16) have suggested that SCH alters the metabolic environment and thus leads to adverse pregnancy complications while IH affects fetal neuronal development due to impaired fT4 availability. Animal studies support the latter by showing atypical neuronal migration and structural changes in brain regions such as somatosensory cortex and hippocampus among the offspring of rat mothers subjected to hypothyroxinemia as a result of low iodine intake (17). However, it is important to note that these are observational studies where correlation does not equal causation. Arguably, some studies show SCH is also associated with lower intellectual development of the infant marked by low performance of neuropsychological and behavioral tests (1, 18, 19). Of note, there are lesser number of studies that examine women with IH compared to that of SCH (16). It could be because in some countries, TSH is analyzed first and only those women with TSH aberrations are next considered for analysis, there by missing a proportion of women with IH. An evolving hypothesis - pathogenesis of gestational hypothyroidism

It is well apparent the existence of differential mechanisms for adverse outcomes observed in women with GHT. It is important to dissect these pregnancy-induced mechanisms not only to reinforce our understanding but also to explore etiology-specific alternative modes of management of GHT. Two overarching schools of hypothesis for underlying pathophysiology of GHT could be considered (20). Firstly, development of GHT directly causes the observed adverse maternal and fetal outcomes. Thus, can be managed timely with prompt administration of levothyroxine treatment. Secondly, pregnancy induced pathophysiological changes, such as mal placentation, lead to both GHT and pregnancy related complications. Thus, the observed adverse maternal and fetal outcomes are not improved with levothyroxine treatment. The latter seems more promising given the recent advances summarized in the sections below. This broad discussion is divided into two sections

- Section 01 – Regulation and feedback loops that includes the role of placenta, role of iodine and the role of estrogen
- Section 02 – Treatment of GHT with levothyroxine – an ongoing debate.

The normal thyroid physiology is regulated *via* the hypothalamus-pituitary-thyroid (HPT) axis. During pregnancy demand and supply of thyroid hormone increases due to various pregnancy induced changes. We hypothesize that in some vulnerable individuals, HPT axis fail to adjust to these

additional requirements - physiological or pathological, thus leading to GHT. The role of placenta in thyroid hormone regulation is of great importance in this suggested hypothesis (**Figure 1**). The sections below attempt to unravel pregnancy induced changes that could possibly impact the thyroid homeostasis, thus inducing GHT.

SECTION 01 – REGULATION AND FEEDBACK LOOPS

Role of Placenta

Effect of Thyroid Hormones on the Placenta

It is important to identify that during pregnancy; placenta may play a central role in responding to and regulating maternal thyroid hormones. A recent review by Adu-Gyamfi et al. (21) well explains the available evidence on the effect of thyroid hormones on the placenta. Of interest, in established conditions which are due to abnormal placentation, such as pre-eclampsia, miscarriage, IUGR; studies have found that these women show abnormal levels of thyroid hormone as well. It is understood that optimal level of thyroid hormone is necessary for proliferation and differentiation of cytotrophoblasts (21). Furthermore, thyroid hormone might also regulate extravillous trophoblast invasion as shown by failure of extravillous trophoblasts to migrate and invade the decidua in pregnancies of hypothyroid rats (22). mRNA expression of molecules such as metalloproteinases (MMP2 and MMP3), oncofetal fibronectin and integrin alpha5beta1, which contribute to invasiveness of extravillous trophoblasts, have found to be increased in extravillous trophoblasts in-vitro when treated with T3. In maternal hypothyroid state, there is also alteration to the anti-inflammatory environment within the placenta, marked by reduced expression of IL-4, IL10 in the decidua (21). If any of the above processes are dysregulated, due to alternation to thyroid hormone availability, it may lead to a dysfunctional placenta.

Regulation of Thyroid Hormones Within the Placenta

Availability of the thyroid hormone within the placenta is governed by regulation of iodine, thyroid hormone metabolism and transport. A recent study by Peng et al. (23), examines the divergence of iodine and thyroid hormones in a term placenta. Placenta is the only other organ, other than the thyroid gland, that stores iodine. It is believed this strict regulation of iodine transport is to ensure that fetus is protected from excess exposure to iodine. However, fetus does require iodine to produce its own thyroid hormones. Iodine is obtained *via* uptake of maternal iodine, placental deiodination of thyroid hormones and the capacity of the placenta to store and transport iodine to fetal circulation. It is believed that there is a similar mechanism to thyroid gland by which placenta transport iodine. Influx of iodine is thought to be governed by Sodium/Iodine symporter (NIS) while efflux is governed by pendrin. In their study by Peng et al. (23), NIS was highly expressed in the apical membranes of syncytiotrophoblasts, the fetal side of the placenta while pendrin was detected syncytiotrophoblasts as well as cytotrophoblasts layers of placenta.

Deiodinases are set of enzymes that metabolize thyroid hormones. Deiodinases type 2 and 3 are found in the placenta, while D3 is predominant. D3 converts T4 to rT3 by inner ring deiodination of T4 and converts T3 to T2, there by releasing iodine (21). D3 is highly expressed in the syncytiotrophoblasts and cytotrophoblast layers of term placenta, fetal endothelium and decidua which in contact with the maternal circulation. This optimal position is to ensure that fetus obtains adequate iodine supply and release to fetal circulation (23). During the first

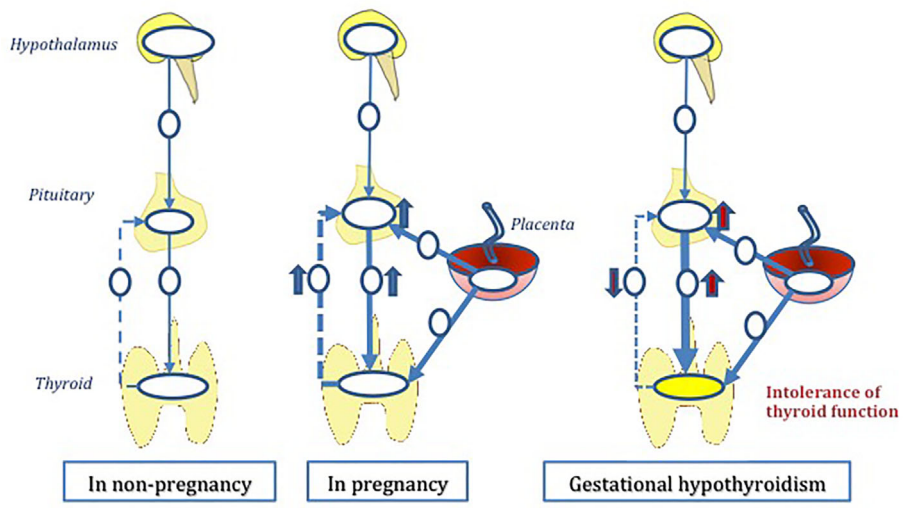


FIGURE 1 | The hypothesis of 'gestational hypothyroidism' (GHT). GHT is developed due to thyroid regulatory mechanisms during pregnancy. Hypothalamus-pituitary-thyroid axis (HPT axis) is a deviated from its normal function as a result of multiple pregnancy induced pathophysiological insults. Mal placentation plays a central role in this multifactorial hypothesis.

trimester, differential expression was observed where D2 is strongly expressed in the cytotrophoblasts layer and weakly in the syncytiotrophoblast layer. On the other hand, D3 is weakly expressed in the cytotrophoblast layer and strongly in the syncytiotrophoblast layer (21). Given that D3 is responsible for inactivation of thyroid hormone, it can be argued that over expression of D3 lead to a reduction in the placental thyroid hormone availability.

With regards to the placental regulation of thyroid hormone transport, thyroid hormone must pass through apical layer, basal layer of syncytiotrophoblasts, pass intracellularly through cytotrophoblasts and reach the fetal endothelium. On the maternal side, high levels of T3 and T4 than the fetal side of placenta are found. Minimal amounts of T3 and T4 is transported from maternal to fetal side. On the fetal side of the placenta, rT3 levels are increased, which plays a main role in ensuring low levels of T3 (23). Considering all together, it shows that thyroid hormone availability to the fetus is tightly regulated by the placenta. Six thyroid hormone transporters are known to exist within the placenta; namely, large amino acid transporter -1 (LAT1), Large amino acid transporter - 2 (LAT2), Organic anion transporting polypeptide 1A2 (OATP1A2), Organic anion transporting polypeptide 4A1 (OATP4A1), Monocarborxylate transporter - 8 (MCT8), Monocarboxylate transporter - 10 (MCT10). Differential expression of these transporters between maternal and fetal side of the placenta is found and abnormal expression of these transporters are thought to contribute to abnormal placentation (21).

Response of Maternal Thyroid Function to Placental Derived Factors

The above mentioned are the placental response to thyroid hormone levels. Strong evidence exists to show dysfunctional placentation due to overt hypothyroidism and hyperthyroidism in pregnant women (21). The question remains whether any factors secreted by the placenta can alter the maternal thyroid function, perhaps lead to gestational hypothyroidism. Presence of GHT in turn could lead to associated maternal and fetal complications. Indeed, a promising set of studies within the last few years, attempt to clarify this notion. Thus far, placental human chorionic gonadotropin (hCG), Placental growth factor (PIGF), pro-angiogenic factor and soluble FMS-like tyrosine kinase-1 (s-Flt1 or soluble vascular endothelial growth factor receptor-1), an antagonist of VEGF and PIGF signaling are identified as potential regulators of maternal thyroid function.

Human Chorionic Gonadotropin (hCG)

Placenta produces hCG which shares molecular similarity with TSH. This homology between the beta subunit of hCG and TSH allows for stimulation of the thyroid gland by binding and activating the TSH receptors of thyroid follicular cells and exerting its effects *via* intracellular messengers, such as cAMP (9). It is believed that both the amplitude and duration of hCG peak plays a role in the degree of the stimulation of the thyroid gland. A transient suppression of TSH and elevation of serum fT4 is expected in those with high concentration of hCG lasting for a longer duration. This may be evident from studies that suggest hCG has a causative role in hyperemesis gravidarum, where excessive vomiting and associated 5% weight loss is considered to be due to transient mild gestational hyperthyroidism state (24). Korevaar and colleagues (25) also support this notion by demonstrating that serum hCG concentration is associated significantly with increasing risk of SCH and OH. As discussed previously, the requirement for thyroid hormone production markedly increases during early stages of the pregnancy, thus hCG plays a major role in supplying for this increased demand (6). Question thus arises whether the sensitivity of TSH reduces at the thyroid gland and whether those vulnerable individuals fail to adapt at a pituitary level. Of interest, a recent analysis of population based prospective cohort study of more than 5000 pregnant women, also mentioned above, demonstrates that serum hCG concentration is not associated with risk of SCH during pregnancy (measured by TSH levels) but it is associated with lower risk of IH (25). However, those women with SCH is presumed to have a lack of thyroidal capacity to respond to hCG stimulation, as shown by an attenuated response to hCG mediated increased fT4 concentrations compared to euthyroid women. Although, there are no clinical trials showing a direct impact due to obvious ethical reasons, it can be speculated that this impaired rise of fT4 in response to increasing hCG, could further dysregulate TSH secretion by the pituitary gland, potentially worsening the SCH state in these women. Interestingly, external factors such as higher BMI, male fetal sex, and parity >2, were associated with reduced thyroidal response to increase in hCG concentration.

Placental Growth Factor (PIGF) and Soluble FMS-Like Tyrosine Kinase-1 (s-Flt-1)

PIGF is a pro-angiogenic factor produced by the placenta which shares 53% molecular homology with vascular endothelial growth factor (VEGF) while s-Flt1 is an anti-angiogenic factor produced by the placenta that can antagonise the effects of PIGF and VEGF. These two factors are potent regulators of vascular endothelial homeostasis within the placenta. Dysregulation of these factors, such as high sFLT1 to low PIGF ratio, has been implicated in pregnancy complications such as pre-eclampsia, IUGR and small for gestational age (26, 27). Thyroid gland is a highly vascularized organ, where animal studies have shown that there is a significant reduction in thyroid vasculature in the presence of VEGF blockade (28). More interestingly, in those patients who underwent VEGF inhibition as anti-angiogenic cancer therapy, have shown to develop OH or SCH as a side effect (29). Firstly, Korevaar and colleagues (30) determined the effects of placental derived factors PIGF and sFlt1 on newborn thyroid function. They found that while elevation of PIGF was associated with fT4 positively, increasing levels of sFlt1 was associated with decreasing levels of fT4 and increasing levels of TSH in newborn cord blood samples. Upon significant association with newborn thyroid function, Korevaar and colleagues (31) then determined the effects on maternal thyroid function. Interestingly, while they indeed found sFlt1 to show a significant negative linear association with fT4 levels, there was a non-significant positive linear association with TSH level. However, very high levels of sFlt1

were associated with a 2.4 fold increased risk of SCH, suggesting a threshold effect of sFlt1 on TSH levels. Supportive evidence by a nested case control and population-based study in pre-eclamptic women by Levine et al. (32) shows that maternal higher sFlt1 level was significantly associated with larger increases in TSH levels between early pregnancy and pre-delivery, suggesting SCH in women with pre-eclampsia. PIGF, on the other hand, was associated with a negative trend in both TSH levels and fT4 levels (31). This is different to the observed effect on the fetal thyroid gland. Authors speculate a differential stimulation of angiogenesis by PIGF between a fully formed maternal thyroid and newly forming fetal thyroid gland. In addition, when stratified according to TPOAb positive pregnant women, effects of sFlt1 and PIGF on TSH levels were found to be stronger, supporting a multi-risk factor model on maternal thyroid function. Furthermore, supporting this model, Korevaar and colleagues (31) analyzed the effect of sFlt1 and PIGF alongside high and low levels of hCG levels. They found that anti-angiogenic effects of high sFlt1 levels could be rescued by increasing concentrations of hCG, which is also known to be a stimulator of angiogenesis. Of interest, they found that overall effect of increasing levels of PIGF and hCG leading to a decrease in thyroid function, possibly due to a hyper stimulatory effect through a similar mechanism of action *via* stimulation of the cAMP/PKA pathway.

Role of Iodine

Iodine is an essential micronutrient for the synthesis of thyroid hormone. Pregnancy, a state of many physiological changes, increases the demand for iodine around 50% (33). This is due to increased renal clearance of iodine as the glomerular filtration rate increases, relative increase in production of maternal thyroid hormone and increased fetal requirements for iodine during second and third trimester for production of its own thyroid hormones (9). There is an active transport of iodine to the thyroid gland which is regulated by TSH and by the concentration of iodine in blood. Thus, an adequate supply of dietary iodine is essential to supply for this increased demand. According to guidelines by WHO/UNICEF/ICCIDD the recommended iodine intake for pregnant and lactating women was increased by 50ug/L from normal adult range of 100-199g/L.

Iodine Deficiency During Pregnancy

A recent systematic review by Candido et al. (34) clearly classify the adverse effects of light, moderate and severe iodine deficiency amongst pregnant women between the three trimesters. In pregnant women with moderate iodine deficiency, subclinical hypothyroidism, pre-eclampsia, anemia, fetal growth restrictions, congenital anomalies were observed while in pregnant women with severe iodine deficiency, eclampsia, placenta previa, cretinism, miscarriage, hemorrhage and fetal demise were observed. The review found that some authors suggest during light iodine deficiency there is an increased iodine uptake to increase thyroxine secretion, noted by the hyperplasia by the thyroid gland, thus reducing the damages. However, other studies suggest even during light iodine deficiency there is an increase in circulating TSH, which is associated with increased oxidative stress probably through antagonizing effects of insulin and thus inducing hyperglycemia (35, 36). Iodine intake is grouped according to WHO criteria by taking measurements of median urinary iodine concertation (UIC). This is because more than 90% of dietary iodine intake is excreted *via* urine. These groups of iodine intake are as follows; adequate intake (UIC 150-249 μg/L), mild/light deficiency (UIC, 100-150 μg/L), moderate and severe deficiency (UIC, <100 μg/L), and more than adequate and excessive intake (UIC, ≥250 μg/L).

Association of SCH with severe and moderate iodine deficiency is evident with studies conducted over many different regions (37–40). The emerging idea is that due to iodine deficiency (UIC < 100g/L) there is an accumulation of oxidative stress that leads to an inflammatory response that in turn triggers thyroid autoimmunity. A recent study by Sun and his colleagues (41), have found an association of thyroid autoantibodies such as TPOAb and TgAb with iodine deficiency. In addition, this cross-sectional study in an iodine adequate region in China of over 7000 pregnant women has shown that in those pregnant women with isolated positivity for TgAb had higher risk of OH and SCH. In addition, as the titers of these antibodies TPOAb and TgAb increased, upward trend of TSH level and downward trend of fT4 levels were clearly observed. Serum thyroglobulin, which is an important component of thyroid hormone synthesis, was also found to be reduced in those antibody positive pregnant women, while associated with increased TSH level and decreased fT4 levels. The study suggests that serum thyrogobulin could possibly be considered as biomarker to assess the extent of thyroid damage in pregnant women. Considering it all, we can postulate that due to the increased demand for iodine and presence of masked yet sustained thyroid autoimmunity, more pronounced adverse effects are observed during pregnancy thus causing SCH or OH.

On the other hand, some studies conducted in other iodine-adequate regions, have not found a significant association between UIC and thyroid function in pregnant women (42, 43). Due to discrepancies of measurements of UIC and thyroid function between laboratories, specifically during pregnancy, outcome of all these studies should be considered with care.

Excess Iodine Intake During Pregnancy

At the present time, with introduction of universal salt iodization, many regions of the world have adequate supply of dietary iodine. Consensus however is that there is a chronic excess intake of dietary iodine. Interestingly, a systematic review and meta-analysis including observational studies by (44), shows that excess iodine intake is significantly associated with SCH in general population. Transient Wolff-Chaikoff effect describes how thyroid hormones level would decrease under high serum iodide concentration and return to normal level after a few days. However, it is believed that in vulnerable individuals, an escape of this adaption could occur, thus resulting in iodine-induced thyroid dysfunction with or without autoimmune thyroiditis eventually leading to SCH (44, 45). A study by Su et al. (46) have identified a group of such vulnerable reproductive-age women, defined by gene polymorphism of PDE4D in Shanxi province, China. Several different studies support the notion that

dietary iodine excess during pregnancy leads to subsequent SCH (33, 35, 45, 47, 48). Due to high risk of SCH, Shi and his colleagues (48) suggests, upper limit of iodine intake during early pregnancy in iodine-sufficient regions should not exceed UIC 250g/L while a UIC of 500g/L should not be exceeded due to significantly high risk of IH. Of interest, studies supporting both mild iodine deficiency and excess iodine intake leading to SCH during pregnancy have found pre-conception higher body mass index (BMI) to be a risk factor (40, 47).

Role of Estrogen

Changes to circulating estrogen during pregnancy could be another factor that might be contributing to development of GHT. It is known that there is relative increase in basal level of thyrogobulin in response to elevated estrogen levels. This decreases availability of free thyroid hormones, which in turn stimulates pituitary-thyroid axis. Thus, the thyroid gland is challenged to secrete more thyroid hormones to ensure a normal availability of free thyroid hormone. A study by De Geyter et al. (49) shows that women with SCH and on a fixed daily low dose (50 ug) supplementation with levothyroxine, still showed an increase in the TSH levels, reaching statistical significance compared to euthyroid women between gestational week 6-8. This closely parallels the pattern of rising estrogen levels during early pregnancy.

A retrospective cohort study at a fertility clinic by Hammond et al. (50) reports that 24% of women who were previously found to be euthyroid, developed mild hypothyroidism within 6 weeks of gestation. A strong association was found between gonadotropin stimulation in the conception cycle and mean elevated TSH level and mean low free T4 level during early pregnancy compared to those women who did not receive gonadotropin treatment. This is also supported by Benaglia et al. (51) where an increase in serum TSH level is found after ovulation trigger, in previously euthyroid women receiving controlled ovarian hyper stimulation. It is suspected that exogenous gonadotropin treatment and associated higher than normal E2 level in the normal cycle, may lead to a further increase in thyroglobulin level. Thus it could be assumed to lead to a fall in the availability of free circulating thyroid hormone (52). However, further studies are required to comment on the equilibrium state of T4 in case of increased thyroglobulin levels.

On the contrary, in a review by Mintziori et al. (53) that outlines the thyroid function and IVF outcome, it notes current in-vivo experimental evidence where 17[beta]-estradiol is shown to directly down regulate paraventricular-TRH mRNA concentration. On the other hand, higher levels of estrogen has shown to suppress T-helper cell responses type 1 (Th1), which is thought to be important for developing thyroid autoimmunity marked by increased serum concentrations of anti-thyroid peroxidase (anti-TPO) and antithyroglobulin (anti-TG) antibodies (54). Presence of thyroid autoimmune antibodies prior to conception can be considered a risk factor for GHT. Considering it all, in euthyroid women undergoing in-vitro fertilization, exist a hyperestrogenism state. For some, it may lead to an increase in serum TSH thus risking the development of GHT while others may not be vulnerable.

SECTION 02 – TREATMENT OF GHT WITH LEVOTHYROXINE - AN ONGOING DEBATE

Evidence From Studies Thus Far

Evidence From Randomized Controlled Trials (RCTs)

Currently there are three different randomized controlled trials that have assessed whether the treatment with levothyroxine was beneficial or not for women with GHT (55–57). When Nazapour et al. (58) stratified their cohort for thyroid antibody positivity, significant reduction in rate of preterm delivery and neonatal admission rate was noted in women with TPOAb (+) and a TSH cut off above 4.0uIU/ml. In the following year, Nazapour et al. (57) assessed their cohort based on two different TSH cut off values, 2.5mIU/ml and 4.0uIU/ml. They noted that in TPOAb (-) women with cut off value of TSH>2.5uIU/ml did not show lower rate of adverse pregnancy outcomes. However, rate of preterm delivery was reduced in TPOAb (-) women with TSH> 4.0uIU/ml who received treatment. Meta-analysis and a systemic review of these RCT trials by Yamamoto et al. (20) states that there was no significant difference between treatment with levothyroxine vs control (either placebo or no treatment) for the adverse outcomes assessed; which are preterm delivery, placental abruption, gestational age at delivery, NICU admission and infant head circumference.

Evidence From Combined Analysis of Cohort Studies and RCTs

On the contrary, systematic review and meta-analysis of 13 cohort studies and RCTs with total of 11,503 participants, noted that levothyroxine treatment reduced the odds of pregnancy loss and increased the chances of live birth rates in women with SCH (59). This is supported by an early systematic review and meta-analysis which found that with levothyroxine treatment for SCH, there is a significant decrease in pregnancy loss and increase in chance of live birth rates, among women who underwent assisted reproductive technologies to facilitate their pregnancy (60). However, as Nazapour et al. (59) suggest, results of their meta-analysis are to be interpreted with caution, as there are not enough RCTs. Also, there is not enough power to exclude the effect of thyroid autoimmunity among the participants (59). Similar to the meta-analysis of only RCTs by Yamamoto et al. (20), meta-analysis by Nazapour et al. (59) which also included cohort studies, did not find significance with chances of adverse maternal complications, obstetrical hemorrhage, neonatal ICU admission among women who got treated for SCH.

Evidence on Effect of Treatment on Neurological Development

Interestingly, all these RCT trials investigated the effect of levothyroxine treatment on neurocognitive and neurobehavioral development of the child born to mothers diagnosed with GHT (55–58). It is of importance to note that these trials did not find benefit of treatment on neurological development of the child at 3, 5 and 9 years of age. In addition, no benefit of time of initiation of therapy (prior to 14 weeks and prior to 11 weeks) on childhood IQ was noted (20). This was further supported by a recent prospective

cohort study where preconception treatment with levothyroxine shown to be not associated with improved neurocognitive function in children up to 2 years of age (61). Recently, CATS-I study by Lazarus et al. (56), the first RCT trial to be performed, was re-assessed for ADHD and ASD in children of mothers who were treated and not treated for GHT (62). No significant difference was seen even when stratified for SCH and IH separately. These studies are summarized in **Table 1** and **Table 2**.

Choice of Treatment of GHT?

Recommendations From Endocrine Society and American Thyroid Association

Therefore, the choice of treatment of GHT with levothyroxine is becoming increasingly controversial. It was long believed that levothyroxine therapy is within physiological range; as such, there is no harm if chosen to treat women presenting with SCH during pregnancy. Thus, it has been the recommendation from the Endocrine Society to treat all women with SCH irrespective of the presence of thyroid autoantibodies (5). Of note, RCT by Casey et al. (55) does not report negative outcomes amongst the women treated with levothyroxine. However, this contrasts with the recommendation from American Thyroid Association (ATA) which recommends treatment only for SCH with TPOAb (+) and not for IH (4).

Risk of Over-Treatment With Levothyroxine

In the recent past, studies have emerged challenging the treatment with levothyroxine for SCH during pregnancy. Over-treatment with levothyroxine that lead to an increase in fT4 levels, though at a subclinical level, is associated with increased risk of pre-eclampsia (64). It is further supported by an USA national survey that demonstrates while there is a reduced risk of miscarriage, other complications such as preterm delivery, gestational diabetes and pre-eclampsia show an increased risk among the women who were treated with levothyroxine for SCH (65). In addition, increased concentration of fT4 has been found in fetal blood during cordocentesis (14). Of note, paradoxically, it is shown that such supra-normal levels are associated with low IQ, low grey matter and cortex volume in the child (66). Supporting this notion, the re-assessment of CATS-I RCT trial, showed that over treatment with levothyroxine, defined as an increase in fT4 levels above the 97.5th percentile, led to more conduct, ADHD features and ASD in children of mothers treated (62). Furthermore, in support, a cohort study in Denmark demonstrated that the children of mothers with subclinical hyperthyroidism throughout their pregnancy had increased the risk of being diagnosed with ADHD (67).

Monitor TSH and fT4 Upon Initiation of Treatment

This apparent 'bi-phasic' effect of fT4 both low and high levels, though subclinical, causing adverse outcomes, stresses the importance of careful monitoring of TSH as well as fT4 levels if chosen to treat women with GHT. It is further facilitated by these RCT studies that shows no benefit of treatment of GHT, questioning the reasoning for initiation of treatment (20). In the light of emerging findings, a recent review by Taylor et al. (16) attempts to introduce a stepwise algorithm yet specific, when initiating the treatment for either SCH or IH during gestation. Importantly they suggest monitoring TSH and fT4 levels every 4 weeks to maintain optimal circulating concentrations of fT4 (16).

DISCUSSION

GHT is a separate entity of thyroid hormone dysregulation occurring during pregnancy. Aberrations of iodine regulation, elevated estrogen levels, mal placentation or physiological placental derived factors insult the maternal thyroid hormone regulation and function, possibly leading to GHT. Current research studies do not fully elucidate the pathological mechanisms behind the development of GHT. It is evident

TABLE 1 | Summary (part 1) of the studies that have assessed neurocognitive and neurodevelopment of the child born to mothers with Gestational Hypothyroidism (GHT) and treated with appropriate dose of levothyroxine throughout pregnancy.

Type	Name of the study	Definition of GHT	Sample size	Treatment	Time of initiation of therapy
RCT*	Lazarus et al. (56) CATS-I trial	TSH* > 97.5th percentile Or/and fT4* < 2.5th percentile	794	Starting dose of 150ug of levothyroxine per day (adjusted as necessary)	Median gestational age of 13 weeks + 3days
RCT	Hales et al. (63) CATS-II trial	TSH > 97.5th percentile Or/and fT4 < 2.5th percentile	449	Starting dose of 150ug of levothyroxine per day (adjusted as necessary)	Median gestational age of 13 weeks + 3days
RCT	Hales et al. (62) (CATS-I trial cohort)	TSH > 97.5th percentile Or/and fT4 < 2.5th percentile	475	Starting dose of 150ug of levothyroxine per day (adjusted as necessary)	Median gestational age of 13 weeks + 3days
RCT	Casey et al. (55)	SCH* –> TSH >= 4.0mU/L with normal fT4	677	Starting dose of 100ug of levothyroxine (adjusted as necessary)	Mean gestational age of 16.7 weeks
		IH*–> fT4 < 0.86ng/dL with normal TSH	526	Starting dose of 50ug of levothyroxine (adjusted as necessary)	Mean gestational age of 17.8 weeks
Prospective Cohort	Zhou et al. (61)	TSH > 97.5th percentile and TPOAb (+) *	466	Levothyroxine treatment targeting TSH values of 0.1-2.5mU/L	Before conception and after conception (mean gestational age 13.5 weeks)

**RCT, Randomised Controlled Trial; TSH, Thyroid-Stimulating Hormone; fT4, Free Thyroxine; SCH, Subclinical Hypothyroidism; IH, Isolated Hypothyroxinemia; TPOAb (+), Thyroid Peroxidases antibodies.*

TABLE 2 | Summary (part 2) of the studies that have assessed neurocognitive and neurodevelopment of the child born to mothers with Gestational Hypothyroidism (GHT) and treated with appropriate dose of levothyroxine throughout pregnancy.

Name of the study	Child neurological outcome measured	Assessments used	Result comparing intervention vs control/placebo groups
Lazarus et al. (56) 'CATS-I trial'	IQ at age 3	Wechsler preschool and primary scale of intelligence (2003 edition) Child behavior aspects that may affect IQ is measured by 1. Child Behaviors Checklist (CBCL 2000) Achenbach System of empirically based assessment, University of Vermont 2. Behaviors Rating Inventory of Executive Function, preschool version, 2003 3. Child Behavior Checklist at 36months and 60 months of age - behavior and social competency assessment	No significant difference of the IQ scores of children
Hales et al. (63) CATS-II trial	Full Scale IQ at age group 7-10 years old	1. Wechsler Intelligence Scale for Children, Fourth Edition UK (WISC-IV) calculated equally from verbal, perceptual reasoning, working memory IQ and processing speed IQ domains.	No significant difference in IQ scores of children
	Long term memory, working memory, fine motor coordination	1. Developmental Neuropsychological Assessment (NEPSY), Second Edition	
Hales et al. (62) (CATS-I trial cohort)	ADHD features in children 7-10 years of age (emotions, conduct, hyperactivity, peer problems, prosocial, inattention, impulsivity, social communication)	1. The Strengths and Difficulties Questionnaire (SDQ) 2. The Child ADHD Questionnaire (ADHDq) 3. Social Communication Questionnaire (SCQ) All 3 questionnaires were completed by the mothers of the children	No significant differences of ADHD features between the children
Casey et al. (55)	Full Scale IQ at 5 years of age Secondary Outcomes - Cognitive, motor, language, behavior and social scores	1. Wechsler Preschool and Primary Scale of Intelligence III (WPPSI-III) 2. Differential Ability Scales - II (DAS) at 3 years of age if the WPPSI-III score was not available at 5years. 3. Bayley Scales of Infant Development, Third Edition (Bayley - III) at 12 months, 24months 4. Conner's Rating Scales - revised at 48months of age for assessment of attention	No significant differences seen with cognitive and behavioral outcomes
Zhou et al. (61)	Psychological Development assessment at age 6, 12, 24 months	Chinese Version of Gesell development Diagnosis Scale (GDDS) with 4 domains - motor, adaptability, language and social emotional responses	No significant difference of neurocognitive development of children between different time of initiation of therapy (before conception and after conception)

that more RCT trials, more *in vitro* and *in vivo* studies are required to unravel the complex hormonal dysregulations and their downstream effects leading to GHT. This review identifies the following research gaps in the field of GHT.

- Although adverse maternal and fetal effects of OH are well described, complications occurring with the existence of SCH and IH are not fully understood. In fact, differential modes of analysis of neurodevelopment of the offspring between studies, especially the RCTs, creates discrepancies and questions the outcome of the studies. Thus, a consensus has to be reached with the mode of analysis. Also presenting results with combined techniques such as MRI and genetic testing could be considered.
- For other endocrine abnormalities during pregnancy such as gestational diabetes, glucose control and treatment is well outlined. However, the aim of treatment of GHT with levothyroxine remains controversial. There appears a fine balance of over treatment and under treatment. Furthermore, time of initiation of screening and therapy remains to be understood.
- It is also important to consider whether to continue treatment postpartum, if levothyroxine treatment is initiated for GHT. Prospective cohort studies on continuation of treatment postpartum could shed light on thyroid function and other related complications of those women on therapy.
- In addition, there is inadequate advice on timeline of follow up of these women identified with SCH and IH during their pregnancy. Other pregnancy related endocrine abnormalities such as gestational diabetes is strictly followed up with glucose level many years post pregnancy. However, there is a lack of research studies following up the maternal thyroid function, endocrine and metabolic abnormalities of women with GHT later in their life.

AUTHOR CONTRIBUTIONS

XL -Proposing the idea of gestational hypothyroidism and identifying discrepancies among treatment options for women, highlighting the need for review, final editing of the article. QZ - Outline of the article, setting rationale, continuous guidance,

identifying gaps in research, discussion, figures, final editing of the article. OM - Gathering primary research articles, analyzing and writing up the review. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Shanghai Key Program of Clinical Science and Technology Innovation (No. 17411950500, No. 17411950501 and No. 18511105602), Clinical Research Plan of SHDC (SHDC2020CR1047B and SHDC2020CR6021), the Shanghai Excellent Young Scholar Plan of Public Health (2020-2022, GWV-10.2-YQ13), Elite Young Scholar 2025 of Fudan University (2020-2023), National Science Foundation of China (81741047), Shanghai Medical Center of Key Programs for Female Reproductive Diseases (2017ZZ01016). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Database reported in this publication was supported by Chinese government, a national nonprofit project which benefit rural reproductive aged population.

REFERENCES

1. Shinohara DR, da Silva Santos T, de Carvalho HC, Lopes LCB, Günther LSA, Aristides SMA, et al. Pregnancy Complications Associated With Maternal Hypothyroidism: A Systematic Review. *Obstetrical Gynecol Survey* (2018) 73 (4):219–30. doi: 10.1097/OGX.0000000000000547
2. Maraka S, Ospina NMS, O'Keeffe DT, Espinosa De Ycaza AE, Gionfriddo MR, Erwin PJ, et al. Subclinical Hypothyroidism in Pregnancy: A Systematic Review and Meta-Analysis. *Thyroid* (2016) 26(4):580–90. doi: 10.1089/thy.2015.0418
3. Dosiou C, Medici M. Management of Endocrine Disease: Isolated Maternal Hypothyroxinemia During Pregnancy: Knowns and Unknowns. *Eur J Endocrinol* (2017) 176(1):R21–38. doi: 10.1530/EJE-16-0354
4. Alexander EK, Pearce EN, Brent GA, Brown RS, Chen H, Dosiou C, et al. 2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum. *Thyroid* (2017) 27(3):315–89. doi: 10.1089/thy.2016.0457
5. De Groot L, Abalovich M, Alexander EK, Amino N, Barbour L, Cobin RH, et al. Management of Thyroid Dysfunction During Pregnancy and Postpartum: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2012) 97(8):2543–65. doi: 10.1210/jc.2011-2803
6. Krassas G, Karras SN, Pontikides N. Thyroid Diseases During Pregnancy: A Number of Important Issues. *Hormones* (2015) 14(1):59–69. doi: 10.1007/BF03401381
7. López-Muñoz E, Mateos-Sánchez L, Mejía-Terrazas GE, Bedwell-Cordero SE. Hypothyroidism and Isolated Hypothyroxinemia in Pregnancy, From Physiology to the Clinic. *Taiwanese J Obstetr Gynecol* (2019) 58(6):757–63. doi: 10.1016/j.tjog.2019.09.005
8. Casey BM, Dashe JS, Wells CE, McIntire DD, Byrd W, Leveno KJ, et al. Subclinical Hypothyroidism and Pregnancy Outcomes. *Obstetr Gynecol* (2005) 105(2):239–45. doi: 10.1097/01.AOG.0000152345.99421.22
9. Brabant G, Peeters RP, Chan SY, Bernal J, Bouchard P, Salvatore D, et al. Management of Subclinical Hypothyroidism in Pregnancy: Are We Too Simplistic? *Eur J Endocrinol* (2015) 173(1):P1–P11. doi: 10.1530/EJE-14-1005
10. Su PY, Huang K, Hao JH, Xu YQ, Yan SQ, Li T, et al. Maternal Thyroid Function in the First Twenty Weeks of Pregnancy and Subsequent Fetal and Infant Development: A Prospective Population-Based Cohort Study in China. *J Clin Endocrinol Metab* (2011) 96(10):3234–41. doi: 10.1210/jc.2011-0274
11. Henrichs J, Bongers-Schokking JJ, Schenk JJ, Ghassabian A, Schmidt HG, Visser TJ, et al. Maternal Thyroid Function During Early Pregnancy and Cognitive Functioning in Early Childhood: The Generation R Study. *J Clin Endocrinol Metab* (2010) 95(9):4227–34. doi: 10.1210/jc.2010-0415
12. Modesto T, Tiemeier H, Peeters RP, Jaddoe VW, Hofman A, Verhulst FC, et al. Maternal Mild Thyroid Hormone Insufficiency in Early Pregnancy and Attention-Deficit/Hyperactivity Disorder Symptoms in Children. *JAMA Pediatr* (2015) 169(9):838–45. doi: 10.1001/jamapediatrics.2015.0498
13. Román GC, Ghassabian A, Bongers-Schokking JJ, Jaddoe VW, Hofman A, De Rijke YB, et al. Association of Gestational Maternal Hypothyroxinemia and Increased Autism Risk. *Ann Neurol* (2013) 74(5):733–42. doi: 10.1002/ana.23976
14. Korevaar TI, Muetzel R, Medici M, Chaker L, Jaddoe VW, de Rijke YB, et al. Association of Maternal Thyroid Function During Early Pregnancy With Offspring IQ and Brain Morphology in Childhood: A Population-Based Prospective Cohort Study. *Lancet Diabetes Endocrinol* (2016) 4(1):35–43. doi: 10.1016/S2213-8587(15)00327-7
15. Korevaar TI, Schalekamp-Timmermans S, de Rijke YB, Visser WE, Visser W, de Muinck Keizer-Schrama SM, et al. Hypothyroxinemia and TPO-antibody Positivity are Risk Factors for Premature Delivery: The Generation R Study. *J Clin Endocrinol Metab* (2013) 98(11):4382–90. doi: 10.1210/jc.2013-2855
16. Taylor PN, Muller I, Nana M, Velasco I, Lazarus, JH. Indications for Treatment of Subclinical Hypothyroidism and Isolated Hypothyroxinaemia in Pregnancy. *Best Pract Res Clin Endocrinol Metab* (2020) 34(4):101436. doi: 10.1016/j.beem.2020.101436
17. Lavado-Autric R, Ausó E, García-Velasco JV, del Carmen Arufe M, del Rey FE, Berbel P, et al. Early Maternal Hypothyroxinemia Alters Histogenesis and Cerebral Cortex Cytoarchitecture of the Progeny. *J Clin Invest* (2003) 111 (7):1073–82. doi: 10.1172/JCI16262
18. Li Y, Shan Z, Teng W, Yu X, Li Y, Fan C, et al. Abnormalities of Maternal Thyroid Function During Pregnancy Affect Neuropsychological Development of Their Children At 25–30 Months. *Clin Endocrinol* (2010) 72(6):825–9. doi: 10.1111/j.1365-2265.2009.03743.x
19. Williams F, Watson J, Ogston S, Hume R, Willatts P, Visser T, et al. Mild Maternal Thyroid Dysfunction At Delivery of Infants Born≤ 34 Weeks and Neurodevelopmental Outcome At 5.5 Years. *J Clin Endocrinol Metab* (2012) 97(6):1977–85. doi: 10.1210/jc.2011-2451
20. Yamamoto JM, Benham JL, Nerenberg KA, Donovan LE. Impact of Levothyroxine Therapy on Obstetric, Neonatal and Childhood Outcomes in Women With Subclinical Hypothyroidism Diagnosed in Pregnancy: A Systematic Review and Meta-Analysis of Randomised Controlled Trials. *BMJ Open* (2018) 8(9):e022837. doi: 10.1136/bmjopen-2018-022837
21. Adu-Gyamfi EA, Wang YX, Ding YB. The Interplay Between Thyroid Hormones and the Placenta: A Comprehensive Review. *Biol Reprod* (2020) 102(1):8–17. doi: 10.1093/biolre/ioz182
22. Silva JF, Vidigal PN, Galvão DD, Boeloni JN, Nunes PP, Ocarino NM, et al. Fetal Growth Restriction in Hypothyroidism is Associated With Changes in Proliferative Activity, Apoptosis and Vascularisation of the Placenta. *Reproduct Fertil Dev* (2012) 24(7):923–31. doi: 10.1071/RD11219
23. Peng S, Li C, Xie X, Zhang X, Wang D, Lu X, et al. Divergence of Iodine and Thyroid Hormones in the Fetal and Maternal Parts of Human-Term Placenta. *Biol Trace Element Res* (2019) 195(1):1–12. doi: 10.1007/s12011-019-01834-z
24. Delitala AP, Capobianco G, Cherchi PL, Dessole S, Delitala G. Thyroid Function and Thyroid Disorders During Pregnancy: A Review and Care Pathway. *Arch Gynecol Obstetr* (2019) 299(2):327–38. doi: 10.1007/s00404-018-5018-8
25. Korevaar TI, de Rijke YB, Chaker L, Medici M, Jaddoe VW, Steegers EA, et al. Stimulation of Thyroid Function by Human Chorionic Gonadotropin During Pregnancy: A Risk Factor for Thyroid Disease and a Mechanism for Known Risk Factors. *Thyroid* (2017) 27(3):440–50. doi: 10.1089/thy.2016.0527
26. Verlohren S, Galindo A, Schlembach D, Zeisler H, Herraiz I, Moertl MG, et al. An Automated Method for the Determination of the sFlt-1/PIGF Ratio in the Assessment of Preeclampsia. *Am J Obstetr Gynecol* (2010) 202(2):161–e1. doi: 10.1016/j.ajog.2009.09.016
27. Coolman M, Timmermans S, de Groot CJ, Russcher H, Lindemans J, Hofman A, et al. Angiogenic and Fibrinolytic Factors in Blood During the First Half of Pregnancy and Adverse Pregnancy Outcomes. *Obstetr Gynecol* (2012) 119 (6):1190–200. doi: 10.1097/AOG.0b013e318256187f

28. Yang Y, Zhang Y, Cao Z, Ji H, Yang X, Iwamoto H, et al. Anti-VEGF–and anti-VEGF Receptor–Induced Vascular Alteration in Mouse Healthy Tissues. *Proc Natl Acad Sci* (2013) 110(29):12018–23. doi: 10.1073/pnas.1301331110
29. Makita N, Iiri T. Tyrosine Kinase Inhibitor–Induced Thyroid Disorders: A Review and Hypothesis. *Thyroid* (2013) 23(2):151–9. doi: 10.1089/thy.2012.0456
30. Korevaar TI, Steegers EA, Schalekamp-Timmermans S, Ligthart S, de Rijke YB, Visser WE, et al. Soluble Flt1 and Placental Growth Factor are Novel Determinants of Newborn Thyroid (Dys) Function: The Generation R Study. *J Clin Endocrinol Metab* (2014) 99(9):E1627–34. doi: 10.1210/jc.2014-1884
31. Korevaar TI, Steegers EA, de Rijke YB, Visser WE, Jaddoe VW, Visser TJ, et al. Placental Angiogenic Factors are Associated With Maternal Thyroid Function and Modify Hcg-Mediated FT4 Stimulation. *J Clin Endocrinol Metab* (2015) 100(10):E1328–34. doi: 10.1210/jc.2015-2553
32. Levine RJ, Vatten LJ, Horowitz GL, Qian C, Romundstad PR, Kai FY, et al. Pre-Eclampsia, Soluble Fms-Like Tyrosine Kinase 1, and the Risk of Reduced Thyroid Function: Nested Case-Control and Population-Based Study. *Bmj* (2009) 339:b4336. doi: 10.1136/bmj.b4336
33. Rebagliato M, Murcia M, Espada M, Álvarez-Pedrerol M, Bolúmar F, Vioque J, et al. Iodine Intake and Maternal Thyroid Function During Pregnancy. *Epidemiology* (2010) 21(3):62–9. doi: 10.1097/EDE.0b013e3181c1592b
34. Candido AC, Azevedo FM, Machamba AAL, Pinto CA, Lopes SO, Macedo MDS, et al. Implications of Iodine Deficiency by Gestational Trimester: A Systematic Review. *Arch Endocrinol Metabol (AHEAD)* (2020) 64(5):507–13. doi: 10.20945/2359-3997000000289
35. Xiao Y, Sun H, Li C, Li Y, Peng S, Fan C, et al. Effect of Iodine Nutrition on Pregnancy Outcomes in an Iodine-Sufficient Area in China. *Biol Trace Element Res* (2018) 182(2):231–7. doi: 10.1007/s12011-017-1101-4
36. Vidal ZEO, Rufino SC, Tlaxcalteco EH, Trejo CH, Campos RM, Meza MN, et al. Oxidative Stress Increased in Pregnant Women With Iodine Deficiency. *Biol Trace Element Res* (2014) 157(3):211–7. doi: 10.1007/s12011-014-9898-6
37. Yang J, Liu Y, Liu H, Zheng H, Li X, Zhu L, et al. Associations of Maternal Iodine Status and Thyroid Function With Adverse Pregnancy Outcomes in Henan Province of China. *J Trace Elements Med Biol* (2018) 47:104–10. doi: 10.1016/j.jtemb.2018.01.013
38. Stagnaro-Green A, Dogo-Isonaige E, Pearce EN, Spencer C, Gaba ND. Marginal Iodine Status and High Rate of Subclinical Hypothyroidism in Washington DC Women Planning Conception. *Thyroid* (2015) 25(10):1151–4. doi: 10.1089/thy.2015.0063
39. Nazarpour S, Tehrani FR, Behboudi-Gandevani S, Yarandi RB, Azizi F. Maternal Urinary Iodine Concentration and Pregnancy Outcomes in Euthyroid Pregnant Women: A Systematic Review and Meta-Analysis. *Biol Trace Element Res* (2019) 197(2):1–10. doi: 10.1007/s12011-019-02020-x
40. Gowachirapant S, Melse-Boonstra A, Winichagoon P, Zimmermann MB. Overweight Increases Risk of First Trimester Hypothyroxinaemia in Iodine-Deficient Pregnant Women. *Maternal Child Nutr* (2014) 10(1):61–71. doi: 10.1111/mcn.12040
41. Sun J, Teng D, Li C, Peng S, Mao J, Wang W, et al. Association Between Iodine Intake and Thyroid Autoantibodies: A Cross-Sectional Study of 7073 Early Pregnant Women in an Iodine-Adequate Region. *J Endocrinol Invest* (2020) 43(1):43–51. doi: 10.1007/s40618-019-01070-1
42. Blumenthal N, Byth K, Eastman CJ. Iodine Intake and Thyroid Function in Pregnant Women in a Private Clinical Practice in Northwestern Sydney Before Mandatory Fortification of Bread With Iodised Salt. *J Thyroid Res* (2012) 2012. doi: 10.1155/2012/798963
43. Etemadi A, Amouzegar A, Mehran L, Tohidi M, Azizi F, Moradi K, et al. Isolated Hypothyroxinemia in Iranian Pregnant Women, the Role of Iodine Deficiency: A Population-Based Cross-Sectional Study. *Thyroid* (2020) 30(2):262–9. doi: 10.1089/thy.2019.0047
44. Katagiri R, Yuan X, Kobayashi S, Sasaki S. Effect of Excess Iodine Intake on Thyroid Diseases in Different Populations: A Systematic Review and Meta-Analyses Including Observational Studies. *PloS One* (2017) 12(3):e0173722. doi: 10.1371/journal.pone.0173722
45. Wang K, Zhang J, Li F, Zhang W, Wang H, Ding L, et al. Urinary Iodine in Early Pregnancy is Associated With Subclinical Hypothyroidism in Tianjin, China: An Observational Study. *BMC Endocrine Disord* (2017) 17(1):1–7. doi: 10.1186/s12902-017-0162-x
46. Su C, Yu T, Zhao R, Wang Y, Jia H, Jing M, et al. Subclinical Thyroid Disease and Single Nucleotide Polymorphisms in Reproductive-Age Women in Areas of Shanxi Province, China, Where Iodine Exposure is Excessive. *Asia Pacific J Clin Nutr* (2018) 27(6):1366. doi: 10.6133/apjcn.201811_27(6).0024
47. Corcino CM, Berbara TMBL, Saraiva DA, Silva de Morais NAOE, Schtscherbyna A, Gertrudes LN, et al. Variation of Iodine Status During Pregnancy and its Associations With Thyroid Function in Women From Rio De Janeiro, Brazil. *Public Health Nutr* (2019) 22(7):1232–40. doi: 10.1017/S1368980019000399
48. Shi X, Han C, Li C, Mao J, Wang W, Xie X, et al. Optimal and Safe Upper Limits of Iodine Intake for Early Pregnancy in Iodine-Sufficient Regions: A Cross-Sectional Study of 7190 Pregnant Women in China. *J Clin Endocrinol Metab* (2015) 100(4):1630–8. doi: 10.1210/jc.2014-3704
49. De Geyter C, Steimann S, Müller B, Kränzlin ME, Meier C. Pattern of Thyroid Function During Early Pregnancy in Women Diagnosed With Subclinical Hypothyroidism and Treated With L-Thyroxine is Similar to That in Euthyroid Controls. *Thyroid* (2009) 19(1):53–9. doi: 10.1089/thy.2007.0355
50. Hammond KR, Cataldo NA, Hubbard JA, Malizia BA, Steinkampf MP. Gestational Hypothyroidism: Development of Mild Hypothyroidism in Early Pregnancy in Previously Euthyroid Women. *Fertil Sterility* (2015) 103(6):1532–6. doi: 10.1016/j.fertnstert.2015.03.006
51. Benaglia L, Busnelli A, Somigliana E, Leonardi M, Vannucchi G, De Leo S, et al. Incidence of Elevation of Serum Thyroid-Stimulating Hormone During Controlled Ovarian Hyperstimulation for In Vitro Fertilization. *Eur J Obstetr Gynecol Reprod Biol* (2014) 173:53–7. doi: 10.1016/j.ejogrb.2013.11.003
52. Gracia CR, Morse CB, Chan G, Schilling S, Prewitt M, Sammel MD, et al. Thyroid Function During Controlled Ovarian Hyperstimulation as Part of In Vitro Fertilization. *Fertil Sterility* (2012) 97(3):585–91. doi: 10.1016/j.fertnstert.2011.12.023.doi:10.1210/clinem/dgz098
53. Mintziori G, Goulis DG, Kolibianakis EM. Thyroid Function and IVF Outcome: When to Investigate and When to Intervene? *Curr Opin Obstetr Gynecol* (2016) 28(3):191–7. doi: 10.1097/GCO.0000000000000263
54. Mintziori G, Goulis DG, Toulis KA, Venetis CA, Kolibianakis EM, Tarlatzis BC. Thyroid Function During Ovarian Stimulation: A Systematic Review. *Fertil Sterility* (2011) 96(3):780–5. doi: 10.1016/j.fertnstert.2011.06.020
55. Casey BM, Thom EA, Peaceman AM, Varner MW, Sorokin Y, Hirtz DG, et al. Treatment of Subclinical Hypothyroidism or Hypothyroxinemia in Pregnancy. *New Engl J Med* (2017) 376(9):815–25. doi: 10.1056/NEJMoa1606205
56. Lazarus JH, Bestwick JP, Channon S, Paradice R, Maina A, Rees R, et al. Antenatal Screening for Hypothyroidism and Childhood Cognitive Impairment. *New Engl J Med* (2012) 306(6):493–501. doi: 10.1056/NEJMoa1106104
57. Nazarpour S, Ramezani Tehrani F, Simbar M, Tohidi M, Minooee S, Rahmati M, et al. Effects of Levothyroxine on Pregnant Women With Subclinical Hypothyroidism, Negative for Thyroid Peroxidase Antibodies. *J Clin Endocrinol Metab* (2018) 103(3):926–35. doi: 10.1210/jc.2017-01850
58. Nazarpour S, Ramezani Tehrani F, Simbar M, Tohidi M, Alavi Majd H, Azizi F. Effects of Levothyroxine Treatment on Pregnancy Outcomes in Pregnant Women With Autoimmune Thyroid Disease. *Eur J Endocrinol* (2017) 176(2):253–65. doi: 10.1530/EJE-16-0548
59. Nazarpour S, Tehrani FR, Amiri M, Yarandi RB, Azizi F. Levothyroxine Treatment and Pregnancy Outcomes in Women With Subclinical Hypothyroidism: A Systematic Review and Meta-Analysis. *Arch Gynecol Obstetr* (2019) 300(4):805–19. doi: 10.1007/s00404-019-05245-2
60. Velkeniers B, Van Meerhaeghe A, Poppe K, Unuane D, Tournaye H, Haentjens P. Levothyroxine Treatment and Pregnancy Outcome in Women With Subclinical Hypothyroidism Undergoing Assisted Reproduction Technologies: Systematic Review and Meta-Analysis of Rcts. *Hum Reprod Update* (2013) 19(3):251–8. doi: 10.1093/humupd/dms052
61. Zhou Q, Wang C, Xu H, Li X. Impact of Preconception Treatment Initiation for Hypothyroidism on Neurocognitive Function in Children. *J Clin Endocrinol Metab* (2020) 105(11):e3919–28. doi: 10.1210/clinem/dgaa565
62. Hales C, Taylor PN, Channon S, McEwan K, Thapar A, Langley K, et al. Controlled Antenatal Thyroid Screening Ii: Effect of Treating Maternal Suboptimal Thyroid Function on Child Behavior. *J Clin Endocrinol Metab* (2020) 105(3):e417–27. doi: 10.1210/clinem/dgz098
63. Hales C, Taylor PN, Channon S, Paradice R, McEwan K, Zhang L, et al. Controlled antenatal thyroid screening II: effect of treating maternal

suboptimal thyroid function on child cognition. *J Clin Endocrinol Metab* (2018) 103(4):1583–91. doi: 10.1210/jc.2017-02378
64. Medici M, Korevaar TI, Schalekamp-Timmermans S, Gaillard R, de Rijke YB, Visser WE, et al. Maternal Early-Pregnancy Thyroid Function is Associated With Subsequent Hypertensive Disorders of Pregnancy: The Generation R Study. *J Clin Endocrinol Metab* (2014) 99(12):E2591–8. doi: 10.1210/jc.2014-1505
65. Maraka S, Mwangi R, McCoy RG, Yao X, Sangaralingham LR, Ospina NMS, et al. Thyroid Hormone Treatment Among Pregnant Women With Subclinical Hypothyroidism: US National Assessment. *Bmj* (2017) 356: i6865. doi: 10.1136/bmj.i6865
66. Spremovic-Radjenovic S, Gudovic A, Lazovic G, Marinkovic J, Radunovic N, Ljubic A. Fetal Free Thyroxine Concentrations in Pregnant Women With Autoimmune Thyroid Disease. *J Clin Endocrinol Metab* (2012) 97(11):4014–21. doi: 10.1210/jc.2012-1392
67. Andersen SL, Laurberg P, Wu CS, Olsen J. Attention Deficit Hyperactivity Disorder and Autism Spectrum Disorder in Children Born to Mothers With Thyroid Dysfunction: A Danish Nationwide Cohort Study. *BJOG: Int J Obstetr Gynaecol* (2014) 121(11):1365–74. doi: 10.1111/1471-0528.12681

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 08 June 2021
doi: 10.3389/fendo.2021.635321

Silencing of ANGPTL8 Alleviates Insulin Resistance in Trophoblast Cells

*Yu Bai, Qiang Du, Le Zhang, Ling Li, Nana Wang, Bo Wu, Ping Li and Ling Li**

Department of Endocrinology, Shengjing Hospital of China Medical University, Shenyang, China

This study aims to investigate the effect of angiopoietin like 8 (ANGPTL8) on gestational diabetes mellitus (GDM) and insulin resistance (IR). The GDM model was induced by high fat diet in mice, and IR was observed. The expression and secretion of ANGPTL8 were promoted in placenta of GDM mice. IR was induced in trophoblast cell HTR-8/SVneo by treatment of high concentration of insulin, and the expression levels of ANGPTL8 were increased. Silencing of ANGPTL8 alleviated IR and decreased glucose uptake in HTR-8/SVneo cells. However, the inflammation and oxidative stress in IR cells were not restrained by ANGPTL8 knockdown. In addition, c-Jun N-terminal kinase (JNK) signaling was activated by IR, which was inhibited by silencing of ANGPTL8. The effect of ANGPTL8 knockdown on IR was attenuated by JNK antagonist, and aggravated by JNK agonist, suggesting that ANGPTL8 affected IR by regulating JNK signaling. In conclusion, we demonstrated that the silencing of ANGPTL8 ameliorated IR by inhibiting JNK signaling in trophoblast cells. These findings may provide novel insights for diagnosis and treatment of GDM in clinic.

Keywords: gestational diabetes mellitus, insulin resistance, trophoblast cells, angiopoietin like 8, c-Jun N-terminal kinase

OPEN ACCESS

Edited by:
Qiongjie Zhou, Fudan University, China

Reviewed by:
Ming-Qing Li, Fudan University, China
Hua Zhang, First Affiliated Hospital of Chongqing Medical University, China

***Correspondence:**
Ling Li
liling8864@hotmail.com

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *30 November 2020*
Accepted: *06 May 2021*
Published: *08 June 2021*

Citation:
Bai Y, Du Q, Zhang L, Li L, Wang N, Wu B, Li P and Li L (2021) Silencing of ANGPTL8 Alleviates Insulin Resistance in Trophoblast Cells. Front. Endocrinol. 12:635321. doi: 10.3389/fendo.2021.635321

INTRODUCTION

Gestational diabetes mellitus (GDM) is the most common metabolic disorder of pregnancy, defined as "the type of glucose intolerance that develops in the second and third trimester of pregnancy, resulting in hyperglycemia of variable severity" (1, 2). GDM is an increasing risk factor for severe pregnancy complications for both mother and child, including cesarean delivery, shoulder dystocia, macrosomia and neonatal hypoglycemia (3, 4). Women with GDM also have an increasing risk factor to develop type 2 diabetes mellitus (T2DM) and cardiovascular disease after pregnancy, and their offspring are at increasing risk for the development of obesity and T2DM in later life (5). The etiology of GDM remains unclear. However, pancreatic β-cell secretory impairment and insulin resistance (IR) are pivotal during pathogenesis of GDM (1).

IR is an impaired response to insulin that characterizes normal pregnancy. Physiologic IR results in increased insulin secretion. Women with GDM are unable to increase insulin production to compensate for the increased IR (6, 7). Pancreatic β-cell deterioration-induced IR and relative insulin deficiency are the primary metabolic changes in GDM. As β-cell function further declines, hyperglycemia becomes more severe. In addition, gluconeogenesis is increased as a result of IR and

insulin deficiency. As a consequence, hyperglycemia is worsened (7). IR and insulin deficiency are often considered as therapeutic targets for GDM.

The expression of multiple genes involved in glucose and lipid metabolism are dysregulated in GDM. Angiopoietin like 8 (ANGPTL8) is a secretory protein, highly expressed in liver and fat, and it is involved in lipid metabolism (8, 9). ANGPTL8 has been reported to be increased in high fat diet (HFD)-induced diabetic mice, and the overexpression of ANGPTL8 alleviated hyperlipemia, glucose tolerance, IR and inflammation in diabetic mice (10). However, in another report, authors found that ANGPTL8 was highly expressed in omental fat of obese individuals with fatty and IR, and antisense oligonucleotide against *Angptl8* prevented hepatic IR in high-fat-fed rats (11). These studies suggested that the roles of ANGPTL8 in diabetes and IR remain uncertain. Its effect on GDM has not been investigated. Recently, ANGPTL8 was found higher in serum of women with GDM, compared with normal pregnancies (12), suggesting that ANGPTL8 may be involved in development of GDM.

In this study, we induced a GDM model *via* high fat diet (HFD) in mice. The effect of ANGPTL8 on GDM and IR was investigated.

MATERIAL AND METHODS

Animal Model

Healthy C57BL/6J male and female mice were kept in controlled environment (12 h/12 h light/dark cycles, 22 ± 1°C) with free access to food and water. After accommodation for one week, the female mice were randomly divided into two groups: HFD and normal fat diet (NFD). The mice in NFD group were fed with mouse standard chow purchased HUANYU BIO (Henan, China) including protein ≥18%, fat ≥4%, fiber ≤5%, ash ≤8%, carbohydrate ≤36%, calcium 1.0-1.8%, phosphorus 0.6-1.2%, and essential amino acid, vitamin and microelement. High fat chow was mixed with 15% lard oil, 10% yelk, 10% sugar and 65% standard chow. After dietary intervention for one week, breeding was conducted overnight in a 1:2 ratio; mating was confirmed by presence of a vagina mucous plug in the following morning, which represented gestation day (GD) 0.5. Oral glucose tolerance test (OGTT) was performed at GD0.5, 11.5 and 16.5. The weight of female mice was measured at GD0.5, 6.5, 12.5 and 18.5. The female mice were sacrificed at GD18.5, and the blood and placenta tissues were collected for subsequent detections.

The taken care of and conduct were line with Guide for the Care and Use of Laboratory Animals (8th, NIH), and the experimental procedure was approved by Ethical Committee of Shengjing Hospital of China Medical University.

OGTT

After fasting overnight, the blood glucose of mice was detected. Then the mice were underwent gavage of glucose solution with 2 g/kg (400 mg/ml), and the blood glucose was determined after gavage for 30, 60 and 90 min.

Homeostasis Model Assessment-Insulin Resistance (HOMA-IR)

After fasting overnight, the blood insulin was detected using an ELISA kit (USCN, Wuhan, Hubei, China) according to the manufacturer's protocol. The serum was incubated with reagent A in ELISA plate well at 37°C for 1 h. Then the solution was removed, and reagent B was added to incubate for 30 min. After solution movement, TMB reagent was added into wells to react in the dark. About 10-20 minutes later, the reaction was stopped with stopping buffer. OD450 was determined with a microplate reader, and the insulin concentration was calculated according to the standard curve. The HOMA-IR value was calculated as follow:

HOMA-IR= blood glucose (mM)×blood insulin (mU/l)/22.5

Detection of Lipid and Lipoprotein

The content of triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) in the serum was determined by kits (Jiancheng, Nanjing, Jiangsu, China) according to the manufacturer's protocol.

HE Staining

HE staining was performed to detect the histological changes in labyrinth zone of placenta tissue. The tissue was fixed with 4% paraformaldehyde overnight. After washing with flow water for 4 h, the tissue was dehydrated with grading concentration of ethanol (70% for 2 h, 80% overnight, 90% for 2 h and 100% for 1 h twice) and xylene for 30 min. Then the tissue was embedded with paraffin at 60°C for 2 h, and the paraffin mass was cut into sections of 5 μm, which were underwent deparaffinization with xylene for 15 min twice, 100% ethanol for 5 min twice, and 95%, 85%, 75% ethanol for 2 min, respectively. The sections were stained with hematoxylin (Solarbio, Beijing, China) for 5 min, soaked with 1% hydrochloric acid/ethanol for several seconds, and counterstained with eosin (Sangon, Shanghai, China) for 3 min. Finally, the sections were dehydrated again with grading concentration of ethanol (75% for 2 min, 85% for 2 min, 95% for 2 min, 100% for 5 min twice) and xylene (for 10 min twice), mounted with gum and observed with a microscope (Olympus, Tokyo, Japan) at 200× magnification.

Periodic Acid Schiff (PAS) Staining

PAS staining was used for detection of glycogen content in labyrinth zone of placenta tissue. The tissue was made into paraffin sections as previous description, and deparaffinization was performed with xylene and ethanol. The sections were incubated with periodic acid solution (Leagene, Beijing, China) for 10 min, washed with water for 5 min, stained with schiff buffer (Leagene) for 15 min, washed with water for 5 min, and countered with hematoxylin for 2 min. After dehydration with ethanol and xylene, the sections were mounted with gum and photographed with a microscope at 200× magnification.

Real-Time PCR

The total RNA was extracted with RNApure extraction kit (BioTeke, Beijing, China), and the concentration was measured with NANO 2000 ultraviolet spectrophotometer (Thermo Scientific, Waltham, MA, USA). The RNA was reversely transcribed into cDNA with M-MLV reverse transcription (TAKARA, Japan) with Oligo(dT) and random primer. The instruments and reagents used in reverse transcription were RNase-free. The cDNA was used for real-time PCR with Taq HS Perfect Mix (TAKARA) and SYBR Green (BioTeke) to detect the mRNA levels of ANGPTL8, tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6. β-actin served as the internal control. The PCR procedure was set as follow: 94°C for 5 min 20 s, 60°C for 30 s, 72°C for 40 s, and 40 cycles of 72°C for 5 min 30 s, 40°C for 4 min 30 s, melting 60-90°C every 1°C for 1 s, and incubated at 25°C for several minutes. The PCR ran in Exicycler TM96 quantitative thermal block. The data were calculated using $2^{-\Delta\Delta Ct}$ method. The primers were purchased from Genscript (Nanjing, Jiangsu, China), and the information was shown in **Table 1**.

Western Blot

The protein was extracted with RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) on ice, and the concentration was determined with BCA protein concentration kit according to the manufacturer's protocol. After denaturation in boiling for 5 min, equal amount of protein was separated with SDS-PAGE, and the concentration of polyacrylamide gel varied from 5% to 12% according to the protein size. After electrophoresis for about 2.5 h, the protein was transferred onto PVDF membrane (Thermo Scientific). After blocking with skim milk at room temperature for 1 h, the membrane was incubated with one of the following antibodies at 4°C overnight: rabbit anti-ANGPTL8 (1:1000; cat. no. A18133, ABclonal, Wuhan, Hubei, China), rabbit anti-insulin receptor β (IRβ) (1:1000; cat. no. AF6099, Affinity, Changzhou, Jiangsu, China), rabbit anti-p-IRβ(Tyr1361) (1:1000; cat. no. AF3099, Affinity), rabbit anti-insulin receptor substrate-1 (IRS-1) (1:1000; cat. no. AF6273, Affinity), rabbit anti-p-IRS-1(Ser307) (1:1000; cat. no. AF3272, Affinity), rabbit anti-p-IRS1(Tyr895) (1:1000; cat. no. 3070, CST, Boston, MA, USA), rabbit anti-Akt (1:1000; cat. no. 4685, CST), rabbit anti-p-Akt(Ser473) (1:1000; cat. no. 4060, CST), rabbit anti-glucose transporter (GLUT)1 (1:1000; cat. no. AF0173, Affinity), mouse anti-GLUT4 (1:1000; cat. no. BF1001, Affinity), rabbit anti-Rho associated coiled-coil containing protein kinase (ROCK)I (1:2000; cat. no. 21850-1-AP, Proteintech, Wuhan, Hubei, China), rabbit anti-ROCKII (1:2000; cat. no. 21645-1-AP, Proteintech), rabbit anti-MYPT1 (1:1000; cat. no. AF5444, Affinity), rabbit anti-p-MYTP1 (Thr853) (1:1000; cat. no. AF5445, Affinity), rabbit anti-c-Jun N-terminal kinase (JNK) (1:500; cat. no. AF6318, Affinity), rabbit anti-p-JNK(Thr183/Tyr185) (1:500; cat. no. AF3318, Affinity) and mouse anti-β-actin (1:2000; cat. no. 60008-1-Ig, Proteintech). After rinsing with TBST, the membrane was incubated with goat anti-mouse or rabbit secondary labeled with HRP (1:10000; Proteintech) at 37°C for 40 min. Finally, the membrane was reacted with ECL reagent for 5 min, followed with signal exposure in the dark. The optical density of the bands was analyzed with Gel-Pro-Analyzer software. β-actin served as the internal control.

TABLE 1 | The information of primers in this study.

Name	Sequence (5'-3')
Mus ANGPTL8 F	CGGTCAAGCCCACCAAGA
Mus ANGPTL8 R	TGCTGCTCTGCCATCTCCC
Mus β-actin F	CTGTGCCCATCTACGAGGGCTAT
Mus β-actin R	TTTGATGTCACGCACGATTTCC
Homo ANGPTL8 F	CTTCGGGCAAGCCTGTT
Homo ANGPTL8 R	GCTGTCCCGTAGCACCTTC
Homo IL-1β F	GAATCTCCGACCACCACTAC
Homo IL-1β R	CACATAAGCCTCGTTATCCC
Homo IL-6 F	TGCCTTCCCTGCCCCAGT
Homo IL-6 R	GTGCCTCTTTGCTGCTTTCA
Homo TNF-α F	CGAGTGACAAGCCTGTAGCC
Homo TNF-α R	TTGAAGAGGACCTGGGAGTAG
Homo β-actin F	CACTGTGCCCATCTACGAGG
Homo β-actin R	TAATGTCACGCACGATTTCC

Cell Culture and Treatment

Immortalized human chorionic trophoblast line HTR-8/SVneo was purchased from Procell (Wuhan, Hubei, China), and cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS) (Biological industries, Kibbutz Beit-Haemek, Israel) at 37°C with 5% CO_2.

The transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with serum-free medium.

The cells were incubated with insulin (Solarbio) of 10^{-6} mol/l for 48 h to induce IR, and insulin of 10^{-7} mol/l was used to stimulate cells to verify the IR.

JNK signaling agonist Anisomycin (1 μg/ml) (Yuanye, Shanghai, China) and antagonist SP600125 (20 μM) (Aladdin, Shanghai, China) was used to treat cells for 30 min to activate or inactivate the JNK signaling.

Immunofluorescent Staining

Immunofluorescent staining was used to detect the expression and distribution of GLUT1 and GLUT4. The cells were pre-seeded on glass slides, and fixed with 4% paraformaldehyde for 15 min. After washing with PBS to remove the residual paraformaldehyde, the cells were permeated with 0.1% TritonX-100 for 30 min to permeate cytomembrane. Then the cells were blocked with goat serum at room temperature for 15 min to block the non-specific antigens, and incubated with antibody against GLUT1 or GLUT4 (1:200; cat. no. AF0173, BF1001, Affinity) at 4°C overnight. After washing with PBS, the cells were incubated with Cy3-labeled secondary body for 60 min in the dark, and incubated with DAPI (Aladdin) to stain nuclei. Finally, the cells were mounted with anti-fading reagent, and photographed with a fluorescence microscope (Olympus) at 400× magnificance.

ELISA

The ANGPTL8 content in serum was detected with an ELISA kit (USCN) according to manufacturer's protocol. First, the serum

sample or cell supernatant was incubated in the ELISA plate well at 37°C for 1 h. Then the solution was removed, and reagent A was added into the ELISA plate well to incubate for 1 h. Subsequently, the well was washed with washing buffer, and incubated with reagent B for 1 h. Finally, TMB substrate solution was added into the well to react for 20 min in the dark, and OD450 was measured. The concentration was calculated according to standard curve.

The content of inflammatory factors, TNF-α, IL-1β and IL-6 in serum were determined with ELISA kits (USCN) according to the manufacturer's introductions. The detail steps were similar to above description.

Detection of Reactive Oxygen Species (ROS)

The content of ROS in cells was detected with an ROS assay kit (Beyotime). The cells were collected and washed with PBS. The cells were incubated with DCFH-DA solution (1:1000 diluted with serum-free medium) at 37°C for 20 min, and the reaction system was mixed upside-down every 3 min. Then the cells were washed with PBS to remove the free DCFH-DA, and fluorescence was detected by flow cytometer (ACEA, San Diego, USA).

Detection of Superoxide Dismutase (SOD) Activity

The SOD activity in the cells was detected by kit (Jiancheng) according to the manufacturer's protocol. The cells were lysed with ultrasound, and the total protein was extracted. The protein concentration was determined with BCA kit, and standard curve was drawn. The protein sample was incubated with color developing agent, and OD550 was measured.

SOD activity (U/mgprot) = ((contro OD550 - sample OD550)/control OD550)/50% × (volume of reaction solution (ml)/sample volume (ml)/protein sample volume (ml))

Statistical Analysis

The data in this study were present as mean ± SD in six (*in vivo*) or three individuals (*in vitro*). The data from animal experiments were analyzed with Student's t test, and data from cell experiments were analyzed with one-way or two-way analysis of variance followed with Bonferroni post-hoc test. A p value less than 0.05 was considered as statistically significant (*p<0.05, **p<0.01, ***p<0.001, ns, no significance).

RESULTS

ANGPTL8 Was Increased in Serum and Placenta Tissues of GDM Mice

GDM was induced by HFD in mice, and the treatments were described in the **Figure 1A**. HFD induced more significant weight gain than NFD during pregnancy (**Figure 1B**). **Figure 1C** revealed that HFD led to glucose tolerance at GD11.5 and GD16.5. The fasting blood glucose and insulin levels were detected at GD18.5. The results showed that HFD caused significant elevation of fasting blood and insulin levels and HOMA-IR index in pregnant mice (**Figures 1D–F**). In addition, the content of triglyceride, cholesterol and low density lipoprotein in serum was increased and that of high density lipoprotein was decreased in HFD mice (**Figure 1G**).

Next, HE and PAS staining revealed that HFD induced inflammatory infiltration and glycogen accumulation in placenta tissues of pregnant mice (**Figures 1H, I**). Then insulin signaling-related molecules were detected. As shown in **Figure 1J**, the phosphorylation of IRβ(Tyr1361), IRS-1 (Tyr896) and Akt was decreased, and that of IRS-1(Ser307) was increased in placenta of HFD mice, which suggested the deactivation of insulin signaling. However, the previous results showed higher blood insulin level in HFD. These results demonstrated the occurrence of IR in placenta of HFD mice. The expression level of a glucose transporter GLUT1 was increased, while that of another glucose transporter GLUT4 was decreased in placenta (**Figure 1K**). In addition, ANGPTL8 was detected. The results showed that the content of ANGTPL8 in serum was increased, and its mRNA and protein levels in placenta were also increased in HFD mice (**Figures 1L–N**).

Silencing of ANGPTL8 Inhibited IR in Trophoblast Cells

In order to investigate the role of ANGPTL8 in IR during pregnancy, IR was induced by high concentration of insulin treatment (10^{-6} mol/l) in HTR-8/SVneo cells. Western blot results showed that ANGPTL8 was increased in trophoblast cells with IR (**Figure 2A**). Then ANGPTL8 was silenced with interference RNAs, and ectopic expressed with overexpression plasmid. The effectiveness of knockdown or overexpression was confirmed with western blot (**Figure 2B**). To investigate the effect of ANGPTL8 on IR, glucose consumption was measured. As shown in **Figure 2C**, the treatment of high concentration of insulin induced obvious IR in trophoblast cells, which was significantly alleviated by ANGPTL8 knockdown. However, the effect of ANGPTL8 overexpression on IR was mild (**Figure 2C**). Thereafter, the insulin signaling was examined. As shown in **Figures 2D, E**, the insulin signaling was rapidly activated by physiological concentration of insulin (10^{-7} mol/l) in untreated cells, evidenced by increased levels of p-IRβ(Tyr1361), p-IRS-1 (Tyr896) and p-Akt, and decreased level of p-IRS-1(Ser307). After IR induction, the cells did not respond to physiological concentration of insulin, and the levels of insulin signaling related molecules almost unchanged. The IR was effectively ameliorated in ANGPTL8-silenced cells, evidenced by the enhanced response to physiological concentration of insulin (**Figure 2D**). However, the overexpression of ANGPTL8 did not affect IR in trophoblast cells (**Figure 2E**). Immunofluorescent staining revealed that IR induced increase of GLUT1 and decrease of GLUT4, which was consistence with those results *in vivo*. After silencing of ANGPTL8, the expression of GLUT1 was reduced, and that of GLUT4 was elevated (**Figures 2F**, **G**), suggesting the enhanced glucose consumption.

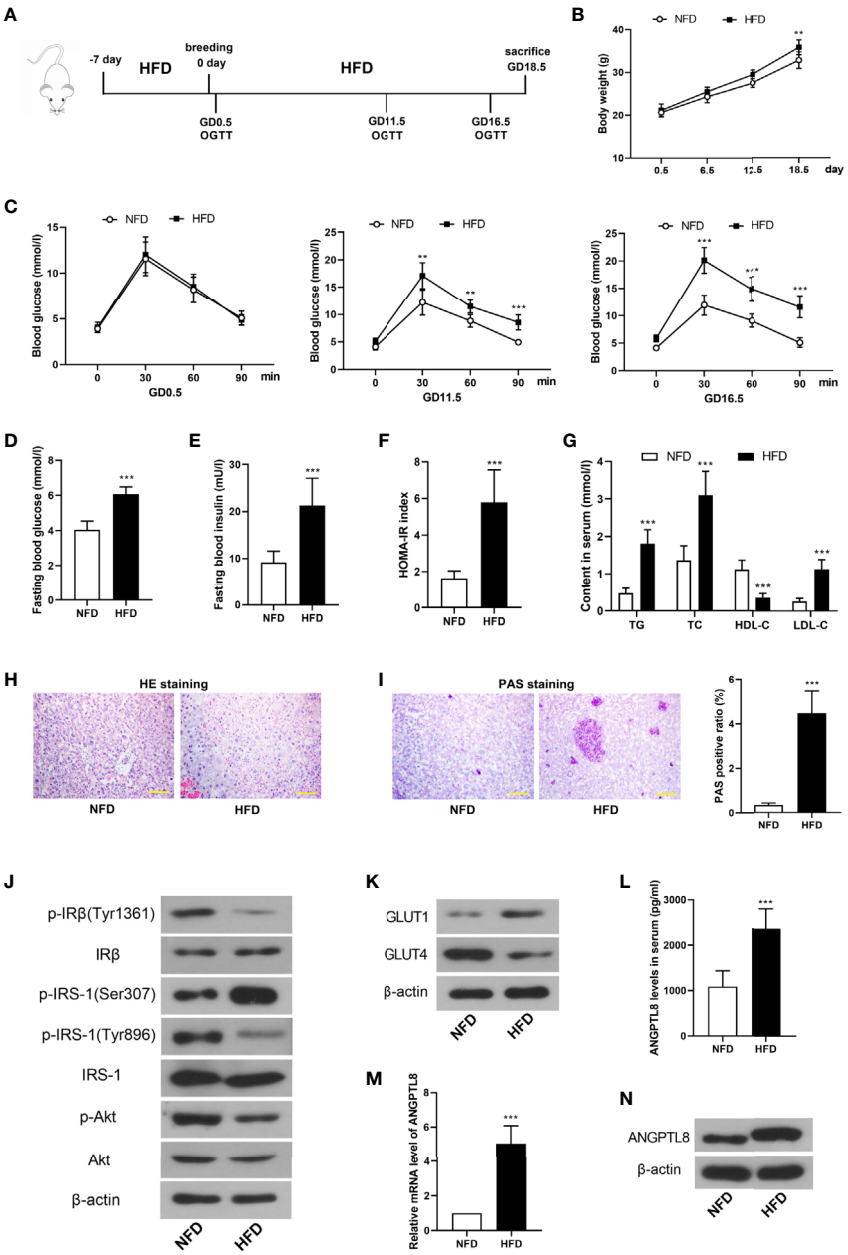


FIGURE 1 | Angiopoietin like-8 (ANGPTL8) was increased in serum and placenta tissues of gestational diabetes mellitus (GDM) mice. **(A)** The mice were treated as described in the chart. **(B)** The body weight of mice in normal fat diet (NFD) and high fat diet (HFD) groups. **(C)** Oral glucose tolerance test (OGTT) was performed at gestational day (GD)0.5, 11.5 and 16.5. **(D, E)** Fasting blood glucose and insulin levels were measured at GD18.5. **(F)** Homeostasis model assessment insulin resistance (HOMA-IR) was calculated as follow: HOMA-IR= blood glucose (mM)×blood insulin (mU/l)/22.5. **(G)** The contents of triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL-C) and low density lipoprotein (LDL-C) in serum were detected. **(H)** HE staining was performed to detect the pathological changes in labyrinth zone of placenta tissues. **(I)** Periodic acid Schiff (PAS) staining was carried out to detect the glycogen accumulation in labyrinth zone of placenta tissues. **(J)** Western blot was used to determine the levels of insulin signaling related molecules, p-IRβ(Tyr1361), IRβ, p-IRS-1(Ser307), p-IRS-1(Tyr896), IRS-1, p-Akt and Akt in placenta tissues. **(K)** The expression levels of glucose transporter 1 (GLUT1) and GLUT4 in placenta tissues. **(L)** The serum level of ANGPTL8 in mice. **(M, N)** The mRNA and protein levels of ANGPTL8 in placenta tissues. (the scale bar represents 100 μm; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NFD).

ANGPTL8 Activated JNK and ROCK Signaling

Since the important roles of JNK and ROCK signaling in diabetes mellitus, molecules in these two signaling pathways were detected. As shown in **Figure 3**, both JNK and ROCK signaling were activated in trophoblast cells with IR, evidenced by increased levels of p-JNK and p-MYPT1. The expression levels of ROCKI and ROCKII were also increased after IR

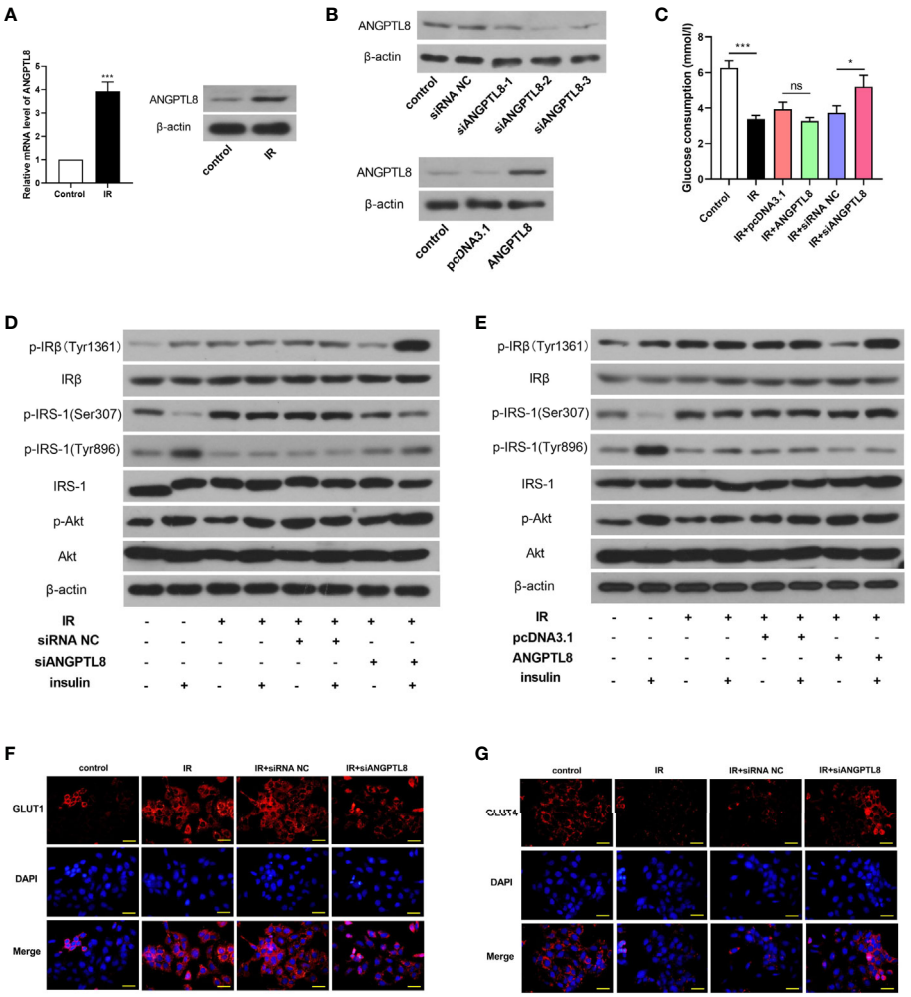


FIGURE 2 | Silencing of ANGPTL8 inhibited IR in trophoblast cells. **(A)** The mRNA and protein levels of ANGPTL8 in HTR-8/SVneo cells with IR. **(B)** The expression levels of ANGTPL8 were detected by western blot after overexpression or knockdown of ANGPTL8 in HTR-8/SVneo cells. **(C)** The glucose consumption in HTR-8/SVneo cells with overexpression or silence of ANGPTL8 was measured. **(D, E)** The levels of insulin signal related molecules, p-IRβ(Tyr1361), IRβ, p-IRS-1(Ser307), p-IRS-1(Tyr896), IRS-1, p-Akt and Akt in HTR-8/SVneo cells with IR, overexpression or knockdown of ANGPTL8 or/and insulin stimulation. **(F, G)** Immunofluorescent staining was used to detect the expression and distribution of GLUT1 and GLUT4 in HTR-8/SVneo cells. (the scale bar represents 50 μm; *$p < 0.05$, ***$p < 0.001$, ns, no significance).

induction, which may promote the activity of ROCK signaling (**Figures 3C, D**). After ANGPTL8 overexpression, the activity of JNK and ROCK was further increased, and it was declined after ANGPTL8 knockdown (**Figures 3A–D**). To explore the detail mechanism of ANGPTL8 function, a JNK signaling agonist Anisomycin and a JNK signaling antagonist SP600125 were used in trophoblast cells. As shown in **Figure 3E**, silencing of ANGPTL8 significantly restrained IR-induced glucose uptake obstruction. The effect of ANGPTL8 knockdown was attenuated by Anisomycin, but strengthened by SP600125 (**Figure 3E**). In addition, the insulin signaling was determined. As shown in **Figure 3F**, the mitigation effect of siANGPTL8 on IR was relieved by Anisomycin, but strengthened by SP600125, evidenced by the changes of insulin signaling related molecules. The results in this section suggested that silencing of ANGPTL8 restrained IR by inhibiting JNK signaling in trophoblast cells.

Silencing of ANGPTL8 did not Ameliorate Inflammation and Oxidative Stress in HTR-8/SVneo Cells With IR

Inflammation and oxidative stress response in placenta play crucial roles in GDM, so inflammation and oxidative stress were detected in trophoblast cells after IR induction. From the data shown in **Figure 4**, we found that the expression and secretion of IL-1β, IL-6 and TNF-α were all increased in trophoblast cells with IR. The inflammatory response induced by IR was not attenuated by silencing of ANGPTL8 (**Figures 4A, B**). IR also led to ROS production and decrease of SOD activity, suggesting oxidative stress response. After silencing of ANGPTL8, the ROS content

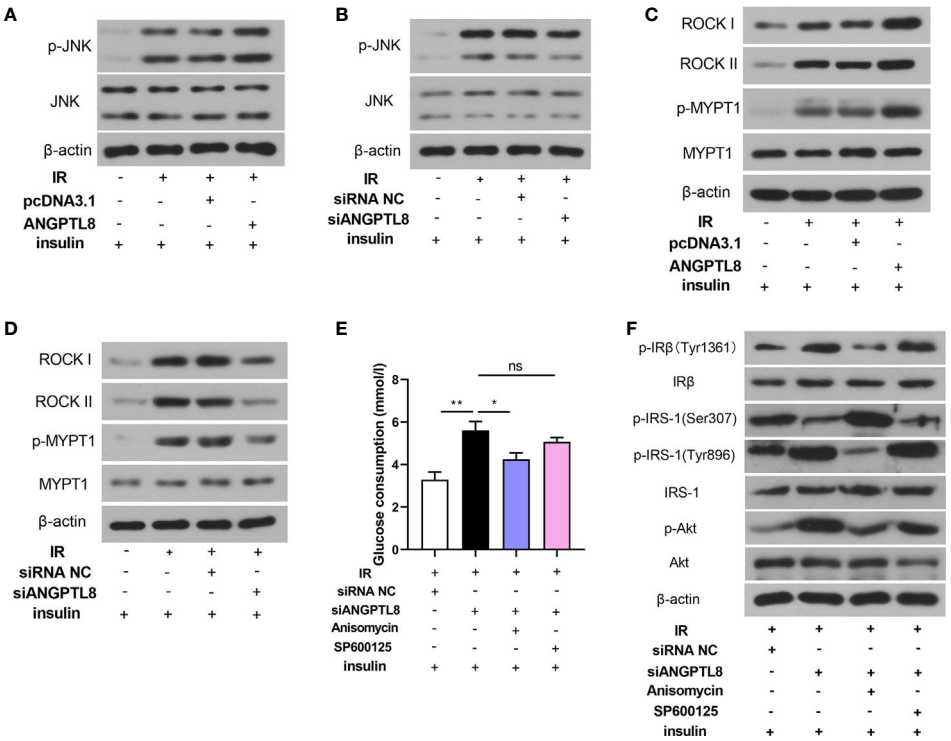


FIGURE 3 | ANGPTL8 activated JNK and ROCK signaling. **(A, B)** The levels of p-JNK and JNK in HTR-8/SVneo cells with IR, overexpression or silence of ANGPTL8 or/and insulin stimulation. **(C, D)** The levels of ROCK I, ROCK II, p-MYPT1 and MYPT1 in HTR-8/SVneo cells. **(E)** Glucose consumption was determined in HTR-8/SVneo cells with ANGPTL8 knockdown, JNK agonist Anisomycin (1 μg/ml) or JNK antagonist SP600125 (20 μM), and insulin stimulation. **(F)** The levels of insulin signaling related molecules were determined in HTR-8/SVneo cells. (*$p < 0.05$, **$p < 0.01$, ns. no significance).

was further increased, and the SOD activity did not changed (**Figures 4C, D**). The results in this section suggested that ANGPTL8 knockdown did not alleviated inflammation and oxidative stress response in trophoblast cells with IR.

DISCUSSION

Insulin is synthesized and secreted in pancreatic β cells, which cooperates with glucagon to maintain plasma glucose homeostasis. Insulin signaling is initiated with insulin binding to the insulin receptor, which is internalized and phosphorylated. The phosphorylated insulin receptor activated IRS family of proteins, and PI3K/Akt signaling was subsequently activated (13, 14). Impaired insulin receptor activities lead to IR, the key factor in the pathology of metabolic disorders including diabetes (15).

During pregnancy, the mother's body undergoes a series of physiological changes in order to support the demands of the growing fetus, and one important metabolic adaptation is insulin sensitivity. During early gestation, insulin sensitivity increases, promoting the uptake of glucose into adipose stores in preparation for the energy demands of later pregnancy (16). However, as pregnancy progresses, a surge of local and placenta hormones together promote a stage of IR (17). As a result, blood glucose is slightly elevated, and this glucose is readily transported across the placenta to fuel the growth of the fetus (18). In order to maintain glucose homeostasis, pregnant women compensate for these changes through hypertrophy and hyperplasia of pancreatic β cells, as well as increased glucose-stimulated insulin secretion (19). Moreover, the maternal insulin sensitivity returns to pre-pregnancy levels within a few days of delivery (20). For some reasons, the normal metabolic adaptations to pregnancy do not adequately occur in all pregnancies, resulting in GDM.

In our study, HFD induced GDM in pregnancy mice, shown as hyperglycemia, hyperinsulinemia, and pathological IR in placenta. The transcription, translation and secretion of ANGPTL8 were enhanced in GDM mice. At the same time, IR was induced in HTR-8/SVneo cells by treatment of high concentration of insulin (10^{-6} mol/l) *in vitro*, and the expression of ANGPTL8 was also increased. Silencing of ANGPTL8 significantly restrained IR in trophoblast cells, while the effect of ANGPTL8 overexpression on IR was negligible. We hypothesized that ANGPTL8 may be essential for IR, but it functioned independently of dosage. The roles of ANGPTL8 in T2DM are contradictory in previous reports (10, 11). We firstly investigated its role in GDM, and demonstrated that silencing of ANGPTL8 alleviated IR in trophoblast cells. However, its effect on GDM mice needs to be further studied by experiments *in vivo*.

Excess glycogen accumulation was found in placenta of GDM mice, suggesting abnormal glucose uptake and gluconeogenesis. At

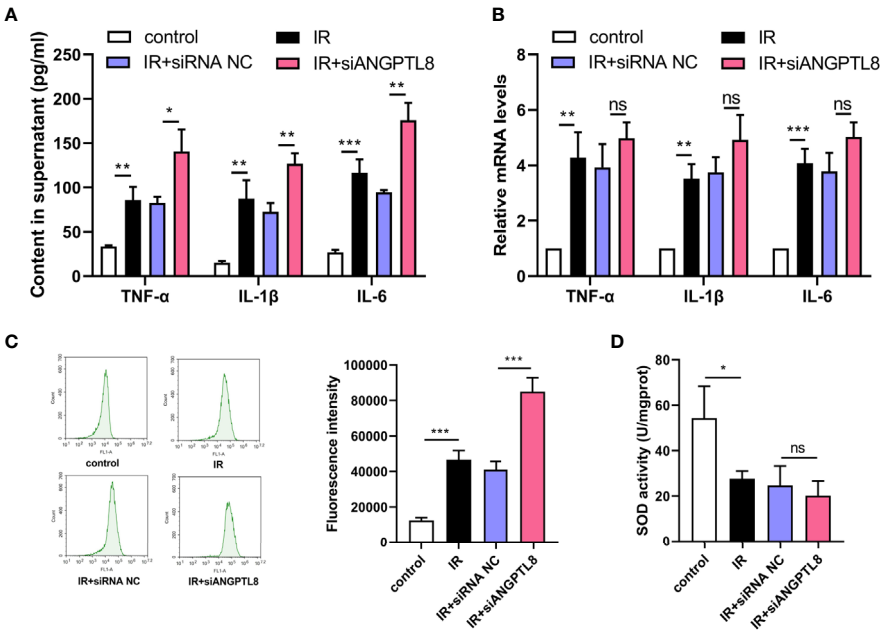


FIGURE 4 | Silencing of ANGPTL8 did not ameliorate inflammation and oxidative stress in HTR-8/SVneo cells with IR. **(A)** The levels of TNF-α, IL-1β and IL-6 in supernatant of HTR-8/SVneo cells with IR and ANGPTL8 knockdown. **(B)** The mRNA levels of TNF-α, IL-1β and IL-6 in HTR-8/SVneo cells with IR and ANGPTL8 knockdown. **(C)** ROS content in HTR-8/SVneo cells was measured with DCFH-DA staining. **(D)** The SOD activity in HTR-8/SVneo cells. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, no significance).

the same time, the expression of GLUT1 was increased, while that of GLUT4 was decreased in placenta. GLUT1 is highly expressed in placenta, and the glucose uptake in placenta is performed mainly *via* GLUT1. GLUT1 acts independently of insulin, and it is usually activated in placenta with IR (21). GLUT1 was reported highly expressed in placenta of women with GDM, especially those with macrosomia offspring (22). Macrosomia is a common complication of GDM, may be result from excessive uptake of glucose and lipid. We hypothesized that the increased expression of GLUT1 may lead to excess glucose uptake and gluconeogenesis in placenta. However, the expression of GLUT4 was decreased in GDM mice. GLUT4 was mainly expressed in fat and muscle. It is an insulin-responsive glucose transport (23). GLUT4 was low expressed in adipocytes of experimental diabetic rats with IR (24). It was also found to be decreased in placentas of women with GDM, and the decrease was most prominent in women receiving insulin (25). This may be result from inactivation of insulin signaling. In trophoblast cells with IR induction, the expression of GLUT1 was increased and that of GLUT4 was decreased. Moreover, the GLUT4 could not respond to insulin stimulation in cells with IR. These were similar with results in mice. However, the glucose uptake was reduced in IR cells, which was inconsistent with the excess glycogen accumulation *in vivo*. We hypothesized that GLUT4 may be more abundantly distributed in HTR-8/SVneo cells, compared with placenta tissue. The glucose uptake of HTR-8/SVneo cells may be mainly performed by GLUT4, the insulin-responsive transporter. Our hypothesis was supported by the subsequent results, which showed that after silencing of ANGPTL8, the IR was restrained, accompanied with the promoted expression of GLUT4 and recovery of glucose uptake. Certainly, this hypothesis needs more experiments to confirm.

JNK signaling has been reported to be activated in STZ-induced diabetic rats (26), and JNK inhibitor alleviated 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced IR in adipocytes (27). In our study, JNK agonist aggravated IR induced by high concentration of insulin treatment, and JNK antagonist attenuated IR in trophoblast cells. The effect of ANGPTL8 knockdown was abolished by JNK antagonist. So we deduced that silencing of ANGPTL8 ameliorated IR by suppressing JNK signaling in trophoblast cells. ROCK signaling was also activated in diabetic rats (26), and a ROCK inhibitor, fasudil, has been demonstrated to retrained experimental IR in trophoblast cells in our another paper (28).

In addition, IR is often accompanied with inflammation in diabetic patients or animals. In our study, the inflammatory reaction was observed in insulin resistant cells, accompanied with the oxidative stress response. After silencing of ANGPLT8, the inflammation and oxidative stress were not inhibited, which were not consistent with results of IR. Although IR and inflammation often occur at the same time, their mechanisms are not exactly the same. In our study, the silencing of ANGPTL8 alleviated IR but not inflammation in trophoblast cells. Its effect on GDM animal *in vivo* still needs to be further studied by more experiments.

In conclusion, we demonstrated that ANGPTL8 was highly expressed in placenta of GDM mice and IR trophoblast cells. The silencing of ANGPTL8 alleviated high concentration of insulin treatment-induced IR by inhibiting JNK signaling. But the inflammation in IR trophoblast cells was not restrained. These

results may provide novel insights for diagnosis and treatment of GDM in clinic.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of Shengjing Hospital of China Medical University.

AUTHOR CONTRIBUTION

YB and LL (8th author) designed the project. YB, QD, LZ, LL (4th author), NW, BW, and PL performed the experimental and analyzed the data. YB drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the 345 talent project plan of Shengjing Hospital of China Medical University and the Department of Education Foundation of Liaoning Province [No. L2015568].

REFERENCES

1. Chiefari E, Arcidiacono B, Foti D, Brunetti A. Gestational Diabetes Mellitus: An Updated Overview. *J Endocrinol Invest* (2017) 40(9):899–909. doi: 10.1007/s40618-016-0607-5
2. American Diabetes A. 2. Classification and Diagnosis of Diabetes. *Diabetes Care* (2016) 39:S13–22. doi: 10.2337/dc16-S005
3. Bellamy L, Casas JP, Hingorani AD, Williams D. Type 2 Diabetes Mellitus After Gestational Diabetes: A Systematic Review and Meta-Analysis. *Lancet* (2009) 373(9677):1773–9. doi: 10.1016/S0140-6736(09)60731-5
4. Sullivan SD, Umans JG, Ratner R. Gestational Diabetes: Implications for Cardiovascular Health. *Curr Diabetes Rep* (2012) 12(1):43–52. doi: 10.1007/s11892-011-0238-3
5. Group HSCR. The Hyperglycemia and Adverse Pregnancy Outcome (Hapo) Study. *Int J Gynaecol Obstet* (2002) 78(1):69–77. doi: 10.1016/S0020-7292(02)00092-9
6. Reece EA, Leguizamon G, Wiznitzer A. Gestational Diabetes: The Need for a Common Ground. *Lancet* (2009) 373(9677):1789–97. doi: 10.1016/S0140-6736(09)60515-8
7. Harlev A, Wiznitzer A. New Insights on Glucose Pathophysiology in Gestational Diabetes and Insulin Resistance. *Curr Diabetes Rep* (2010) 10(3):242–7. doi: 10.1007/s11892-010-0113-7
8. Ren G, Kim JY, Smas CM. Identification of RIFL, a Novel Adipocyte-Enriched Insulin Target Gene With a Role in Lipid Metabolism. *Am J Physiol Endocrinol Metab* (2012) 303(3):E334–51. doi: 10.1152/ajpendo.00084.2012
9. Zhang R. Lipasin, a Novel Nutritionally-Regulated Liver-Enriched Factor That Regulates Serum Triglyceride Levels. *Biochem Biophys Res Commun* (2012) 424(4):786–92. doi: 10.1016/j.bbrc.2012.07.038
10. Luo D, Chen X, Yang W, Ran W, Wen Z. Angiopoietin-Like 8 Improves Insulin Resistance and Attenuates Adipose Tissue Inflammation in Diet-Induced Obese Mice. *Exp Clin Endocrinol Diabetes* (2020) 128(5):290–6. doi: 10.1055/a-0725-7897
11. Vatner DF, Goedeke L, Camporez JG, Lyu K, Nasiri AR, Zhang D, et al. Angptl8 Antisense Oligonucleotide Improves Adipose Lipid Metabolism and Prevents Diet-Induced NAFLD and Hepatic Insulin Resistance in Rodents. *Diabetologia* (2018) 61(6):1435–46. doi: 10.1007/s00125-018-4579-1
12. Huang Y, Chen X, Chen X, Feng Y, Guo H, Li S, et al. Angiopoietin-Like Protein 8 in Early Pregnancy Improves the Prediction of Gestational Diabetes. *Diabetologia* (2018) 61(3):574–80. doi: 10.1007/s00125-017-4505-y
13. Hall C, Yu H, Choi E. Insulin Receptor Endocytosis in the Pathophysiology of Insulin Resistance. *Exp Mol Med* (2020) 52(6):911–20. doi: 10.1038/s12276-020-0456-3
14. Kulkarni RN. Receptors for Insulin and Insulin-Like Growth Factor-1 and Insulin Receptor Substrate-1 Mediate Pathways That Regulate Islet Function. *Biochem Soc Trans* (2002) 30(2):317–22. doi: 10.1042/bst0300317
15. Chen Y, Huang L, Qi X, Chen C. Insulin Receptor Trafficking: Consequences for Insulin Sensitivity and Diabetes. *Int J Mol Sci* (2019) 20(20):5007. doi: 10.3390/ijms20205007
16. Di Cianni G, Miccoli R, Volpe L, Lencioni C, Del Prato S. Intermediate Metabolism in Normal Pregnancy and in Gestational Diabetes. *Diabetes Metab Res Rev* (2003) 19(4):259–70. doi: 10.1002/dmrr.390
17. Catalano PM, Tyzbir ED, Roman NM, Amini SB, Sims EA. Longitudinal Changes in Insulin Release and Insulin Resistance in Nonobese Pregnant Women. *Am J Obstet Gynecol* (1991) 165(6 Pt 1):1667–72. doi: 10.1016/0002-9378(91)90012-G
18. Phelps RL, Metzger BE, Freinkel N. Carbohydrate Metabolism in Pregnancy. XVII. Diurnal Profiles of Plasma Glucose, Insulin, Free Fatty Acids, Triglycerides, Cholesterol, and Individual Amino Acids in Late Normal Pregnancy. *Am J Obstet Gynecol* (1981) 140(7):730–6. doi: 10.1016/0002-9378(81)90731-6
19. Parsons JA, Brelje TC, Sorenson RL. Adaptation of Islets of Langerhans to Pregnancy: Increased Islet Cell Proliferation and Insulin Secretion Correlates With the Onset of Placental Lactogen Secretion. *Endocrinology* (1992) 130(3):1459–66. doi: 10.1210/en.130.3.1459
20. Plows JF, Stanley JL, Baker PN, Reynolds CM, Vickers MH. The Pathophysiology of Gestational Diabetes Mellitus. *Int J Mol Sci* (2018) 19(11):3342. doi: 10.3390/ijms19113342
21. Ruiz-Palacios M, Ruiz-Alcaraz AJ, Sanchez-Campillo M, Larque E. Role of Insulin in Placental Transport of Nutrients in Gestational Diabetes Mellitus. *Ann Nutr Metab* (2017) 70(1):16–25. doi: 10.1159/000455904
22. Yao G, Zhang Y, Wang D, Yang R, Sang H, Han L, et al. Gdm-Induced Macrosomia is Reversed by Cav-1 *Via* AMPK-Mediated Fatty Acid Transport and GLUT1-Mediated Glucose Transport in Placenta. *PloS One* (2017) 12(1):e0170490. doi: 10.1371/journal.pone.0170490
23. Chadt A, Al-Hasani H. Glucose Transporters in Adipose Tissue, Liver, and Skeletal Muscle in Metabolic Health and Disease. *Pflugers Arch* (2020) 472(9):1273–98. doi: 10.1007/s00424-020-02417-x
24. Guo L, He B, Fang P, Bo P, Zhang Z. Activation of Central Galanin Receptor 2 Mitigated Insulin Resistance in Adipocytes of Diabetic Rats. *J Endocrinol Invest* (2021) 44(3):515–22. doi: 10.1007/s40618-020-01336-z
25. Colomiere M, Permezel M, Riley C, Desoye G, Lappas M. Defective Insulin Signaling in Placenta From Pregnancies Complicated by Gestational Diabetes Mellitus. *Eur J Endocrinol* (2009) 160(4):567–78. doi: 10.1530/EJE-09-0031
26. Peng X, Su H, Liang D, Li J, Ting WJ, Liao SC, et al. Ramipril and Resveratrol Co-Treatment Attenuates RhoA/ROCK Pathway-Regulated Early-Stage Diabetic Nephropathy-Associated Glomerulosclerosis in Streptozotocin-

Induced Diabetic Rats. *Environ Toxicol* (2019) 34(7):861–8. doi: 10.1002/tox.22758

27. Nishiumi S, Yoshida M, Azuma T, Yoshida K, Ashida H. 2,3,7,8-Tetrachlorodibenzo-P-Dioxin Impairs an Insulin Signaling Pathway Through the Induction of Tumor Necrosis Factor-Alpha in Adipocytes. *Toxicol Sci* (2010) 115(2):482–91. doi: 10.1093/toxsci/kfq052
28. Bai Y, Du Q, Zhang L, Li L, Tang L, Zhang W, et al. Fasudil Alleviated Insulin Resistance Through Promotion of Proliferation, Attenuation of Cell Apoptosis and Inflammation and Regulation of RhoA/Rho Kinase/Insulin/Nuclear Factor-kappaB Signaling Pathway in HTR-8/SVneo Cells. *J Pharm Pharmacol* (2021). doi: 10.1093/jpp/rgab033

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 10 June 2021
doi: 10.3389/fendo.2021.689888

Investigation Into the Predictive Potential of Three-Dimensional Ultrasonographic Placental Volume and Vascular Indices in Gestational Diabetes Mellitus

Zhenyan Han[1†], Yuan Zhang[1†], Xuelan Li[1,2], Wei-Hsiu Chiu[3,4], Yuzhu Yin[1] and Hongying Hou[1*]*

[1] Department of Obstetrics and Gynecology, Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, China, [2] Health Care Center of Minzhi Community, Shenzhen, China, [3] Department of Obstetrics and Gynecology, Guangzhou iBorn Women's Hospital, Guangzhou, China, [4] Department of Ultrasound in Obstetrics and Gynecology, Xuzhou Women and Children's Peace Hospital, Xuzhou, China

OPEN ACCESS

Edited by:
Qiongjie Zhou,
Fudan University, China

Reviewed by:
Xiaocan Lei,
University of South China, China
Shun Zhang,
Affiliated Hospital of Guilin Medical University, China
Tao Tang,
The Chinese University of Hong Kong, China

***Correspondence:**
Hongying Hou
houhongying123@126.com
Yuzhu Yin
yzzst2011@163.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *01 April 2021*
Accepted: *24 May 2021*
Published: *10 June 2021*

Citation:
Han Z, Zhang Y, Li X, Chiu W-H, Yin Y and Hou H (2021) Investigation Into the Predictive Potential of Three-Dimensional Ultrasonographic Placental Volume and Vascular Indices in Gestational Diabetes Mellitus. Front. Endocrinol. 12:689888. doi: 10.3389/fendo.2021.689888

Background: The use of ultrasonography in pregnancies complicated with gestational diabetes mellitus (GDM) can vary according to clinical practice. This study aims to compare the changes of placental volume (PV) and vascular indices measured by three-dimensional (3D) Power Doppler between pregnant women with and without GDM.

Materials and Methods: This was a prospective study of singleton pregnancies who took the early nuchal translucency examination from January 2018 to September 2019. Data on PV and vascular indices including vascularization index (VI), flow index (FI), and vascularization flow index (VFI) between pregnant women with and without GDM were measured by 3D Power Doppler ultrasound machine. Univariate and multivariate logistic regression determined the association between risk factors and GDM. Receiver operating characteristic (ROC) and area under the ROC curve (AUC) were applied to evaluate the diagnostic value of different parameters for GDM.

Results: Of the 141 pregnant women enrolled, 35 developed GDM and 106 did not. The maternal age and gravida in the GDM group were significantly higher than that in the non-GDM group. The PV, VI, FI, and VFI in the GDM group were significantly lower than that in the non-GDM group. There were no significant differences in other clinical parameters between the two groups. After adjustments in multivariate logistic regression analysis, significant differences were observed in VI [odds ratio (OR) = 0.98, 95% confidence interval (CI) = 0.951–1.002], FI (OR = 0.93, 955 CI: 0.86–1.00), and VFI (OR = 0.67, 95% CI = 0.52–0.87). ROC analysis indicated that the combination of maternal age, gravida, PV, and VFI was more accurate as a marker for detecting GDM than the PV, VI, FI, or VFI alone.

Conclusions: The 3D ultrasonography results suggest that PV and vascular indices (VI, FI, and VFI) during the first trimester may serve as potential markers for GDM diagnosis.

The combination of maternal age, gravida, and sonographic markers may have good diagnostic values for GDM, which should be confirmed by further investigations.

Keywords: gestational diabetes mellitus, three-dimensional power Doppler, placental volume, vascularization index, flow index, vascularization flow index

INTRODUCTION

Gestational diabetes mellitus (GDM) is featured by abnormal glucose intolerance during pregnancy (1). GDM affects about 7% of pregnancies, where advanced maternal age, obesity, previous history of GDM, previously giving birth to macrosomic infants, and family history of diabetes mellitus are regarded as major risk factors (2–4). Oral glucose tolerance test is often used for screening GDM during 24–28 gestational weeks (5, 6). However, GDM screening at 24–28 gestational weeks is too late for the physicians to make dietary or pharmacology therapy, which may ultimately affect placental integrity and fetal growth (7, 8). Thus, identifying novel markers for early screening of GDM is of great clinical significance.

Although clinical manifestations of GDM usually occur in the second or third trimester, evidence of abnormal placental development from as early as the first trimester has been reported, and dysregulation of various hormones and cytokines caused by GDM during early pregnancy has been reported to lead to placental dysfunction (9). Furthermore, increases in inflammatory markers due to persistent hyperglycemia can cause damage within the placenta such as villous maturation, vascularization, and branching (10). Ultrasonography is a non-invasive, readily available tool to examine and assess the fetus, which is helpful in instituting early therapeutic interventions for pregnancies complicated by diabetes (11). Improvements in three-dimensional (3D) ultrasonography have pointed out the role of placental volume (PV) and the potentially associated factors contributing to pregnancy complications. Pala et al., used Virtual Organ Computer-aided AnaLysis (VOCAL) to evaluate PV and placental mean gray value, and found that PV was significantly increased in GDM, whereas mean gray values did not alter (12). Saha et al., observed that the placentae in GDM were significantly bigger in size, weight, volume, area, thickness, diameter, and circumference than those in normal pregnant women, and there was significant increase in villous edema, fibrin deposition, calcification, and congestion of blood vessels in GDM (13). Studies from Wong et al., showed that placental vascular indices can provide an insight into placental vascularization in GDM during early pregnancy, and vascularization flow index (VFI) rather than placental volume may be a sensitive sonographic marker in the first trimester of GDM placentas (14), which still need to be confirmed with larger sample size. Desoye et al., proposed that the placentae in diabetic pregnancies increased levels of thromboxane and tumor necrosis factor alfa leading to vasoconstriction that may contribute to the decrease in vascularization index (VI) and VFI (15).

Based on the above evidence, the association between morphometric changes of placenta and GDM during early pregnancy remains elusive. Therefore, this study aims to determine the PV and vascular indices [vascularization index (VI), flow index (FI), and VFI] by using VOCAL during pregnancy, and to explore the association between these sonographic markers in combination with other clinical parameters and GDM. The present study may identify sensitive sonographic markers for the early diagnosis of GDM, which may be important to avoid the potential complications of GDM.

MATERIALS AND METHODS

This prospective study of singleton pregnancies who took the early nuchal translucency examination was performed at Guangzhou iBorn Women's Hospital from January 2018 to September 2019. A total of 161 pregnant women received ultrasonographic examination during 11^{+0} and 13^{+6} weeks. The inclusion criteria were: (1) gestational age between $11 + {}^{0}$ and $13 + {}^{6}$ weeks; (2) crown-lump length (CRL) between 45 and 80 mm; (3) singleton pregnancy. The exclusion criteria were: (1) fetal chromosomal or structural anomalies detected during karyotyping or sonographic examinations; (2) multifetal pregnancy; (3) history of hypertension or preeclampsia, thyroid disease, chronic kidney disease, autoimmune disease, or a diagnosis of these diseases in the current pregnancy; (4) pregestational diabetes mellitus; (5) long-term use of aspirin or glucocorticoids. This study was approved by the Ethical Committee of Guangzhou iBorn Women's Hospital, and each patient signed the written informed consent.

A Voluson E8 ultrasound machine (GE Medical Systems, Milwaukee, WI, USA) with a 1–6 MHz transabdominal RAB6-1D probe was used in this study. The ultrasound scan was performed according to the ISUOG practice guidelines (16). The nuchal translucency (NT), biparietal diameter (BPD), CRL, abdominal circumference (AC), femur length (FL), uterine artery doppler pulsatility index (UTPI), uterine artery resistance index (UTRI), VI, FI, and VFI were measured between 11^{+0} and 13^{+6} gestational weeks.

To obtain optimal PV, the probe was placed along the alignment of the placenta. Each woman was asked to hold her breath for 10 s, and the margin of the placenta was outlined to obtain its maximum area. This procedure was repeated six times after rotating the probe 30 degrees around the axis each time to acquire the full volume of the placenta. For laterally and posteriorly located placentas, the position of the probe was adjusted to fit the placental alignment as far as possible to obtain the optimal volume (**Figure 1**). All of the examined cases were measured using the same ultrasound instrument settings (Power Doppler map, 6; frequency, low; smoothing, rise 2/fall 4; flow resolution, mild 1; line density, 7; balance, 210; ensemble, 10; line filter, 3; artifact suppression, on; quality, high1; wall motion filter, med 1; pulse repetition frequency, 1.3 kHz). All the ultrasound scans were performed by the same examiner, and the placental vascular indices and PV were measured twice to inspect the intrarater reliability.

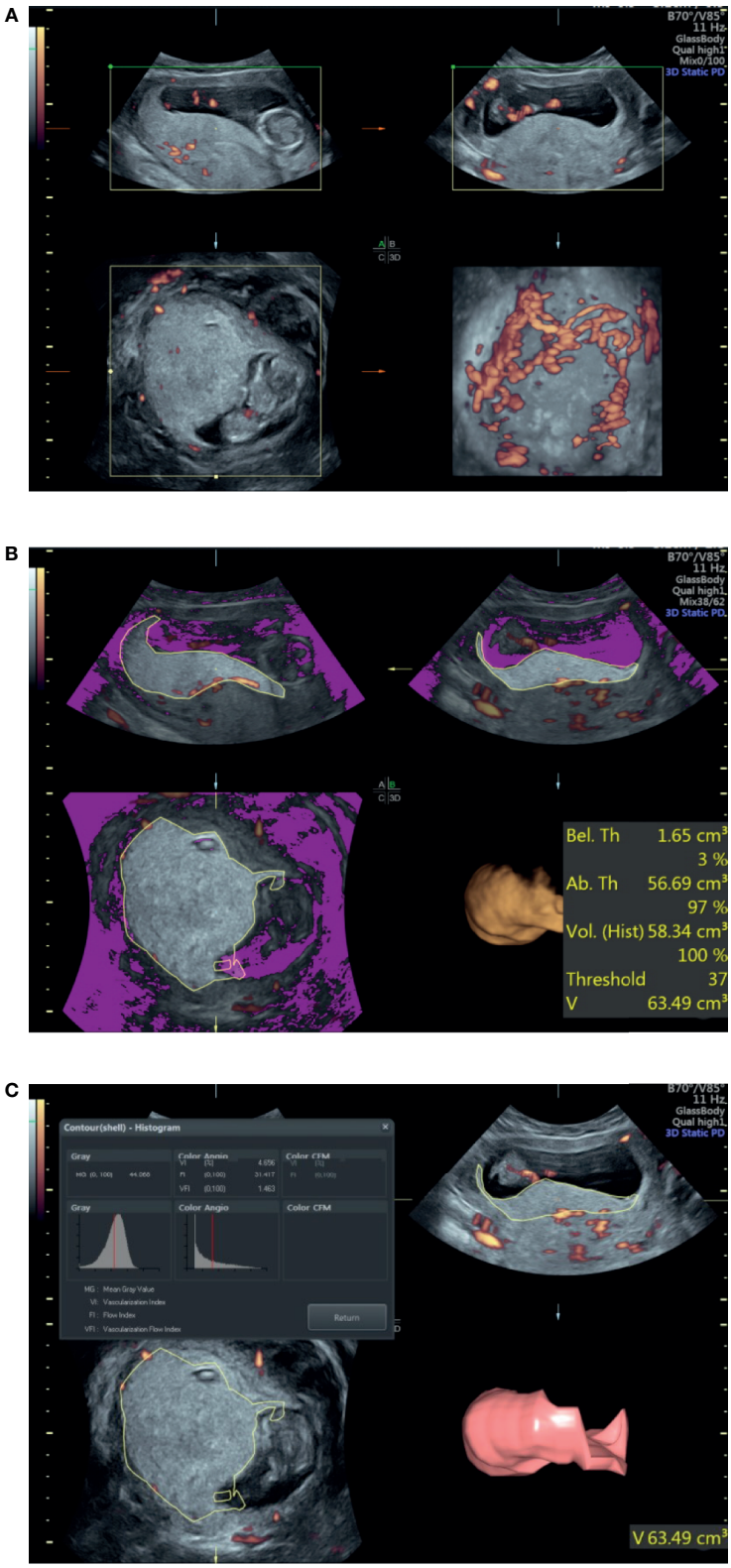


FIGURE 1 | Ultrasound examination of the placental volume and vascular perfusion. **(A)** Placental volume as determined by three-dimensional power Doppler. **(B)** Measurement of placental volume by VOCAL. **(C)** Placental vascular indices determined by three-dimensional power Doppler.

The three vascular indices (VI, FI, and VFI) were developed through specific algorithms based on signal intensity and the relative proportion of color voxels (three-dimensional pixels) within the defined volume, where VFI is a combination of VI and FI information (17). The placental vascular indices were automatically calculated using VOCAL^{TM} imaging software (GE Medical Systems) and expressed on a scale of 0–100 (**Figure 1**).

All pregnant women were followed up after delivery. Screening for GDM was universally performed at 24–28 gestational weeks according to Guideline for Diagnosis and Treatment of Gestational Diabetes Mellitus (2014) (6). The GDM is diagnosed if any of the oral glucose tolerance test plasma glucose values meets or exceeds the following cut-off values: 5.1 mmol/L at fasting; 10.0 mmol/L at 1 h; and 8.5 mmol/L at 2 h. The follow-up data were collected as follows: pregnant complications, gestational age, modes of delivery, 1 and 5 min Apgar scores, birth weight, placental weight, and placental volume after delivery. The placental weight and placental volume after delivery were determined according to previous methods (18).

All the data analyses were performed using SPSS software (version 22.0, IBM, Armonk, USA). The normality of the data was examined by the Kolmogorov-Smirnov test. The Chi-square test was used for categorical variables. The Student's t-test was used to evaluate differences in the continuous data between non-GDM and GDM group, and the continuous data were presented as mean ± standard deviation. Univariate and multivariate logistic regression analyses of individual confounding factor were also performed. Receiver operating characteristic (ROC) and the area under the ROC curve (AUC) were applied to evaluate the diagnostic value and the Youden index is a measure of a diagnostic test's ability to balance sensitivity (detecting disease) and specificity (detecting health or no disease) (19). Youden index was applied to determine the optimal cut-off points of different parameters in the diagnosis of GDM. $P < 0.05$ was considered statistical significance.

RESULTS

Clinical Characteristics of Pregnant Women in the Non-GDM and GDM Group

In this study, a total of 161 pregnant women received ultrasonographic examination. Among these pregnancies, four women had miscarriage during the second trimester; nine women had pregnant complications (hypertension, preeclampsia, or thyroid disease); seven women lost to follow-up. Thus, 106 pregnant women without GDM and 35 pregnant women with GDM were included in this study. As shown in **Table 1**, the maternal age and gravida in the GDM group were significantly higher than that in the non-GDM group (**Table 1**). For the ultrasonographic examination, no significant difference was detected in NT, BPD, CRL, AC, FL, UTPI, and UTRI between non-GDM and GDM group (**Table 1**). There was no significant difference in gestational age at delivery, Caesarean section, and premature birth rates between non-GDM and GDM group (**Table 1**). For the newborns, no significant difference was identified in birth weight, placental weight, PV

TABLE 1 | Comparison of clinical characteristics between non-GDM group and GDM group.

	Non-GDM group (n = 106)	GDM group (n = 35)	*P* value
Maternal			
Maternal age (years old)	30.04 ± 4.24	32.40 ± 4.56	0.004
BMI (kg/m^2)	21.36 ± 2.54	22.34 ± 2.98	0.062
Gravida	1.88 ± 1.03	2.34 ± 1.37	0.035
Parity			
Primiparity	61	17	0.4336
Multiparity	45	18	
Ultrasonography			
NT (mm)	1.45 ± 0.40	1.35 ± 0.38	0.972
BPD (cm)	2.04 ± 0.26	2.03 ± 0.27	0.706
CRL (cm)	6.29 ± 0.82	6.06 ± 0.79	0.160
AC (cm)	6.19 ± 0.82	5.95 ± 0.86	0.128
FL (cm)	0.75 ± 0.20	0.72 ± 0.22	0.335
UTPI	1.43 ± 0.51	1.40 ± 0.45	0.776
UTRI	0.67 ± 0.13	0.64 ± 0.16	0.305
Outcomes			
Gestational age at delivery (weeks)	39.31 ± 1.32	38.89 ± 1.83	0.141
Caesarean section (%)	36.79% (39/106)	40.00% (14/35)	0.841
Premature birth (%)	5.66% (6/106)	2.86% (1/35)	0.681
Birth weight (g)	3203 ± 394	3229 ± 496	0.182
Placental weight (g)	561.50 ± 83.74	548.56 ± 68.31	0.471
Placental volume after delivery (cm^3)	462.83 ± 123.65	462.55 ± 104.36	0.991
1 min Apgar score	9.95 ± 0.25	10.00 ± 0.00	0.275
5 min Apgar score	10.00 ± 0.00	10.00 ± 0.00	–

AC, abdominal circumference; BMI, body mass index; BPD, biparietal diameter; CRL, crown-rump length; FL, Femur length; GDM, gestational diabetes mellitus; NT, nuchal translucency; UTPI, uterine artery doppler pulsatility index; UTRI, uterine artery resistance index.

after delivery, 1 and 5 min Apgar scores, between non-GDM and GDM group (**Table 1**).

Comparison of Placental Volume and Vascular Perfusion Between Non-GDM Group and GDM Group

The gestational age at ultrasound examination was 12.76 ± 0.48 and 12.64 ± 0.43 weeks in non-GDM and GDM group, respectively (**Table 2**). In the non-GDM group, PV (cm^3), VI, FI, and VFI were 50.49 ± 18.53, 13.08 ± 6.30, 31.71 ± 5.71, and 4.15 ± 2.17, respectively (**Table 2**); in the GDM group, the PV (cm^3), VI, FI, and VFI were 43.20 ± 14.07, 9.49 ± 6.46, 29.18 ± 5.46, and 2.82 ± 2.05 (**Table 2**). PV, VI, FI, and VFI in the GDM group were significantly lower than that in the non-GDM group (**Table 2**). The distribution of pregnant women based on maternal age, maternal BMI, gestational age at examination, PV, VI, FI, VFI, gestational age at delivery, placental weight, and PV after delivery was shown in **Figure 2**. In terms of maternal age, the distribution of maternal age was mainly between 25 and 35 years old; while the proportion of patients >35 years old was higher in the GDM group (**Figure 2**). The distribution of BMI, PV, and FI was mainly between 18 and 24 kg/m^2, 30–60 cm^3, and 20–40, respectively (**Figure 2**). In terms of VFI, the distribution of VFI < 2 in GDM group was higher than that in the non-GDM group (**Figure 2**).

TABLE 2 | Comparison of placental volume and vascular perfusion between non-GDM group and GDM group.

	Non-GDM group (n = 106)	GDM group (n = 35)	*P* value
Gestational age at examination (weeks)	12.76 ± 0.48	12.64 ± 0.43	0.184
PV (cm^3)	50.49 ± 18.53	43.20 ± 14.07	0.025
VI (%)	13.08 ± 6.30	9.49 ± 6.46	0.001
FI	31.71 ± 5.71	29.18 ± 5.46	0.023
VFI	4.15 ± 2.17	2.82 ± 2.05	< 0.001

FI, flow index; GDM, gestational diabetes mellitus; PV, placental volume; VFI, vascularization-flow index; VI, vascularization index.

Univariate and Multivariate Logistic Regression Analyses of Clinical Parameters Associated With GDM

Univariate and multivariate analyses were performed to determine the association between clinical parameters and GDM. The univariate analysis showed that maternal age [odds ratio (OR) = 1.13, 95% confidence interval (CI) = 1.03–1.23, P = 0.008], gravida (OR = 2.55, 95% CI = 1.12–5.80, P = 0.0026), PV (OR = 0.97, 95% CI = 0.95–1.00, P = 0.038), VI (OR = 0.90, 95% CI = 0.83–0.97, P = 0.006), FI (OR = 0.92, 95% CI = 0.86–0.99, P = 0.026), and VFI (OR = 0.68, 95% CI = 0.53–0.87, P = 0.002) were significantly associated with GDM (**Table 3**). Multivariate analysis revealed that VI (OR = 0.89, 95% CI = 0.83–0.96, P = 0.003), FI (OR: 0.93, 95% CI = 0.86–1.00, P = 0.038), and VFI (OR = 0.67, 95% CI = 0.52–0.87, P = 0.002) were significantly associated with GDM (**Table 3**); while PV (OR = 0.98, 95% CI = 0.95–1.00, P = 0.076) had no effect on GDM (**Table 3**).

Diagnostic Values of PV, VI, FI, and VFI for GDM

Subsequently, ROC curves were used to evaluate the ability of PV, VI, FI, and VFI for diagnosing GDM. The ROC analysis revealed that PV, VI, FI, and VFI yielded an AUC of 0.63, 0.69,

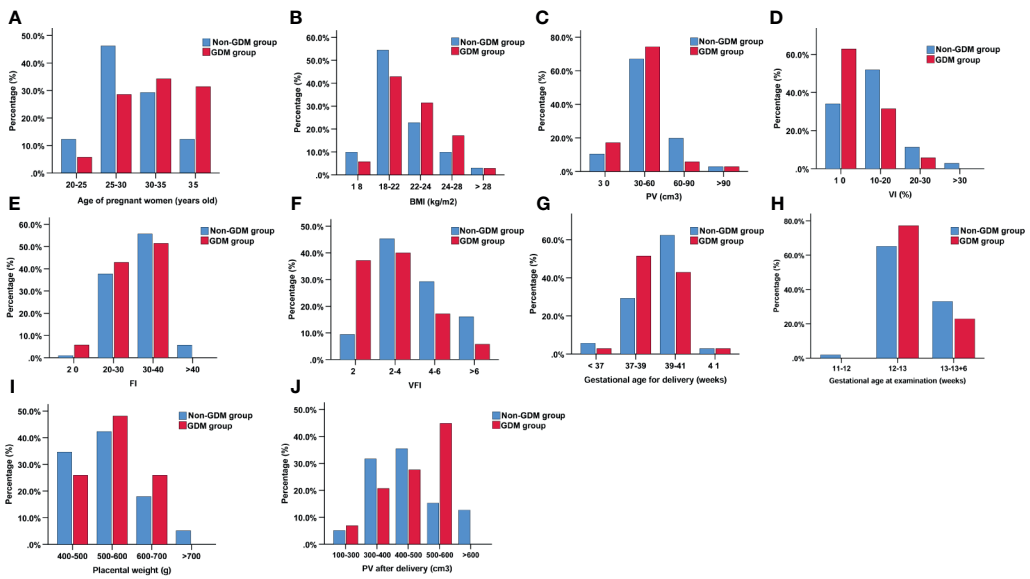


FIGURE 2 | The distribution of pregnant women based on age of pregnant women **(A)**, BMI **(B)**, PV **(C)**, VI (%) **(D)**, FI **(E)**, VFI **(F)**, gestational age for delivery **(G)**, gestational age at examination **(H)**, placental weight **(I)**, and PV after delivery **(J)**.

TABLE 3 | Univariate and multivariate logistic regression analysis of clinical parameters associated with GDM.

Variables	Univariate analysis			Multivariate analysis		
	OR	95% CI	*P* value	OR	95% CI	*P* value
Age	1.13	1.03–1.23	0.008			
Gravida	2.55	1.12–5.80	0.026			
PV (cm^3)	0.97	0.95–1.00	0.038	0.98	0.95–1.00	0.076
VI (%)	0.90	0.83–0.97	0.006	0.89	0.83–0.96	0.003
FI	0.92	0.86–0.99	0.026	0.93	0.86–1.00	0.038
VFI	0.68	0.53–0.87	0.002	0.67	0.52–0.87	0.002

CI, confidence interval; FI, flow index; OR, odds ratio; PV, placental volume; VFI, vascularization-flow index; VI, vascularization index.

0.61, and 0.71, respectively (**Table 4** and **Figure 3**). In addition, based on the Youden index, the results indicated that VFI (Youden index = 0.40) had the best diagnostic value for GDM when compared to VI (Youden index = 0.38), PV (Youden index = 0.23), and FI (Youden index = 0.20; **Table 4** and **Figure 3**). As maternal age and gravida were potential risk factors for GDM in our cohort, we combined maternal age, gravida, PV, and VFI by using the regression model. The ROC analysis revealed an AUC of 0.76 (95% CI = 0.66–0.86, P < 0.001; **Table 4** and **Figure 3**). ROC analysis indicated that the combination of maternal age, gravida, PV and VFI was more accurate as a marker for detecting GDM than the PV, VI, FI, or VFI alone (**Table 4** and **Figure 3E**).

DISCUSSION

According to the demographic data, advanced maternal age is one of the risk factors for GDM. Schaefer et al. found that women older than 35 years old had 3.95-fold increased risk of GDM compared with women aged between 16 and 25 years old (20). A study by analyzing the prevalence of GDM in the USA between 2007 and 2014 indicated that older age was one of the risk factors associated with GDM (21). Shan et al., found that there was a 2–3-fold risk for mothers with advanced maternal age to be diagnosed with GDM in a retrospective cohort study from China (22). Khalil et al., analyzed a population of 22,933 pregnancies, and demonstrated that advanced maternal age was a risk factor for GDM (23). Our results consistently showed that the maternal age in the GDM group was higher than that in the non-GDM group, and maternal age was included in the subsequent ROC analysis.

In this study, we used the VOCAL to determine the PV and placental vascular indices (VI, FI, and VFI). Our results showed that the PV in the GDM group was significantly smaller than that in the non-GDM group during early pregnancy. However, Wong et al., showed that the PV was similar between GDM and non-GDM group during the first trimester, while it was significantly increased in the GDM group during the second trimester (14). Moreover, studies demonstrated that placental calcification and volume increased with advancing gestation in pre-gestational diabetic placentae (12, 24). On the other hand, no significant difference was detected in the PV and placental weight after delivery. These discrepancies may be to the small sample size and the variations in determine the PV after delivery, and future studies should include large samples to confirm our findings. In the analysis of VI, FI, and VFI, previous studies from Hafner et al., showed that VI, FI, and VFI could be used for a quick and reliable first trimester assessment of severe pregnancy risks (25). Consistently, Wong et al., showed that VI, FI, and VFI were significantly lower in the GDM group during the first and second trimesters (14). In addition, VI, FI, and VFI were significantly lower in diabetic pregnancies between 35 and 40 gestational weeks (24). Consistently, our results showed that the above three vascular indices were significantly lower in the GDM group during the early pregnancy. The above results indicated that there was a remarkable reduction in VI, FI, and VFI during GDM.

Based on the logistic regression analysis, our results indicated that reduced PV, VI, FI, and VFI were associated with the increased risk of GDM, which was consistent with the findings from Wong et al. (14). In addition, PV and vascular indices during early pregnancy could be used to predict the pregnant complications (25). Although the PV increases with the advancement in gestational age, the placental vascular index of pregnant women with GDM still decreases during the third

TABLE 4 | Diagnostic values of PV, VI, FI, and VFI for GDM.

Variables	AUC	Sensitivity (%)	Specificity (%)	Cut-off value	95% CI	LR+	LR–	Youden index	*P* value
PV (cm^3)	0.63	65.71	57.55	44.90	0.52–0.73	1.55	0.60	0.23	0.017
VI (%)	0.69	60.00	78.30	8.22	0.57–0.80	2.77	0.51	0.38	0.002
FI	0.61	91.43	28.30	34.93	0.50–0.71	1.28	0.30	0.20	0.053
VFI	0.71	60.00	80.19	2.62	0.60–0.82	3.03	0.50	0.40	<0.001
Regression model	0.761	62.26	82.86	–	0.66–0.86	3.24	0.46	0.45	<0.001

AUC, area under the curve; FI, flow index; LR+, positive likelihood ratio; LR–, negative likelihood ratio; OR, odds ratio; PV, placental volume; VFI, vascularization-flow index; VI, vascularization index.

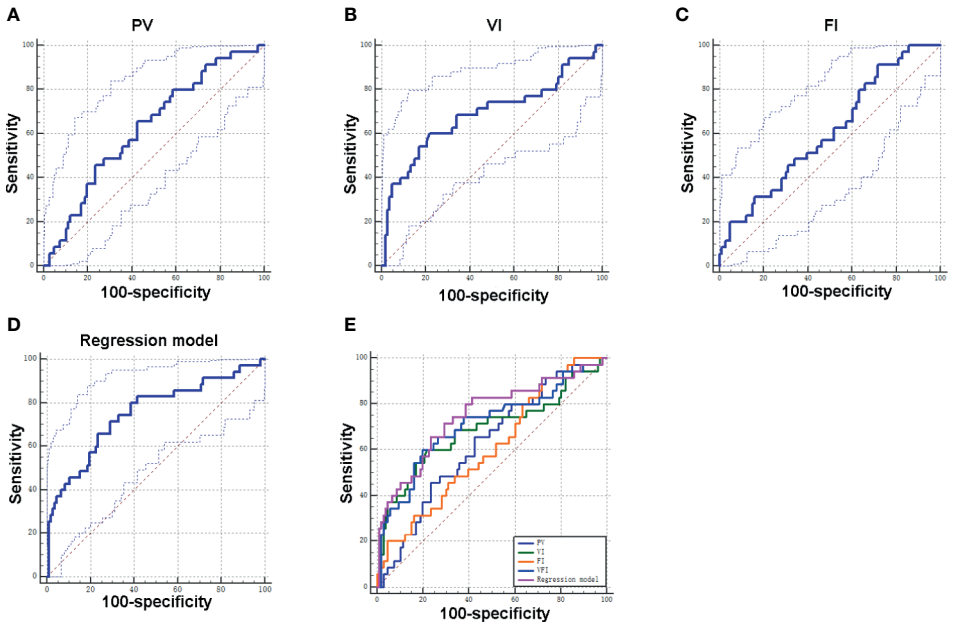


FIGURE 3 | Diagnostic values of PV, VI, FI, and VFI for GDM. **(A)** ROC curve of PV for distinguishing GDM patients from non-GDM patients. **(B)** ROC curve of VI for distinguishing GDM patients from non-GDM patients. **(C)** ROC curve of FI for distinguishing GDM patients from non-GDM patients. **(D)** ROC curve of integrated factors using regression model for distinguishing GDM patients from non-GDM patients. **(E)** ROC curves of individual factor and integrated factors for distinguishing GDM patients from non-GDM patients.

trimester (14). This finding may be related to the abnormal secretion of key mediators in GDM pregnant women in the first trimester, which affects placental vascular remodeling, and finally affects the placental development, leading to increased PV and reduced vascular indices (26, 27). Moran et al., found that PV was significantly larger at all stages of gestation from 12 weeks (24). In our study, the PV was mainly determined between 11^{+0} and 13^{+6} weeks, when the changes in placenta have taken place, thus, suggesting the reliability of our findings.

This study also determined diagnostic potentials of PV, VI, FI, VFI in GDM. According to the results of the ROC curve of each parameter, the AUC of VFI curve is the largest, indicating good predictive value, and its sensitivity and specificity are 60.00 and 80.19%, respectively, which means that the diagnosis rate is high and the missed diagnosis rate is low. In addition, Youden index is the highest for VFI, indicating that it has good clinical practical value. The AUC values of PV, VI, and FI were less than 0.7, indicating that its predictive value is low. FI has the highest sensitivity, but low specificity, which indicates that its misdiagnosis rate is high. Our results were consistent with previous studies showing that VFI may be a more sensitive sonographic marker than VI and FI in the first trimester of GDM placentas (14). Our results of combined maternal age, gravida, PV, and VFI by using the regression model revealed that the combination of these parameters was more accurate as a marker for detecting GDM than the PV, VI, FI, or VFI alone, suggesting that the maternal age, gravida, PV, and VFI may representative important diagnostic markers for GDM.

This study is subjected to several limitations. First of all, the present study is limited to small sample size, and larger population of pregnant women may be included for analysis in the future. Secondly, this study was a single-center prospective study, which may lead to study bias. Multiple-center studies should be considered, in order to confirm our findings. Thirdly, how PV and vascular indices correlate with GDM remains unknown, which still requires the mechanistic investigations.

In conclusion, the 3D ultrasonography results suggest that PV and vascular indices (VI, FI, and VFI) during the first trimester may serve as potential markers for GDM diagnosis. The combination of maternal age, gravida, and sonographic markers may have good diagnostic values for GDM, which should be confirmed by further investigations.

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

This study was approved by the Ethical Committee of Guangzhou iBorn Women's Hospital, and each patient signed

the written informed consent. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HH, YY, and W-HC conceptualized the study and were involved in study design. ZH and YZ collected the data and performed the follow-up. XL and W-HC performed the data analysis. ZH and YZ drafted the manuscript and HH revised it critically. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We would like to thank all the doctors and nurses who facilitated this study at Guangzhou iBorn Women's Hospital and the Third Affiliated Hospital of Sun Yat-Sen University.

REFERENCES

1. Saravanan P. Gestational Diabetes: Opportunities for Improving Maternal and Child Health. *Lancet Diabetes Endocrinol* (2020) 8(9):793–800. doi: 10.1016/S2213-8587(20)30161-3
2. Chiefari E, Arcidiacono B, Foti D, Brunetti A. Gestational Diabetes Mellitus: An Updated Overview. *J Endocrinol Invest* (2017) 40(9):899–909. doi: 10.1007/s40618-016-0607-5
3. Coustan DR. Gestational Diabetes Mellitus. *Clin Chem* (2013) 59(9):1310–21. doi: 10.1373/clinchem.2013.203331
4. Johns EC, Denison FC, Norman JE, Reynolds RM. Gestational Diabetes Mellitus: Mechanisms, Treatment, and Complications. *Trends Endocrinol Metab* (2018) 29(11):743–54. doi: 10.1016/j.tem.2018.09.004
5. Spaight C, Gross J, Horsch A, Puder JJ. Gestational Diabetes Mellitus. *Endocr Dev* (2016) 31:163–78. doi: 10.1159/000439413
6. Juan J, Yang H. Prevalence, Prevention, and Lifestyle Intervention of Gestational Diabetes Mellitus in China. *Int J Environ Res Public Health* (2020) 17(24):9517. doi: 10.3390/ijerph17249517
7. Kc K, Shakya S, Zhang H. Gestational Diabetes Mellitus and Macrosomia: A Literature Review. *Ann Nutr Metab* (2015) 66(Suppl 2):14–20. doi: 10.1159/000371628
8. McIntyre HD, Catalano P, Zhang C, Desoye G, Mathiesen ER, Damm P. Gestational Diabetes Mellitus. *Nat Rev Dis Primers* (2019) 5(1):47. doi: 10.1038/s41572-019-0098-8
9. Plows JF, Stanley JL, Baker PN, Reynolds CM, Vickers MH. The Pathophysiology of Gestational Diabetes Mellitus. *Int J Mol Sci* (2018) 19 (11):3342. doi: 10.3390/ijms19113342
10. Huynh J, Dawson D, Roberts D, Bentley-Lewis R. A Systematic Review of Placental Pathology in Maternal Diabetes Mellitus. *Placenta* (2015) 36(2):101–14. doi: 10.1016/j.placenta.2014.11.021
11. Ahmed B, Abushama M, Khraisheh M, Dudenhausen J. Role of Ultrasound in the Management of Diabetes in Pregnancy. *J Matern Fetal Neonatal Med* (2015) 28(15):1856–63. doi: 10.3109/14767058.2014.971745
12. Pala HG, Artunc-Ulkumen B, Koyuncu FM, Bulbul-Baytur Y. Three-Dimensional Ultrasonographic Placental Volume in Gestational Diabetes Mellitus. *J Matern Fetal Neonatal Med* (2016) 29(4):610–4. doi: 10.3109/14767058.2015.1012066
13. Saha S, Biswas S, Mitra D, Adhikari A, Saha C. Histologic and Morphometric Study of Human Placenta in Gestational Diabetes Mellitus. *Ital J Anat Embryol* (2014) 119(1):1–9. doi: 10.13128/IJAE-14634
14. Wong CH, Chen CP, Sun FJ, Chen CY. Comparison of Placental Three-Dimensional Power Doppler Indices and Volume in the First and the Second Trimesters of Pregnancy Complicated by Gestational Diabetes Mellitus. *J Matern Fetal Neonatal Med* (2019) 32(22):3784–91. doi: 10.1080/14767058.2018.1472226
15. Desoye G, Hauguel-de Mouzon S. The Human Placenta in Gestational Diabetes Mellitus. *Insulin Cytokine Netw Diabetes Care* (2007) 30(Suppl 2): S120–6. doi: 10.2337/dc07-s203
16. Salomon LJ, Alfirevic Z, Bilardo CM, Chalouhi GE, Ghi T, Kagan KO, et al. ISUOG Practice Guidelines: Performance of First-Trimester Fetal Ultrasound Scan. *Ultrasound Obstet Gynecol* (2013) 41(1):102–13. doi: 10.1002/uog.12342
17. Pairleitner H, Steiner H, Hasenoehrl G, Staudach A. Three-Dimensional Power Doppler Sonography: Imaging and Quantifying Blood Flow and Vascularization. *Ultrasound Obstet Gynecol* (1999) 14(2):139–43. doi: 10.1046/j.1469-0705.1999.14020139.x
18. Balihallimath RL, Shirol VS, Gan AM, Tyagi NK, Bandankar MR. Placental Morphometry Determines the Birth Weight. *J Clin Diagn Res* (2013) 7 (11):2428–31. doi: 10.7860/JCDR/2013/7478.3564
19. Martínez-Camblor P, Pardo-Fernández JC. The Youden Index in the Generalized Receiver Operating Characteristic Curve Context. *Int J Biostat* (2019) 15(1):3342. doi: 10.1515/ijb-2018-0060
20. Schaefer KK, Xiao W, Chen Q, He J, Lu J, Chan F, et al. Prediction of Gestational Diabetes Mellitus in the Born in Guangzhou Cohort Study, China. *Int J Gynaecol Obstet* (2018) 143(2):164–71. doi: 10.1002/ijgo.12627
21. Casagrande SS, Linder B, Cowie CC. Prevalence of Gestational Diabetes and Subsequent Type 2 Diabetes Among U. *S Women Diabetes Res Clin Pract* (2018) . 141:200–8. doi: 10.1016/j.diabres.2018.05.010
22. Shan D, Qiu PY, Wu YX, Chen Q, Li AL, Ramadoss S, et al. Pregnancy Outcomes in Women of Advanced Maternal Age: A Retrospective Cohort Study from China. *Sci Rep* (2018) 8(1):12239. doi: 10.1038/s41598-018-29889-3
23. Londero AP, Rossetti E, Pittini C, Cagnacci A, Driul L. Maternal Age and the Risk of Adverse Pregnancy Outcomes: A Retrospective Cohort Study. *BMC Pregnancy Childbirth* (2019) 19(1):261. doi: 10.1186/s12884-019-2400-x
24. Moran M, Mulcahy C, Daly L, Zombori G, Downey P, McAuliffe FM. Novel Placental Ultrasound Assessment: Potential Role in Pre-Gestational Diabetic Pregnancy. *Placenta* (2014) 35(8):639–44. doi: 10.1016/j.placenta.2014.03.007
25. Hafner E, Metzenbauer M, Stümpflen I, Waldhör T. Measurement of Placental Bed Vascularization in the First Trimester, Using 3D-Power-Doppler, for the Detection of Pregnancies At-Risk for Fetal and Maternal Complications. *Placenta* (2013) 34(10):892–8. doi: 10.1016/j.placenta.2013.06.303
26. Thadhani R, Wolf M, Hsu-Blatman K, Sandler L, Nathan D, Ecker JL. First-Trimester Sex Hormone Binding Globulin and Subsequent Gestational Diabetes Mellitus. *Am J Obstet Gynecol* (2003) 189(1):171–6. doi: 10.1067/mob.2003.343
27. Nanda S, Savvidou M, Syngelaki A, Akolekar R, Nicolaides KH. Prediction of Gestational Diabetes Mellitus by Maternal Factors and Biomarkers at 11 to 13 Weeks. *Prenat Diagn* (2011) 31(2):135–41. doi: 10.1002/pd.2636

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 28 July 2021
doi: 10.3389/fendo.2021.694885

Perfluoroalkyl Substance Exposure and the BDNF Pathway in the Placental Trophoblast

Melissa J. Marchese[1†], *Shuman Li*[2†], *Bin Liu*[2], *Jun J. Zhang*[2*] *and Liping Feng*[1,2*]

[1] *Department of Obstetrics and Gynecology, Duke University, Durham, NC, United States,* [2] *Ministry of Education-Shanghai Key Laboratory of Children's Environmental Health, School of Public Health, Shanghai Jiao-Tong University School of Medicine, Shanghai, China*

Background: Per- and polyfluoroalkyl substances (PFAS) are persistent organic pollutants that have become globally ubiquitous in humans and the environment. *In utero* PFAS exposure is associated with neurodevelopmental effects; however, the mechanism is poorly understood. Brain-derived neurotrophic factor (BDNF) signaling is critical to fetal neurodevelopment during pregnancy and maintains important regulatory roles later in life. This study aims to characterize placental BDNF signaling and investigate whether PFAS exposure disrupts the signaling pathway in placental trophoblast cells.

Methods: The expression and localization of BDNF receptors–$p75^{NTR}$ and TrkB–in first trimester and term human placentas and trophoblast cells were investigated by immunofluorescence staining. To assess the effects of PFAS exposure on the BDNF pathway, BeWo cells were treated with PFAS mixtures that mimicked blood levels in a highly exposed population and major PFAS compounds in the mixture at 0.01, 0.1, 1, and 10 μM concentrations. Changes in pro-BDNF levels and phosphorylation of TrkB receptors were examined by Western blot.

Results: In first trimester human placentas, TrkB and $p75^{NTR}$ receptors were primarily localized to syncytiotrophoblast and cytotrophoblast cells. At term, TrkB and $p75^{NTR}$ receptors were primarily observed in the placental villous stroma. TrkB receptor staining in trophoblasts was reduced at term, while $p75^{NTR}$ receptor staining was negative. TrkB receptors were confined to the nuclear and perinuclear spaces, and phosphorylation occurred at the Tyr816 residue in BeWo cells. Exposure to PFOS, PFOA, PFBS, and the six-PFAS mixture did not significantly affect BDNF levels or activation (phosphorylation) of TrkB. Treating cells with 1 μM and 10 μM of PFNA resulted in increased TrkB phosphorylation compared to unexposed controls, but BDNF levels were unchanged.

Conclusions: BDNF receptors are present in different regions of human placental villi, indicating diverse functions of BDNF signaling in placental development. Our findings suggest that the BDNF pathway in placental trophoblast cells is not disrupted by exposures to PFOS, PFOA, PFBS, and a PFAS mixture, but may be affected by PFNA

OPEN ACCESS

Edited by:
Qiongjie Zhou,
Fudan University, China

Reviewed by:
Udo Jeschke,
Ludwig-Maximilians-Universität München, Germany
Gendie Lash,
Guangzhou Medical University, China

****Correspondence:***
Liping Feng
liping.feng@duke.edu
Jun J. Zhang
junjimzhang@sina.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *13 April 2021*
Accepted: *12 July 2021*
Published: *28 July 2021*

Citation:
Marchese MJ, Li S, Liu B, Zhang JJ and Feng L (2021) Perfluoroalkyl Substance Exposure and the BDNF Pathway in the Placental Trophoblast. Front. Endocrinol. 12:694885. doi: 10.3389/fendo.2021.694885

exposures. Further investigation is needed on how PFAS affects other critical signaling pathways during fetal neurodevelopment.

Keywords: Perfluoroalkyl substances, BDNF, TrkB, placenta, trophoblast

INTRODUCTION

Per- and polyfluoroalkyl substances (PFAS) are synthetic carbon fluorine compounds used in industry and commerce since the 1940s (1). The stability and surfactant properties exhibited by PFAS have led to widespread use in products from stain-resistant textiles to firefighting foam—making them ubiquitous in the environment (2, 3). Biomonitoring data have demonstrated global accumulation in humans and biota *via* contaminated drinking water, food and its packaging, outdoor air, house dust, and soil (4–6). Previous studies estimate over 95% of Americans to have detectable levels of PFAS in their blood, but certain communities face disproportionate exposure risk (7–9). One such affected population is in Pittsboro, North Carolina, where sum PFAS concentrations up to 1,076 ng/L and 270.8 ng/L were measured at drinking water intakes and in finished household drinking water, respectively.[1] These numbers are over 30 times higher than those in the neighboring city of Durham and are strikingly higher than surrounding cities.[1] The blood levels of PFAS are between two and four times higher in Pittsboro residents than the general United States population.[2]

Concern surrounding PFAS has risen among affected North Carolina residents due to the potential deleterious human health effects associated with exposure. A growing body of robust laboratory and epidemiologic data link PFAS to increased risk of cancer, immune system dysfunction, poor antibody response to vaccines, hormone disruption, kidney disease, thyroid disorders, high cholesterol, pregnancy-induced hypertension, preeclampsia, asthma, and decreased fertility (10). Fetuses are particularly vulnerable to PFAS exposure because PFAS readily cross the placental barrier and are transferred to developing babies (11–13). Early-life exposure to various PFAS compounds is associated with an array of developmental and neurobehavioral impacts. Previous early childhood studies observed associations between PFAS exposure and delays in gross motor development (14), worsened visual motor abilities (15), lower IQ test scores (16), externalizing behavioral difficulties (17–19), poorer executive functioning (20), neuropsychological developmental problems (21), and attention deficit hyperactivity disorder (ADHD) and related diagnostic symptoms (19, 22, 23). Other observational studies of child neurobehavior report mixed or null associations (24–29). Despite the effects of PFAS on human development gaining more attention, a consensus on the neurodevelopmental consequences of exposure has yet to be reached and further investigation is necessary.

Although the mechanism of neurotoxicity remains unclear, one pathway that may be implicated is the production of brain-derived neurotrophic factor (BDNF). BDNF is essential to mammalian nervous system development and function (30). BDNF primarily interacts with two receptors, $p75^{NTR}$ and tropomyosin sensitive receptor kinase B (TrkB) (31, 32). Abnormalities in the BDNF pathway have been linked to psychiatric disorders such as ADHD, schizophrenia, major depression, autism, bipolar disorder, Alzheimer's, and Parkinson's diseases (1, 5, 33–38). Reduced expression of BDNF has been shown to impair working memory, reduce volume of the cerebellum and hippocampus, and diminish cognitive ability (39). Neurotrophins like BDNF play a role in both prenatal and postnatal brain development and may offer neuroprotective effects (40–42). BDNF supports differentiation of neuronal cell types and influences synaptic properties in the peripheral and central nervous systems during early development (41, 43, 44). During pregnancy, the majority of BDNF is derived from either the mother or the placenta and regulates fetal and placental development (45–48). BDNF contributes to fetal growth by promoting survival, proliferation, migration, and differentiation of cytotrophoblasts in addition to angiogenesis, vessel stabilization, regulation of growth factors, and control of energy homeostasis (48–51). Changes in the BDNF pathway may lead to abnormal placental development and pregnancy complications such as preeclampsia (52, 53). However, the characteristics of the BDNF expression in the human placenta are poorly understood. *BDNF* is a plastic gene whose expression is highly responsive to external stimuli and environmental exposures (30, 35, 37, 54). Significant PFAS-attributable alterations in BDNF expression have been observed in several animal model and cell studies, but conclusions are largely inconsistent (55–59).

This study aims to characterize the BDNF pathway in human placental tissues and trophoblast cell lines. Further, we seek to investigate how the BDNF pathway in placental trophoblast cells is affected by exposures to a PFAS mixture—which models the exposure of Pittsboro, North Carolina residents (based on blood levels)—and major PFAS compounds in this mixture.

MATERIALS AND METHODS

Chemicals and Reagents

Potassium nonafluoro-1-butanesulfonate (K+PFBS, CAS No. 29420-49-3), tridecafluorohexane-1-sulfonic acid potassium salt (K+PFHxS), and heptadecafluorooatanesulfonic acid potassium salt (K+PFOS) were purchased from Sigma-Aldrich (St. Louis,

[1] https://www.northcarolinahealthnews.org/2019/07/30/pfas-shows-up-in-haw-river-pittsboro-water-but-little-local-outcry/

[2] https://www.northcarolinahealthnews.org/2020/10/29/duke-study-finds-high-pfas-levels-in-pittsboro-residents-blood/

MO). Oerfluorohexanoic acid (PFHxA), perfluoro-n-octanoic acid (PFOA), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) were purchased from Synquest Laboratories (Alachua, FL). Product purity is above 95%. Stock solutions of PFAS at 10, 1, 0.1, 0.01 mM were prepared by dissolving PFAS compounds in ultrapure distilled water. A PFAS mixture was made based on the PFAS levels in blood samples collected from people living in Pittsboro, NC, in 2019 (**Table 1**).[3] Forskolin (Wako Chemicals, Japan) stock solution was reconstituted in DMSO at 80 mM.

Cell Culture and PFAS Treatments

An immortalized human choriocarcinoma cell line (BeWo cells, gifted by Dr. Sallie Permar, Duke University, Durham, North Carolina) was cultured in Dulbecco's Modified Eagle Medium-Hams/F12 (DMEM/F12) media supplemented with 10% Fetal Bovine Serum (FBS) and 1x antibiotic-antimycotic solution (Thermo Fisher Scientific). Cell culture and treatment conditions are described in our previous publications (60, 61). In short, after 60-70% confluency was reached, cells were treated for 48 hours with DMSO (1:2000 dilution) or 40 μM forskolin (FSK) to induce cell fusion, thereby modeling syncytialization of cytotrophoblasts (CTB) during placental development. Culture media supplemented with fresh 40 μM FSK was changed every 24 hours to ensure complete cell fusion. BeWo cells with and without FSK treatments were then treated with individual PFAS compounds at 0, 0.01, 0.1, 1, and 10 μM concentrations or the PFAS mixture (1:1, 1:10, 1:50, 1:100 dilutions; **Table 1**) for 24 hours. These doses were chosen for cellular function studies based on human exposure levels.[3] To ensure the effects on cellular functions are not due to cytotoxicity, we used doses at least 10 times lower than LD10 (61). The following methods for immunofluorescence staining and Western blot were performed as described in Marinello et al. (62).

Immunofluorescence Staining of Placental Tissues

First trimester placental tissues were collected from elective pregnancy termination patients without notifiable complications at 8-10 gestational weeks. The study protocol was approved by the Ethics Committees of Xinhua Hospital affiliated with Shanghai Jiao Tong University School of Medicine (IRB# XHEC-C-2018-089) with the consent waiver to obtain de-identified tissue that was not to be used for clinical purposes. Term placental tissues were collected under the Duke University Institutional Review Board approval (IRB# PRO00014627) with the consent waiver to obtain de-identified tissue that was not to be used for clinical purposes. The placentas were collected from women who underwent planned cesarean delivery at term (39 to 40 gestational weeks), without labor and current or previous pregnancy complications.

Sections of placental tissues were fixed and paraffin embedded. Tissue slides were deparaffinized with xylene followed by graded rehydration in ethanol and distilled water.

[3] https://sites.nicholas.duke.edu/pfas/pfas-research-in-pittsboro-nc/pfas-study-results/

TABLE 1 | PFAS mixture (ng/g) administered to cell cultures based on levels in blood samples collected from residents of Pittsboro, North Carolina.

PFOS	12.10
PFOA	7.00
PFHxS	3.10
PFNA	1.80
PFDA	0.75
PFHxA	0.60
Total	25.35

Subsequently, the sections were subjected to heat-induced epitope retrieval by heating in antigen unmarking solution (Vector Laboratories, Inc, Burlingame, California) preheated to boiling for 20 minutes, followed by a 20-minute cool-down period at room temperature. Slides were permeabilized and blocked with 1% BSA, 10% normal goat serum and 0.01% tween-20 in PBS for 60 min at room temperature. After blocking, the slides were incubated with primary antibodies in blocking buffer overnight at 4°C in humidified chambers. Primary rabbit anti-human syndecan 1 (SDC-1) antibody (Sigma, catalog no. HPA006185), mouse anti-human TrkB antibody (R & D System, cat#: MAB397), or p75NTR antibody (R & D System, cat#: MAB3671) was used at a 1:200 dilution. Goat anti-rabbit Alexa Fluo 488 secondary antibody and Goat anti-mouse Alexa Fluo 594 secondary antibody (Life Technologies, Carlsbad, CA) were used at 1:300 dilution. Nuclei were stained using NucBlue Fixed Cell ReadyProbes Reagent (DAPI, Invitrogen). Slides were mounted using ProLong Diamond Antifade Mountant (Invitrogen) and examined with a Leica SP5 inverted confocal fluorescence microscope.

Immunofluorescence Staining of BeWo Cells

BeWo cells were seeded at a density of 2 x 10^4 cells per well in glass chamber slides (ibidi GmbH, Germany) then incubated for 24 hours. Cells were fixed with cold methanol for 10 minutes and blocked with 1% BSA, 5% goat serum, and 0.1% Tween-20 in PBS for 1 hour at room temperature. Next, cells were incubated at 4°C overnight with primary antibodies in humidified chambers. 1:100 dilutions of primary rabbit anti-human TrkB antibodies (Abcam, cat#: ab18987) and p75NTR antibodies (Abcam, cat#52987) were used. Anti-rabbit IgG antibodies served as a negative control (R & D system, Minneapolis, MN). 1:300 dilutions of goat anti-rabbit secondary antibodies and Alexa Fluo 594 (Life Technologies, Carlsbad, CA) were used. F-actin was stained using fluorescein Phalloidin (Invitrogen, cat# F432) at 1:300 dilution. Nuclei were stained using NucBlue Fixed Cell ReadyProbes Reagent (DAPI, Invitrogen). Slides were mounted using ProLong Diamond Antifade Mountant (Invitrogen) and examined with a Leica SP5 inverted confocal fluorescence microscope.

Cell Viability Assay

BeWo cells were seeded at a density of 1 x 10^4 cells per well in 96-well plates and incubated for 24 hours. The cells were then treated with each individual PFAS compound at 0, 0.01, 0.1, 1, 10 μM or a PFAS mixture (**Table 1**) at 1:1, 1:10, 1:50, 1:100 dilutions

in quadruplicate for 24 hours. Cell viability was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit as instructed by the manufacturer instructions (G5421; Promega, Madison, WI). The optical density at 490 nm (OD490) was used to quantitatively compare cell viability between treatments after incubating the cells with MTS reagents for 4 hours. These experiments were repeated five times (N=5).

Western Blot

To harvest cell lysates after treatment, we used RIPA buffer (Sigma Aldrich, St. Louis, MO) with the complete mini-protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were determined with the Bradford assay (Bio-Rad Laboratories, Hercules, CA). 25µg of total protein samples were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels, separated, and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% milk in Tris-Buffered Saline and Tween-20 (TBST) buffer. Membranes were probed in blocking buffer at 4°C with primary antibodies overnight. The following primary antibodies were used in the experiments: rabbit anti-human TrkB antibody (Abcam, cat#: ab18987), phosphor-TrkB (Tyr816) antibody (Cell Signaling Technology, cat#4168), and rabbit anti-human pro-BDNF antibody (Invitrogen, cat# PA1-18360) at 1:1000 dilution; rabbit anti-human GAPDH antibody at 1:20 000 dilution. The secondary antibody was diluted at a ratio of 1:2000. To visualize and photograph the membranes, we used the ChemiDoc MP Imaging System with Image Lab Software (Bio-Rad, Berkeley, CA). Band intensity was quantified with ImageJ (NIH, Bethesda, MD). Data were normalized to the internal control (GAPDH) and are presented as ratios. Experiments were repeated four times (N=4).

Statistical Analysis

Data are presented as means ± SD. Multiple comparisons between treatments were performed with a one-way ANOVA and the post-hoc Dunnett's test using GraphPad Prism 6.0 (La Jolla, CA). Results from treatment groups are compared to unexposed control cells. A p-value smaller than 0.05 is considered statistically significant.

RESULTS

Localization of BDNF Receptors in Placental Tissues

Syndican-1 (SDC-1), a biomarker for syncytiotrophoblast (STB) was stained green on their epical membrane, TrkB and $p75^{NTR}$ receptors were stained red, and nuclei were stained blue (**Figure 1**). In the first trimester villous placenta, both TrkB and $p75^{NTR}$ staining were most pronounced in the STB and cytotrophoblast cells (CTBs) (**Figure 1A**). Staining was more intense in the STB compared to the CTBs for the $p75^{NTR}$. Spot staining was observed in placental villous stroma tissue for the $p75^{NTR}$ and TrkB.

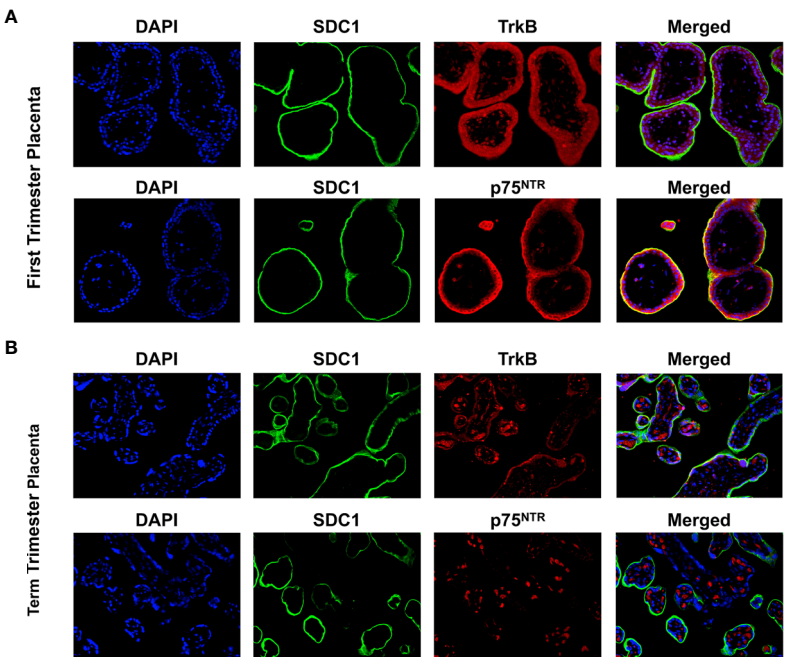


FIGURE 1 | Expression of BDNF receptors in human placental tissues. **(A)** Representative confocal immunofluorescence staining images of showing DAPI (blue) and SDC1 (a syncytiotrophoblast biomarker, green) along with TrkB (red) and $p75^{NTR}$ (red) receptors in the first trimester placenta. **(B)** Confocal immunofluorescence staining images showing DAPI, SDC1, TrkB, and $p75^{NTR}$ in the term placenta.

In term villous placenta, TrkB and $p75^{NTR}$ receptors were primarily observed in the placental villous stroma distinguished it from the first trimester placenta (**Figure 1B**). TrkB receptor staining in STB was reduced, while $p75^{NTR}$ receptor staining was negative. Given these results and our focus on trophoblast cells, TrkB receptors in BeWo cells were examined further in this study.

Localization and Expression of TrkB in BeWo Cells

The presence of TrkB receptors in placental BeWo cells was evaluated through immunofluorescence staining. BeWo cells were cultured with and without forskolin treatments. Forskolin treatment was administered to induce fusion of the cells, modeling the villous trophoblast syncytialization (63). F-actin staining distinguished individual BeWo cells before forskolin treatment and diminished in fused cells after treatment. TrkB receptors appeared to be localized in the nuclei, and some appeared to be translocated into the perinuclear region after forskolin-induced fusion. Representative images of TrkB staining in BeWo cells are presented in **Figure 2**.

PFAS-Induced Disruption of the BDNF Pathway in BeWo Cells

After confirming the presence of TrkB receptors, we examined the effects of PFAS exposure on BDNF production and the ratio of phosphorylated TrkB to total TrkB in BeWo cells with and without forskolin treatment by Western blots. First, the MTS cell viability assay confirmed that the doses used in this study did not cause cell death. Next, we screened the phosphorylation sites, Tyr516, Tyr706/707, and Tyr816 of TrkB receptors in BeWo cells. The phospho-TrkB (Tyr816) was widely detected in these cells with and without forskolin treatment. Finally, Western blot results demonstrated that exposure to PFOS, PFOA, PFBS, and the six-PFAS mixture did not significantly alter BDNF production or activation of TrkB, manifested by the constant ratio of phospho-TrkB (Tyr816) to total TrkB at any given dose (**Figures 3–7**). Although BDNF production was not changed by PFNA exposures, the ratio of phospho-TrkB (Tyr816) to total TrkB was significantly higher in forskolin-treated BeWo cells exposed to 10 μM and 1 μM of PFNA than in unexposed forskolin-treated BeWo cells (P=0.02; **Figure 3**). The same trend was observed in BeWo cells without forskolin treatment but was not significant (**Figure 3**).

DISCUSSION

This study investigates the BDNF signaling pathway in placental cells and how it is affected by exposure to various PFAS compounds and a PFAS mixture mimicking residents' blood levels in Pittsboro, North Carolina, USA. Confocal immunofluorescence staining results show that TrkB receptors are present in trophoblasts in the first trimester and term placental villi. Similar expression of $p75^{NTR}$ receptors was observed in first trimester placental tissues. At term, however, $p75^{NTR}$ receptors were primarily located in the placental villous stroma. Immunofluorescence staining results reveal that TrkB receptors are localized to the nuclear and perinuclear regions in BeWo cells. Western blot results indicate that TrkB receptors are activated in BeWo cells by phosphorylation at the Tyr816 site. Additionally, Western blots demonstrate that PFNA exposure at 1 μM and 10 μM doses increased the ratio of phospho-TrkB (Tyr816) to total TrkB. Exposure to other PFAS compounds (PFOS, PFOA, PFBS) and the PFAS mixture did not significantly alter BDNF signaling, as evidenced by unchanged levels of BDNF and phospho-TrkB. Thus, the impacts of PFAS exposure on fetal neurodevelopment previously observed in cohort studies (14–23) do not necessarily occur *via* the placental BDNF pathway.

Although BDNF signaling has been extensively studied in neuronal tissues and cells, little is known about this pathway in

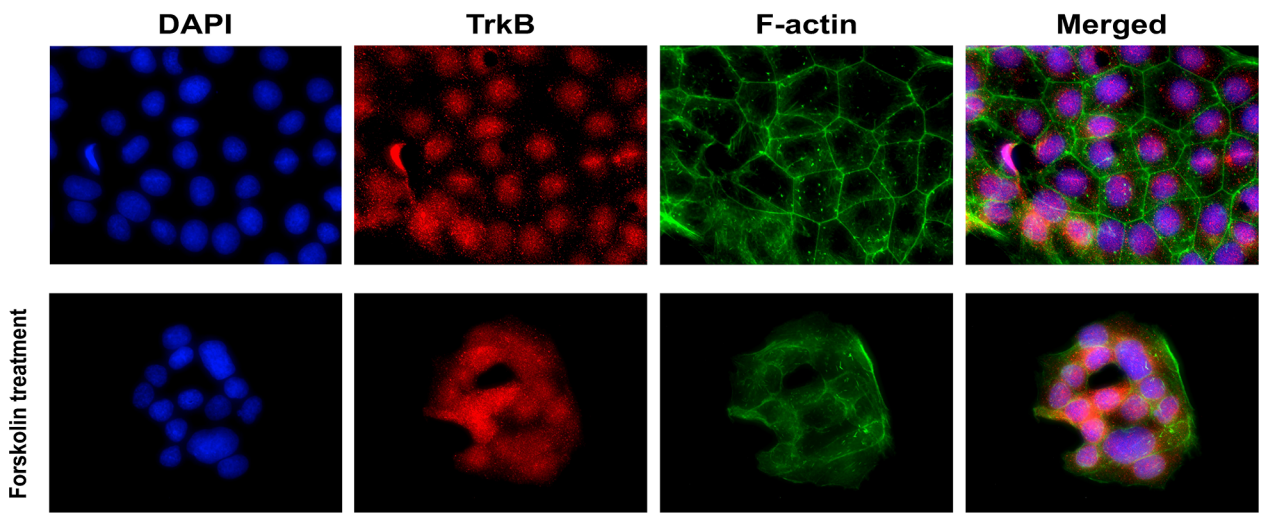


FIGURE 2 | Localization of BDNF receptors in placental BeWo cells. Representative immunofluorescent images showing DAPI, TrkB, and f-actin.

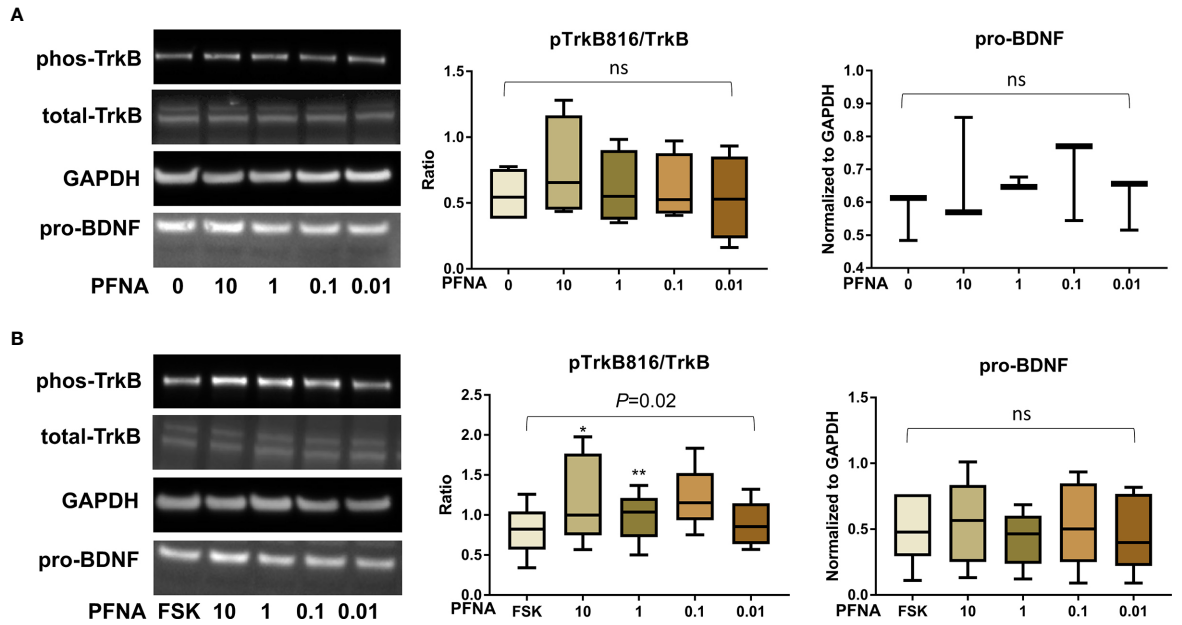


FIGURE 3 | Altered TrkB phosphorylation at high concentrations of PFNA. **(A)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells without forskolin (FSK) treatment exposed to PFNA (0, 0.01, 0.1, 1, and 10 μM) and box plots for densitometry analysis; **(B)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells with FSK treatment exposed to PFNA (0, 0.01, 0.1, 1, and 10 μM) and box plots for densitometry analysis. ns indicates differences in protein levels were not significant. pTrkB816 represents for phosphorylation of TrkB at Tyr816 residue. *P < 0.05 **P < 0.01 compared to unexposed forskolin-treated cells.

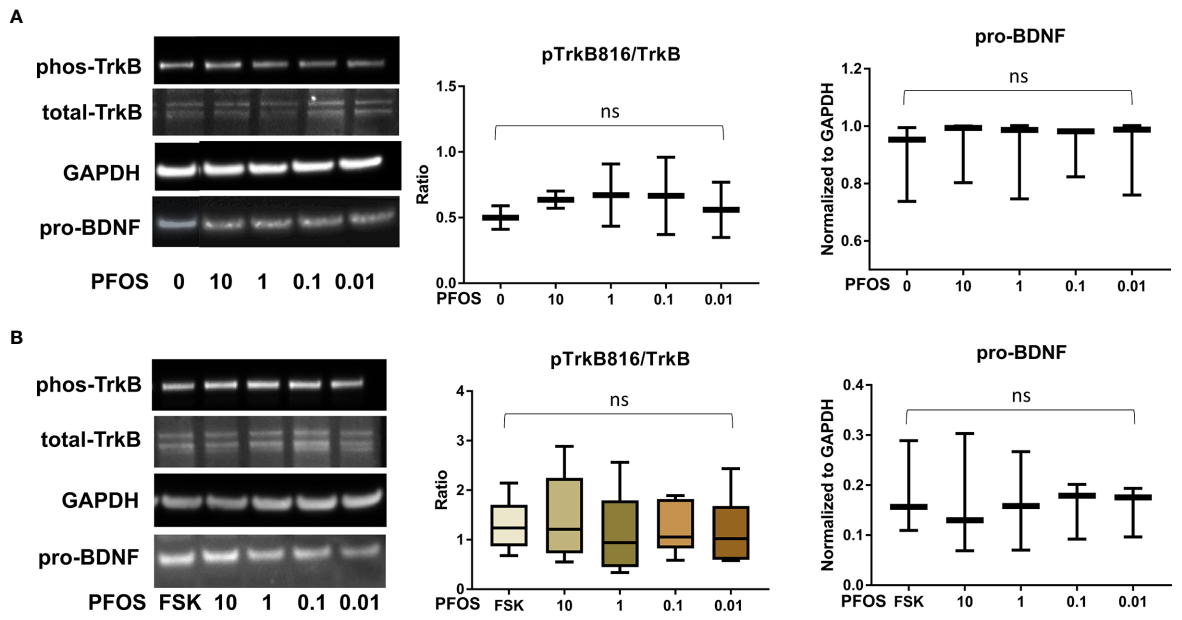


FIGURE 4 | PFOS exposure did not significantly disrupt the BDNF-TrkB pathway. **(A)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells without forskolin (FSK) treatment exposed to PFOS (0, 0.01, 0.1, 1, and 10 μM) and box plots for densitometry analysis; **(B)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells with FSK treatment exposed to PFOS (0, 0.01, 0.1, 1, and 10 μM) and box plots for densitometry analysis. ns indicates differences in protein levels were not significant. pTrkB816 represents for phosphorylation of TrkB at Tyr816 residue.

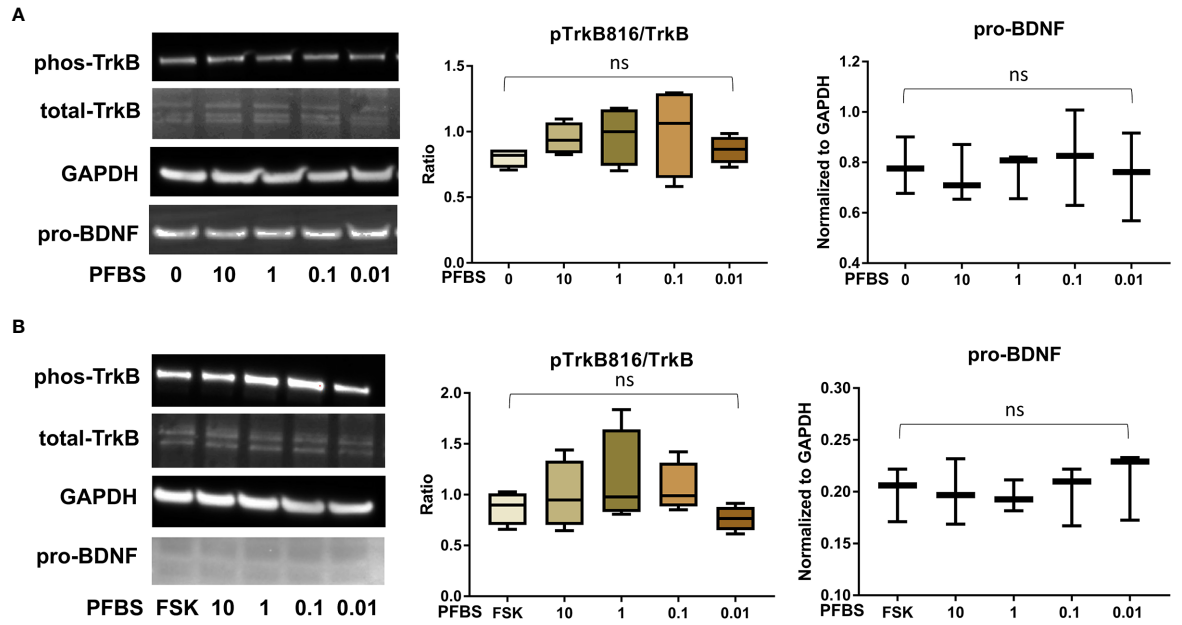


FIGURE 5 | PFBS exposure did not significantly disrupt the BDNF-TrkB pathway. **(A)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells without forskolin (FSK) treatment exposed to PFBS (0, 0.01, 0.1, 1, and 10 μM) and box plots for densitometry analysis; **(B)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells with FSK treatment exposed to PFBS (0, 0.01, 0.1, 1, and 10 μM) and box plots for densitometry analysis. ns indicates differences in protein levels were not significant. pTrkB816 represents for phosphorylation of TrkB at Tyr816 residue.

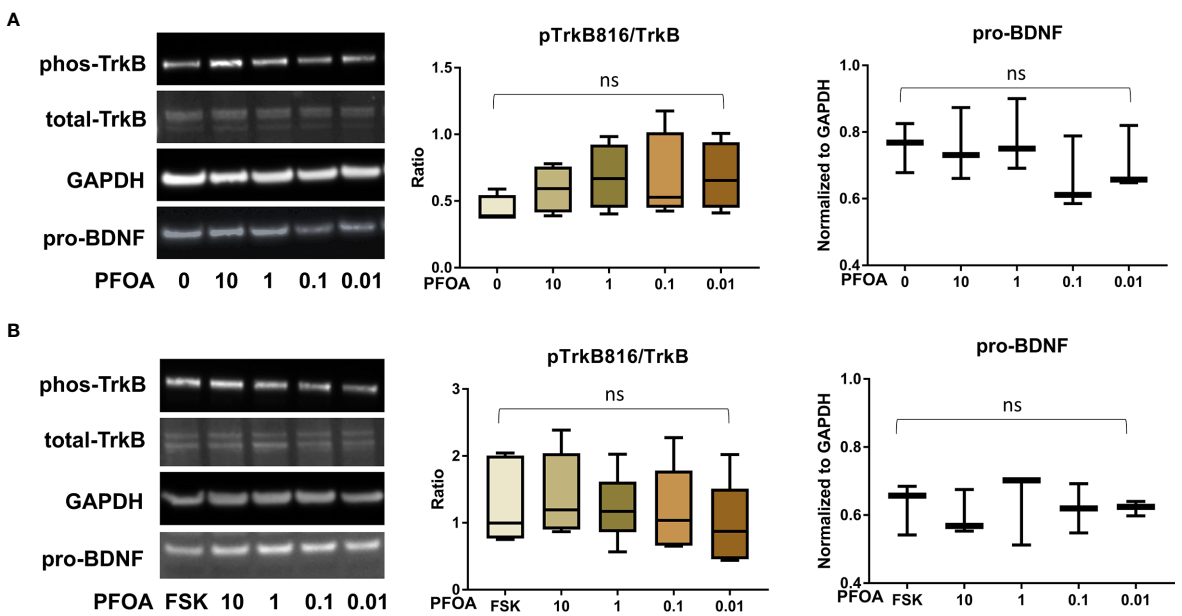


FIGURE 6 | PFOA exposure did not significantly disrupt the BDNF-TrkB pathway. **(A)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells without forskolin (FSK) treatment exposed to PFOA (0, 0.01, 0.1, 1, and 10 μM) and box plots for densitometry analysis; **(B)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells with FSK treatment exposed to PFOA (0, 0.01, 0.1, 1, and 10 μM) and box plots for densitometry analysis. ns indicates differences in protein levels were not significant. pTrkB816 represents for phosphorylation of TrkB at Tyr816 residue.

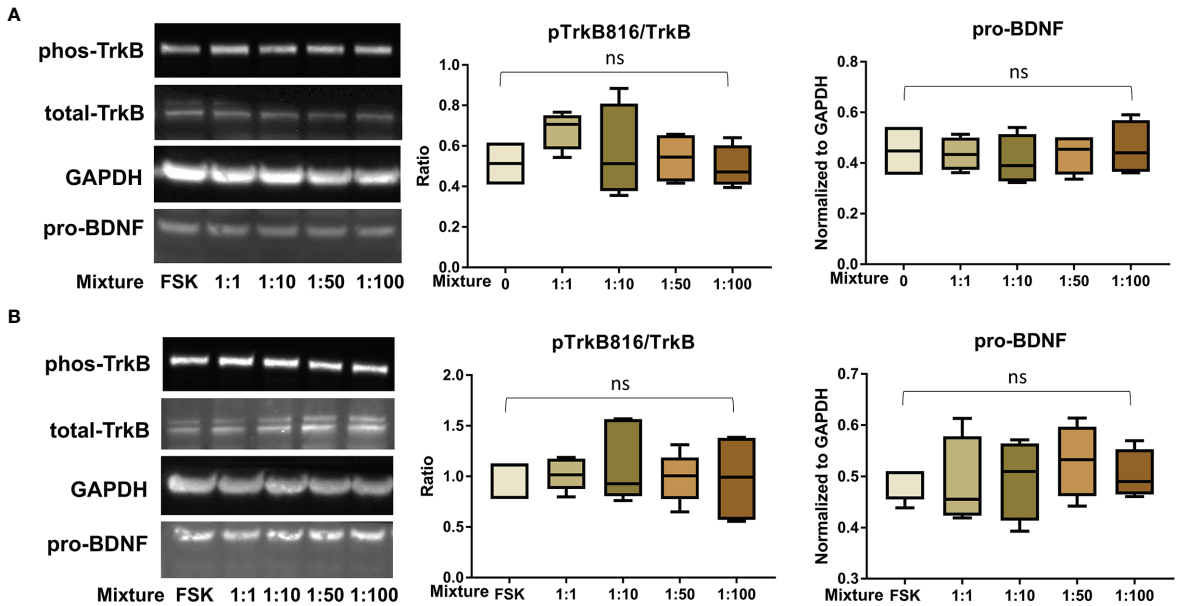


FIGURE 7 | PFAS mixture exposure (detailed in **Table 1**) did not significantly disrupt the BDNF-TrkB pathway. **(A)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells without forskolin (FSK) treatment exposed to mixture dilutions (0, 1:1, 1:10, 1:50, 1:100) and box plots for densitometry analysis; **(B)** Representative Western blot images of phos-TrkB, total-TrkB, GAPDH, and pro-BDNF in BeWo cells with FSK treatment exposed to mixture dilutions (0, 1:1, 1:10, 1:50, 1:100) and box plots for densitometry analysis. ns indicates differences in protein levels were not significant. pTrkB816 represents for phosphorylation of TrkB at Tyr816 residue.

gestational tissues. The current study further characterizes the BDNF pathway in placental tissues. BDNF is first synthesized as a precursor protein, prepro-BDNF, which is converted into pro-BDNF by removal of the signal peptide (64). Once the protein is secreted, pro-BDNF is converted to mature BDNF (mBDNF) through proteolytic cleavage by furin and other proprotein convertases (65). We were able to detect pro-BDNF by Western blots in BeWo cell lysates. However, mBDNF levels were close to the limits of detection in BeWo cell culture media, which could be due to limited pro-BDNF secretion or cleavage in our current culture condition. mBDNF is preferentially released *via* a tightly controlled pathway driven by activity-dependent depolarization and Ca^{2+} entry (66). BDNF binds to specific receptors and regulates distinct biological functions. The BDNF receptors in this study were localized in discrete placental domains, indicating that BDNF exhibits distinct functions in different placental cells. BDNF binding to TrkB results in TrkB activation and autophosphorylation at Tyr sites (67). The activated TrkB receptors can induce an array of intracellular signaling cascades (43). TrkB receptor activation at the Tyr490 and Tyr515 residues regulates MAPK/ERK kinases and the PI3K/Akt pathway (68). Both of these pathways activate transcription factors (CREB and C-myc) which trigger neurotrophic functions of cell survival, growth, and differentiation (68). Phosphorylation of TrkB receptors at the Tyr816 site activates phospholipase C γ (PLC γ) (68, 69). The phosphorylation of Tyr706/707 sites can lead to the transphosphorylation of Tyr515 and Tyr816 residues (69, 70). These signaling pathways support many aspects of growth and development such as cell survival, synaptic structure, and synaptic plasticity (43, 59). In BeWo cell cultures, the phosphorylation of the TrkB receptor at its Tyr816 residue was robustly detected, while its Tyr515 and Tyr707 residues were seldom observed.

Expression of neurotrophins like BDNF is susceptible to change in response to perturbations in the maternal environment (40). Exposure to environmental pollutants is a maternal challenge that may affect BDNF expression and signaling in offspring. In rodents, prenatal stress can lead to decreased BDNF expression in offspring from weaning to adulthood (54). Moreover, mouse prenatal exposure to synthetic organic compounds such as bisphenol A (BPA) can induce lasting DNA methylations in transcriptionally relevant BDNF regions (54). Other studies have observed altered cord blood concentrations in humans following acute BPA exposure (35). Given these previous findings, the continued investigation of PFAS-induced BDNF pathway disruptions is imperative. Li et al. found increased TrkB expression in response to 10 μM doses of PFOS in human neuroblastoma cells (58). However, this observation was a proposed compensatory response to a simultaneous PFOS-related decrease in BDNF protein levels, which was seen in other reports (58, 59). Generally, the PFAS mixture and individual PFAS compounds did not significantly regulate the BDNF pathway in BeWo cells in this study. The phosphorylation of TrkB at the Tyr816 site was only increased by PFNA exposure at 1 and 10 μM levels. We speculate that the

activation of TrkB receptors is a secondary effect of PFNA exposure on these cells because the protein levels of pro-BDNF were not altered by PFNA exposure and mBDNF was not detected. Further, the activation of TrkB receptors can be triggered by other pathways or ligands unrelated to BDNF (71, 72). Additional studies are needed to investigate the mechanism by which PFNA induces changes in the BDNF signaling pathway and what other neurohormonal pathways could be disrupted by PFAS in both the placenta and fetal brain.

The present study has several limitations. First, the PFAS mixture in this study was based on blood concentrations of Pittsboro residents who were not pregnant. Maternal and placental blood levels of PFAS are unknown at this time. Second, the timeline and dosing allowed us to study the effects of acute exposures but preclude us from drawing conclusions about chronic low-level exposure. Third, our findings are limited to *in vitro* observations. However, we found a strong correlation between *in vitro* (BeWo cells) and *in vivo* (mice placenta and fetal brain tissues) observations of the BDNF pathway upon PM2.5 exposure [unpublished data]. Finally, our findings are based on a single cell model due to the limited availability of STB cell models. JAR, JEG-3—other *in vitro* models of the trophoblast barrier—were unsuitable for this study because they fail to undergo substantial syncytial fusion (73, 74).

This study is the first to localize p75 and TrkB receptors in the human placenta at different stages of gestation and in placental cells originating from choriocarcinoma (BeWo cells). Three individually tested PFAS compounds (PFOA, PFOS, PFBS) and a six-PFAS mixture mimicking residential exposure in Pittsboro, North Carolina, did not induce increased TrkB phosphorylation or alter pro-BDNF levels. Additionally, our results showed that in cells exposed to high concentrations of PFNA, phosphorylation of TrkB receptors increased while pro-BDNF levels remained stable. Although BDNF plays a critical role in brain development throughout all stages of life, it is unlikely to be primarily responsible for the observed neurodevelopmental consequences associated with *in utero* PFAS exposure. Additional investigation is required to understand whether adverse effects arise *via* an alternative placental pathway or are enacted directly on the fetus. These findings contribute to an improved understanding of the understudied BDNF signaling pathway in the gestational tissues and how the pathway is altered by PFAS exposure.

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 Committees of Xinhua Hospital affiliated with Shanghai Jiao Tong University School of Medicine and the Duke University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MM drafted the manuscript. SL performed research and data analysis. JZ contributed to the study design and drafting the manuscript. LF designed research, performed experiments and data analysis, and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Fogarty International Center of the National Institutes of Health under Award Number 1K01TW010828-01 (PI: LF). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Rappazzo KM, Coffman E, Hines EP. Exposure to Perfluorinated Alkyl Substances and Health Outcomes in Children: A Systematic Review of the Epidemiologic Literature. *Int J Environ Res Public Health* (2017) 14(7):1–22. doi: 10.3390/ijerph14070691
2. Birnbaum ,LS. *Hearing on "The Federal Role in the Toxic PFAS Chemical Crisis" Testimony. Senate Committee on Homeland Security and Governmental Affairs Subcommittee on Federal Spending Oversight and Emergency Management* (2018). https://doi.org/10.1093/.
3. Pelch KE, Reade A, Wolffe TAM, Kwiatkowski CF. PFAS Health Effects Database: Protocol for a Systematic Evidence Map. *Environ Inter* (2019) 130:104851. 10.1016/j.envint.2019.05.045
4. Houde M, Martin JW, Letcher RJ, Solomon KR, Muir DCG. Biological Monitoring of Polyfluoroalkyl Substances: A Review. *Environ Sci Technol* (2006) 40(11):3463–73. doi: 10.1021/es052580b
5. Liew Z, Goudarzi H, Oulhote Y. Developmental Exposures to Perfluoroalkyl Substances (PFASs): An Update of Associated Health Outcomes. *Curr Environ Health Rep* (2018) 5(1):1–19. doi: 10.1007/s40572-018-0173-4
6. Fromme H, Tittlemier SA, Völkel W, Wilhelm M, Twardella D. Perfluorinated Compounds – Exposure Assessment for the General Population in Western Countries. *Int J Hyg Environ Health* (2009) 212 (3):239–70. doi: 10.1016/j.ijheh.2008.04.007
7. Lewis RC, Johns LE, Meeker JD. Serum Biomarkers of Exposure to Perfluoroalkyl Substances in Relation to Serum Testosterone and Measures of Thyroid Function Among Adults and Adolescents From NHANES 2011–2012. *Int J Environ Res Public Health* (2015) 12(6):6098–114. doi: 10.3390/ijerph120606098
8. Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Tully JS, Needham LL. Serum Concentrations of 11 Polyfluoroalkyl Compounds in the U.S. Population: Data From the National Health and Nutrition Examination Survey (NHANES) 1999-2000. *Environ Sci Technol* (2007) 41(7):2237–42. doi: 10.1021/es062686m
9. Khalil N, Chen A, Lee M, Czerwinski SA, Ebert JR, De Witt JC, et al. Association of Perfluoroalkyl Substances, Bone Mineral Density, and Osteoporosis in the U.S. Population in NHANES 2009–2010. *Environ Health Perspect* (2016) 124(1):81–7. doi: 10.1289/ehp.1307909
10. Agency for Toxic Substances and Disease Registry. Toxicological Profile for Perfluoroalkyls. *Oxid Med Cell Longev* (2018) 1–882.

11. Austin ME, Kasturi BS, Barber M, Kannan K, MohanKumar PS, MohanKumar SMJ. Neuroendocrine Effects of Perflurooactane Sulfonate in Rats. *Environ Health Perspect* (2003) 11(12):1485–9. doi: 10.1289/ehp.6128
12. Apelberg BJ, Goldman LR, Calafat AM, Herbstman JB, Kuklenyik Z, Heidler J, et al. Determinants of Fetal Exposure to Polyfluoroalkyl Compounds in Baltimore, Maryland. *Environ Sci Technol* (2007) 41(11):3891–7. doi: 10.1021/es0700911
13. Kärrman A, Ericson I, VanBavel B, Ola Darnerud P, Aune M, Glynn A, et al. Exposure of Perfluorinated Chemicals Through Lactation: Levels of Matched Human Milk and Serum and a Temporal Trend, 1996-2004, in Sweden. *Environ Health Perspect* (2007) 115(2):226–30. doi: 10.1289/ehp.9491
14. Chen MH, Ha EH, Liao HF, Jeng SF, Su YN, Wen TW, et al. Perfluorinated Compound Levels in Cord Blood and Neurodevelopment at 2 Years of Age. *Epidemiology* (2013) 24(6):800–8. doi: 10.1097/EDE.0b013e3182a6dd46
15. Harris MH, Oken E, Rifas-Shiman SL, Calafat AM, Ye X, Bellinger DC, et al. Prenatal and Childhood Exposure to Per- and Polyfluoroalkyl Substances (PFASs) and Child Cognition. *Environ Int* (2018) 115:358–69. doi: 10.1016/j.envint.2018.03.025
16. Wang Y, Rogan WJ, Chen HY, Chen PC, Su PH, Chen HY, et al. Prenatal Exposure to Perfluroalkyl Substances and Children's IQ: The Taiwan Maternal and Infant Cohort Study. *Int J Hyg Environ Health* (2015) 218(7):639–44. doi: 10.1016/j.ijheh.2015.07.002
17. Høyer BB, Bonde JP, Tøttenborg SS, Ramlau-Hansen CH, Lindh C, Pedersen HS, et al. Exposure to Perfluoroalkyl Substances During Pregnancy and Child Behaviour at 5 to 9 Years of Age. *Horm Behav* (2018) 101:105–12. doi: 10.1016/j.yhbeh.2017.11.007
18. Luo J, Xiao J, Gao Y, Ramlau-Hansen CH, Toft G, Li J, et al. Prenatal Exposure to Perfluoroalkyl Substances and Behavioral Difficulties in Childhood at 7 and 11 Years. *Environ Res* (2020) 191:110111. doi: 10.1289/isee.2020.virtual.P-0872
19. Vuong AM, Webster GM, Yolton K, Calafat AM, Muckle G, Lanphear BP, et al. Prenatal Exposure to Per- and Polyfluoroalkyl Substances (PFAS) and Neurobehavior in US Children Through 8 Years of Age: The HOME Study. *Environ Res* (2021) 195:110825. doi: 10.1016/j.envres.2021.110825
20. Vuong AM, Yolton K, Wang Z, Xie C, Webster GM, Ye X, et al. Childhood Perfluoroalkyl Substance Exposure and Executive Function in Children at 8 Years. *Environ Int* (2018) 119:212–9. doi: 10.1016/j.envint.2018.06.028
21. Niu J, Liang H, Tian Y, Yuan W, Xiao H, Hu H, et al. Prenatal Plasma Concentrations of Perfluoroalkyl and Polyfluoroalkyl Substances and Neuropsychological Development in Children at Four Years of Age. *Environ Health* (2019) 18(1):53. doi: 10.1186/s12940-019-0493-3
22. Hoffman K, Webster TF, Weisskopf MG, Weinberg J, Vieira VM. Exposure to Polyfluoroalkyl Chemicals and Attention Deficit/Hyperactivity Disorder in U.S. Children 12–15 Years of Age. *Environ Health Perspect* (2010) 118(12):1762–7. doi: 10.1289/ehp.1001898
23. Lien GW, Huang CC, Shiu JS, Chen MH, Hsieh WS, Guo YL, et al. Perfluoroalkyl Substances in Cord Blood and Attention Deficit/Hyperactivity Disorder Symptoms in Seven-Year-Old Children. *Chemosphere* (2016) 156:118–27. doi: 10.1016/j.chemosphere.2016.04.102
24. Forns J, Verner MA, Iszatt N, Nowack N, Bach CC, Vrijheid M, et al. Early Life Exposure to Perfluoroalkyl Substances (PFAS) and ADHD: A Meta-Analysis of Nine European Population-Based Studies. *Environ Health Perspect* (2020) 128(5):57002. doi: 10.1289/EHP5444
25. Liew Z, Ritz B, Bach CC, Asarnow RF, Bech BH, Nohr EA, et al. Prenatal Exposure to Perfluoroalkyl Substances and IQ Scores at Age 5; A Study in the Danish National Birth Cohort. *Environ Health Perspect* (2018) 126(6):067004. doi: 10.1289/EHP2754
26. Fei C, Olsen J. Prenatal Exposure to Perfluorinated Chemicals and Behavioral or Coordination Problems at Age 7 Years. *Environ Health Perspect* (2011) 119(4):573–8. doi: 10.1289/ehp.1002026
27. Strøm M, Hansen S, Olsen SF, Haug LS, Rantakokko P, Kiviranta H, et al. Persistent Organic Pollutants Measured in Maternal Serum and Offspring Neurodevelopmental Outcomes - A Prospective Study With Long-Term Follow-Up. *Environ Int* (2014) 68:41–8. doi: 10.1016/j.envint.2014.03.002
28. Forns J, Iszatt N, White RA, Mandal S, Sabaredzovic A, Lamoree M, et al. Perfluoroalkyl Substances Measured in Breast Milk and Child Neuropsychological Development in a Norwegian Birth Cohort Study. *Environ Int* (2015) 83:176–82. doi: 10.1016/j.envint.2015.06.013
29. Vuong AM, Braun JM, Yolton K, Wang Z, Xie C, Webster GM, et al. Prenatal and Childhood Exposure to Perfluoroalkyl Substances (PFAS) and Measures of Attention, Impulse Control, and Visual Spatial Abilities. *Environ Int* (2018) 119:413–20. doi: 10.1016/j.envint.2018.07.013
30. Zheleznyakova GY, Cao H, Schiöth HB. BDNF DNA Methylation Changes as a Biomarker of Psychiatric Disorders: Literature Review and Open Access Database Analysis. *Behav Brain Funct* (2016) 12(1):17. doi: 10.1186/s12993-016-0101-4
31. De La Cruz-Morcillo MA, Berger J, Sánchez-Prieto R, Saada S, Naves T, Guillaudeau A, et al. P75 Neurotrophin Receptor and Pro-BDNF Promote Cell Survival and Migration in Clear Cell Renal Cell Carcinoma. *Oncotarget* (2016) 7(23):34480–97. doi: 10.18632/oncotarget.8911
32. Mayeur S, Silhol M, Moitrot E, Barbaux S, Breton C, Gabory A, et al. Placental BDNF/TrkB Signaling System is Modulated by Fetal Growth Disturbances in Rat and Human. *Placenta* (2010) 31(9):785–91. doi: 10.1016/j.placenta.2010.06.008
33. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ. Sustained Hippocampal Chromatin Regulation in a Mouse Model of Depression and Antidepressant Action. *Nat Neurosci* (2006) 9:519–25. doi: 10.1038/nn1659
34. Jha S, Dong B, Sakata K. Enriched Environment Treatment Reverses Depression-Like Behavior and Restores Reduced Hippocampal Neurogenesis and Protein Levels of Brain-Derived Neurotrophic Factor in Mice Lacking its Expression Through Promoter IV. *Transl Psychiatry* (2011) 1484(1):9–31. doi: 10.1038/tp.2011.33
35. Kundakovic M, Gudsnuk K, Herbstman JB, Tang D, Perera FP, Champagne FA. DNA Methylation of BDNF as a Biomarker of Early-Life Adversity. *Proc Natl Acad Sci U S A* (2015) 112(22):6807–13. doi: 10.1073/pnas.1408355111
36. Notaras M, Hill R, Van den Buuse M. A Role for the BDNF Gene Val66Met Polymorphism in Schizophrenia? A Comprehensive Review. *Neurosci Biobehav Rev* (2015) 51:15–30. doi: 10.1016/j.neubiorev.2014.12.016
37. Kang H-J, Kim J-M, Lee J-Y, Kim S-Y, Bae K-Y, Kim S-W, et al. BDNF Promoter Methylation and Suicidal Behavior in Depressive Patients. *J Affect Disord* (2013) 151(2):679–85. doi: 10.1016/j.jad.2013.08.001
38. Lee Y, Lim SW, Kim SY, Chung JW, Kim J, Myung W, et al. Association Between the BDNF Val66Met Polymorphism and Chronicity of Depression. *Psychiatry Investig* (2013) 10(1):56–61. doi: 10.4306/pi.2013.10.1.56
39. Brooks SJ, Nilsson EK, Jacobsson JA, Stein DJ, Fredriksson R, Lind L, et al. BDNF Polymorphisms are Linked to Poorer Working Memory Performance, Reduced Cerebellar and Hippocampal Volumes and Differences in Prefrontal Cortex in a Swedish Elderly Population. *PloS One* (2014) 9(1):e82707. doi: 10.1371/journal.pone.0082707
40. Zeltser LM, Leibel RL. Roles of the Placenta in Fetal Brain Development. *Proc Natl Acad Sci U S A* (2011) 108(38):15667–8. doi: 10.1073/pnas.1112239108
41. Sahay A, Kale A, Joshi S. Role of Neurotrophins in Pregnancy and Offspring Brain Development. *Neuropeptides* (2020) 83:102075. Churchill Livingstone. doi: 10.1016/j.npep.2020.102075
42. Nikolaou KE, Malamitsi-Puchner A, Boutsikou T, Economou E, Boutsikou M, Puchner K-P, et al. The Varying Patterns of Neurotrophin Changes in the Perinatal Period. *Ann N Y Acad Sci* (2006) 1092(1):426–33. doi: 10.1196/annals.1365.041
43. Binder DK, Scharfman HE. Brain-Derived Neurotrophic Factor. *Growth Factors* (2004) 22(3):123–31. doi: 10.1016/B0-12-341103-3/00037-1
44. Zagrebelsky M, Korte M. Form Follows Function: BDNF and its Involvement in Sculpting the Function and Structure of Synapses. *Neuropharmacology* (2014) 76:628–38. Pergamon. doi: 10.1016/j.neuropharm.2013.05.029
45. Chao MV, Hempstead BL. P75 and Trk: A Two-Receptor System. *Trends Neurosci* (1995) 18(7):321–6. doi: 10.1016/0166-2236(95)93922-K
46. Kodomari I, Wada E, Nakamura S, Wada K. Maternal Supply of BDNF to Mouse Fetal Brain Through the Placenta. *Neurochem Int* (2009) 54(2):95–8. doi: 10.1016/j.neuint.2008.11.005
47. Wada E, Wada K. Bio-Communication Between Mother and Offspring. In: *Reproductive and Developmental Toxicology*. Elsevier Inc (2011). p. 33–8.
48. Mayeur S, Lukaszewski MA, Breton C, Storme L, Vieau D, Lesage J. Do Neurotrophins Regulate the Feto-Placental Development? *Med Hypotheses* (2011) 76(5):726–8. doi: 10.1016/j.mehy.2011.02.008
49. Kawamura K, Kawamura N, Kumazawa Y, Kumagai J, Fujimoto T, Tanaka T. Brain-Derived Neurotrophic Factor/Tyrosine Kinase B Signaling Regulates

Human Trophoblast Growth in an *In Vivo* Animal Model of Ectopic Pregnancy. *Endocrinology* (2011) 152(3):1090–100. doi: 10.1210/en.2010-1124
50. Kertes DA, Bhatt SS, Kamin HS, Hughes DA, Rodney NC, Mulligan CJ. BNDF Methylation in Mothers and Newborns is Associated With Maternal Exposure to War Trauma. *Clin Epigenet* (2017) 9(1):68. doi: 10.1186/s13148-017-0367-x
51. Kermani P, Hempstead B. Brain-Derived Neurotrophic Factor: A Newly Described Mediator of Angiogenesis. *Trends Cardiovasc Med* (2007) 17 (4):140–3. doi: 10.1016/j.tcm.2007.03.002
52. D'Souza V, Patil V, Pisal H, Randhir K, Joshi A, Mehendale S, et al. Levels of Brain Derived Neurotrophic Factors Across Gestation in Women With Preeclampsia. *Int J Dev Neurosci* (2014) 37:36–40. doi: 10.1016/j.ijdevneu.2014.06.008
53. Kawamura K, Kawamura N, Sato W, Fukuda J, Kumagai J, Tanaka T. Brain-Derived Neurotrophic Factor Promotes Implantation and Subsequent Placental Development by Stimulating Trophoblast Cell Growth and Survival. *Endocrinology* (2009) 150(8):3774–82. doi: 10.1210/en.2009-0213
54. Boersma GJ, Lee RS, Cordner ZA, Ewald ER, Purcell RH, Moghadam AA, et al. Prenatal Stress Decreases Bdnf Expression and Increases Methylation of Bdnf Exon IV in Rats. *Epigenetics* (2013) 9(3):437–47. doi: 10.4161/epi.27558
55. Annunziato KM, Jantzen CE, Gronske MC, Cooper KR. Subtle Morphometric, Behavioral and Gene Expression Effects in Larval Zebrafish Exposed to PFHxA, PFHxS and 6:2 FTOH. *Aquat Toxicol* (2019) 208 (January):126–37. doi: 10.1016/j.aquatox.2019.01.009
56. Wang Y, Liu W, Zhang Q, Zhao H, Quan X. Effects of Developmental Perfluorooctane Sulfonate Exposure on Spatial Learning and Memory Ability of Rats and Mechanism Associated With Synaptic Plasticity. *Food Chem Toxicol* (2015) 76:70–6. doi: 10.1016/j.fct.2014.12.008
57. Jantzen CE, Annunziato KM, Cooper KR. Behavioral, Morphometric, and Gene Expression Effects in Adult Zebrafish (Danio Rerio) Embryonically Exposed to PFOA, PFOS, and PFNA. *Aquat Toxicol* (2016) 180:123–30. doi: 10.1016/j.aquatox.2016.09.011
58. Li W, He QZ, Wu CQ, Pan XY, Wang J, Tan Y, et al. PFOS Disturbs BDNF-ERK-CREB Signalling in Association With Increased MicroRNA-22 in SH-SY5Y Cells. *BioMed Res Int* (2015) 2015(7):1–10. doi: 10.1155/2015/302653
59. Guo XX, He QZ, Li W, Long DX, Pan XY, Chen C, et al. Brain-Derived Neurotrophic Factor Mediated Perfluorooctane Sulfonate Induced-Neurotoxicity *Via* Epigenetics Regulation in SK-N-SH Cells. *Int J Mol Sci* (2017) 18(4):1–13. doi: 10.3390/ijms18040893
60. Zhang Y, Le T, Grabau R, Mohseni Z, Kim H, Natale DR, et al. TMEM16F Phospholipid Scramblase Mediates Trophoblast Fusion and Placental Development. *Sci Adv* (2020) 6(19):eaba0310. doi: 10.1126/sciadv.aba0310
61. Pham A, Zhang J, Feng L. Exposure to Perfluorobutane Sulfonate and Perfluorooctanesulfonic Acid Disrupts the Production of Angiogenesis Factors and Stress Responses in Human Placental Syncytiotrophoblast. *Reprod Toxicol* (2020) 98:269–77. doi: 10.1016/j.reprotox.2020.10.013
62. Marinello WP, Mohseni ZS, Cunningham SJ, Crute C, Huang R, Zhang JJ, et al. Perfluorobutane Sulfonate Exposure Disrupted Human Placental Cytotrophoblast Cell Proliferation and Invasion Involving in Dysregulating Preeclampsia Related Genes. *FASEB J* (2020) 34(11):14182–99. doi: 10.1096/fj.202000716RR
63. Orendi K, Gauster M, Moser G, Meiri H, Huppertz B. The Choriocarcinoma Cell Line BeWo: Syncytial Fusion and Expression of Syncytium-Specific Proteins. *Reproduction* (2010) 140(5):759–66. doi: 10.1530/REP-10-0221
64. Yang B, Ren Q, Zhang JC, Chen QX, Hashimoto K. Altered Expression of BDNF, BDNF Pro-Peptide and Their Precursor proBDNF in Brain and Liver Tissues From Psychiatric Disorders: Rethinking the Brain-Liver Axis. *Transl Psychiatry* (2017) 7(5):e1128. doi: 10.1038/tp.2017.95
65. Mowla SJ, Farhadi HF, Pareek S, Atwal JK, Morris SJ, Seidah NG, et al. Biosynthesis and Post-Translational Processing of the Precursor to Brain-Derived Neurotrophic Factor. *J Biol Chem* (2001) 276(16):12660–6. doi: 10.1074/jbc.M008104200
66. Balkowiec A, Katz DM. Cellular Mechanisms Regulating Activity-Dependent Release of Native Brain-Derived Neurotrophic Factor From Hippocampal Neurons. *J Neurosci* (2002) 22(23):10399–407. doi: 10.1523/JNEUROSCI.22-23-10399.2002
67. Lieto A, Rantamäki T, Vesa L, Yanpallewar S, Antila H, Lindholm J, et al. The Responsiveness of Trkb to Bdnf and Antidepressant Drugs is Differentially Regulated During Mouse Development. *PloS One* (2012) 7(3):1–11. doi: 10.1371/journal.pone.0032869
68. Pradhan J, Noakes PG, Bellingham MC. The Role of Altered BDNF/TrkB Signaling in Amyotrophic Lateral Sclerosis. *Front Cell Neurosci* (2019) 13:368. Frontiers Media S.A. doi: 10.3389/fncel.2019.00368
69. Middlemas DS, Meisenhelder J, Hunter T. Identification of TrkB Autophosphorylation Sites and Evidence That Phospholipase C-γ1 Is a Substrate of the TrkB Receptor. *J Biol Chem* (1994) 269(7):5458–66. doi: 10.1016/S0021-9258(17)37708-6
70. Segal RA, Bhattacharyya A, Rua LA, Alberta JA, Stephens RM, Kaplan DR, et al. Differential Utilization of Trk Autophosphorylation Sites. *J Biol Chem* (1996) 271(33):20175–81. doi: 10.1074/jbc.271.33.20175
71. Gupta VK, You Y, Gupta VB, Klistorner A, Graham SL. TrkB Receptor Signalling: Implications in Neurodegenerative, Psychiatric and Proliferative Disorders. *Int J Mol Sci* (2013) 14(5):10122–42. doi: 10.3390/ijms140510122
72. Nagappan G, Woo NH, Lu B. Ama"Zinc" Link Between TrkB Transactivation and Synaptic Plasticity. *Neuron* (2008) 57(4):477–9. doi: 10.1016/j.neuron.2008.02.004
73. Msheik H, El Hayek S, Bari MF, Azar J, Abou-Kheir W, Kobeissy F, et al. Transcriptomic Profiling of Trophoblast Fusion Using BeWo and JEG-3 Cell Lines. *Mol Hum Reprod* (2019) 25(12):811–24. doi: 10.1093/molehr/gaz061
74. Rothbauer M, Patel N, Gondola H, Siwetz M, Huppertz B, Ertl P. A Comparative Study of Five Physiological Key Parameters Between Four Different Human Trophoblast-Derived Cell Lines. *Sci Rep* (2017) 7(1):5892. doi: 10.1038/s41598-017-06364-z

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 06 August 2021
doi: 10.3389/fendo.2021.664766

Collagen I Induces Preeclampsia-Like Symptoms by Suppressing Proliferation and Invasion of Trophoblasts

Yinglin Feng[1†], Xia Chen[2†], Huiqiao Wang[3], Xueping Chen[1], Zixin Lan[3], Pan Li[4], Yingshi Cao[1], Mian Liu[1], Jin Lv[5], Yun Chen[1], Yu Wang[1], Chao Sheng[1], Yingying Huang[1], Mei Zhong[1], Zhijian Wang[1], Xiaojing Yue[1] and Liping Huang[1*]*

[1] Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University, Guangzhou, China, [2] Department of Obstetrics and Gynecology, Foshan First People's Hospital, Foshan, China, [3] Zhujiang Hospital, Southern Medical University, Guangzhou, China, [4] Microbiome Research Center, University of New South Wales, Sydney, NSW, Australia, [5] Department of Pathology, Foshan First People's Hospital, Foshan, China

Preeclampsia is a common obstetric disorder affecting 2-8% of pregnancy worldwide. Fibrosis is an important histological change occurring in preeclamptic placenta, and might depend on the excess deposition of collagen I. However, the role of fibrotic placenta and collagen I in the pathogenesis of preeclampsia remains unclear. Therefore, we analyzed the collagen deposition and the expression of Collagen I in human placenta by Masson staining, Sirius red staining and western blotting. Further, the role of collagen I in preeclampsia pathogenesis was studied in C57BL/6 mice. HTR-8/SVneo cells were used to investigate the mechanisms underlying the effects of collagen I in trophoblasts by transcriptome sequencing and pharmacological agonists. Human preeclamptic placenta exhibited a significantly higher degree of fibrosis in stem villi and terminal villi than normal placenta, and was characterized by collagen I deposition. *In vivo*, a single injection of collagen I on gestational day 0.5 led to an increase in systolic pressure of pregnant mice from gestational days 4.5–17.5, to a decrease in weight and number of embryos, and to enhanced placental collagen I expression and degree of fibrosis compared with control mice. *In vitro*, collagen I attenuated the proliferation and invasion of HTR-8SV/neo cells. This effect could be reversed by treatment with agonists of ERK and β-catenin. Moreover, transcriptome sequencing demonstrated that signaling pathways related to cell proliferation and invasion were significantly downregulated in HTR-8SV/neo cells. Thus, we propose that collagen I induced preeclampsia-like symptoms by suppressing the proliferation and invasion of trophoblasts through inhibition of the ERK phosphorylation and WNT/β-catenin signaling pathways. Our findings could pave the way to the discovery of small-molecule inhibitors for preeclampsia treatment and future studies with larger sample size are required.

Keywords: fibrosis, collagen I, preeclampsia, placenta, pathogenesis

OPEN ACCESS

Edited by:
Ganesh Acharya,
Karolinska Institute, Sweden

Reviewed by:
Rahul Pal,
National Institute of Immunology (NII), India
Wei Wu,
Nanjing Medical University, China
Mona Nystad,
University Hospital of North Norway, Norway

***Correspondence:**
Liping Huang
lphuang2006@126.com
Xiaojing Yue
yuexj1986@163.com

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *09 March 2021*
Accepted: *14 June 2021*
Published: *06 August 2021*

Citation:
Feng Y, Chen X, Wang H, Chen Y, Lan Z, Li P, Cao Y, Liu M, Lv J, Chen X, Wang Y, Sheng C, Huang Y, Zhong M, Wang Z, Yue X and Huang L (2021) Collagen I Induces Preeclampsia-Like Symptoms by Suppressing Proliferation and Invasion of Trophoblasts. Front. Endocrinol. 12:664766. doi: 10.3389/fendo.2021.664766

INTRODUCTION

Preeclampsia is a common, pregnancy-specific disorder that threatens 2–8% of pregnancies globally and constitutes one of the leading causes of maternal and perinatal mortality worldwide (1). It is characterized by the occurrence of new-onset hypertension, accompanied by proteinuria or other end-organ dysfunction after 20 weeks of gestation in previously normotensive women (2). To date, the pathogenesis of this morbidity is poorly understood. The placenta is generally perceived as the primary organ during disease development because the removal of placenta is necessary for symptoms to regress (3). Previous research has shown that histological changes exist in pre-eclampsia (PE) placenta include chronic inflammation, vascular lesions, villous coagulation, and villous fibrosis. Among the changes occurring in these placentae, fibrosis is an important feature (4). In particular, microscopic comparison with control placenta has revealed that villous fibrosis is more frequent in preeclamptic placentas (4, 5). Notably, fibrosis in preeclamptic placenta is reported to be related to the activation of stromal fibroblasts (6). Whether placental fibrosis is involved in the pathogenesis of preeclampsia is still unclear.

Fibrosis is a well-known pathogenic process resulting in the progressive loss of organ structure and function. It is defined by overproduction of the extracellular matrix (ECM) in connective tissues and plays a significant role in the impairment of organ function, such as during liver cirrhosis and cardiac fibrosis (7, 8). It is noteworthy that diverse diseases in different organs are associated with common pathogenic pathways, as excessive ECM deposition causes physical organ deformation, which impairs organ function and ultimately induces organ failure (9).

Collagens are the predominant components of the ECM (8, 10). Consequently, increases in collagen expression and deposition are directly associated with fibrosis (11). The family of collagens comprises twenty-eight members and regulates cell proliferation and migration, cell-matrix interactions, and cell signaling (12). Furthermore, emerging studies have indicated that collagen exerts contrasting effects on the physiology of different organs. For example, collagen could stimulate angiogenesis in a myocardial infarction model (13). Moreover, collagens have been demonstrated to reduce melanoma cell proliferation and invasion by inhibiting the FAK/PI3K/AKT pathway (14).

Collagen I is well known as one of the main member of the collagen family, constituting over 90% of the collagen of the body (15, 16). Higher production of Collagen I was once detected in preeclamptic placenta (17). Takako and his colleagues indicated that the fibroblasts isolated from preeclamptic placenta showed a higher expression of collagen I (6). Although the similar studies were limited, that collagen I may be the characteristic collagen deposited in preeclamptic placenta, and may influences the placental function, warrants further investigation.

To date, detailed investigation of the collagen I in preeclamptic placenta is lacking. Whether placental fibrosis is involved in the pathogenesis of preeclampsia is still unclear. In this study, we hypothesize that exacerbation of fibrosis leads to impairment of placental function and is involved in the pathogenesis of preeclampsia. Therefore, we performed *in vivo* and *in vitro* experiments to clarify the relationship between placental fibrosis and preeclampsia.

MATERIALS AND METHODS

Collection of Human Placenta

Women with PE and normotensive pregnant (NP) women without previous treatments were recruited in the third trimester from March 2018 to 2019 in the Department of Obstetrics of the Nanfang Hospital, Southern Medical University, China. According to the current American College of Obstetricians and Gynecologists criteria (1), preeclampsia is diagnosed as maternal systolic blood pressure blood pressure ≥140mmHg and/or diastolic blood pressure ≥90mmHg on at least two occasions, four hours apart, with proteinuria, after 20 weeks of gestation in previously normotensive women. In the absence of proteinuria, patients with new-onset of hypertension with the new onset of any of the following: thrombocytopenia, renal insufficiency, impaired liver function, or pulmonary edema can be induced. The clinical characteristics of placenta donors are summarized in **Supplementary Table S1**.

Fresh placentae were obtained immediately after delivery. The tissue was collected from the area near the umbilical cord (1 cm from the cord insert), from both the maternal and fetal side of the placenta.

A sample size of 10 placentae in each group was used for the identification of collagen and quantification of collagen I by Masson staining, Sirius red staining, and Western blotting. The experimental procedures were detailed in the **Supplementary Material**. Following staining, the placentae were assessed for the degree of fibrosis in a blinded fashion by a trained pathologist. For Masson trichrome straining, collagen fiber was stained blue, while cytoplasm and red blood cells were stained red and nucleus blue and brown. Fibrosis area% was calculated in μm digitally using the software NDP.view2 (Hamamatsu Photonics, Hamamatsu, Japan). Sirius Red staining is a method used for collagen identification. Collagen I appears red-yellow when observed under a polarizing microscope. Each analyzed field was chosen randomly and the positive red-stained areas and red-yellow density were quantified using computerized image analysis software (NIH, MD, USA).

The collection of placentae was approved by the Ethics Committee of Nanfang Hospital(NFEC-2017-055). All participants gave written consent prior to donating their placenta.

Collagen I Preparation

Collagen I (C7774, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.1 mM acetic acid and emulsified in an equal volume incomplete Freund's adjuvant (IFA) (F5506, Sigma-Aldrich, St. Louis, MO, USA) to obtain a 1000 μg/ml solution as the stock solution.

Animals and Experimental Protocol

This project was performed in accordance with animal protocol procedures approved by the Department of Laboratory Animal Sciences, Southern Medical University (L-2019216), and the

animals were handled according to the guiding principles published in the National Institutes of Health Guide for the Care of Animals.

Female C57/BL6 mice strain (8 weeks, weight 18–20 g, purchased from the Animal Laboratory Center of Southern Medical University) were used in the experiments. They were maintained on a 12 hour/12hour light/dark schedule with free access to food and water. All female mice were housed with male mice at a 2:1 ratio overnight, and pregnancy was confirmed by the presence of vaginal spermatozoa. The presence of spermatozoa was indicative of gestational day 0.5 (E0.5d).

Pregnant female mice were randomly divided into five groups on E0.5d(six mice per group): pregnant control group, 0.5 mg/kg collagen I-treated group, 5 mg/kg collagen I-treated group, IFA-treated group, and NG-nitro-L-arginine methyl ester (L-NAME)-treated group. In the collagen I-treated groups, 100 μL of collagen I (0.5 mg/kg and 5 mg/kg) was injected intradermally at the base of the tail of each mouse on E0.5d. The mice in IFA-treated group were injected 100μL intradermally at the base of the tail on E0.5.

The mice in the L-NAME group were treated with continuous administration of 125 mg/kg/d of L-NAME, a common vasoconstrictor, starting at 10.5 days of gestation *via* subcutaneous injection on the nucha (18). Control mice were not injected with any solution. Systolic blood pressure (SBP) was recorded every four days. All mice were sacrificed, and placental tissues were harvested on gestational day 17.5 (E17.5d). The numbers of fetus were counted and recorded. We cut open the uterus along the uterine membrane to remove the fetus. Then, we washed the fetus with PBS and placed into a clean dish. The excess liquid was removed by filter paper before weighting.

Measurements of Blood Pressure in Mice

Monitor of blood pressure were to evaluate the preeclampsia symptom of animal. We determined the blood pressure in conscious mice by tail cuff plethysmography using the Softron BP 2010 (Softron Biotechnology, Beijing, China). All the mice were habituated to the measurement procedure 10 times a day before caging with male mice. At least 9 consecutive measurements were recorded, but only when the condition of the mice was stable. Six time point (pre-pregnancy, E0.5, E4.5, E8.5, E12.5, and E16.5) were selected for assessing systolic blood pressure level throughout the pregnancy. Serial blood pressure measures analyzed by two-way repeated measures ANOVA.

Cell Culture

HTR-8SV/neo cells (purchased from ATCC, Rockville, USA) were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. Cells were maintained at 37°C, with 5% CO_2. Liguid were changed every other day until cells reached 90% confluence.

Precoating of Cell Culture Plates With Collagen I

The surfaces of six-well cell culture plates were coated with different doses of collagen I, which was diluted in a minimal volume (500 μL per well). After 4 hours of incubation with collagen I, the solution was removed from the plate surface; plates were then dried overnight at room temperature and washed with phosphate buffered saline to remove the unattached protein.

Cell Stimulation

HTR-8SV/neo cells were seeded in six-well plate which were incubated with different concentration of collagen I for 48h. CCK-8 (ab228554, Abcam, MA, USA) assay was conducted to detect cell viability and cell cycle analysis cell cycle stage was analyzed using flow cytometry. Western blotting was used to measure the relative protein level. The experimental procedures were detailed in the **Supplementary Material**.

Honokiol, which enhance the phosphorylation of ERK phosphorylation, (HY-N0003, MedChemExpress, USA) dissolved at a concentration of 10μM. In the co-treatment group, HTR-8/SVneo cells were treated with Honokiol and collagen I 48h. SKL-2001 (HY-101085, MedChemExpress, USA), an agonist of β-catenin which was used at a concentration of 5μM. Co-treament with collagen I 24h. Cell viability, cell cycle and relative protein level were analyzed by CCK-8, cycle cell and western blotting.

Protein Expression and Biochemical Analysis

Protein was extracted from cells and placental tissue with commercial lysis buffer RIPA (89900, Thermo Scientific, USA). Western blotting was performed as described in **Supplementary Material**. Primary antibodies against collagen I (ab260043, Abcam, Cambridge, UK), E-cadherin (#14472, Cell Signaling, USA), N-cadherin (#13116, Cell Signaling, USA), MMP-9(ab38898, Abcam, Cambridge, UK), Vimentin (ab92547, Abcam, Cambridge, UK), GAPDH (ab8245, Abcam, Cambridge, UK), ERK (#4695, Cell Signaling, USA), p-ERK(#4370, Cell Signaling, USA), β-Catenin (ab32572, Abcam, Cambridge, UK).

Transwell Assay

Transwell assays were performed to test the invasion ability of HTR-8/SVneo Matrigel (356234, BD, USA) and Transwell (3422, Costa, USA) were purchased and used as per manufacturer's instructions (19). We precoated the upper chamber of each Transwell (Costa, USA) insert with different doses of collagen I (100 or 1000 μg/mL). Cells were grown in the upper chamber in medium containing 3% FBS and assessed for their invasion ability through collagen I toward a chemoattractant (10% FBS) in the lower chamber for 48 h.

Transcriptome Sequencing and Data Analysis

Differential gene expression analysis of 30,945 expressed genes was performed using DESeq2 (19, 20), and yielded 1237 differentially expressed genes (an adjusted p value < 0.05). We collected PE-related genes from six meta-analyses (21–26), two microarray-based studies (27, 28), one review paper (29), and one research paper based on RNA-seq (30) published since 2009. A total of 3553 PE-associated genes were obtained. We found 2347 of these PE-related genes to be expressed in our dataset, 227 of which were differentially expressed. KEGG (Kyoto

Encyclopedia of Genes and Genomes) pathway enrichment analysis for 227 genes was performed with the clusterProfiler package (**Supplementary Table S2**) (31). A cutoff of adjusted P value < 0.05 was chosen to select the most significantly enriched pathways. Finally, pathview (32) was used to visualize the differentially expressed genes belonging to significantly enriched pathways.

Statistics

Statistical analyses were performed using Prism 7.0 software (GraphPad Software, Inc. San Diego, CA, USA). After testing for normal distribution, differences among groups were analyzed by two-tailed, unpaired Student's *t*-tests or one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. All data are presented as mean ± standard error of mean (SEM).

Enrichment analyses were performed on the R platform, and a two-tailed Fisher's exact test was used. Error bars were used to represent the standard error of the fraction, estimated using a bootstrapping method with 100 re-samplings. The data relative to the experimental validations are presented as mean ± SEM of two independent experiments. Comparisons between two independent groups were conducted using Student's *t*-test. Statistical significance was described as *P < 0.05, or **P < 0.01.

RESULTS

Elevated Collagen I Levels in Human Placenta From Women Diagnosed With Preeclampsia

We collected placental samples from 20 patients, 10 of whom were diagnosed with preeclampsia and 10 represented normotensive controls. The characteristics of the study cohort are presented in **Supplementary Table S1**. As expected, increased blood pressure and proteinuria were evident in the preeclampsia-diagnosed group.

The degree of fibrosis of the various placental samples is shown in **Figure 1A**. Collagen deposited in villous tissues and wrapped in blood vessels. According to the Masson's trichome staining the collagen expression, a high degree of fibrosis of stem villi and terminal villi was more frequent in preeclamptic placenta. Sirius Red staining is a method used for collagen identification. The elongated axis of dye molecules are attached parallel to collagen, resulting in enhanced birefringency and specificity when combined with polarized light detection methods (33). Collagen I appears red-yellow when observed under a polarizing microscope (34). Notably, the red-yellow color density was much higher in preeclamptic placentas than in control placenta (**Figure 1B**). Further, the relative gene and protein expression levels of collagen I were higher in preeclamptic placenta (**Figures 1C, D**). These results indicated that collagen I may be the characteristic collagen deposited in human preeclamptic placenta.

Collagen I Induces Preeclampsia-Like Symptoms in Pregnant Mice

To further test whether excessive collagen I can cause PE progression *in vivo*, pregnant mice were randomly divided into five groups on E0.5d (**Figure 2A**).

At E4.5 d, collagen I-injected mice exhibited significantly higher SBP than did control and IFA mice ($p < 0.05$). Moreover, the SBP of the collagen I- and L-NAME-treated groups was elevated from the second trimester to the end of gestation ($p < 0.01$ **Figure 2B**). Placental tissue was harvested at E17.5d to examine the weight of foetuses and the structure of the placenta. Fetal weight was decreased in both the collagen I- and L-NAME-treated groups ($p < 0.01$ **Figure 2C**). However, the number of fetus only decreased in the group treated with 5 mg/kg of collagen I ($p < 0.01$ **Figure 2C**). Nevertheless, histomorphometric analysis revealed structural changes in the placenta of mice in the collagen I- and L-NAME-treated groups; particularly, infarction was observed in the labyrinth layer (**Figure 2D**). Moreover, in both the labyrinth and junctional zones, a higher degree of fibrosis was observed in the collagen I- and L-NAME-treated groups than in the control group (**Figure 2E**). However, the relative protein expression of collagen I was higher only in the collagen I-treated groups ($p < 0.01$ in the 5mg/kg group and $p < 0.05$ in the 0.5mg/kg group, **Figure 2F**). These results indicated that exposure to collagen I injection during early pregnancy may result in an increased deposition of collagen I in the placenta, and in subsequent preeclampsia-like adverse reproductive outcomes.

In addition to histological changes, defective trophoblast invasion is involved in the pathogenesis of preeclampsia (35). Therefore, we analyzed the relative protein expression of MMP-9, E-cadherin, N-cadherin, and vimentin, which regulate trophoblast invasion (36–38), using western blotting. Upon exposure to collagen I, the relative protein expression of MMP-9, N-cadherin, and vimentin in the placenta decreased compared to that of the control placenta, while E-cadherin expression increased. These results suggested that collagen I induced preeclampsia-like symptoms by suppressing the invasive ability of trophoblasts.

Collagen I Suppress the Proliferation and Invasive Ability of HTR-8SV/neo Cells

Defective trophoblast proliferation and invasive ability are important factors contributing to pathogenesis of preeclampsia (35). At collagen I doses of 100 and 1000 μg/mL, the viability of cells decreased compared with that of cells grown in control plates ($p < 0.05$ **Figure 3A**). Particularly, collagen I significantly induced cell cycle arrest at the G2/M phase at a dose of 1000 μg/mL ($p < 0.01$ in the 1000μg/mL group, **Figure 3B**). Further, cell invasion was significantly attenuated by collagen I ($p < 0.01$ **Figure 3C**). Because MMP-9, E-cadherin, N-cadherin, and vimentin have been implicated in preeclampsia pathogenesis, we investigated their relative expression by western blot analysis and found that collagen I significantly decreased MMP-9, vimentin, and N-cadherin expression, but increased E-cadherin protein levels (**Figure 3D**).

Altogether, our *in vitro* experiments suggest that collagen I induced trophoblast dysfunction by suppressing the proliferation and invasive ability of this cell type.

Collagen I Affect Gene Expression in Trophoblasts

To identify collagen I-dependent changes in gene expression patterns of trophoblasts at the transcriptional level, we analyzed

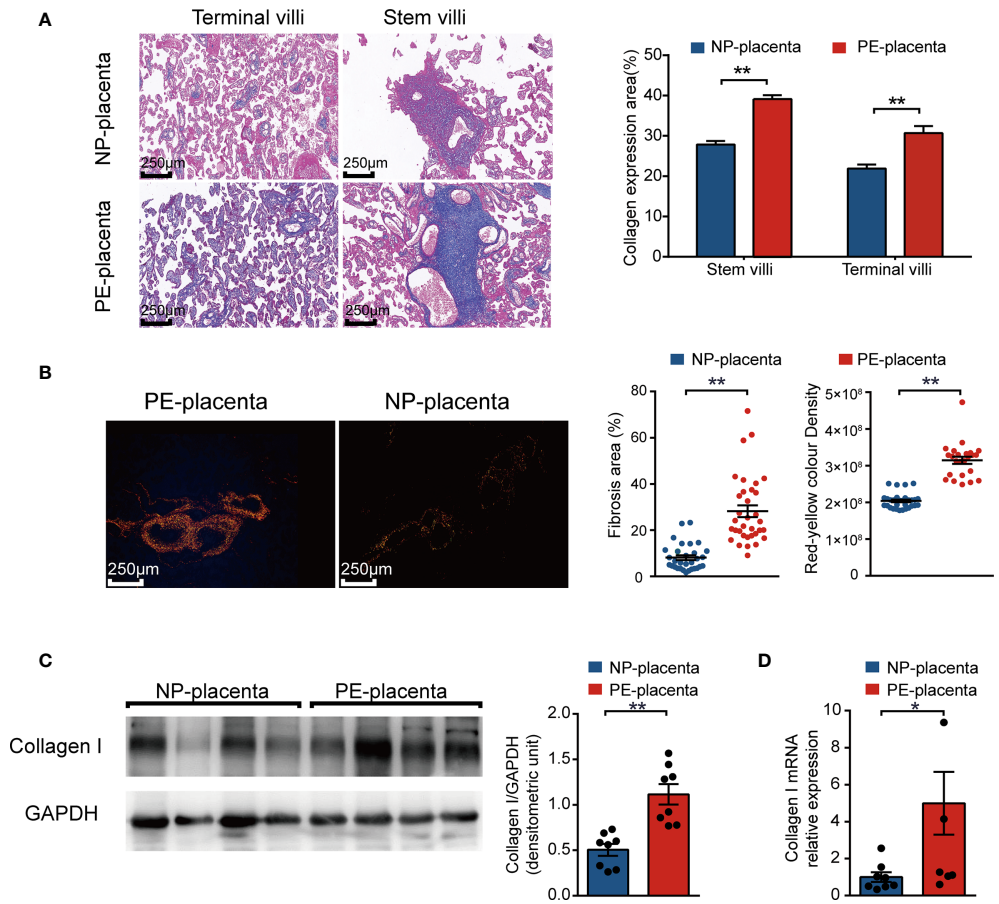


FIGURE 1 | Identification and quantification of collagen types in human preeclamptic (PE) placentas. **(A)** Masson's trichrome staining of placentas in the stem and terminal villus region from normotensive pregnant women (NP placenta) and from PE patients (PE placenta; n = 6 per group, scale bar = 250 nm). Collagen expression area of PE and NP placentas. Collagen fibers were stained blue, while cytoplasm and red blood cells were stained red, and the nucleus blue and brown. **(B)** Representative image of Sirius red staining of placenta (magnification, 200×). Measured area and red-yellow color intensity of Sirius Red staining in PE and NP placenta (three visual fields for each placental sample, n = 6 per group). **(C)** Representative image of western blotting and quantitative analysis of collagen I in PE and NP placenta (bar chart, n = 8 per group). **(D)** Relative gene expression of collagen I (bar chart, n = 8 per group). Data are presented as mean ± standard error of mean (SEM). *p < 0.05, **p < 0.01 by two-tailed unpaired *t*-test analysis.

total RNA samples from 100μg/mL collagen I-treated and untreated HTR-8SV/neo cells by RNA-seq. We found that the expression of 801 genes increased while that of 436 genes decreased under collagen I treatment compared with gene expression in the control group (**Figure 4A**). The relative expression of upregulated and downregulated genes was illustrated in a heat map (**Figure 4B**). Additionally, we found 2347 PE-related genes to be expressed in our dataset, among which 227 were differentially expressed (**Figure 4C**). With the latter gene set, we carried out KEGG pathway enrichment analysis; the significantly enriched pathways are summarized in **Figure 4D**. Interestingly, genes involved in proteoglycans in cancer, cell cycle, and the PI3K-AKT signaling pathway (which is related to cell proliferation and invasion), were significantly downregulated in our dataset. Consistently, collagen I treatment downregulated the expression of *ERK2, MET, PI3K, β-catenin*, and *WNT5A*, genes related to cell proliferation and invasion in trophoblasts (**Figure 4E**).

Collagen I Suppress Trophoblast Proliferation and Invasion

Together with transcriptome sequencing analysis, western blotting was used to detect p-ERK/ERK and β-catenin expression in collagen I-treated and untreated mouse placenta and HTR-8SV/neo cells. In both types of samples, we observed decreased expression of p-ERK and β-catenin after collagen I treatment (**Figures 5A, B**). ERK is a member of the MAPK signaling pathway. Alterations in ERK phosphorylation can lead to changes in cell proliferation (39). The present results demonstrated that the ERK phosphorylation level was decreased following treatment with collagen I. Furthermore, the combination of the ERK activator, honokiol, with collagen I to treat

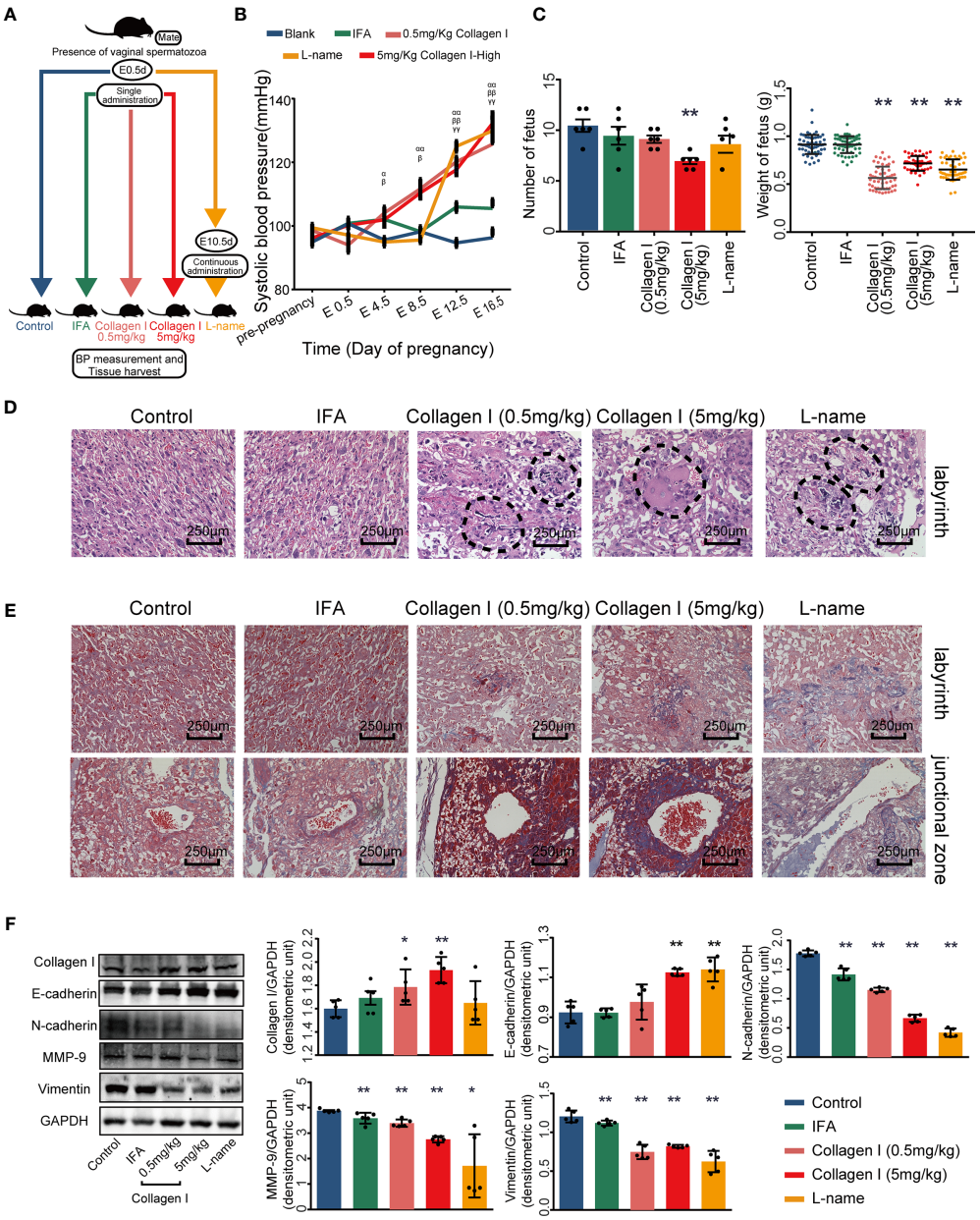


FIGURE 2 | Collagen I induces preeclampsia-like symptoms in mice. Effects of collagen I on the reproductive outcomes of pregnant mice. **(A)** Schematic representation of the experimental groups. **(B)** The systolic blood pressure of members of the five groups during pregnancy (n = 6 per group, α indicates mice treated with 5 mg/kg of collagen I *vs.* control, β indicates mice treated with 0.5 mg/kg of collagen I *vs.* control, γ indicates L-NAME-treated mice *vs.* control, with $^{\alpha,\beta,\gamma}p < 0.05$ and $^{\alpha\alpha,\beta\beta,\gamma\gamma}p < 0.01$, according to two-way repeated measures ANOVA). **(C)** The number of offspring significantly decreased in the group treated with 5 mg/kg of collagen I (n = 6 per group) compared with the control at 17.5 days of gestation. Fetal weights in the groups treated with 0.5 mg/kg of collagen I (n = 54), 5 mg/kg of collagen I (n = 46), and L-NAME (n = 49) were significantly decreased compared with those of the control (n = 59) and IFA-treated groups (n = 56). **(D)** Representative image of the H&E-stained labyrinth zone of mouse placenta. Typical lesions are marked with dotted lines (original magnification, ×200; scale bar = 250 μm, n = 4). **(E)** Representative image of Masson's trichrome staining in the labyrinth and junctional zone of the mouse placenta. (original magnification, ×200; scale bar = 250 μm, n = 4). **(F)** Western blot analysis of the protein level of collagen I and the proteins related to PE pathogenesis including MMP-9, E-cadherin, N-cadherin, and vimentin (n = 5, asterisks indicate differences between groups *vs.* control, with $^{*}p < 0.05$, $^{**}p < 0.01$, according to one-way ANOVA followed by Dunnett post-hoc test).

HTR-8/SVneo cells, reversed the decline in proliferation and the G2/M arrest induced by collagen I (**Figures 5C, D**).

Wnt/β-catenin signaling is an essential pathway promoting blastocyst activation and implantation. In particular, β-catenin plays a central role in canonical Wnt/β-catenin signaling. β-catenin levels decreased in both collagen I-treated mouse placenta and HTR-8/SVneo cells. Therefore, SKL-2001, an agonist of the Wnt/β-catenin pathway that can stabilize

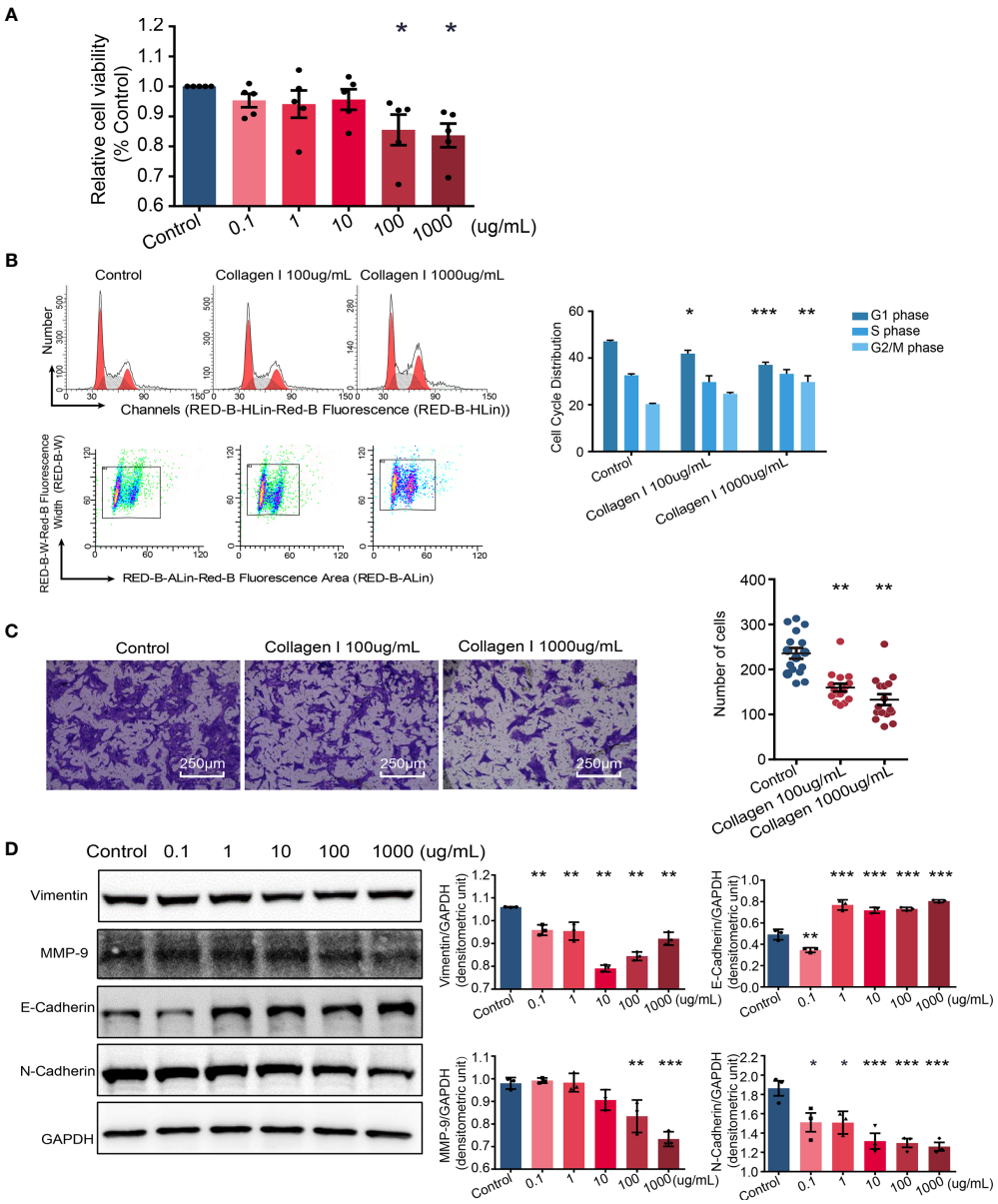


FIGURE 3 | Collagen I induce trophoblast dysfunction in HTR-8/SVneo cells. Effect of collagen I on proliferation and invasion abilities of HTR-8/SVneo cells. **(A)** HTR-8/SVneo cells were cultured at different concentrations of collagen I (0 μg/mL, 0.1 μg/mL, 1 μg/mL, 10 μg/mL, 100 μg/mL, and 1000 μg/mL). Cell proliferation of HTR-8SV/neo cells was analyzed by CCK-8 assay after 48 hours of incubation (n = 5 per group). **(B)** Effect of collagen I on the cell cycle profile of HTR-8SV/neo cells. After cultured at 100 μg/mL and 1000 μg/mL of collagen I for 48 hours, cells were labeled with propidium iodide and the stain was detected by laser scanning cytometry(LSC). Bar plot representing the proportion of cells at each phase of the cell cycle (G1, S, and G2/M phases) in three different cell groups (cells grown in presence of 100 μg/mL or 1000 μg/mL of collagen I and control cells; n = 5 per group). **(C)** Migration of HTR-8/SVneo cells. The total number of invading cells was counted in four representative fields under ×200 magnification (n = 4 per group). **(D)** Representative western blot image and quantitative analysis of vimentin, MMP-9, E-cadherin, and N-cadherin expression in the three cell groups (n = 3 per group). Asterisks indicate differences between groups *vs.* control, with *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, according to one-way ANOVA followed by Dunnett post-hoc test.

intracellular β-catenin levels, was used to clarify the role of β-catenin in collagen I-dependent remodulation of trophoblast properties. The relative protein levels of MMP-9, vimentin, N-cadherin, and E-cadherin were also recovered compared with those of collagen I-treated cells (**Figure 5E**).

Overall, our results showed that activators of ERK and β-catenin (honokiol and SKL-2001) reversed the decline in cell proliferation and invasion induced by collagen I, demonstrating that collagen I suppressed proliferation and invasion through inhibition of ERK phosphorylation, and the Wnt/β-catenin signal pathway.

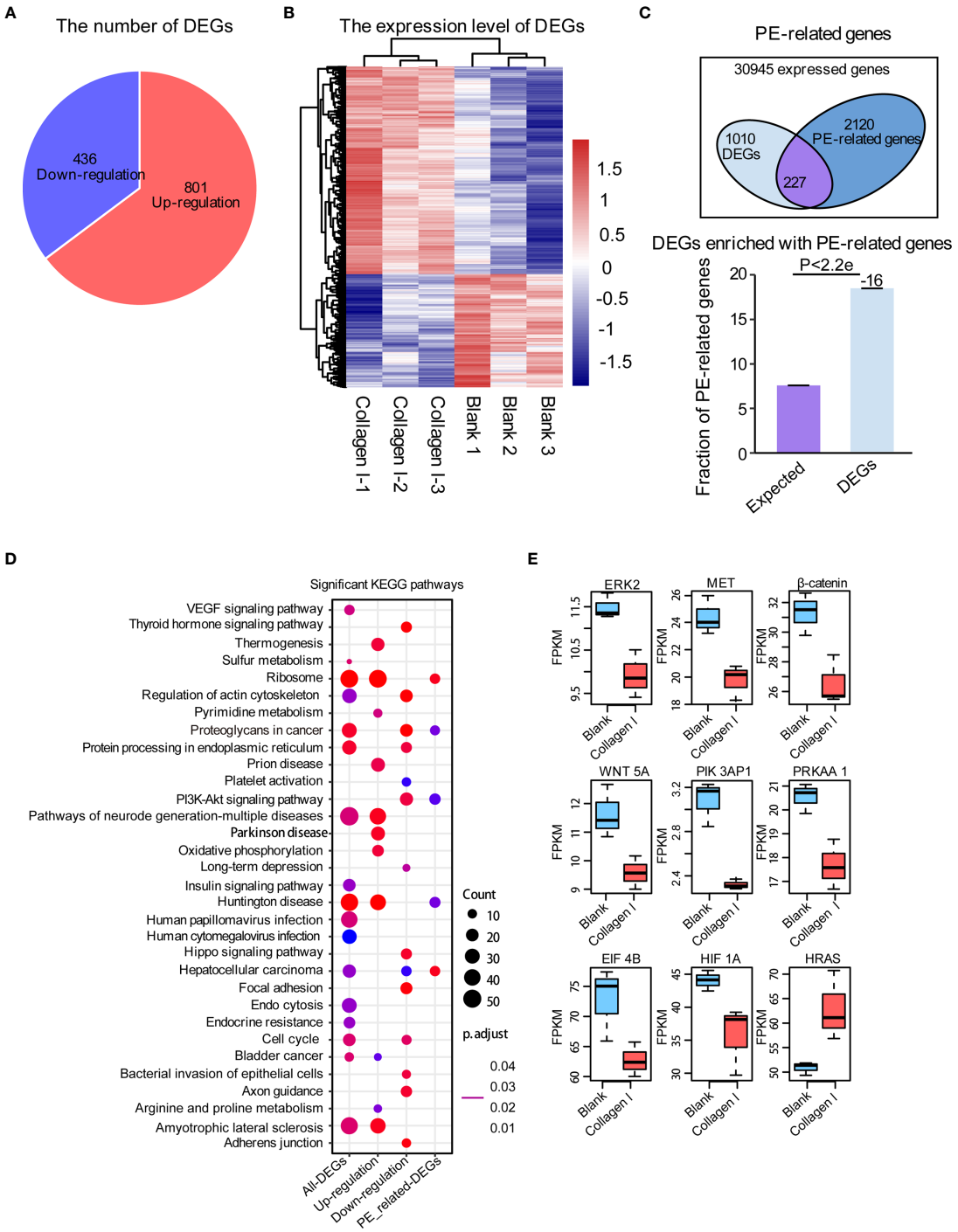


FIGURE 4 | Collagen I induce transcriptional changes in HTR-8SV/neo cells. Overview of differentially expressed genes upon collagen I treatment of HTR-8SV/neo cells. **(A)** Pie chart showing the number of differentially expressed genes between collagen I-treated cells and the control group. **(B)** Heatmap showing the expression levels of differentially expressed genes. **(C)** Venn diagram showing the PE-related genes expressed in our dataset. Differentially expressed genes were enriched in PE-related genes ($p < 0.05$). **(D)** Significantly enriched KEGG pathways within total differentially expressed genes, including upregulated genes, downregulated genes, and differentially expressed PE-related genes. **(E)** Boxplots showing the relative expression levels of *ERK2 (MAPK1)*, *MET*, *β-catenin*, *WNT5A*, *PIK3AP1*, *PRKAA1*, *EIF4B*, *HIF1A*, and *EIF4B*. Enrichment analyses were performed on the R platform, and two-tailed Fisher's exact test was used. Error bars represent the standard error of the fraction, estimated using a bootstrapping method with 100 re-samplings. The data of the experimental validations are presented as mean ± standard error of mean (SEM) of two independent experiments. Comparisons between two independent groups were conducted using Student's *t*-test.

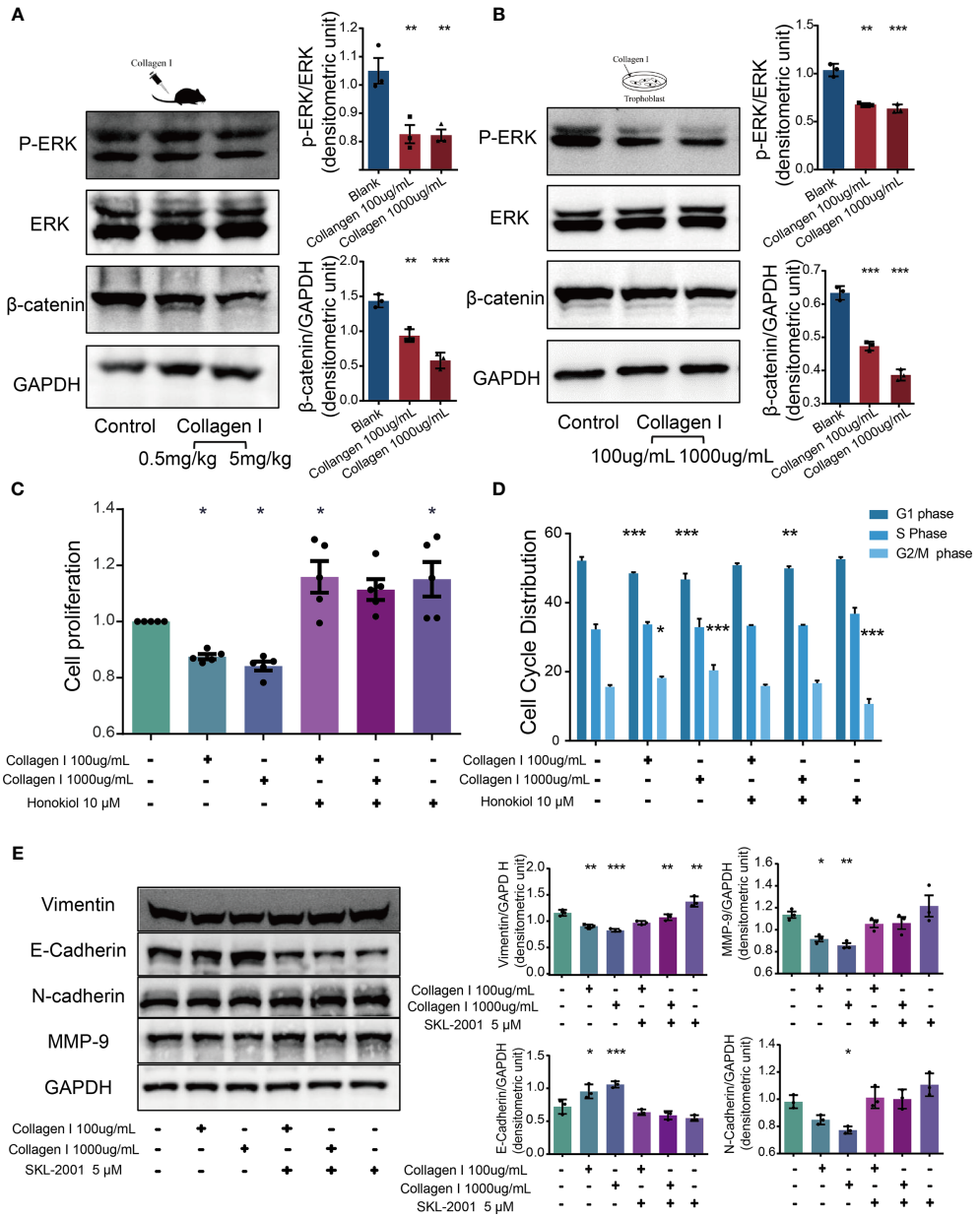


FIGURE 5 | Collagen I suppressed proliferation and invasion of trophoblasts through inhibition of ERK phosphorylation and the WNT/β-catenin signaling pathway. Honokiol and SKL-2001 reversed the decrease in proliferation and invasion ability of trophoblasts induced by collagen I. **(A)** Representative western blot image and quantitative analysis of p-ERK, ERK, and β-catenin levels in collagen I-treated mouse placentas (n = 3 per group). **(B)** Representative western blot image and quantitative analysis of p-ERK, ERK, and β-catenin levels in collagen I-treated HTR-8/SVneo cells (n = 3 per group). **(C)** After incubation with honokiol and collagen I for 48 hours, HTR-8/SVneo cell proliferation was measured by CCK-8 assay (n = 5 per group). **(D)** After incubation with honokiol and collagen I for 48 hours, cells were labelled with propidium iodide (PI) and the stain was detected by LSC. Bar plots representing the proportion of cells at each phase of the cell cycle (G1, S, and G2/M phases) in three different cell groups (n = 5 per group). **(E)** Representative western blot image and quantitative analysis of MMP-9, vimentin, E-cadherin, and N-cadherin levels in collagen I- and/or SKL-2001-treated HTR-8/SVneo cells (n = 3 per group). Asterisks indicate differences between groups *vs.* control, with *p < 0.05, **p < 0.01, and ***p < 0.001, according to one-way ANOVA followed by Dunnett's post-hoc test.

DISCUSSION

Herein, we proved the characteristic role of Collagen I in preeclamptic placenta is consistent with previous reports (17). Moreover, we determined that excess collagen I accumulation is associated with preeclampsia-like symptoms in pregnant mice. Indeed, a single injection of collagen I in early pregnancy led to increased blood pressure on E4.5d, which continued to stay elevated until term. Furthermore, the weight and number of offspring decreased when the tissue was harvested at term.

This phenomenon might depend on the decreased proliferation and invasion ability of trophoblasts in the presence of excess collagen I. In summary, we propose that excessive collagen I deposition in the placenta plays a crucial role in preeclampsia pathogenesis (summarized in **Figure 6**).

The pathogenesis of preeclampsia is still unclear. A possible reason for this is the difficulty in performing high-quality research on the etiology of placental pathologies in humans because of ethical challenges associated with the examination of pregnant women in the first trimester. Thus, animal models of preeclampsia are the most important tool for longitudinal investigation of adverse pregnancy outcomes (40). For instance, treating third-trimester pregnant mice with L-NAME, a common vasoconstrictor, to induce PE symptoms is a common practice, as once drug treatment is stopped, blood pressure returns to normal (41). In our study, a single injection of collagen I at early pregnancy led to changes in placental structure at term. In addition to a higher degree of fibrosis, collagen I-treated placentas showed a significantly higher collagen I protein level than the control group. Moreover, preeclampsia-like symptoms were induced in later trimesters of pregnancy. In comparison with L-NAME treatment, the symptoms induced by collagen I did not depend on continuous medication, but occurred in the second trimester and continued through the third. These disease dynamics were close to those observed in clinical practice.

It is widely accepted that primary defective trophoblast invasion, leading to inadequate transformation of maternal uterine vasculature, probably drives preeclampsia pathogenesis. It has also been indicated that early pregnancy placentation is closely related to trophoblast function and behavior (42). In our *in vitro* study, excessive collagen I induced trophoblast dysfunction by direct stimulation. It decreased the proliferation and invasion of HTR-8/SVneo cells. Surprisingly, we found that preeclampsia-like symptoms emerged in later pregnancy and infarction and a high degree of fibrosis were observed in the placenta after a single injection of collagen I on E0.5 d *in vivo*. Moreover, changes in MMP-9, vimentin, E-cadherin and N-cadherin expression both in placental and HTR-8SV/neo cells were consistent with the phenotypes of preeclampsia (36–38) after treatment with collagen I. Thus, we suggest that the single injection of collagen I in early pregnancy induced preeclampsia development by interfering with placentation in early pregnancy due to impaired trophoblast invasion. Collagen I is the most abundant structural protein in most tissues (43). However, excessive accumulation of collagen I may lead to fibrosis, which in turn impairs normal organ function. Trigonelline hydrocholoride, targeting collagen I fibrillation, could ameliorate fibrosis of the myocardium (44). Therefore, collagen I may also be a novel target for therapeutic interventions in patients with preeclampsia.

The ERK/MAPK pathway is an important signaling cascades that is involved in cell proliferation and embryo development (45). We found that collagen I downregulated ERK phosphorylation in HTR-8/SVneo cells and mouse placenta compared with the control. Honokiol which enhances the

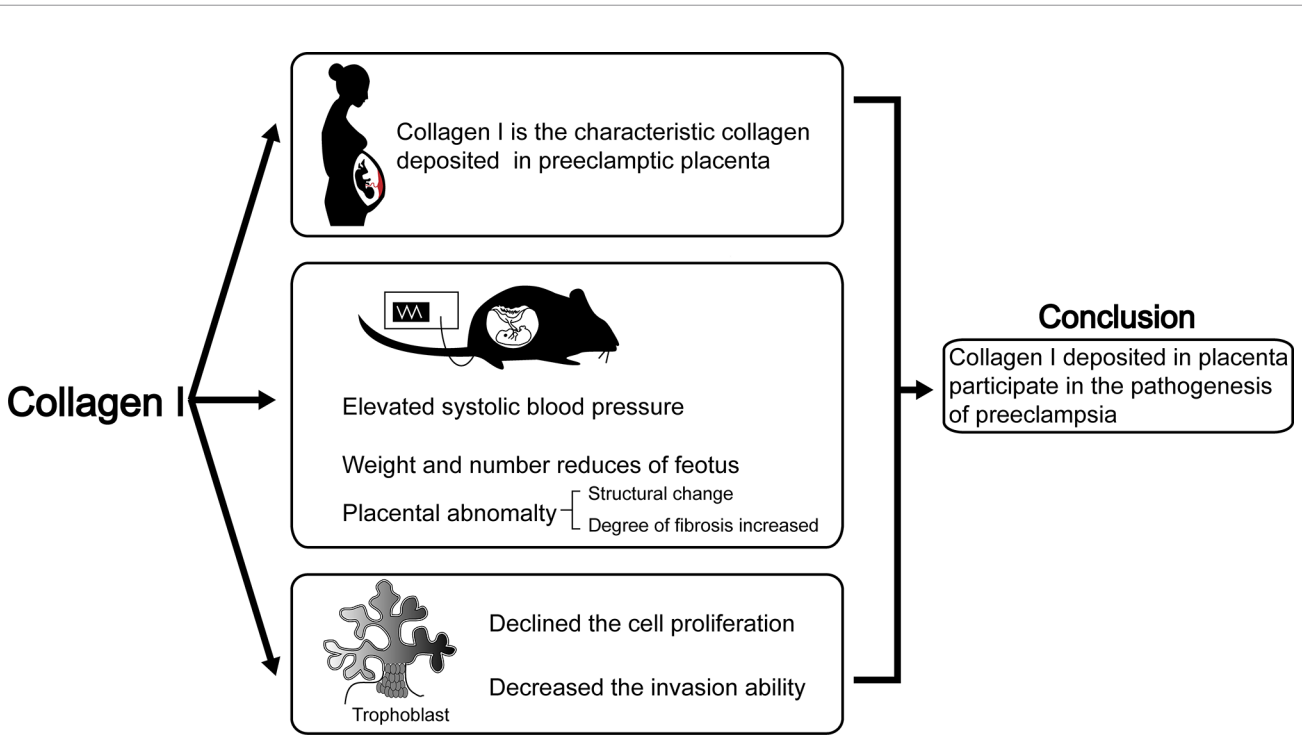


FIGURE 6 | The abstract graphic. Collagen I deposited in placenta participate in the pathogenesis of preeclampsia: Collagen I is the characteristic collagen deposited in preeclamptic placenta. Collagen I administration is associated with suppressed proliferation and invasion of trophoblasts, and with preeclampsia-like symptoms in pregnant mice.

phosphorylation of ERK phosphorylation, could reverse the weakened proliferation induced by collagen I. Further, the canonical "Wnt/β-catenin" pathway regulates cell invasion, and abnormal Wnt/β-catenin expression contributes to preeclampsia development (46). β-catenin and GSK-3β were proteins levels were reportedly significantly decreased in severe preeclamptic placenta (47). In our study, β-catenin expression in collagen I-treated placentas was less than that in the pregnant control Transcriptome analysis suggested β-catenin and GSK-3β expression decreased in HTR-8/SVneo cells incubated with 100μg/mL collagen I. To verify these findings, SKL-2001, an agonist of that β-catenin was used. *In vitro*, SKL-2001 could reverse the downregulation of MMP-9, N-cadherin and vimentin induced by collagen I. Thus, the results suggested that collagen I suppresses trophoblast proliferation and invasion through inhibition of ERK phosphorylation, and the WNT/β-catenin signaling pathway.

There were some limitations of this study. First, our experiments demonstrated that the dysfunction of HTR-8/SVneo cells induced by collagen I can be reversed by ERK and β-catenin agonists. Therefore, the possible role of upstream regulators of the ERK/MAPK and WNT/β-catenin pathways in preeclampsia pathogenesis should be investigated. Second, placental Masson's and Sirius red staining showed that the degree of fibrosis in PE patients was more serious than that in normal patients after delivery. However, whether the extent of placental fibrosis and its alteration can be determined by ultrasound or other diagnostic methods during pregnancy, calls for further clinical study and discussion. Third, small-molecule inhibitors against placental collagen I deposition are still lacking and should be explored as a future research avenue. Finally, the sample is relatively small, larger sample size is required in our coming study.

Overall, in this study we confirmed that the preeclamptic placenta shows significant histological signs of fibrosis and is characterized by collagen I deposition. Furthermore, an excess of collagen I caused preeclampsia-like features in pregnant mice by suppressing proliferation and invasion of trophoblasts. *In vitro*, we detected altered expression of genes related to proliferation and invasion upon collagen I stimulation. In conclusion, our study points at the relevant role of collagen I in the development of preeclamptic placentas, providing new insights into the pathogenesis of preeclampsia.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://data.mendeley.com/datasets/hcd2c3n9tc/1, The Mendeley dataset DOI: 10.17632/hcd2c3n9tc.1. https://github.com/YingLin-Feng/Collagen-I-and-preeclampsia.

ETHICS STATEMENT

The collection of placentae was approved by the Ethics Committee of Nanfang Hospital (NFEC-2017-055). All participants gave written consent prior to donating their placenta. The patients/participants provided their written informed consent to participate in this study. This project was performed in accordance with animal protocol procedures approved by the Department of Laboratory Animal Sciences, Southern Medical University (L-2019216).

AUTHOR CONTRIBUTIONS

YF, XiC, and LH designed the study. Data collection was performed by HW, YiC, ML, ZL, XuC, YuC, YW, CS, and YH. Data analysis was done by PL, JL, MZ, ZW, and XY. YF wrote the manuscript and designed the figures, whilst all other authors revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the National Natural Science Foundation of China (82071669), Foshan Dengfeng project (2020B002), the Natural Science Foundation of Guangdong Province (2019A1515010637) and Science and Technology Planning Project of Guangdong Province (2017A010105025).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.664766/full#supplementary-material

REFERENCES

1. Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. *Obstet Gynecol* (2020) 135(6):e237–60. doi: 10.1097/AOG.0000000000003891
2. Hypertension in Pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstet Gynecol* (2013) 122(5):1122–31. doi: 10.1097/01.AOG.0000437382.03963.88
3. Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia: An Endothelial Cell Disorder. *Am J Obstet Gynecol* (1989) 161(5):1200–4. doi: 10.1016/0002-9378(89)90665-0
4. Ducray JF, Naicker T, Moodley J. Pilot Study of Comparative Placental Morphometry in Pre-Eclamptic and Normotensive Pregnancies Suggests Possible Maladaptations of the Fetal Component of the Placenta. *Eur J Obstet Gynecol Reprod Biol* (2011) 156(1):29–34. doi: 10.1016/j.ejogrb.2010.12.038
5. Devisme L, Merlot B, Ego A, Houfflin-Debarge V, Deruelle P, Subtil D. A Case-Control Study of Placental Lesions Associated With Pre-Eclampsia. *Int J Gynaecol Obstet* (2013) 120(2):165–8. doi: 10.1016/j.ijgo.2012.08.023
6. Ohmaru-Nakanishi T, Asanoma K, Fujikawa M, Fujita Y, Yagi H, Onoyama I, et al. Fibrosis in Preeclamptic Placentas Is Associated With Stromal Fibroblasts Activated by the Transforming Growth Factor-Beta1 Signaling Pathway. *Am J Pathol* (2018) 188(3):683–95. doi: 10.1016/j.ajpath.2017.11.008
7. Zeisberg M, Kalluri R. Cellular Mechanisms of Tissue Fibrosis. 1. Common and Organ-Specific Mechanisms Associated With Tissue

Fibrosis. *Am J Physiol Cell Physiol* (2013) 304(3):C216–25. doi: 10.1152/ajpcell.00328.2012
8. Rockey DC, Bell PD, Hill JA. Fibrosis–A Common Pathway to Organ Injury and Failure. *N Engl J Med* (2015) 372(12):1138–49. doi: 10.1056/NEJMra1300575
9. Weiskirchen R, Weiskirchen S, Tacke F. Organ and Tissue Fibrosis: Molecular Signals, Cellular Mechanisms and Translational Implications. *Mol Aspects Med* (2019) 65:2–15. doi: 10.1016/j.mam.2018.06.003
10. Wahyudi H, Reynolds AA, Li Y, Owen SC, Yu SM. Targeting Collagen for Diagnostic Imaging and Therapeutic Delivery. *J Control Release* (2016) 240:323–31. doi: 10.1016/j.jconrel.2016.01.007
11. Zhang Y, Stefanovic B. LARP6 Meets Collagen mRNA: Specific Regulation of Type I Collagen Expression. *Int J Mol Sci* (2016) 17(3):419. doi: 10.3390/ijms17030419
12. Ricard-Blum S, Baffet G, Theret N. Molecular and Tissue Alterations of Collagens in Fibrosis. *Matrix Biol* (2018) 68-69:122–49. doi: 10.1016/j.matbio.2018.02.004
13. Lindsey ML, Iyer RP, Zamilpa R, Yabluchanskiy A, DeLeon-Pennell KY, Hall ME, et al. A Novel Collagen Matricryptin Reduces Left Ventricular Dilation Post-Myocardial Infarction by Promoting Scar Formation and Angiogenesis. *J Am Coll Cardiol* (2015) 66(12):1364–74. doi: 10.1016/j.jacc.2015.07.035
14. Lambert E, Fuselier E, Ramont L, Brassart B, Dukic S, Oudart JB, et al. Conformation-Dependent Binding of a Tetrastatin Peptide to Alphavbeta3 Integrin Decreases Melanoma Progression Through FAK/PI3K/Akt Pathway Inhibition. *Sci Rep* (2018) 8(1):9837. doi: 10.1038/s41598-018-28003-x
15. Stefanovic B, Manojlovic Z, Vied C, Badger CD, Stefanovic L. Discovery and Evaluation of Inhibitor of LARP6 as Specific Antifibrotic Compound. *Sci Rep* (2019) 9(1):326. doi: 10.1038/s41598-018-36841-y
16. Sadeghi-Avalshahr AR, Nokhasteh S, Molavi AM, Mohammad-Pour N, Sadeghi M. Tailored PCL Scaffolds as Skin Substitutes Using Sacrificial PVP Fibers and Collagen/Chitosan Blend. *Int J Mol Sci* (2020) 21(7):2311. doi: 10.3390/ijms21072311
17. Xu XH, Jia Y, Zhou X, Xie D, Huang X, Jia L, et al. Downregulation of Lysyl Oxidase and Lysyl Oxidase-Like Protein 2 Suppressed the Migration and Invasion of Trophoblasts by Activating the TGF-Beta/Collagen Pathway in Preeclampsia. *Exp Mol Med* (2019) 51(2):1–12. doi: 10.1038/s12276-019-0211-9
18. Shu W, Li H, Gong H, Zhang M, Niu X, Ma Y, et al. Evaluation of Blood Vessel Injury, Oxidative Stress and Circulating Inflammatory Factors in an L-NAME-Induced Preeclampsia-Like Rat Model. *Exp Ther Med* (2018) 16 (2):585–94. doi: 10.3892/etm.2018.6217
19. Marshall J. Transwell((R)) Invasion Assays. *Methods Mol Biol* (2011) 769:97–110. doi: 10.1007/978-1-61779-207-6_8
20. Love MI, Huber W, Anders S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data With Deseq2. *Genome Biol* (2014) 15(12):550. doi: 10.1186/s13059-014-0550-8
21. Kleinrouweler CE, van Uitert M, Moerland PD, Ris-Stalpers C, van der Post JA, Afink GB. Differentially Expressed Genes in the Pre-Eclamptic Placenta: A Systematic Review and Meta-Analysis. *PloS One* (2013) 8(7):e68991. doi: 10.1371/journal.pone.0068991
22. Vaiman D, Calicchio R, Miralles F. Landscape of Transcriptional Deregulations in the Preeclamptic Placenta. *PloS One* (2013) 8(6):e65498. doi: 10.1371/journal.pone.0065498
23. Brew O, Sullivan MH, Woodman A. Comparison of Normal and Pre-Eclamptic Placental Gene Expression: A Systematic Review With Meta-Analysis. *PloS One* (2016) 11(8):e161504. doi: 10.1371/journal.pone.0161504
24. Vaiman D, Miralles F. An Integrative Analysis of Preeclampsia Based on the Construction of an Extended Composite Network Featuring Protein-Protein Physical Interactions and Transcriptional Relationship. *PloS One* (2016) 11 (11):e165849. doi: 10.1371/journal.pone.0165849
25. Tejera E, Cruz-Monteagudo M, Burgos G, Sanchez ME, Sanchez-Rodriguez A, Perez-Castillo Y, et al. Consensus Strategy in Genes Prioritization and Combined Bioinformatics Analysis for Preeclampsia Pathogenesis. *BMC Med Genomics* (2017) 10(1):50. doi: 10.1186/s12920-017-0286-x
26. Moslehi R, Mills JL, Signore C, Kumar A, Ambroggio X, Dzutsev A. Integrative Transcriptome Analysis Reveals Dysregulation of Canonical Cancer Molecular Pathways in Placenta Leading to Preeclampsia. *Sci Rep* (2013) 3:2407. doi: 10.1038/srep02407
27. Song Y, Liu J, Huang S, Zhang L. Analysis of Differentially Expressed Genes in Placental Tissues of Preeclampsia Patients Using Microarray Combined With the Connectivity Map Database. *Placenta* (2013) 34(12):1190–5. doi: 10.1016/j.placenta.2013.09.013
28. Sitras V, Paulssen RH, Gronaas H, Leirvik J, Hanssen TA, Vartun A, et al. Differential Placental Gene Expression in Severe Preeclampsia. *Placenta* (2009) 30(5):424–33. doi: 10.1016/j.placenta.2009.01.012
29. Kobayashi H. Characterization of the Down-Regulated Genes Identified in Preeclampsia Placenta. *Hypertens Pregnancy* (2016) 35(1):15–21. doi: 10.3109/10641955.2015.1116555
30. Kaartokallio T, Cervera A, Kyllonen A, Laivuori K, Kere J, Laivuori H. Gene Expression Profiling of Pre-Eclamptic Placentae by RNA Sequencing. *Sci Rep* (2015) 5:14107. doi: 10.1038/srep14107
31. Yu G, Wang LG, Han Y, He QY. Clusterprofiler: An R Package for Comparing Biological Themes Among Gene Clusters. *Omics* (2012) 16(5):284–7. doi: 10.1089/omi.2011.0118
32. Luo W, Brouwer C. Pathview: An R/Bioconductor Package for Pathway-Based Data Integration and Visualization. *Bioinformatics* (2013) 29(14):1830–1. doi: 10.1093/bioinformatics/btt285
33. Winters J, von Braunmuhl ME, Zeemering S, Gilbers M, Brink TT, Scaf B, et al. JavaCyte, A Novel Open-Source Tool for Automated Quantification of Key Hallmarks Of Cardiac Structural Remodeling. *Sci Rep* (2020) 10(1):20074. doi: 10.1038/s41598-020-76932-3
34. Liang X, Yang LX, Guo R, Shi Y, Hou X, Yang Z, et al. Atorvastatin Attenuates Plaque Vulnerability by Downregulation of EMMPRIN Expression *via* COX-2/PGE2 Pathway. *Exp Ther Med* (2017) 13(3):835–44. doi: 10.3892/etm.2017.4062
35. Cui Y, Wang W, Dong N, Lou J, Srinivasan DK, Cheng W, et al. Role of Corin in Trophoblast Invasion and Uterine Spiral Artery Remodelling in Pregnancy. *Nature* (2012) 484(7393):246–50. doi: 10.1038/nature10897
36. Espino YSS, Flores-Pliego A, Espejel-Nunez A, Medina-Bastidas D, Vadillo-Ortega F, Zaga-Clavellina V, et al. New Insights Into the Role of Matrix Metalloproteinases in Preeclampsia. *Int J Mol Sci* (2017) 18(7):1488. doi: 10.3390/ijms18071448
37. Vicovac L, Aplin JD. Epithelial-Mesenchymal Transition During Trophoblast Differentiation. *Acta Anat (Basel)* (1996) 156(3):202–16. doi: 10.1159/000147847
38. Yue D, Li H, Che J, Zhang Y, Tseng HH, Jin JQ, et al. Hedgehog/Gli Promotes Epithelial-Mesenchymal Transition in Lung Squamous Cell Carcinomas. *J Exp Clin Cancer Res* (2014) 33:34. doi: 10.1186/1756-9966-33-34
39. Liu F, Feng XX, Zhu SL, Huang HY, Chen YD, Pan YF, et al. Sonic Hedgehog Signaling Pathway Mediates Proliferation and Migration of Fibroblast-Like Synoviocytes in Rheumatoid Arthritis via MAPK/ERK Signaling Pathway. *Front Immunol* (2018) 9:2847. doi: 10.3389/fimmu.2018.02847
40. Sones JL, Davisson RL. Preeclampsia, of Mice and Women. *Physiol Genomics* (2016) 48(8):565–72. doi: 10.1152/physiolgenomics.00125.2015
41. Kanashiro CA, Cockrell KL, Alexander BT, Granger JP, Khalil RA. Pregnancy-Associated Reduction in Vascular Protein Kinase C Activity Rebounds During Inhibition of NO Synthesis. *Am J Physiol Regul Integr Comp Physiol* (2000) 278(2):R295–303. doi: 10.1152/ajpregu.2000.278.2.R295
42. Ridder A, Giorgione V, Khalil A, Thilaganathan B. Preeclampsia: The Relationship Between Uterine Artery Blood Flow and Trophoblast Function. *Int J Mol Sci* (2019) 20(13):2363. doi: 10.3390/ijms20133263
43. Yamauchi M, Sricholpech M, Terajima M, Tomer KB, Perdivara I. Glycosylation of Type I Collage. *Methods Mol Biol* (2019) 1934:127–44. doi: 10.1007/978-1-4939-9055-9_9
44. Rasheeda K, Fathima NN. Trigonelline Hydrochloride: A Promising Inhibitor for Type I Collagen Fibrillation. *Colloids Surf B Biointerfaces* (2018) 170:273–9. doi: 10.1016/j.colsurfb.2018.06.030
45. Xing CY, Zhang DX, Gui SQ, Tao MF. Kidney-Replenishing Herb Induces SOCS-3 Expression *via* ERK/MAPK Pathway and Improves Growth of the First-Trimester Human Trophoblast Cell. *Evid Based Complement Alternat Med* (2017) 2017:2473431. doi: 10.1155/2017/2473431
46. Zhang Z, Wang X, Zhang L, Shi Y, Wang J, Yan H. Wnt/beta-Catenin Signaling Pathway in Trophoblasts and Abnormal Activation in Preeclampsia (Review). *Mol Med Rep* (2017) 16(2):1007–13. doi: 10.3892/mmr.2017.6718

47. Wang X, Zhang Z, Zeng X, Wang J, Zhang L, Song W, et al. Wnt/beta-Catenin Signaling Pathway in Severe Preeclampsia. *J Mol Histol* (2018) 49(3):317–27. doi: 10.1007/s10735-018-9770-7

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 15 September 2021
doi: 10.3389/fendo.2021.742145

Gestational Hormone Concentrations Are Associated With Timing of Delivery in a Fetal Sex-Dependent Manner

*Amber L. Cathey[1], Deborah J. Watkins[1], Zaira Y. Rosario[2], Carmen M. Vélez Vega[2], Bhramar Mukherjee[3], Marie S. O'Neill[1,4], Rita Loch-Caruso[1], Akram N. Alshawabkeh[5], José F. Cordero[6] and John D. Meeker[1]**

[1] Department of Environmental Health Sciences, University of Michigan School of Public Health, Ann Arbor, MI, United States, [2] Graduate School of Public Health, University of Puerto Rico, San Juan, PR, United States, [3] Department of Biostatistics, University of Michigan School of Public Health, Ann Arbor, MI, United States, [4] Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, MI, United States, [5] College of Engineering, Northeastern University, Boston, MA, United States, [6] College of Public Health, University of Georgia, Athens, GA, United States

OPEN ACCESS

Edited by:
Qiongjie Zhou,
Fudan University, China

Reviewed by:
Rajakumar Anbazhagan,
Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), United States
Irene Scavello,
Careggi University Hospital, Italy

***Correspondence:**
John D. Meeker
meekerj@umich.edu

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: 15 July 2021
Accepted: 25 August 2021
Published: 15 September 2021

Citation:
Cathey AL, Watkins DJ, Rosario ZY, Vega CMV, Mukherjee B, O'Neill MS, Loch-Caruso R, Alshawabkeh AN, Cordero JF and Meeker JD (2021) Gestational Hormone Concentrations Are Associated With Timing of Delivery in a Fetal Sex-Dependent Manner. Front. Endocrinol. 12:742145. doi: 10.3389/fendo.2021.742145

Background: Early delivery remains a significant public health problem that has long-lasting impacts on mother and child. Understanding biological mechanisms underlying timing of labor, including endocrine disruption, can inform prevention efforts.

Methods: Gestational hormones were measured among 976 women in PROTECT, a longitudinal birth cohort in Puerto Rico. We evaluated associations between hormone concentrations at 18 and 26 weeks gestation and gestational age at birth, while assessing effect modification by fetal sex. Exploratory analyses assessed binary outcomes of overall preterm birth (PTB, <37 weeks gestation) and the spontaneous PTB subtype, defined as preterm premature rupture of membranes, spontaneous preterm labor, or both. Multivariable logistic and linear regressions were fit using visit-specific hormone concentrations, and fetal sex-specific effects were estimated using interaction terms. Main outcome models were adjusted for maternal age, education, marital status, alcohol consumption, environmental tobacco smoke exposure, and pre-pregnancy body mass index (BMI). Exploratory models adjusted for maternal age and education.

Results: We observed reduced gestational age at birth with higher circulating CRH (β: -2.73 days, 95% CI: -4.97, -0.42), progesterone (β: -4.90 days, 95% CI: -7.07, -2.73), and fT4 concentrations (β: -2.73 days, 95% CI: -4.76, -0.70) at 18 weeks specifically among male fetuses. Greater odds of overall and spontaneous PTB were observed among males with higher CRH, estriol, progesterone, total triiodothyronine (T3), and free thyroxine (fT4) concentrations. Greater odds of PTB among females was observed with higher testosterone concentrations.

Conclusions: Various associations between hormones and timing of delivery were modified by fetal sex and timing of hormone measurement. Future studies are needed to understand differential mechanisms involved with timing of labor between fetal sexes.

Keywords: birth cohort, corticotropin releasing hormone, gestational age, pregnancy, preterm birth, progesterone

INTRODUCTION

Delivery before 37 weeks of gestation, or preterm birth (PTB), is the leading cause of neonatal mortality worldwide (1). The preterm birth rate in the United States is higher than that of most other high-income nations and increased to 9.85% from 2014 to 2016 (2). This increase was primarily driven by late PTBs, which occur between 34 and 36 weeks gestation, suggesting that development of targeted prediction strategies for detecting pregnancies destined for late preterm delivery could have marked impacts on the public health burden of early delivery. Infants born preterm are at increased risk for adverse health outcomes later in life including reduced renal function (3), neurodevelopmental impairments (4), cerebral palsy (5), and reduced myocardial function (6). Despite being a common public health problem, the causes of early delivery are largely unknown.

Preterm birth can be subcategorized into spontaneous and indicated groups based on obstetric presentation, and some have suggested that assessing these subtypes separately may better inform their etiologies. The spontaneous subtype occurs from spontaneous premature initiation of labor or rupture of membranes, and is characterized by an inflammatory uterine environment which is not present in medically indicated PTBs (7). The physiological changes that occur upstream of clinical detection of PTB may be distinct between subgroups and could help clinicians determine pregnancies that are at high risk for early delivery.

The maternal and fetal endocrine milieus change and interact in unique ways at different points throughout gestation. Numerous hormones play critical roles during pregnancy and so understanding their effects on the uterine environment and fetal growth could shed light on mechanisms involved with early delivery. Hormones of special interest during pregnancy can be broken into two categories: reproductive hormones and thyroid hormones. Reproductive hormones include testosterone, sex hormone binding globulin (SHBG), estriol, and progesterone. Elevated concentrations of testosterone are observed in women with polycystic ovarian syndrome (PCOS), as well as women with recurring miscarriages. Testosterone may reduce levels of endometrial secretory proteins which are positively associated with length of gestation (8), or it may act antagonistically with estrogens (9). Testosterone circulates through the body bound to SHBG, so changing SHBG concentrations could affect the concentration of bioavailable testosterone. The coordination of concentrations of estriol and progesterone are critically important for the timing of labor, as estriol primes the uterus for contractions (10) and progesterone promotes quiescence of the uterus until the time of labor (11, 12). Progesterone keeps the actions of estriol in check through pregnancy, but an untimely shift in dominance from progesterone to estriol could result in early delivery.

Thyroid hormones are critical early in pregnancy for proper brain and skeletal development of the fetus (13). The maternal supply of thyroxine (T4) is particularly important in the first half of pregnancy, before the fetal thyroid gland has matured enough to produce adequate hormones (14). Through gestation, thyroid hormones are important for fetal growth and are correlated with infant weight and length at birth (15). Previous studies have demonstrated associations between clinical hyper- and hypothyroidism and adverse birth outcomes (16–19), but much less is known about subclinical thyroid disruption and gestational length.

Finally, corticotropin releasing hormone (CRH), which is secreted from the hypothalamus and normally involved in stress response, may also be key in understanding the endocrine role in early delivery. CRH concentrations are low in the first half of pregnancy and then begin to exponentially increase around the 20th week of gestation to peak at birth (20). An earlier and more rapid increase of CRH concentrations has been observed in women who experience early delivery (21), suggesting that CRH may be involved in a placental clock and that monitoring CRH concentrations early in pregnancy could provide clues about pregnancies at risk for early delivery.

Few large epidemiological studies exist that assess a wide array of hormone concentrations and timing of delivery. The majority of existing research focuses on one hormone/class of hormones, which makes it challenging to gain a broad understanding of the endocrine pathways implicated in the etiology of early delivery (22–24). Importantly, the spontaneous subtype of PTB has not been well studied. Few previous studies have investigated hormone concentrations at more than one time point during gestation, which is critical because some hormone concentrations change dramatically throughout gestation. Previous work also has not widely assessed the impact of fetal sex on these associations, despite existing literature showing male fetuses conferring greater risk of early delivery, perhaps *via* a greater pro-inflammatory environment (25) or relatively greater weight for gestational age at birth (26). Further, concentrations of hormones such as testosterone, estriol, and progesterone have been shown to be influenced by the sex of the fetus (27–29). Because of these gaps in the literature, the aim of this study was to investigate associations between various hormone concentrations and gestational age at birth, specific to timing of hormone assessment and fetal sex. Additionally, we conducted an exploratory analysis in which binary outcomes of overall PTB and spontaneous PTB were assessed.

MATERIALS AND METHODS

Study Population

Pregnant women were recruited into the PROTECT birth cohort between 2011 and 2018 at 14±2 weeks' gestation from seven hospitals and prenatal clinics in northern Puerto Rico. Study design and recruitment protocols have been described elsewhere (30). Briefly, women were recruited at 14±2 weeks gestation and were eligible to participate if they were between the ages of 18 and 40 years, participated in their first clinic visit before their 20th week of pregnancy, had not taken oral contraceptives within 3 months of getting pregnant, had not used *in vitro* fertilization to get pregnant, and had no known preexisting medical or obstetric conditions. Demographic and self-reported health information was provided at the first clinic visit. This study was approved by the research and ethics committees of the University of Michigan School of Public Health, University of Puerto Rico, Northeastern University, and participating hospitals and clinics. All methods reported in this study were performed in accordance with relevant guidelines and regulations imposed by those institutions. All study participants provided full informed consent prior to participation.

Hormone Measurements

All women provided serum samples at their first and third clinic visits, aligning with median 18 (range 16-20) and 26 (range 24-28) weeks gestation which are periods of rapid fetal growth and development. Serum samples were analyzed at the Central Ligand Assay Satellite Services (CLASS) laboratory in the Department of Epidemiology at the University of Michigan School of Public Health. Progesterone, sex hormone-binding globulin (SHBG), testosterone, total triiodothyronine (T3), total thyroxine (T4), free thyroxine (fT4), and thyroid-stimulating hormone (TSH) were measured using a chemiluminescence immunoassay. Estriol and corticotropin releasing hormone (CRH) were measured using an enzyme immunoassay. Some hormone concentrations were not available for all participants due to sample volume limitations. The ratios of progesterone to estriol (Prog/E3) and T3 to T4 (T3/T4) were assessed in addition to measured hormones because of previous research indicating that the ratios may be better indices of gestational age at birth than single hormone measurements (23, 31, 32). All hormone concentrations below the limit of detection (LOD) were replaced by the LOD divided by the square root of two.

Birth Outcome Assessment

Based on recommendations from the American College of Obstetricians and Gynecologists, self-reported date of the last menstrual period was collected at the first study visit and used in combination with early ultrasound measurements to determine gestational age at birth (33). Briefly, the LMP was used as the gold standard and was compared to ultrasound measurements taken primarily before 14 weeks gestation. Gestational age was changed from the LMP estimate to the ultrasound estimate if the difference between the two methods was greater than a certain number of days, depending on which week the ultrasound was performed. PTB was defined as delivery before 37 weeks gestation. We also assessed spontaneous PTB, defined as PTB presenting with premature rupture of membranes, spontaneous preterm labor, or both (7).

Statistical Methods

Distributions of demographic, health, and pregnancy characteristics were calculated. Distributions of hormone concentrations were assessed individually at each study visit. Summary measures of gestational hormone concentrations were also assessed for descriptive purposes using arithmetic means of all available concentrations for each study participant, or geometric means for log-normally distributed hormones. Intraclass correlation coefficients (ICCs) were used to assess between- and within-individual variability of hormone concentrations across study visits.

Linear regression was used to model gestational age at birth, and logistic regression was used to model overall and spontaneous PTB. All statistical models utilized visit-specific hormone concentrations as opposed to summary measures. Effect estimates specific to fetal sex and study visit were derived using two interaction terms: one between the exposure variable and an indicator for fetal sex, and one between the exposure variable and an indicator for study visit. Interaction term p-values less than 0.05 were deemed significant. Sandwich estimators were used in these models to correct for biased standard errors due to the non-repeating nature of outcome variables. Possible confounders were first explored by evaluating their associations with exposure and outcome variables. Then, possible confounders were added into statistical models in a forward stepwise manner and retained in the model if the resultant change in the main effect estimate was greater than 10%. Main gestational age at birth models adjusted for categorical forms of maternal age, maternal education, marital status, exposure to environmental tobacco smoke, alcohol consumption, and pre-pregnancy BMI. All models assessing testosterone also included SHBG to adjust for bound testosterone. In exploratory analyses assessing binary outcomes of overall and spontaneous PTB, statistical models were adjusted only for maternal age and maternal education to maximize sample size.

RESULTS

Demographics of the study population are shown in **Table 1**. The majority of mothers were under the age of 30 (67.1%), had at least some college education (79%), were employed (63%), had an annual household income under $30,000 (63.1%), were married (53.1%), reported never smoking (86%) or being exposed to environmental tobacco smoke (88.7%), did not drink alcohol during pregnancy (93.6%), had given birth to less than 2 previous children (86.9%), and had a pre-pregnancy BMI of less than 25 (56.1%).

Distributions of hormone concentrations are shown in **Supplementary Table S1**. ICCs for most hormones were high (0.65-0.86), indicating only modest variability in hormone measurements within individuals across study visits.

TABLE 1 | Maternal demographic characteristics of the study population (N=976).

	N (%)
Maternal Age (years)	
18-24	354 (36.3%)
25-29	301 (30.8%)
30-34	206 (21.1%)
35-41	115 (11.8%)
Maternal Education	
GED or less	203 (21%)
Some College	331 (34.2%)
Bachelors or Higher	433 (44.8%)
Employment Status	
No	357 (37%)
Yes	608 (63%)
Annual Household Income	
<10k	269 (31.6%)
10k-<30k	268 (31.5%)
30k-<50k	203 (23.8%)
>=50k	112 (13.1%)
Marital Status	
Single	197 (20.4%)
Married	521 (53.9%)
Cohabitating	249 (25.7%)
Smoking Status	
Never	833 (86%)
Ever	121 (12.5%)
Current	15 (1.55%)
Daily Environmental Tobacco Smoke Exposure	
Never	808 (88.7%)
1 Hour or less	40 (4.39%)
>1 Hour	63 (6.92%)
Alcohol Use	
Never	504 (52.2%)
Yes, before Pregnancy	400 (41.4%)
Yes, currently	62 (6.42%)
Number of Previous Children	
0	355 (42.7%)
1	367 (44.2%)
2 to 5	109 (13.1%)
Pre-Pregnancy BMI	
[0,25]	520 (56.1%)
(25, 30]	240 (25.9%)
Above 30	167 (18%)
Fetal Sex	
Female	464 (48%)
Male	502 (52%)

TABLE 2 | Distributions of gestational age at birth, overall preterm birth, and spontaneous preterm birth.

Gestational Age (wks)	Min	10th	25th	50th	75th	90th	Max
Male Fetuses	23.3	36.6	38.3	39.1	40.0	40.7	42.7
Female Fetuses	21.1	37.0	38.1	39.1	40.1	40.7	42.7
	Male Fetuses	**Female Fetuses**					
Preterm Birth	**N (%)**	**N (%)**					
Yes	53 (10.7%)	41 (8.9%)					
No	444 (89.3%)	419 (91.1%)					
Spontaneous Preterm Birth	**N (%)**	**N (%)**					
Yes	32 (6.6%)	22 (4.9%)					
No	451 (93.4%)	428 (95.1%)					

Exceptions were estriol (ICC: -0.22, 95% CI: -0.35, -0.11) and progesterone (ICC: 0.07, 95% CI: -0.04, 0.17), both of which displayed significantly higher concentrations at 26 weeks compared to 18 weeks.

Distributions of birth outcomes, stratified by fetal sex, are shown in **Table 2**. The median gestational age at birth was 39.1 weeks for pregnancies carrying either a male or a female fetus. Both overall and spontaneous PTB occurred more frequently among pregnancies with a male fetus (10.7% and 6.6%, respectively) than those with a female fetus (8.9% and 4.9%, respectively).

CRH and Reproductive Hormones

Figure 1A shows the associations between CRH and reproductive hormone concentrations and gestational age at birth, specific to fetal sex and study visit (effect estimates, confidence intervals, and p-values for fetal sex interaction terms can be found in **Supplementary Table S2**). Fetal sex significantly modified the association between progesterone and gestational age at birth (interaction p=0.015); among male fetuses only, an IQR increase in progesterone at 18 weeks was associated with a decrease in gestational age at delivery of 4.9 days (95% CI: 2.73, 7.07). Other significant findings were observed without significant effect modification by fetal sex. An IQR increase in CRH among male fetuses at 18 weeks was associated with a reduction in gestational age at delivery of 2.73 days (95% CI: 0.42, 4.97). Male fetuses also had a marginal reduction in gestational age at birth with an IQR increase in the ratio of progesterone to estriol at both visits (visit 1 β: -1.75 days, 95% CI: -3.5, -0.07; visit 3 β: -2.24 days, 95% CI: -4.27, -0.28). Finally, female fetuses experienced an increase in gestational age at birth of 3.92 days (95% CI: 0.77, 7.07) with an IQR increase in estriol at 26 weeks.

Thyroid Hormones

Figure 1B shows the associations between thyroid hormone concentrations and gestational age at birth, specific to fetal sexes and study visits (effect estimates, confidence intervals, and p-values for fetal sex interaction terms can be found in **Supplementary Table S2**). We did observe differences in these associations between fetal sexes, though none of them reached statistical significance. Generally, greater reductions in gestational age at birth were observed among male fetuses, but the only finding to reach statistical significance was for fT4 at 18 weeks (β: -2.73 days, 95% CI: -4.76, -0.70).

Exploratory Analyses of Binary Outcomes

Results for exploratory analyses assessing the binary outcomes of overall and spontaneous PTB and their associations with CRH and reproductive hormones are shown in **Figure 2** (effect estimates, confidence intervals, and p-values for fetal sex interaction terms can be found in **Supplementary Table S2**). Fetal sex effect modification was much more apparent for these outcomes than for continuous gestational age at delivery. The associations between CRH and both overall and spontaneous PTB were significantly modified by fetal sex (interaction p=0.002 and 0.003, respectively); effect estimates were inverse among female fetuses and positive among male fetuses for both

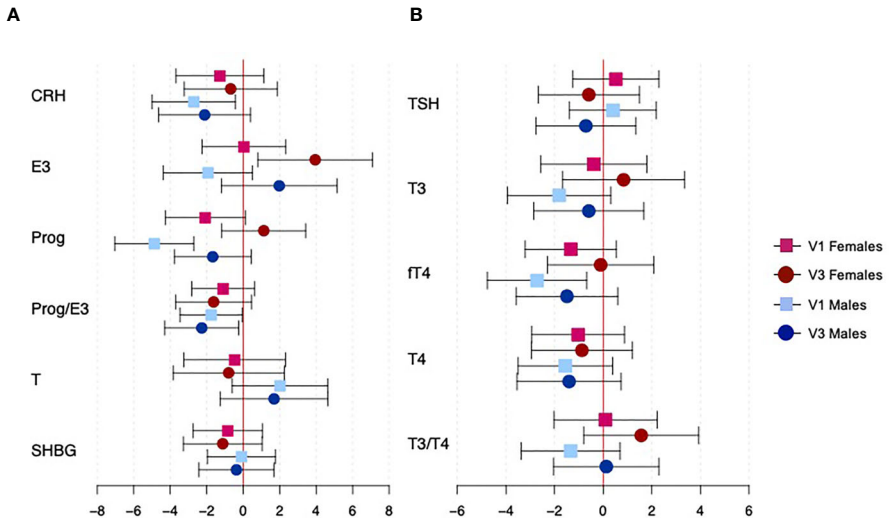


FIGURE 1 | Associations between hormone concentrations and gestational age at delivery, specific to fetal sexes and study visit. Panel **(A)** shows CRH and reproductive hormones, and panel **(B)** shows thyroid hormones. Effect estimates represent the change in gestational age at birth (in days) with an interquartile range increase in hormone concentration. Red and blue estimates correspond to female and male fetuses, respectively. Squares denote effects from hormones at visit 1, while circles denote effects from hormones at visit 3.

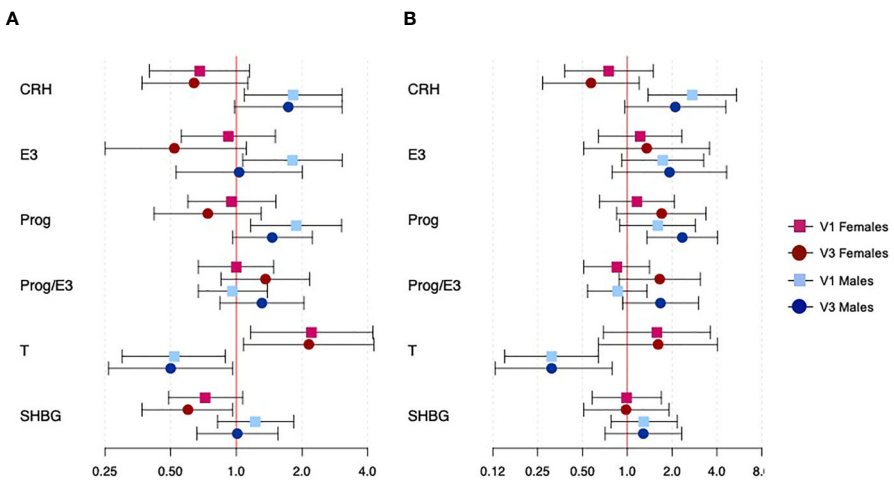


FIGURE 2 | Associations between CRH and reproductive hormone concentrations and binary outcomes of overall and spontaneous PTB, specific to fetal sexes and study visit. Panel **(A)** shows overall PTB and panel **(B)** shows spontaneous PTB. Effect estimates represent the odds of overall or spontaneous PTB with an interquartile range increase in hormone concentration. Red and blue estimates correspond to female and male fetuses, respectively. Squares denote effects from hormones at visit 1, while circles denote effects from hormones at visit 3.

outcomes at both study visits, though only effects with hormones at 18 weeks among male fetuses were significant (overall PTB OR: 1.82, 95% CI: 1.09, 3.05; spontaneous PTB OR: 2.73, 95% CI: 1.38, 5.43). Fetal sex also modified the association between estriol and overall PTB (interaction p=0.022); female fetuses saw a marginal reduction in the odds of PTB with an IQR increase in estriol at 26 weeks (OR: 0.52, 95% CI: 0.25, 1.11), while male fetuses saw an increase in odds of PTB with an IQR increase in estriol at 18 weeks (OR: 1.81, 95% CI: 1.07, 3.06). Results for progesterone were similar; fetal sex modified only associations with overall PTB (interaction p=0.011), and significant increased odds of PTB were observed for male fetuses at 18 weeks (OR: 1.88, 95% CI: 1.16, 3.04). Though effect estimates were not significant, study visit was important for determining the associations between the ratio of progesterone to estriol and both overall and spontaneous PTB; effect estimates were positive

and larger in magnitude at 26 weeks compared to 18 weeks among both male and female fetuses. Testosterone showed highly significant effect modification by fetal sex for both overall and spontaneous PTB (both interaction p<0.001), with an IQR increase in testosterone conferring lower risk of PTB among male fetuses and higher risk among female fetuses which did not differ between study visits. Female fetuses saw 2.21 times the odds (95% CI: 1.16, 4.23) of overall PTB with an IQR increase in testosterone at 18 weeks, while males saw 0.52 times the odds (95% CI: 0.30, 0.89) at the same study visit. Finally, fetal sex modified the association between SHBG and overall PTB; an IQR increase in SHBG was associated with 0.60 times the odds (95% CI: 0.37, 0.96) of PTB only among female fetuses at 26 weeks.

Results for exploratory analyses assessing the binary outcomes of overall and spontaneous PTB and their associations with thyroid hormones are shown in **Figure 3** (effect estimates, confidence intervals, and p-values for fetal sex interaction terms can be found in **Supplementary Table S2**). Though most associations were not significant, effect modification by fetal sex was observed for T3 on both overall and spontaneous PTB (both interaction p=0.013), and the ratio of T3 to T4 on overall PTB (interaction p=0.032). An IQR increase in T3 at both study visits resulted in reduced odds of PTB among female fetuses and increased odds of PTB among male fetuses, though only results for spontaneous PTB among male fetuses were significant (visit 1 OR: 2.01, 95% CI: 1.10, 3.65; visit 3 OR: 2.05, 95% CI: 1.10, 3.84). An IQR increase in fT4 at both study visits resulted in marginally increased odds of overall and spontaneous PTB among only male fetuses. Lastly, an IQR increase in the ratio of T3 to T4 resulted in reduced odds of PTB among female fetuses and increased odds of PTB among male fetuses, though none of these associations reached statistical significance.

DISCUSSION

In the present analysis we explored associations between hormone concentrations measured at two time points during mid-gestation and timing of delivery. Because of previous work indicating dramatic changes in some hormone concentrations through pregnancy, as well as differential susceptibility to adverse pregnancy outcomes based on fetal sex, we determined effect estimates specific to hormones measured at 18 *versus* 26 weeks gestation and male *versus* female fetal sexes. Overall, our results suggest that some hormones, particularly reproductive hormones, have different effects on timing of delivery depending on the sex of the fetus, and most associations show male fetuses being at greater risk for early delivery. We also observed that some associations differed when hormones were assessed at 18 *versus* 26 weeks gestation. An exploratory analysis of the binary outcomes overall and spontaneous PTB revealed similar findings with greater significance. Interestingly, results for the spontaneous subtype of PTB were less affected by study visit than results for overall PTB, supporting separation of these outcomes into more homogenous subgroups.

CRH and Reproductive Hormones

Reduced gestational age at birth was observed among only male fetuses with increased concentrations of CRH at 18 weeks. While very little epidemiologic work has been done to understand the roles of CRH in adverse birth outcomes, researchers have speculated its influence on labor and delivery for decades. Detection of elevated concentrations of CRH during the second trimester in women who eventually delivered preterm compared to those who delivered after 37 weeks was first described by Campbell et al. in 1987 (34). Since then, CRH has been described as being involved in a "placental clock," with

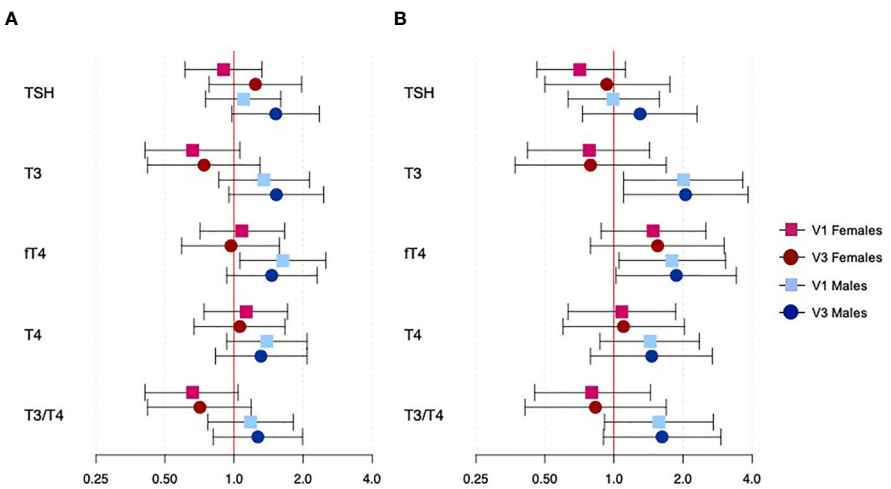


FIGURE 3 | Associations between thyroid hormone concentrations and binary outcomes of overall and spontaneous PTB, specific to fetal sexes and study visit. Panel **(A)** shows overall PTB and panel **(B)** shows spontaneous PTB. Effect estimates represent the odds of overall or spontaneous PTB with an interquartile range increase in hormone concentration. Red and blue estimates correspond to female and male fetuses, respectively. Squares denote effects from hormones at visit 1, while circles denote effects from hormones at visit 3.

CRH concentrations as early as 20 weeks possibly setting the stage for future preterm delivery (21). CRH receptors are present in the myometrium (35) and in the fetal zone of the fetal adrenal gland (36), so CRH could exert its effects on labor by interacting with these receptors.

We observed reduced gestational age, and greater odds of overall and spontaneous PTB, with increasing progesterone concentrations when fetal sex was male, but other studies demonstrating similar significant associations are lacking. One study observed progesterone concentrations measured between 28 and 32 weeks gestation to be higher among women who delivered preterm compared to full term (22). We observed higher progesterone concentrations among PTB cases when fetal sex was male, but only around 18 weeks gestation. We also observed higher progesterone concentrations around 26 weeks among women who spontaneously delivered preterm compared to women who carried to term, regardless of fetal sex.

Previous work has shown that a ratio favoring estriol in mid-pregnancy (23) and at delivery (37) is associated with earlier time of labor. Progesterone concentrations rise steadily during pregnancy, contributing to uterine quiescence, downregulation of prostaglandin production, and immune tolerance of the fetus (11, 12). At the onset of human labor, progesterone concentrations do not notably decrease; rather, the body's response to progesterone is dampened. It is not clear exactly how this occurs, but possibilities include reduction in progesterone receptor expression, changes in receptor isoforms, and local progesterone metabolism (38). As term approaches, the ratio of progesterone to estriol shifts to favor estrogens, with the functional decrease in progesterone driving initiation of labor (39). The new dominance of estrogens promotes an increase in prostaglandin and oxytocin receptors and enzymes responsible for muscle contractions, which work together to help promote labor (10). We observed a positive association between odds of PTB and estriol concentrations when fetal sex was male, but we also unexpectedly observed later gestational age at birth with higher concentrations of estriol at 26 weeks gestation when the fetus was female. In contrast with previous studies, we observed that a higher ratio of progesterone to estriol was associated with reduced gestational age at birth. Interestingly, among women who delivered preterm, a previous study observed a lower ratio of progesterone to estriol among only those without premature rupture of membranes (40), possibly implicating different endocrine pathways in the occurrence of the spontaneous subtype of PTB.

We observed significant effect modification by fetal sex on the associations between testosterone and overall and spontaneous PTB, for which the effect was positive among female fetuses and protective among male fetuses. Previous research on the effects of fetal sex on circulating maternal testosterone has been mixed. Though earlier research suggested that male fetuses conferred higher testosterone concentrations in maternal plasma during gestation (27, 41), more recent findings suggest that there is no significant difference (42–45). Based on more recent evidence, we believe that the fetal sex differences we have observed for testosterone are not due to elevated fetal testosterone in males impacting maternal testosterone. Nevertheless, it remains unclear what biological mechanisms may be differentially at play between fetal sexes. One group has proposed that high concentrations of testosterone may act on the endometrium to disrupt its production of secretory proteins, which may result in elevated risk for preterm delivery (8), but how this process may differ between male and female fetuses is unknown.

Thyroid Hormones

Decreased odds of PTB have been shown with increased concentrations of fT4 in the second (24) and third (46) trimesters, which contradicts our finding that fT4 was inversely associated with gestational age at birth and positively associated with odds of overall and spontaneous PTB. One prior study also found increased odds of PTB with greater T3 concentrations at 10 and 26 weeks gestation (46). Similarly, we found that T3 was associated with increased odds of spontaneous PTB when the fetus was male. Mechanisms of the association between thyroid hormones and PTB are poorly understood, but previous research has suggested that altered thyroid hormone concentrations may be involved in other disease states or exposures for which we have evidence of associations with PTB, such as oxidative stress and inflammation (47–49), or environmental exposures such as phthalates (50–52).

Thyroid hormones are critical for proper brain and skeletal development of the fetus (13). The fetus requires maternal thyroid hormones in the first half of pregnancy, during which time the fetal thyroid gland is immature and cannot produce its own hormones (14). During the second half of pregnancy, both maternal and fetal thyroid hormones are at play. Thyroid hormones cross the placenta *via* thyroid hormone transporters (15), and differential expression of these transporters can affect the levels of thyroid hormones available to the fetus. Thus, assessment of expression patterns of hormone transporters may be necessary to fully understand thyroid hormone associations with timing of labor.

Several previous studies have observed that male fetal sex is associated with a greater risk of delivering preterm. Proposed biological explanations for this observation include a pro-inflammatory environment generated by a male fetus (25) and larger size at birth for males relative to females (26). We observed significant associations with timing of labor unique to women carrying a male fetus for CRH, estriol, progesterone, and fT4, providing further evidence that the effect of fetal sex on the timing of labor is complex, possibly involving diverse endocrine pathways.

Strengths and Limitations

The present study was subject to several limitations. We were not able to measure hCG, for which previous work has suggested a fetal sex difference is present (53). We also did not assess thyroid autoantibody status which could be clouding our thyroid hormone results due to unmeasured confounding. Some critical changes in the maternal endocrine environment occur later in gestation than we were able to measure, such as the exponential increase in CRH right before the onset of labor. Although the goal of this study was to determine whether mid-

pregnancy hormone levels were indicative of increased risk of adverse pregnancy outcomes, measurements at later time points could shed additional light on the various endocrine pathways implicated in adverse birth outcomes. We observed low rates of the spontaneous subtype of PTB, which reduces the reliability of effect estimates, particularly when investigating effects specific to study visit and fetal sex. However, these lower rates may have been observed because we excluded women with preexisting conditions from our cohort to allow more precise examination of associations between hormone concentrations and birth outcomes, since preexisting conditions can influence hormone concentrations and susceptibility to adverse birth outcomes. Furthermore, excluding women with preexisting conditions may limit the generalizability of our findings.

Despite the aforementioned limitations, this study was also strong in various ways. This is one of few studies to assess a broad panel of hormone concentrations in relation to gestational age, which is advantageous because the disease state may implicate multiple endocrine pathways. This is also one of few studies to assess hormones at more than one time point during gestation to investigate different windows of susceptibility to hormonal disruption. Assessment of hormones at multiple time points is also necessary for hormones whose concentrations change dramatically through pregnancy, including CRH, estriol, and progesterone. We are also one of few groups to assess interactions between gestational hormone concentrations and fetal sex, despite existing research suggesting differential susceptibility to early delivery between male and female fetuses. Finally, our study was strengthened by a higher sample size of mothers than was seen in most previously published cohorts, which is particularly important when studying rare outcomes such as spontaneous PTB.

In conclusion, we observed a range of associations between hormones and timing of delivery. We found differences based on the timing of hormone assessment, and many significant findings were unique to mothers carrying a male fetus. Future work will attempt to place these findings in the context of relevant environmental contaminants on the island of Puerto Rico by exploring possibilities of endocrine disruption as a mediator between chemical exposures and pregnancy outcomes. Additional studies are needed to more fully elucidate the role of altered hormone concentrations in the etiology of adverse birth 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

This study was approved by the research and ethics committees of the University of Michigan School of Public Health, University of Puerto Rico, Northeastern University, and participating hospitals and clinics. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC analyzed and interpreted data and drafted the original article. JM, JC, AA, RL-C, and CV made substantial contributions to the conception and design of the study. DW and ZR contributed to acquisition of data. BM, RL-C, and MO'n assisted with interpretation of data. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grant number P42ES017198 from the NIH; grant numbers R01ES031591, R01ES032203, and P30ES017885 from the NIEHS; and program grant number UH3OD023251 for the Environmental influences on Child Health Outcomes (ECHO) from the NIH, Office of the Director.

ACKNOWLEDGMENTS

We would like to extend our gratitude to all PROTECT study participants and their families. The authors also thank the nurses and research staff who participated in cohort recruitment and follow up, as well as the Federally Qualified Health Centers (FQHC) in Puerto Rico that facilitated participant recruitment, including Morovis Community Health Center, Prymed in Ciales, Camuy Health Services, Inc. and the Delta OBGyn Group in Manati, as well as the Manati Medical Center and the Metro Pavia Hospital in Arecibo.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.742145/full#supplementary-material

REFERENCES

1. Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller A-B, Narwal R, et al. National, Regional, and Worldwide Estimates of Preterm Birth Rates in the Year 2010 With Time Trends Since 1990 for Selected Countries: A Systematic Analysis and Implications. *Lancet (London England)* (2012) 379:2162–72. doi: 10.1016/S0140-6736(12)60820-4
2. Martin JA, Hamilton BE, Osterman MJKS, Driscoll AK, Drake P. Births: Final Data for 2016 National Vital Statistics Reports. *Natl Vital Stat Rep* (2018) 67:1–55.
3. Luyckx VA. Preterm Birth and its Impact on Renal Health. *Semin Nephrol* (2017) 37:311–9. doi: 10.1016/j.semnephrol.2017.05.002
4. Leung MP, Thompson B, Black J, Dai S, Alsweiler JM. The Effects of Preterm Birth on Visual Development. *Clin Exp Optom* (2018) 101:4–12. doi: 10.1111/cxo.12578

5. Spittle AJ, Morgan C, Olsen JE, Novak I, Cheong JLY. Early Diagnosis and Treatment of Cerebral Palsy in Children With a History of Preterm Birth. *Clin Perinatol* (2018) 45:409–20. doi: 10.1016/j.clp.2018.05.011
6. Schubert U, Muller M, Abdul-Khaliq H, Norman M. Preterm Birth Is Associated With Altered Myocardial Function in Infancy. *J Am Soc Echocardiogr* (2016) 29:670–8. doi: 10.1016/j.echo.2016.03.011
7. McElrath TF, Hecht JL, Dammann O, Boggess K, Onderdonk A, Markenson G, et al. Pregnancy Disorders That Lead to Delivery Before the 28th Week of Gestation: An Epidemiologic Approach to Classification. *Am J Epidemiol* (2008) 168:980–9. doi: 10.1093/aje/kwn202
8. Ruge S, Sørensen S, Pedersen JF, Lange AP, Byrjalsen I, Bohn H. Secretory Endometrial Protein PP14 in Women With Early Pregnancy Bleeding. *Hum Reprod* (1991) 6:885–8. doi: 10.1093/oxfordjournals.humrep.a137445
9. Okon MA, Laird SM, Tuckerman EM, Li TC. Serum Androgen Levels in Women Who Have Recurrent Miscarriages and Their Correlation With Markers of Endometrial Function. *Fertil Steril* (1998) 69:682–90. doi: 10.1016/s0015-0282(98)00007-7
10. Challis JRG, Matthews SG, Gibb W, Lye SJ. C DPJRG. Endocrine and Paracrine Regulation of Birth at Term and Preterm. *Endocr Rev* (2000) 21:514–50. doi: 10.1210/edrv.21.5.0407
11. Carlo Di Renzo G, Giardina I, Clerici G, Brillo E, Gerli S. Progesterone in Normal and Pathological Pregnancy. *Horm Mol Biol Clin Investig* (2016) 27:35–48. doi: 10.1515/hmbci-2016-0038
12. Byrns MC. Regulation of Progesterone Signaling During Pregnancy: Implications for the Use of Progestins for the Prevention of Preterm Birth. *J Steroid Biochem Mol Biol* (2014) 139:173–81. doi: 10.1016/j.jsbmb.2013.01.015
13. Yen PM. Physiological and Molecular Basis of Thyroid Hormone Action. *Physiol Rev* (2001) 81:1097–142. doi: 10.1152/physrev.2001.81.3.1097
14. Springer D, Jiskra J, Limanova Z, Zima T, Potlukova E. Thyroid in Pregnancy: From Physiology to Screening. *Crit Rev Clin Lab Sci* (2017) 54:102–16. doi: 10.1080/10408363.2016.1269309
15. Forhead AJ, Fowden AL. Thyroid Hormones in Fetal Growth and Prepartum Maturation. *J Endocrinol* (2014) 221:R87–R103. doi: 10.1530/JOE-14-0025
16. Mannisto T, Mendola P, Grewal J, Xie Y, Chen Z, Laughon SK. Thyroid Diseases and Adverse Pregnancy Outcomes in a Contemporary US Cohort. *J Clin Endocrinol Metab* (2013) 98:2725–33. doi: 10.1210/jc.2012-4233
17. Allan WC, Haddow JE, Palomaki GE, Williams JR, Mitchell ML, Hermos RJ, et al. Maternal Thyroid Deficiency and Pregnancy Complications: Implications for Population Screening. *J Med Screen* (2000) 7:127–30. doi: 10.1136/jms.7.3.127
18. Abalovich M, Gutierrez S, Alcaraz G, Maccallini G, Garcia A, Levalle O. Overt and Subclinical Hypothyroidism Complicating Pregnancy. *Thyroid* (2002) 12:63–8. doi: 10.1089/105072502753451986
19. Nazarpour S, Ramezani Tehrani F, Simbar M, Azizi F. Thyroid Dysfunction and Pregnancy Outcomes. *Iran J Reprod Med* (2015) 13:387–96.
20. Smith R, Nicholson RC. Corticotrophin Releasing Hormone and the Timing of Birth. *Front Biosci* (2007) 12:912–8. doi: 10.2741/2113
21. McLean M, Bisits A, Davies J, Woods R, Lowry P, Smith R. A Placental Clock Controlling the Length of Human Pregnancy. *Nat Med* (1995) 1:460–3. doi: 10.1038/nm0595-460
22. Feng T, Allen JC, Ng MJ, Yeo GSH, Kwek KYC, Chern BSM, et al. The Association Between Serum Progesterone Level and Preterm Delivery. *Int J Gynaecol Obstet* (2018) 142:308–14. doi: 10.1002/ijgo.12564
23. Ruiz RJ, Saade GR, Brown CEL, Nelson-Becker C, Tan A, Bishop S, et al. The Effect of Acculturation on Progesterone/Estriol Ratios and Preterm Birth in Hispanics. *Obstet Gynecol* (2008) 111:309–16. doi: 10.1097/01.AOG.0000297896.00491.2c
24. Korevaar TIM, Chaker L, Medici M, de Rijke YB, Jaddoe VWV, Steegers EAP, et al. Maternal Total T4 During the First Half of Pregnancy: Physiologic Aspects and the Risk of Adverse Outcomes in Comparison With Free T4. *Clin Endocrinol (Oxf)* (2016) 85:757–63. doi: 10.1111/cen.13106
25. Challis J, Newnham J, Petraglia F, Yeganegi M, Bocking A. Fetal Sex and Preterm Birth. *Placenta* (2013) 34:95–9. doi: 10.1016/j.placenta.2012.11.007
26. McGregor JA, Leff M, Orleans M, Baron A. Fetal Gender Differences in Preterm Birth: Findings in a North American Cohort. *Am J Perinatol* (1992) 9:43–8. doi: 10.1055/s-2007-994668
27. Meulenberg PM, Hofman JA. Maternal Testosterone and Fetal Sex. *J Steroid Biochem Mol Biol* (1991) 39:51–4. doi: 10.1016/0960-0760(91)90012-t
28. Bremme K, Eneroth P. Fetal Sex Dependent Hormone Levels in Early Pregnant Women With Elevated Maternal Serum Alpha-Fetoprotein. *Int J Gynaecol Obstet Off Organ Int Fed Gynaecol Obstet* (1983) 21:451–7. doi: 10.1016/0020-7292(83)90034-6
29. Takagi S, Yoshida T, Tsubata K, Ozaki H, Fujii TK, Nomura Y, et al. Sex Differences in Fetal Gonadotropins and Androgens. *J Steroid Biochem* (1977) 8:609–20. doi: 10.1016/0022-4731(77)90270-9
30. Meeker JD, Cantonwine DE, Rivera-González LO, Ferguson KK, Mukherjee B, Calafat AM, et al. Distribution, Variability, and Predictors of Urinary Concentrations of Phenols and Parabens Among Pregnant Women in Puerto Rico. *Environ Sci Technol* (2013) 47:3439–47. doi: 10.1021/es400510g
31. Dietrich JW, Landgrafe G, Fotiadou EH. TSH and Thyrotropic Agonists: Key Actors in Thyroid Homeostasis. *J Thyroid Res* (2012) 2012:351864. doi: 10.1155/2012/351864
32. Romero R, Scoccia B, Mazor M, Wu YK, Benveniste R. Evidence for a Local Change in the Progesterone/ Estrogen Ratio in Human Parturition at Term. *Am J Obstet Gynecol* (1988) 159:657–60. doi: 10.1016/S0002-9378(88)80029-2
33. Pettker C, Goldberg J, El-Sayed Y, Copel J. Committee Opinion No 700: Methods for Estimating the Due Date. *Obstet Gynecol* (2017) 129:e150–4. doi: 10.1097/AOG.0000000000002046
34. Campbell EA, Linton EA, Wolfe CD, Scraggs PR, Jones MT, Lowry PJ. Plasma Corticotropin-Releasing Hormone Concentrations During Pregnancy and Parturition. *J Clin Endocrinol Metab* (1987) 64:1054–9. doi: 10.1210/jcem-64-5-1054
35. Grammatopoulos DK. The Role of CRH Receptors and Their Agonists in Myometrial Contractility and Quiescence During Pregnancy and Labour. *Front Biosci* (2007) 12:561–71. doi: 10.2741/2082
36. Smith R, Mesiano S, Chan EC, Brown S, Jaffe RB. Corticotropin-Releasing Hormone Directly and Preferentially Stimulates Dehydroepiandrosterone Sulfate Secretion by Human Fetal Adrenal Cortical Cells. *J Clin Endocrinol Metab* (1998) 83:2916–20. doi: 10.1210/jcem.83.8.5020
37. Moran D, Mcgarrigle HH, Lachelin GC. Lack of Normal Increase in Saliva Estriol / Progesterone Ratio in Women With Labor Induced at 42 Weeks ' Gestation. *Am J Obstet Gynecol* (1992) 167(6):1563–4. doi: 10.1016/0002-9378(92)91740-2
38. Mesiano S, Welsh TN. Steroid Hormone Control of Myometrial Contractility and Parturition. *Semin Cell Dev Biol* (2007) 18:321–31. doi: 10.1016/j.semcdb.2007.05.003
39. Snegovskikh V, Park JS. Endocrinology of Parturition. *Endocrinol Metab Clin North Am* (2006) 35:173–91. doi: 10.1016/j.ecl.2005.09.012
40. Darne J, Mcgarrigle H, Lachelin G. Increased Saliva Oestriol to Progesterone Ratio Before Idiopathic Preterm Delivery : A Possible Predictor for Preterm Labour? *Br Med J* (1987) 294:270–2. doi: 10.1136/bmj.294.6567.270
41. Klinga K, Bek E, Runnebaum B. Maternal Peripheral Testosterone Levels During the First Half of Pregnancy. *Am J Obstet Gynecol* (1978) 131:60–2. doi: 10.1016/0002-9378(78)90474-x
42. Voegtline KM, Costigan KA, Kivlighan KT, Henderson JL, DiPietro JA. Sex-Specific Associations of Maternal Prenatal Testosterone Levels With Birth Weight and Weight Gain in Infancy. *J Dev Orig Health Dis* (2013) 4:280–4. doi: 10.1017/S2040174413000135
43. Toriola AT, Vääräsmäki M, Lehtinen M, Zeleniuch-Jacquotte A, Lundin E, Rodgers K-G, et al. Determinants of Maternal Sex Steroids During the First Half of Pregnancy. *Obstet Gynecol* (2011) 118:1029–36. doi: 10.1097/AOG.0b013e3182342b7f
44. Cohen-Bendahan CCC, van Goozen SHM, Buitelaar JK, Cohen-Kettenis PT. Maternal Serum Steroid Levels are Unrelated to Fetal Sex: A Study in Twin Pregnancies. *Twin Res Hum Genet Off J Int Soc Twin Stud* (2005) 8:173–7. doi: 10.1375/1832427053738764
45. Vlková B, Vávrová S, Szemes T, Minárik G, Turna J, Celec P. Testosterone and Estradiol in Maternal Plasma and Their Relation to Fetal Sex. *Prenat Diagn* (2010) 30:806–7. doi: 10.1002/pd.2535
46. Johns LE, Ferguson KK, McElrath TF, Mukherjee B, Seely EW, Meeker JD. Longitudinal Profiles of Thyroid Hormone Parameters in Pregnancy and Associations With Preterm Birth. *PloS One* (2017) 12:1–15. doi: 10.1371/journal.pone.0169542
47. Mancini A, Di Segni C, Raimondo S, Olivieri G, Silvestrini A, Meucci E, et al. Thyroid Hormones, Oxidative Stress, and Inflammation. *Mediators Inflammation* (2016) 2016:6757154. doi: 10.1155/2016/6757154
48. Moore TA, Ahmad IM, Zimmerman MC. Oxidative Stress and Preterm Birth: An Integrative Review. *Biol Res Nurs* (2018) 20:497–512. doi: 10.1177/1099800418791028

49. Cappelletti M, Della Bella S, Ferrazzi E, Mavilio D, Divanovic S. Inflammation and Preterm Birth. *J Leukoc Biol* (2016) 99:67–78. doi: 10.1189/jlb.3MR0615-272RR
50. Cathey AL, Watkins D, Rosario ZY, Velez C, Alshawabkeh AN, Cordero JF, et al. Associations of Phthalates and Phthalate Replacements With CRH and Other Hormones Among Pregnant Women in Puerto Rico. *J Endocr Soc* (2019) 3:1127–49. doi: 10.1210/js.2019-00010
51. Johns LE, Ferguson KK, McElrath TF, Mukherjee B, Meeker JD. Associations Between Repeated Measures of Maternal Urinary Phthalate Metabolites and Thyroid Hormone Parameters During Pregnancy. *Environ Health Perspect* (2016) 124:1808–15. doi: 10.1289/EHP170
52. Ferguson KK, Rosen EM, Rosario Z, Feric Z, Calafat AM, McElrath TF, et al. Environmental Phthalate Exposure and Preterm Birth in the PROTECT Birth Cohort. *Environ Int* (2019) 132:105099. doi: 10.1016/j.envint.2019.105099
53. Adibi JJ, Lee MK, Saha S, Boscardin WJ, Apfel A, Currier RJ. Fetal Sex Differences in Human Chorionic Gonadotropin Fluctuate by Maternal Race, Age, Weight and by Gestational Age. *J Dev Orig Health Dis* (2015) 6:493–500. doi: 10.1017/S2040174415001336

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.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 30 September 2021
doi: 10.3389/fendo.2021.738964

Umbilical Cord Blood Adiponectin, Leptin, Insulin, and Ghrelin in Premature Infants and Their Association With Birth Outcomes

Luyan Han[1,2†], Bo Li[3†], Xiaojing Xu[1], Shufang Liu[1], Zhenghong Li[2], Ming Li[3] and Danhua Wang[2*]*

[1] Department of Pediatrics, The First Hospital of Tsinghua University, Beijing, China, [2] Department of Pediatrics, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China, [3] Department of Endocrinology, NHC Key Laboratory of Endocrinology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China

OPEN ACCESS

Edited by:
Ganesh Acharya, Karolinska Institute, Sweden

Reviewed by:
Yukiko Kohmura-Kobayashi, Hamamatsu University School of Medicine, Japan
Akio Nakamura, Jissen Women's University, Japan

***Correspondence:**
Ming Li
liming@pumch.cn
Danhua Wang
danhuawang10@126.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Pediatric Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *09 July 2021*
Accepted: *26 August 2021*
Published: *30 September 2021*

Citation:
Han L, Li B, Xu X, Liu S, Li Z, Li M and Wang D (2021) Umbilical Cord Blood Adiponectin, Leptin, Insulin, and Ghrelin in Premature Infants and Their Association With Birth Outcomes. Front. Endocrinol. 12:738964. doi: 10.3389/fendo.2021.738964

Background: Premature/low-birth-weight infants are at significant risk of metabolic diseases in adulthood, which may be related to the levels of fetal adipokine. Here, we investigated the differences in the levels of umbilical cord blood adiponectin, leptin, insulin, and ghrelin in preterm and term infants and sought to elucidate the link between these hormones and fetal growth. We also evaluated the interrelationship among these metabolic hormones in both groups of newborns.

Methods: A total of 149 mother–infant pairs (100 in the preterm group and 49 in the term group) were enrolled in the study. The preterm group was further subdivided according to birth weight (≤1,500, 1,501–2,000, 2,001–2,500, and >2,500 g), gestational age (<34 *vs.* ≥34 weeks), and appropriate for gestational age (AGA) *vs.* small for gestational age (SGA). The general condition of the mothers and the growth parameters of the newborns at birth were recorded.

Results: The levels of adiponectin, leptin, and ghrelin were lower in the preterm group than those in the term group ($p < 0.05$). In the preterm group, the leptin levels of infants with gestational age ≥34 weeks were significantly higher than those of infants with gestational age <34 weeks (mean ln leptin = 0.63 *vs.* 0.36 ng/ml, $p = 0.009$). The levels of adiponectin were lower in the SGA group than those in the AGA group (mean ln adiponectin = 2.26 *vs.* 2.84 μg/ml, $p = 0.001$), whereas those of ghrelin displayed the opposite trend (mean ln ghrelin = 6.29 *vs.* 5.71 pg/ml, $p < 0.001$). Leptin was significantly correlated with insulin both in preterm infants with birth weight (BW) >2,000 g and in term infants. Umbilical cord blood leptin was positively correlated with the BW, birth length, and head circumference of newborns ($r = 0.460$, 0.311, and 0.310, respectively, all $p < 0.05$), whereas ghrelin was negatively correlated with the same parameters ($r = –0.372$, –0.415, and –0.373, respectively, all $p > 0.05$).

Conclusions: The lack of maturation of adipose tissue and the gastrointestinal tract by the fetus due to prematurity is associated with changes in the levels of cord blood

adiponectin, leptin, and ghrelin. The dysregulation of these hormones in preterm infants may be a risk factor for fetal growth and future metabolic diseases.

Keywords: adipokine, insulin, umbilical cord blood, premature infants, neonatal growth

INTRODUCTION

Premature infants are at high risk of developing metabolic syndrome-related disorders such as obesity, type 2 diabetes, and cardiovascular diseases in later life (1, 2); however, the mechanisms underlying the link between fetal growth and these metabolic disorders are not well understood. Investigating adipose tissue dysfunction may provide valuable insights into this association. Fetal adipose tissue maturation occurs in the second trimester of gestation, while its accumulation begins during the third trimester (3). As a consequence, prematurity can affect the correct acquisition of adipose tissue, thereby disturbing its metabolic functions and limiting the metabolic adaptation of premature newborns to extrauterine life. The adipokine profile is partially dependent on the amount of adipose tissue accumulated during fetal life, rendering cord blood adipokine levels potential markers of the degree of development and amount of adipose tissue.

Among the numerous adipokines derived from adipose tissue, adiponectin and leptin comprise a crucial signaling link between adiposity and metabolic disorders. Leptin has critical roles in the regulation of food intake and energy expenditure in white adipose tissue (4). Studies on cord blood have demonstrated that the levels of fetal leptin increase with advancing gestation (5, 6). Initially, the levels of fetal leptin are dependent on both transplacental delivery and placental leptin production until approximately 32 weeks of gestation. Subsequently, as it starts to accrue significant amounts of adipose tissue, the fetus becomes the main contributor to its own levels of plasma leptin (7). Adiponectin is an anti-inflammatory adipokine that regulates glucose metabolism and fatty acid oxidation (8). Circulating adiponectin exists in three forms, namely, trimers, hexamers, and high-molecular-weight oligomers, the latter being the most active and a relatively important isoform for promoting insulin sensitivity (9). Several studies have demonstrated that a negative correlation exists between maternal serum adiponectin levels and birth weight (BW) (10, 11).

In addition to the adipokines secreted by adipose tissue, insulin, produced in pancreatic islets, and ghrelin, produced primarily in the gastrointestinal mucosa, also play roles in fetal growth (12). The cross-talk between these hormones may be a contributing factor to fetal development (13, 14). Ghrelin stimulates appetite by binding to its specific receptor, GHS-1A, in the hypothalamus and activating NPY/AgRP-expressing neurons; it also participates in a meal-to-meal control system that is itself sensitive to changes in the levels of insulin and leptin (10). The influence of ghrelin on energy homeostasis raises the possibility that it may also play a role in prenatal growth.

Although numerous studies have explored the adiponectin and leptin levels in the umbilical cord blood of full-term infants (15–17), little is known about their homeostasis in preterm infants. Here, we focused on determining the differences in the levels of umbilical cord blood adiponectin, leptin, insulin, and ghrelin in preterm and term infants and sought to identify their possible influencing factors. Additionally, we investigated the interrelation among these metabolic hormones in both preterm and term infants and examined the relationship between these hormones and the neonatal growth parameters, thereby providing a possible theoretical basis for explaining how premature infants are prone to metabolic syndrome in later life.

MATERIALS AND METHODS

Study Population

Venous cord blood samples were collected from 149 newborns in the Department of Pediatrics and Obstetrics of Peking Union Medical College Hospital between August 2011 and December 2011. The mothers were 16 years of age or older, pregnant with a single fetus, and with no self-reported smoking and drinking history and diabetes, asthma, cancer, or psychiatric illness. According to gestational age (GA), the mother–infant pairs were divided into a preterm group (<37 weeks gestation) or a term group (37–41 weeks gestation). Preterm infants were also divided into different subgroups according to GA (<34 *vs.* ≥34 weeks) since several organ systems reach adequate maturity after 34 weeks (18). We also divided preterm infants according to BW (≤1,500, 1,501–2,000, 2,001–2,500, and >2,500 g), or small for gestational age (SGA) *vs.* appropriate for gestational age (AGA). The study procedures were approved by the Ethics Committee of Peking Union Medical College Hospital, and signed informed consent was obtained from all the participating families.

Assessment of Maternal and Neonatal Characteristics

Measurements of infant BW, birth length, and head circumference were obtained immediately after birth. The GA was confirmed by ultrasound before week 20 of gestation. Maternal age, education, and gravidity were self-reported at the first research-related visit. Infant sex was reported by the mother shortly after delivery or deduced from the medical records. Neonatal umbilical cord blood was collected at delivery. The samples were centrifuged, aliquoted, and immediately frozen at −80°C until analysis of hormone levels. The levels of total ghrelin were tested using the total Human Ghrelin enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Burlington, MA, USA) (19). The intra- and inter-assay coefficients of variation (CVs) for the ghrelin assay were <1.9% and 7.7%, respectively. The concentrations of adiponectin (20), leptin (21), and insulin (22) were measured using monoclonal antibody-based sandwich ELISA developed in the Key Laboratory

of Endocrinology, Peking Union Medical College Hospital (Beijing, China). The intra- and inter-assay CVs were respectively <5.4% and <8.5% for adiponectin and <7.4% and <9.3% for leptin. The insulin assay showed no cross-reactivity to proinsulin (<0.05%), indicating that it was true insulin. The sensitivity of the insulin assay was 0.5 mU/L and the intra- and inter-assay CVs were <4.1% and <7.0%, respectively.

Statistical Analysis

All analyses were performed using SPSS version 20.0 (IBM SPSS Statistics, Armonk, NY, USA). Data displaying skewed distributions were natural logarithmically transformed before analysis. 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 of the demographic characteristics, clinical features, and laboratory results between subjects in the preterm group and those in the term group were performed using Student's *t*-test. A general linear model with adjustment for confounders was used to compare the hormone levels among the subgroups of preterm infants. Multivariate linear regression models were used to estimate the influencing factors of leptin, adiponectin, insulin, and ghrelin in cord blood after adjusting for confounders. Associations between hormones and neonatal growth adjusted for confounders were evaluated by partial correlation analysis. The confounders considered were maternal age, pre-pregnancy body mass index (BMI, in kilograms per square meter), mode of delivery, GA, and sex of the infants. A *p*-value <0.05 was considered statistically significant.

RESULTS

Baseline Characteristics and Anthropometric Indices of the Mother–Infant Pairs

A total of 149 mother–infant pairs were enrolled in this study, including 100 in the preterm group and 49 in the term group. Statistically significant differences were found in GA, BW, birth length, and birth head circumference between the two groups; however, no significant differences were observed for gender, delivery mode, and maternal age (**Table 1**). The preterm group had 43 cases (43%) of GA <34 weeks and 57 (57%) of GA >34 weeks. The BW was <2,000 g in 39 cases (39%) and was ≥2,000 g in 61 cases (61%). SGA occurred in 28 cases (28%) and AGA in 72 (72%). In the preterm group, 15 cases (15%) reached the agreed standards for SGA, BW <2,000 g, and GA <34 weeks (**Figure 1**).

Comparisons of Hormones in the Two Groups and the Subgroups of Preterm Infants

The cord blood concentrations of adiponectin, leptin, and ghrelin in the preterm group were significantly lower than those in the term group; however, no differences in the insulin levels were found between the two groups (**Table 1**). As depicted in **Figure 2**, analysis of the preterm subgroup indicated that the concentrations of leptin were significantly lower in preterm infants with GA <34 weeks than in those with GA ≥34 weeks (mean ln leptin = 0.63 *vs.* 0.36 ng, $p = 0.009$) after adjustments for sex, mother's age, delivery mode, weight gain during pregnancy, and pre-pregnancy BMI (**Figure 2B**). No significant differences were found for the levels of the other hormones.

The levels of leptin, adiponectin, and insulin were substantially lower in preterm infants with very low BW (<1,500 g) relative to other preterm infants (**Figures 2A–D**), whereas those of ghrelin were significantly higher. In addition, compared with infants in the AGA group, those in the SGA group showed significantly lower levels of adiponectin (mean ln adiponectin = 2.26 *vs.* 2.84 μg/ml, $p = 0.001$) (**Figure 2A**). Meanwhile, the levels of ghrelin were higher in infants from the SGA group than in those from the AGA group (mean ln ghrelin = 6.29 *vs.* 5.71 pg/ml, $p < 0.001$) (**Figure 2D**).

Interrelationship Among Metabolic Hormones in Preterm and Term Infants

Serum leptin was significantly correlated with insulin both in preterm infants with BW >2,000 g ($p = 0.019$) and in term infants ($p = 0.009$) after adjusting for GA, sex, mother's age, BW, weight

TABLE 1 | Baseline characteristics of the mother–infant pairs in the two groups.

	Preterm group (*n* = 100)	Term group (*n* = 49)	*p*-value
Maternal age (years)	30.0 (28.0–34.0)	32.5 (30.0–36.5)	0.423
Gestational age (weeks)	34 (32–35)	39 (38–40)	**<0.001*****
Delivery mode (SVD/CS), *n* (%)	46 (46.0)/54 (54.0)	16 (32.7)/33 (67.3)	0.561
Gender (male/female), *n* (%)	48 (48.0)/52 (52.0)	29 (59.2)/20 (40.8)	0.252
Birth weight (kg)	2.20 ± 0.67	3.34 ± 0.4	**<0.001*****
Birth length (cm)	44.5 ± 3.9	49.9 ± 1.6	**<0.001*****
Head circumference (cm)	31.1 ± 2.4	34.3 ± 1.0	**<0.001*****
Adiponectin (μg/ml)	16.7 (10.6–27.4)	28.3 (23.5–36.6)	**<0.001*****
Leptin (ng/ml)	1.56 (0.65–3.23)	5.39 (3.99–7.99)	**<0.001*****
Insulin (μIU/ml)	5.80 (4.15–13.82)	5.73 (3.28–7.41)	0.865
Ghrelin (pg/ml)	374.1 (271.7–527.5)	408.4 (323.5–653.7)	**0.015***

Data presented are medians, mean ± SD for continuous variables, and n (%) for categorical variables. The p-values are from t-tests for differences in means for continuous variables or chi-square tests for differences in proportions for categorical variables between the preterm and term groups. Values in bold are significant at p < 0.05.
SVD, spontaneous vaginal delivery; CS, cesarean section.
**p < 0.05, ***p < 0.001.*

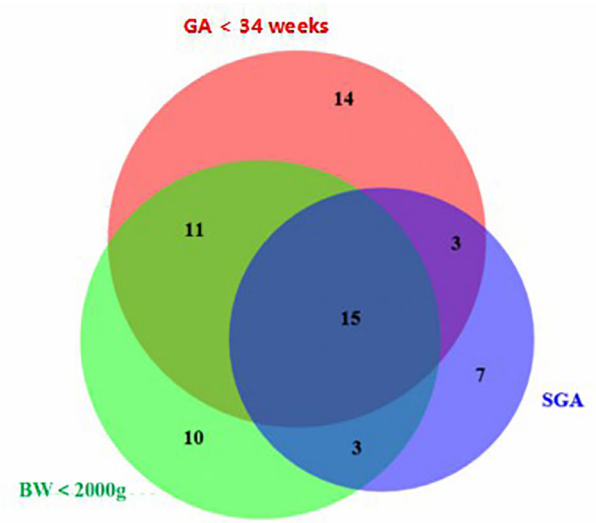


FIGURE 1 | Distribution of premature infants with GA <34 weeks, BW <2,000 g, and SGA and the proportion and overlapping relationships between them. *SGA*, small for gestational age; *GA*, gestational age; *BW*, birth weight.

gain during pregnancy, and pre-pregnancy BMI (**Table 2**). In addition, leptin was only found to have a significant association with serum adiponectin ($p = 0.049$) and a borderline association with serum ghrelin in term infants ($p = 0.061$).

Multivariate Linear Regression Analysis of the Influencing Factors for Different Hormones

GA, sex, and SGA were associated with the levels of adiponectin in umbilical cord blood. GA ($\beta = 0.161$, $p = 0.001$) and being female ($\beta = 0.378$, $p = 0.023$) were positively correlated and SGA negatively correlated ($\beta = -0.642$, $p = 0.002$) with the levels of adiponectin. GA ($\beta = 0.248$, $p < 0.001$) was correlated with higher levels of leptin. A significant correlation was found between vaginal delivery and the levels of insulin ($\beta = 0.622$, $p = 0.041$), while GA was negatively correlated ($\beta = -0.057$, $p = 0.007$) and SGA positively correlated ($\beta = 0.362$, $p < 0.001$) with the concentrations of ghrelin (**Table 3**).

Partial Correlations Between Different Hormones and Neonatal Growth

The partial correlation coefficients between the different hormones and the neonatal growth parameters are shown in **Table 4**. After adjusting for GA, sex, mother's age, delivery mode, and pre-pregnancy BMI, the levels of cord blood leptin were found to be positively correlated with BW ($p < 0.001$), body length ($p = 0.012$), and head circumference ($p = 0.012$) in premature infants, whereas the levels of ghrelin were negatively correlated with these parameters (BW, $p = 0.002$; body length, $p = 0.001$; head circumference, $p = 0.002$). However, no statistically significant associations were detected between the levels of adiponectin or insulin and BW, body length, or head circumference.

DISCUSSION

Main Findings

In this cross-sectional sample of preterm infants, we found that the levels of cord blood leptin were positively correlated with the neonatal growth parameters such as BW, body length, and head circumference, whereas the levels of ghrelin were negatively correlated with these parameters. Our study is the first to demonstrate the association between the levels of cord blood adiponectin, leptin, insulin, and ghrelin and the birth outcomes in premature Chinese infants. When compared with those of other preterm infants, the levels of leptin, adiponectin, and insulin were substantially lower in those with very low BW, whereas the levels of ghrelin were significantly higher.

Data Interpretation and Comparisons With Previous Findings

Adiponectin is secreted only by fully differentiated adipocytes and is related to insulin resistance (23). In our study, we found that the concentrations of cord blood adiponectin were significantly lower in preterm infants than those in full-term newborns, which is consistent with the results of previous studies (24, 25). Terrazzan et al. (26) reported that the concentrations of cord blood adiponectin were lower in preterm infants with BW <1,500 g than those in full-term infants in Brazil. Here, we also found that the concentrations of cord blood adiponectin in preterm infants with very low BW were lower than those in other premature infants. We further observed that, in the preterm group, SGA infants had markedly lower concentrations of cord blood adiponectin compared with AGA infants. The negative influence of SGA on serum adiponectin levels is consistent with previous reports (27, 28). GA was positively correlated with adiponectin, which suggests that the quality of early postnatal growth differed between preterm and term infants. We found a significant difference in the adiponectin levels between female and male preterm neonates. Previous studies have indicated that a higher level of adiponectin in female cord blood compared with that of males could be another indication of the advantages females have regarding the acquisition and functionality of adipose tissue during gestation (25, 29).

Leptin is an anorexigenic peptide secreted by adipocytes that enables communication between lipid tissue and the hypothalamus. Studies have shown that preterm infants have lower levels of leptin than do term infants (1), which is consistent with our findings. GA is the most commonly reported cord blood leptin modulator (30, 31); we also observed that GA was positively correlated with the levels of leptin in the umbilical cord blood of preterm infants. A positive correlation was detected between leptin and BW, birth length, and head circumference, an association that had already been suggested in a previous study in China (32). The level of leptin in umbilical

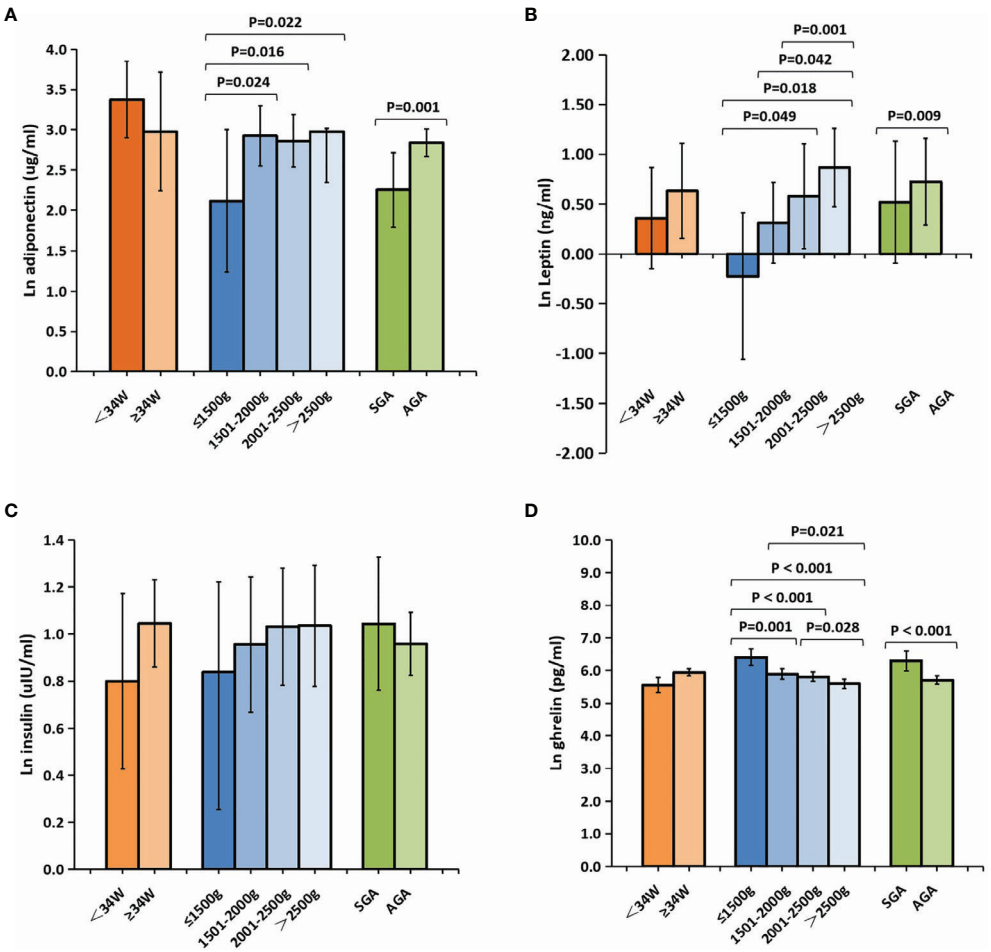


FIGURE 2 | Comparison of the cord plasma concentrations of adiponectin **(A)**, leptin **(B)**, insulin **(C)**, and ghrelin **(D)** among the subgroups of preterm newborns after natural logarithmic (ln) transformation. Data were analyzed after adjusting for sex, mother's age, delivery mode, weight gain during pregnancy, and pre-pregnancy BMI between the different GA groups, and for sex, mother's age, delivery mode, GA, weight gain during pregnancy, and pre-pregnancy BMI among the different BW groups and the SGA/AGA groups. All data are expressed as 95% CIs. *Values in bold* are significant at $p < 0.05$. *BMI*, body mass index; *SGA*, small for gestational age; *AGA*, appropriate for gestational age; *GA*, gestational age; *BW*, birth weight.

cord blood increases with advancing pregnancy (33). A marked increase in the concentrations of circulating leptin after 34 weeks of gestation may be of physiological advantage to newborn infants through limiting the body energy expenditure and conserving nutritional reserves for growth and development (12). It has been suggested that the lower the leptin level, the higher the BMI and the faster the weight growth of premature infants, which is expected to contribute to the catch-up growth of premature infants.

Ghrelin is a strong orexigenic peptide hormone produced primarily in the gastrointestinal tract and, to a lesser extent, in the placenta and other tissues (12). In our study, cord blood ghrelin was detectable and its concentrations were significantly lower in preterm infants than those in term infants, which is inconsistent with previously reported results (34, 35). The association between ghrelin and GA in preterm infants is controversial (13, 25, 35, 36). Here, we found that ghrelin was inversely related to GA in premature infants. The different sampling times for ghrelin and racial differences may have contributed to the discrepant results. SGA preterm infants had higher levels of ghrelin than did their AGA counterparts. Although several studies have demonstrated an association between GA and the level of ghrelin in term infants, evidence for such an association in preterm infants has been limited (37–40), and whether a relationship exists between ghrelin and the anthropometric parameters at birth remains controversial (39, 41, 42). The inverse relationship between ghrelin and the anthropometric indices in preterm infants found in this study suggests that ghrelin might adopt its physiological role in regulating growth and metabolism at a relatively early stage of gestation.

The pancreatic hormone insulin plays a role in fetal growth (14). In this study, we found no significant differences in cord blood insulin between preterm and term infants, which does not agree

TABLE 2 | Interrelationship between the metabolic hormones in preterm and term infants.

	Adiponectin		Leptin		Insulin	
	r	***p*-value**	***r***	***p*-value**	***r***	***p*-value**
	Preterm infants with BW <2,000 g					
Adiponectin						
Leptin	0.223	0.318				
Insulin	–0.079	0.728	0.227	0.310		
Ghrelin	–0.179	0.426	0.204	0.363	–0302	0.172
	Preterm infants with BW ≥2,000 g					
Adiponectin						
Leptin	0.144	0.351				
Insulin	0.122	0.430	0.352	**0.019***		
Ghrelin	0.269	0.077	–0.060	0.701	0.060	0.697
	Term infants					
Adiponectin						
Leptin	0.295	**0.049***				
Insulin	0.136	0.350	0.383	**0.009****		
Ghrelin	0.178	0.241	0.281	0.061	–0.041	0.792

Partial correlation analysis was used with adjustments for maternal age, parity, mode of delivery, gestational age, gender, pre-pregnancy BMI, and weight gain during pregnancy. P-values in bold are significant at p < 0.05.
r, partial correlation coefficient; BMI, body mass index.
**p < 0.05, **p < 0.01.*

TABLE 3 | Multivariate linear regression analysis of the influencing factors for the levels adiponectin, leptin, true insulin, and ghrelin in cord blood.

	β	***p*-value**	**95% CI**	
	ln Adiponectin			
Maternal age	–0.015	0.444	–0.053	0.023
Parity	0.104	0.667	–0.376	0.584
SVD (yes = 1)	–0.204	0.265	–0.566	0.158
Gestational age	0.161	**0.001****	0.066	0.256
Gender (female = 1)	0.378	**0.023***	0.055	0.702
Pre-pregnancy BMI (kg/m^2)	0.001	0.937	–0.016	0.018
Weight gain during pregnancy	0.001	0.937	–0.016	0.018
SGA (yes = 1)	–0.642	**0.002****	–1.049	–0.235
	ln Leptin			
Maternal age	–0.058	0.029	–0.111	–0.006
Parity	0.515	0.124	–0.145	1.175
SVD (yes = 1)	0.083	0.741	–0.416	0.581
Gestational age	0.248	**<0.001*****	0.117	0.379
Gender (female = 1)	0.307	0.174	–0.138	0.752
Pre-pregnancy BMI (kg/m^2)	0.012	0.318	–0.012	0.035
Weight gain during pregnancy	0.012	0.318	–0.012	0.035
SGA (yes = 1)	–0.141	0.617	–0.701	0.419
	ln Insulin			
Maternal age	0.013	0.711	–0.056	0.081
Parity	–0.223	0.608	–1.087	0.641
SVD (yes = 1)	0.622	**0.041***	0.031	1.274
Gestational age	–0.033	0.701	–0.205	0.139
Gender (female = 1)	0.012	0.967	–0.571	0.595
Pre-pregnancy BMI (kg/m^2)	0.021	0.18	–0.01	0.052
Weight gain during pregnancy	0.021	0.18	–0.01	0.052
SGA (yes = 1)	0.216	0.559	–0.517	0.948
	ln Ghrelin			
Maternal age	0	0.961	–0.017	0.016
Parity	0.013	0.9	–0.194	0.22
SVD (yes = 1)	–0.135	0.088	–0.291	0.021
Gestational age	–0.057	**0.007****	–0.098	–0.016
Gender (female = 1)	0.016	0.818	–0.123	0.155
Pre-pregnancy BMI (kg/m^2)	–0.003	0.454	–0.01	0.005
Weight gain during pregnancy	0	0.95	–0.014	0.015
SGA (yes = 1)	0.362	**<0.001*****	0.187	0.538

Multivariate linear regression analysis was used with adjustments for maternal age, parity, mode of delivery, gestational age, sex, SGA, pre-pregnancy BMI, and weight gain during pregnancy. Values in bold are significant at p < 0.05.
SVD, spontaneous vaginal delivery; BMI, body mass index; SGA, small for gestational age; ln, natural logarithmic.
**p < 0.05, **p < 0.01, ***p < 0.001.*

with the findings of a previous study (34). The levels of cord blood insulin were observed to be higher in infants born through vaginal delivery than in those born through cesarean section, and the reason may be that cesarean section and spontaneous vaginal delivery induce different levels of stress. In our study, we did not find a correlation between the insulin levels and the fetal anthropological measurements in preterm infants; however, a positive correlation was identified between the levels of leptin and insulin and GA, BW, height, and head circumference (34).

Because hormone-mediated cross-talk between endocrine organs is thought to contribute to fetal development, we also investigated the interrelationship among these metabolic hormones in our cohort. We identified a significant correlation between cord blood leptin and insulin in both preterm infants with BW >2,000 g and in term infants, supporting that the "adipoinsular axis" is likely to be active in early intrauterine life (43, 44). The strong correlation detected between these hormones suggests that the activity and the function of the "adipoinsular axis" are likely to increase as fetal maturation progresses.

Strengths and Limitations

One of the strengths of this research was that we not only compared the levels of adiponectin, leptin, insulin, and ghrelin in the umbilical cord blood between premature and full-term infants but also divided premature infants into subgroups according to GA, BW, and AGA/SGA. We further investigated the interrelationship among these metabolic hormones in preterm and term infants and explored whether a functional "adipoinsular axis" might exist in preterm newborns. However, there are also some limitations to our study. Firstly, we did not observe a link between ghrelin and the other hormones in either preterm or term infants, which might be due to the relatively small sample size. Secondly, given that adiponectin multimers rather than total adiponectin have been reported to be more strongly related to fetal growth and neonatal body composition (45), we only measured the levels of total adiponectin and not its multimeric forms, which might have contributed to the lack of association between adiponectin and the neonatal growth parameters in our study.

Conclusion

In summary, our results demonstrate that hormone dysregulation occurs in preterm infants, especially those with a BW ≤1,500 g. GA and BW were found to be major determinants of the cord blood adipokine profile and the levels of ghrelin. Furthermore, the interrelationship between leptin and insulin found in term infants and in preterm infants with BW >2,000 g in this study suggests that a fetal adipoinsular axis at birth may influence the "programming" of satiety and body metabolism, thereby determining postnatal weight gain and adiposity. Combined, these findings indicate that the lack

TABLE 4 | Relationship between the levels of adiponectin, leptin, insulin, and ghrelin and the neonatal growth parameters.

	ln Adiponectin		ln Leptin		ln Insulin		ln Ghrelin	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Birth weight (g)	–0.052	0.678	0.460	**<0.001*****	0.143	0.286	–0.372	**0.002****
Birth length (cm)	0.106	0.403	0.311	**0.012***	0.183	0.143	–0.415	**0.001****
Head circumference (cm)	0.132	0.295	0.310	**0.012***	0.131	0.298	–0.373	**0.002****

Partial correlation analysis was used with adjustments for maternal age, parity, mode of delivery, gestational age, gender, pre-pregnancy BMI, and weight gain during pregnancy. P-values in bold are significant at p < 0.05.

r, partial correlation coefficient; BMI, body mass index.

**p < 0.05, **p < 0.01, ***p < 0.001.*

of proper acquisition of adipose tissue by the fetus in prematurity is associated with changes in the cord blood adipokine profile and the levels of ghrelin, which may disturb the metabolic adaptation of the infant to extrauterine life. These observations further suggest that the detection of the levels of cord blood hormones can reflect the development of preterm infants. However, the mechanisms underlying the observed associations need further investigation.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking Union Medical College Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ML and DW conceived the study. LH and ZL contributed significantly to manuscript preparation. LH, BL, ML, XX, and SL performed the data analysis and wrote the manuscript. ML and DW helped with data analysis. All the authors contributed to the manuscript and approved the submitted version.

FUNDING

This work was partly supported by a grant from Capital's Funds for Health Improvement and Research (2020-2Z-40117).

ACKNOWLEDGMENTS

We wish to thank the assistance provided by members of the Department of Pediatrics and Obstetrics of Peking Union Medical College Hospital. We also thank all the mothers who provided samples.

REFERENCES

1. Bouret SG, Simerly RB. Development of Leptin-Sensitive Circuits. *J Neuroendocrinol* (2007) 19:575–82. doi: 10.1111/j.1365-2826.2007.01563.x
2. Koerner A, Kratzsch J, Kiess W. Adipocytokines: Leptin–the Classical, Resistin–the Controversical, Adiponectin–the Promising, and More to Come. *Best Pract Res Clin Endocrinol Metab* (2005) 19:525–46. doi: 10.1016/j.beem.2005.07.008
3. Symonds ME, Mostyn A, Pearce S, Budge H, Stephenson T. Endocrine and Nutritional Regulation of Fetal Adipose Tissue Development. *J Endocrinol* (2003) 179:293–9. doi: 10.1677/joe.0.1790293
4. Logan CA, Bornemann R, Koenig W, Reister F, Walter V, Fantuzzi G, et al. Gestational Weight Gain and Fetal-Maternal Adiponectin, Leptin, and CRP: Results of Two Birth Cohorts Studies. *Sci Rep* (2017) 7:41847. doi: 10.1038/srep41847
5. Stoll-Becker S, Kreuder J, Reiss I, Etspuler J, Blum WF, Gortner L. Influence of Gestational Age and Intrauterine Growth on Leptin Concentrations in Venous Cord Blood of Human Newborns. *Klin Padiatr* (2003) 215:3–8. doi: 10.1055/s-2003-36892
6. Cetin I, Morpurgo PS, Radaelli T, Taricco E, Cortelazzi D, Bellott M, et al. Fetal Plasma Leptin Concentrations: Relationship With Different Intrauterine Growth Patterns From 19 Weeks to Term. *Pediatr Res* (2000) 48:646–51. doi: 10.1203/00006450-200011000-00016
7. Steinbrekera B, Roghair R. Modeling the Impact of Growth and Leptin Deficits on the Neuronal Regulation of Blood Pressure. *J Endocrinol* (2016) 231:R47–60. doi: 10.1530/JOE-16-0273
8. Nakahara K, Nakagawa M, Baba Y, Sato M, Toshinai K, Date Y, et al. Maternal Ghrelin Plays an Important Role in Rat Fetal Development During Pregnancy. *Endocrinology* (2006) 147:1333–42. doi: 10.1210/en.2005-0708
9. Wang ZV, Scherer PE. Adiponectin, the Past Two Decades. *J Mol Cell Biol* (2016) 8:93–100. doi: 10.1093/jmcb/mjw011
10. Bazaes RA, Mericq V. Premature Birth and Insulin Resistance. *N Engl J Med* (2005) 352:939–40. doi: 10.1056/NEJM200503033520920
11. Payal V, Jora R, Sharma P, Gupta PK, Gupta M. Premature Birth and Insulin Resistance in Infancy: A Prospective Cohort Study. *Indian J Endocrinol Metab* (2016) 20:497–505. doi: 10.4103/2230-8210.183470
12. Gualillo O, Caminos J, Blanco M, Garcia-Caballero T, Kojima M, Kangawa K, et al. Casanueva: Ghrelin, a Novel Placental-Derived Hormone. *Endocrinology* (2001) 142(2):788–94. doi: 10.1210/endo.142.2.7987
13. Lanyi E, Varnagy A, Kovacs KA, Csermely T, Szasz M, Szabo TI. Ghrelin and Acyl Ghrelin in Preterm Infants and Maternal Blood: Relationship With Endocrine and Anthropometric Measures. *Eur J Endocrinol* (2008) 158:27–33. doi: 10.1530/EJE-07-0410
14. Ozanne SE, Hales CN. Early Programming of Glucose-Insulin Metabolism. *Trends Endocrinol Metab* (2002) 13:368–73. doi: 10.1016/s1043-2760(02)00666-5
15. Lindsay RS, Walker JD, Havel PJ, Hamilton BA, Calder AA, Johnstone FD. Adiponectin Is Present in Cord Blood But Is Unrelated to Birth Weight. *Diabetes Care* (2003) 26:2244–9. doi: 10.2337/diacare.26.8.2244
16. Rothenbacher D, Nieters A, Weyermann M, Brenner H. Adiponectin Polymorphisms, Cord Blood Levels of Adiponectin, and Body Composition. *J Allergy Clin Immunol* (2007) 120:469–70. doi: 10.1016/j.jaci.2007.03.036

17. Karakosta P, Chatzi L, Plana E, Margioris A, Castanas E, Kogevinas M. Leptin Levels in Cord Blood and Anthropometric Measures at Birth: A Systematic Review and Meta-Analysis. *Paediatr Perinat Epidemiol* (2021) 25(2):150–63. doi: 10.1111/j.1365-3016.2010.01163.x
18. Brown HK. Neonatal Morbidity Associated With Late Preterm and Early Term Birth: The Roles of Gestational Age and Biological Determinants of Preterm Birth. *Int J Epidemiol* (2014) 43(3):802–14. doi: 10.1093/ije/dyt251
19. Yu X, Rong SS, Sun X, Ding G, Wan W, Zou L, et al. Associations of Breast Milk Adiponectin, Leptin, Insulin and Ghrelin With Maternal Characteristics and Early Infant Growth: A Longitudinal Study. *Br J Nutr* (2018) 120:1380–87. doi: 10.1017/S0007114518002933
20. Li Q, Lu Y, Sun L, Yan J, Yan X, Fang L, et al. Plasma Adiponectin Levels in Relation to Prognosis in Patients With Angiographic Coronary Artery Disease. *Metabolism* (2012) 61:1803–8. doi: 10.1016/j.metabol.2012.06.001
21. Li M, Yin JH, Zhang K, Wu CY. A Highly Sensitive Enzyme-Linked Immunosorbent Assay for Measurement of Leptin Secretion in Human Adipocytes. *Zhonghua Yi Xue Za Zhi* (2008) 88(46):3293–7. doi: 10.3321/j.issn:0376-2491.2008.46.015
22. Yin J, Li M, Xu L, Wang Y, Cheng H, Zhao X, et al. Insulin Resistance Determined by Homeostasis Model Assessment (HOMA) and Associations With Metabolic Syndrome Among Chinese Children and Teenagers. *Diabetol Metab Syndr* (2013) 5:71. doi: 10.1186/1758-5996-5-71
23. Aye IL, Powell TL, Jansson T. Review: Adiponectin–the Missing Link Between Maternal Adiposity, Placental Transport and Fetal Growth? *Placenta* (2013) 34:S40–5. doi: 10.1016/j.placenta.2012.11.024
24. Yoshida T, Nagasaki H, Asato Y, Ohta T. The Ratio of High-Molecular Weight Adiponectin and Total Adiponectin Differs in Preterm and Term Infants. *Pediatr Res* (2009) 65:580–3. doi: 10.1203/PDR.0b013e3181995103
25. Martos-Moreno GA, Barrios V, Saenz DPM, Pozo J, Dorronsoro I, Martinez-Biarge M, et al. Influence of Prematurity and Growth Restriction on the Adipokine Profile, IGF1, and Ghrelin Levels in Cord Blood: Relationship With Glucose Metabolism. *Eur J Endocrinol* (2009) 161:381–9. doi: 10.1530/EJE-09-0193
26. Terrazzan AC, Procianoy RS, Silveira RC. Neonatal Cord Blood Adiponectin and Insulin Levels in Very Low Birth Weight Preterm and Healthy Full-Term Infants. *J Matern Fetal Neonatal Med* (2014) 27:616–20. doi: 10.3109/14767058.2013.823939
27. Siahanidou T, Mandyla H, Papassotiriou GP, Papassotiriou I, Chrousos G. Circulating Levels of Adiponectin in Preterm Infants. *Arch Dis Child Fetal Neonatal Ed* (2007) 92:F286–90. doi: 10.1136/adc.2006.106112
28. Kamoda T, Saitoh H, Saito M, Sugiura M, Matsui A. Serum Adiponectin Concentrations in Newborn Infants in Early Postnatal Life. *Pediatr Res* (2004) 56:690–3. doi: 10.1203/01.PDR.0000142711.24999.8A
29. Lau SM, Hng TM, de Vries B, McLean M, Cheung NW. Sexual Dimorphism of High Molecular Weight Adiponectin in Cord Blood. *Clin Endocrinol (Oxf)* (2009) 70:500–1. doi: 10.1111/j.1365-2265.2008.03373.x
30. Jaquet D, Leger J, Levy-Marchal C, Oury JF, Czernichow P. Ontogeny of Leptin in Human Fetuses and Newborns: Effect of Intrauterine Growth Retardation on Serum Leptin Concentrations. *J Clin Endocrinol Metab* (1998) 83:1243–6. doi: 10.1210/jcem.83.4.4731
31. Clapp JR, Kiess W. Cord Blood Leptin Reflects Fetal Fat Mass. *J Soc Gynecol Investig* (1998) 5:300–3. doi: 10.1177/107155769800500604
32. Lo HC, Tsao LY, Hsu WY, Chen HN, Yu WK, Chi CY. Relation of Cord Serum Levels of Growth Hormone, Insulin-Like Growth Factors, Insulin-Like Growth Factor Binding Proteins, Leptin, and Interleukin-6 With Birth Weight, Birth Length, and Head Circumference in Term and Preterm Neonates. *Nutrition* (2002) 18:604–8. doi: 10.1016/s0899-9007(01)00811-5
33. Claris O, Beltrand J, Levy-Marchal C. Consequences of Intrauterine Growth and Early Neonatal Catch-Up Growth. *Semin Perinatol* (2010) 34:207–10. doi: 10.1053/j.semperi.2010.02.005
34. Ozdemir ZC, Aksit MA. The Association of Ghrelin, Leptin, and Insulin Levels in Umbilical Cord Blood With Fetal Anthropometric Measurements and Glucose Levels at Birth. *J Matern Fetal Neonatal Med* (2020) 33:1486–91. doi: 10.1080/14767058.2018.1520828
35. Ng PC, Lee CH, Lam CW, Chan IH, Wong E, Fok TF. Ghrelin in Preterm and Term Newborns: Relation to Anthropometry, Leptin and Insulin. *Clin Endocrinol (Oxf)* (2005) 63:217–22. doi: 10.1111/j.1365-2265.2005.02328.x
36. Shimizu T, Kitamura T, Yoshikawa N, Suganuma H, Hisata K, Tanaka K, et al. Plasma Levels of Active Ghrelin Until 8 Weeks After Birth in Preterm Infants: Relationship With Anthropometric and Biochemical Measures. *Arch Dis Child Fetal Neonatal Ed* (2007) 92:F291–2. doi: 10.1136/adc.2005.092379
37. Kyriakakou M, Malamitsi-Puchner A, Mastorakos G, Boutsikou T, Hassiakos D, Papassotiriou I, et al. The Role of IGF-1 and Ghrelin in the Compensation of Intrauterine Growth Restriction. *Reprod Sci* (2009) 16:1193–200. doi: 10.1177/1933719109344629
38. Mendez-Ramirez F, Barbosa-Sabanero G, Romero-Gutierrez G, Malacara JM. Ghrelin in Small-for-Gestational Age (SGA) Newborn Babies: A Cross-Sectional Study. *Clin Endocrinol (Oxf)* (2009) 70:41–6. doi: 10.1111/j.1365-2265.2008.03278.x
39. Onal EE, Cinaz P, Atalay Y, Turkyilmaz C, Bideci A, Akturk A, et al. Umbilical Cord Ghrelin Concentrations in Small- and Appropriate-for-Gestational Age Newborn Infants: Relationship to Anthropometric Markers. *J Endocrinol* (2004) 180:267–71. doi: 10.1677/joe.0.1800267
40. Kitamura S, Yokota I, Hosoda H, Kotani Y, Matsuda J, Naito E, et al. Ghrelin Concentration in Cord and Neonatal Blood: Relation to Fetal Growth and Energy Balance. *J Clin Endocrinol Metab* (2003) 88:5473–7. doi: 10.1210/jc.2002-021350
41. Bellone S, Rapa A, Vivenza D, Vercellotti A, Petri A, Radetti G, et al. Circulating Ghrelin Levels in the Newborn are Positively Associated With Gestational Age. *Clin Endocrinol (Oxf)* (2004) 60:613–7. doi: 10.1111/j.1365-2265.2004.02014.x
42. Chanoine JP, Yeung LP, Wong AC, Birmingham CL. Immunoreactive Ghrelin in Human Cord Blood: Relation to Anthropometry, Leptin, and Growth Hormone. *J Pediatr Gastroenterol Nutr* (2002) 35:282–6. doi: 10.1097/00005176-200209000-00009
43. Ng PC, Lam CW, Lee CH, Wong GW, Fok TF, Chan IH, et al. Leptin and Metabolic Hormones in Preterm Newborns. *Arch Dis Child Fetal Neonatal Ed* (2000) 83:F198–202. doi: 10.1136/fn.83.3.f198
44. Schwartz MW, Morton GJ. Obesity: Keeping Hunger at Bay. *Nature* (2002) 418:595–7. doi: 10.1038/418595a
45. Simón-Muela I, Näf S, Ballesteros M, Vendrell J, Ceperuelo-Mallafre V, de la Flor M, et al. Gender Determines the Actions of Adiponectin Multimers on Fetal Growth and Adiposity. *Am J Obstet Gynecol* (2013) 208(6):481.e1–7. doi: 10.1016/j.ajog.2013.02.045

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Physiology

ORIGINAL RESEARCH
published: 25 November 2021
doi: 10.3389/fphys.2021.744548

Vitamin D Supplementation Improves Uterine Receptivity in a Rat Model of Vitamin D Deficiency: A Possible Role of HOXA-10/FKBP52 Axis

Hend Ashour[1,2†], Sara Mahmoud Gamal[2], Nermeen Bakr Sadek[2], Laila Ahmed Rashed[3†], Rania Elsayed Hussein[3], Samaa Samir Kamar[4,5†], Hayam Ateyya[6,7], Marwa Nagi Mehesen[7] and Asmaa Mohammed ShamsEldeen[2†]*

[1] Department of Physiology, Faculty of Medicine, King Khalid University, Abha, Saudi Arabia, [2] Department of Physiology, Kasralainy Faculty of Medicine, Cairo University, Giza, Egypt, [3] Department of Biochemistry and Molecular Biology, Kasralainy Faculty of Medicine, Cairo University, Giza, Egypt, [4] Department of Histology and Cell Biology, Kasralainy Faculty of Medicine, Cairo University, Giza, Egypt, [5] Armed Forces College of Medicine, Cairo, Egypt, [6] Department of Pharmacy Practice and Clinical Pharmacy, Faculty of Pharmacy, Future University in Egypt, Cairo, Egypt, [7] Department of Medical Pharmacology, Faculty of Medicine, Cairo University, Giza, Egypt

OPEN ACCESS

Edited by:
Qiongjie Zhou,
Fudan University, China

Reviewed by:
Sangappa B. Chadchan,
Washington University in St. Louis, United States
Winnie Shum,
ShanghaiTech University, China
María Mercedes Milesi,
National University of Litoral, Argentina

***Correspondence:**
Asmaa Mohammed ShamsEldeen
asmaa82shamseldeen@gmail.com;
Asmaa.AbdulWahab@kasralainy.edu.eg

†ORCID:
Asmaa Mohammed ShamsEldeen
orcid.org/0000-0002-4250-0818
Hend Ashour
orcid.org/0000-0002-5423-7228
Laila Ahmed Rashed
orcid.org/0000-0002-0362-1385
Samaa Samir Kamar
orcid.org/0000-0002-9040-584x

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: *20 July 2021*
Accepted: *21 October 2021*
Published: *25 November 2021*

Synchronized uterine receptivity with the time of implantation is crucial for pregnancy continuity. Vitamin D (VD) deficiency has been linked to the failure of implantation. Therefore, we tested the link between the Homeobox transcription factor-10/immunophilin FK506-binding protein 52 (HOXA-10/FKBP52) axis and the uterine receptivity in VD-deficient rats. The effect of VD supplementation at different doses was also investigated. Forty-eight pregnant rats were divided into six groups (eight/group); normal control rats fed with standard chow (control), control rats supplemented with VD (equivalent dose of 400 IU/day) (control-D400). VD-deficient group (DEF) and the three VD deficiency groups with VD supplementation were equivalent to 400, 4,000, and 10,000 IU/day (DEF-D400, DEF-D4000, and DEF-D10000, respectively). The expression levels of HOXA-10/FKBP52, progesterone level, and histological evaluation of decidualization using osteopontin (OSN) and progesterone receptor (PGR) were estimated. An assessment of the uterine contractility was conducted for all rats. This study showed the downregulation of HOXA-10/FKBP52 together with increased amplitude and frequency of the uterine contractility in the DEF group compared to control. VD dose-dependent supplementation restored progesterone/receptor competency, upregulated the expressional response of HOXA-10 and its downstream FKBP52, and improved uterine receptivity and endometrial decidualization at the time of implantation that was documented by increased area% of OSN and the number of implantation beads.

Keywords: vitamin D, contraction, progesterone receptor, decidualization, HOXA-10/FKBP52

INTRODUCTION

Successful implantation is a result of complex molecular interactions between the hormonally primed uterus and mature blastocyst, which is necessary for pregnancy continuity (Ochoa-Bernal and Fazleabas, 2020). Some studies have linked vitamin D (VD) status with pregnancy loss and neonatal outcomes (Mumford et al., 2018; Fernando et al., 2020). However, the precise physiological function of VD during pregnancy is still unclear. This certainly extends far beyond the fetal calcium regulation and bone homeostasis that occurs quite late in gestation (Hewison, 2018). Gestational VD deficiency is becoming a growing concern. A recent evidence emphasized the vital role of VD during the implantation window (Von Websky et al., 2018), and its deficiency is associated with early miscarriage (Sharef et al., 2020).

Uterine receptivity is defined as the time period during which the uterus achieves proper differentiation and is ready for the initiation of implantation. In mice, the uterus becomes fully receptive on the 4th day of pregnancy (Tranguch et al., 2005). Endometrial receptivity could be assessed by several parameters, including endometrial thickness, decidualization, and the uterine blood flow (Cheng et al., 2015). Myometrial contractility regulates the extent of blood flow, if increased, it may interfere with the adequate blood supply and consequently the uterine receptivity (Cheng et al., 2015).

The active form of VD is crucial for the decidualization process, which is the key phase of implantation (Shin et al., 2011). Interestingly, both maternal decidua and fetal trophoblast express 1α-hydroxylase enzyme (CYP27B1) and are capable of producing local amounts of VD to coincide with a significant expression of vitamin D receptor (VDR) (Hou et al., 2020). Therefore, uterine tissues respond to the locally produced besides the humoral VD through the biological binding to its receptor (VDR). In their review, Ganguly et al. (2018) have emphasized the role of VD during the implantation window. The maternal decidua and fetal trophoblast expression of VD system components facilitate extravillous trophoblast invasion and establishment of early pregnancy (Ganguly et al., 2018). In line with these data, the VD level positively affects the implantation rate of the *in vitro* fertilization (Farzadi et al., 2015; Liu et al., 2019).

Homeobox transcription factor-10 (HOXA-10), a molecular multitask protein, belongs to the homeobox family of transcription factors (Guo et al., 2020) and is specifically expressed by differentiating uterine cells during the peri-implantation period (Vitiello et al., 2008) as a part of regulating the decidualization process (Ashary et al., 2020).

One of the downstream targets of HOXA-10 during implantation is the immunophilin FK506-binding protein 52 (FKBP52) (Yang et al., 2012). FKBP52 is a co-chaperone of the progesterone receptor (PGR) and is related to the uterine response to progesterone (Tranguch et al., 2007; Alaee et al., 2014). Around the implantation period, the mouse uterus demonstrates a specific pattern of FKBP52 expression, suggesting an essential role in regulating the implantation process (Daikoku et al., 2005). Meanwhile, FKBP52 protein level was decreased in the chorionic villi of recurrent spontaneous abortion patients, indicating the importance of FKBP52 in uterine receptivity in the early stages of pregnancy (Chen et al., 2015). Therefore, a HOXA-10/FKBP52 axis is thought to be a potential biomarker for predicting endometrial receptivity and blastocyst implantation (Alaee et al., 2014).

However, the link between VD and PGR signaling in the process of implantation remains unclear.

Guided by the previous data, our objectives were to investigate the role of VD in the uterine receptivity and implantation. Applying the different doses of VD was our aim to examine endometrium decidualization and myometrial contractility. Molecular determination of the cross talk between VD and PGR expression and its co-chaperone FKBP52 was investigated.

MATERIALS AND METHODS

Experimental Animals

Forty-eight female Wistar albino rats aged about 3 weeks and weighing 60–65 g with normal plasma VD level were included in this study. All animal procedures were approved by Cairo University, Institutional Animal Care and Use Committee of Cairo University (CU-IACUC) (numbered: CU/III/F/70/17). The animals were purchased from the Animal House of the Research Institute of Ophthalmology (RIO), and the study was conducted in the RIO and Department of Physiology, Faculty of Medicine at Cairo University. The rats were housed in standard cages under room temperature (25 ± 2°C) and relative humidity (**Figure 1**).

Animal Groups and Study Protocol

The rats were divided into six equal groups (eight rats each), and sample size was calculated using one-way ANOVA (G power analysis) regarding the primary outcome (the establishment of VD deficiency). VD analysis was in the normal range of the included rats at the study start point (**Figure 2**).

Sixteen rats were kept on the ordinary living conditions for 6 weeks to acclimatize under a light/dark cycle (light on 06:00–19:00), and then the rats were divided into: (1) Control group (n = 8) in which pregnancy was allowed (the method is described below). These rats were given corn oil (as a vehicle for VD at a dose of 0.5 ml/day/rat orally). Rats were followed up until the 7th day of pregnancy. (2) The control group (control-D400) with the same protocol as the control group but with VD (Euro D 10000, Euro pharm, Saint-Léonard, QC, Canada) supplementation was given in a dose equivalent to 400 IU/day starting from G0 till the time of sacrifice. The chosen dose of VD is the recommended one by the Chief Medical Officers for the United Kingdom for pregnant females (Davies et al., 2012). The dose was converted into the rat dose and calculated to be 7.2 IU/rat/day orally.

The VD deficiency model was induced in the remaining 32 female rats (as described below). Then, the pregnant rats were divided into: (3) VD deficiency group without treatment (DEF). (4) VD-deficient animals with VD supplementation of 7.2 IU/rat/day, equivalent to 400 IU (DEF-D400). (5) VD-deficient animals treated with VD in a dose of 72 IU/rat/day equivalent to human dose 4,000 IU/day (Hollis et al., 2011) (DEF-D4000). (6)

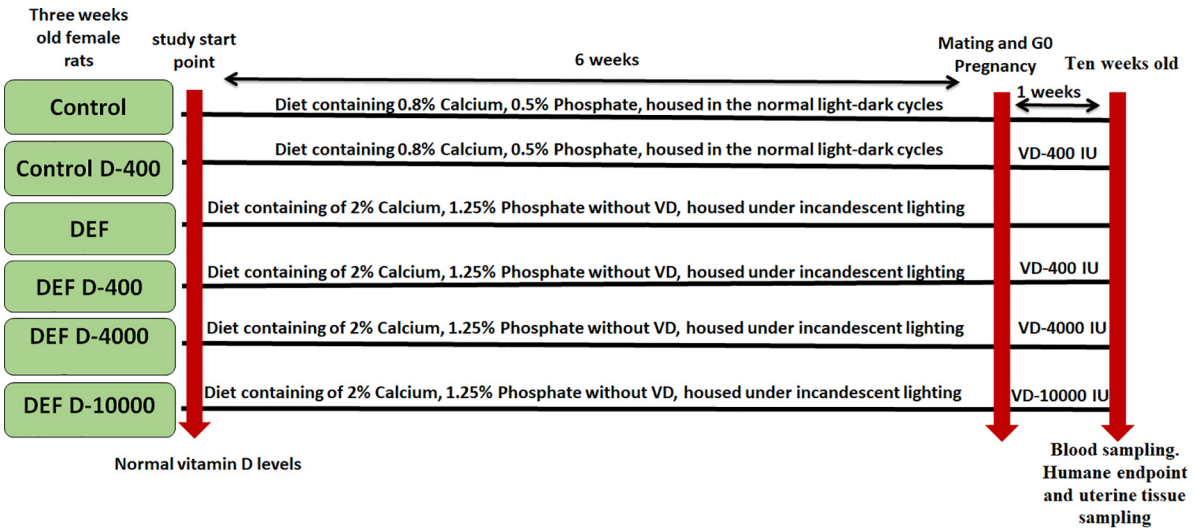


FIGURE 1 | A timeline represents the included groups with the scheduled experimental interventions. D: refers to vitamin D (VD) supplementation in different doses equivalent to 400, 4,000, and 10,000 IU. DEF: refers to VD deficiency.

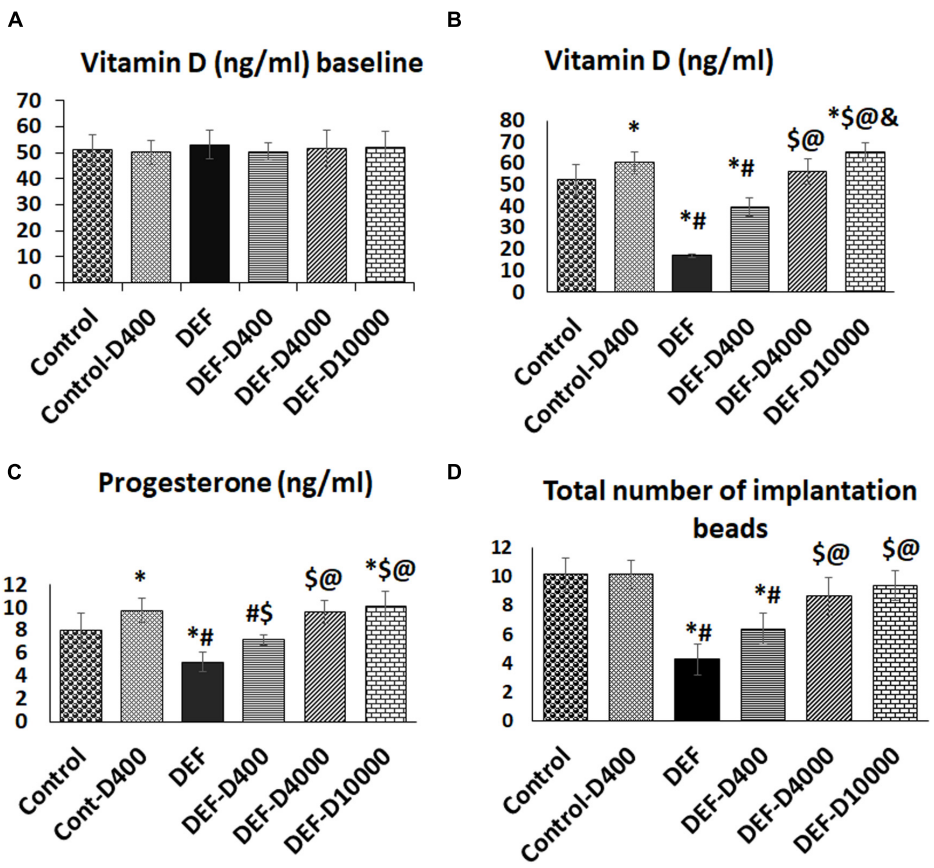


FIGURE 2 | (A) Representation of the baseline level of VD, **(B)** the changes in the serum VD levels at the study end, **(C)** the serum progesterone, and **(D)** the number of uterine implantation sites. D: refers to VD supplementation in different doses equivalent to 400, 4,000, and 10,000 IU. DEF: refers to VD deficiency, *: A statistically significant sign compared to the control group, #: compared to control-400, $: compared to the deficiency group, @: compared to DEF-400, and &: significant compared to the DEF-4000 group ($p < 0.05$).

VD-deficient rats treated with VD (180 IU/rat/day) equivalent to human 10,000 IU/day (Heaney et al., 2003) (DEF-D10000).

The dose conversion from human to rat was done using the Paget table (1964). VD was dissolved in corn oil, and the treatment was started from the 1st to the 7th day of pregnancy.

Vitamin D Deficiency Model

Normal rats were kept for 6 weeks before mating on normal rat chaw diet containing 0.8% calcium, 0.5% phosphate in the normal light-dark cycles. The VD deficiency model was established by maintaining rat housing under incandescent lighting (Harms et al., 2008), to avoid VD activation by UV rays. In addition, diet administration was composed of 2% calcium, and 1.25% phosphate without VD (Stavenuiter et al., 2015). High calcium containing diet was needed to maintain normal calcium level and to prevent hyperparathyroidism (Kollenkirchen et al., 2009).

The model was proven by the low VD levels that were 16.46 ± 2.4 compared to that of control 57.59 ± 11.27 (ng/ml).

In the VD treated groups, calcium and phosphate in the diet were returned to the normal level (0.8%, 0.5%) to avoid excessive absorption and to prevent the disturbed serum levels.

Establishment of Pregnancy

After the determination of their sexual maturity using vaginal smear, the nulliparous 9-week female rats were bred overnight with the same number of fertile males. Then, female rats were subjected to daily morning examination for the detection of the vaginal plug and the presence of spermatozoa in vaginal smear. The + ve smear determined the day 0 of gestation (G0).

Tissue and Blood Sampling

At early morning of the 8th day of gestation, retro-orbital blood samples were withdrawn using fine heparinized catheters. Samples were collected in test tubes; plasma was separated by centrifugation and stored at −80°C until further assessment of 25 Vitamin D3 levels, progesterone, and reactive oxygen species (ROS) levels. The animals were euthanized by an intraperitoneal injection of pentobarbital (50 mg/kg i.p.) anesthesia and were sacrificed. Careful exposure of the uterine and proper dissection of both horns were made. The number of implantation sits (beads) was calculated. Then, the uterine tissues were used for biochemical analysis, assessment of contractility, and histological examination. All samples took the same codes of their included rats, and the handling of the samples and analysis was done blindly.

Estimating Serum Levels of Total 25-Hydroxyvitamin D3 and Progesterone Level

Using ELISA, serum VD was estimated according to the manufacturer's recommendations (Cat. No: DEIA2219, Creative Diagnostics, NY, United States), and progesterone (Cat. No: SE120087, Sigma Aldrich, St. Louis, MO, United States).

Estimation of Serum Calcium and Phosphate Levels

Using the colorimetric assay method supplied by BioVision, Inc., CA, United States, and following the instructions of the manufacturer, the levels of calcium and phosphate were determined by the Calcium Colorimetric Assay kit (Catalog #K380-250; 250 assays) and the Phosphate Colorimetric Assay kit (Catalog #K410-500; 500 assays).

Estimation of Uterine Hydrogen Peroxide

Uterine content of hydrogen peroxide (H_2O_2) levels was measured by the method of Pick (1996). Briefly, 100 μl of tissue homogenate was prepared in Tris–HCl buffer (20 mM, pH7.4), and 100 μl of the prepared assay solution was added to 10 μl of 1.0 N NaOH to get the reaction. Absorbance was recorded using the ELISA reader.

Analysis of Uterine Glutathione Peroxidase Activity

Quantitative assessment of glutathione peroxidase (GSH-Px) activity in the uterine tissues was determined using the colorimetric Glutathione Peroxidase Assay kit (ab102530; Abcam, Cambridge, United Kingdom). The principle of the test is that GSH-Px oxidizes GSH to produce GSSG as a part of the reaction in which it reduces cumene hydroperoxide. Then, glutathione reductase reduces GSSG to produce GSH, and consumes NADPH. The reaction was measured at optical density = 340 nm.

Quantitative Real-Time PCR of HOXA-10 and FKBP52 Expression Level

Samples from the uterine tissue were processed and homogenated, and the RNA was extracted using the SV total RNA isolation system (Promega, Madison, WI, United States). The obtained RNA was determined by spectrophotometry at 260 nm, the absorbance unit (A260) equals 40 μg of single standard RNA/ml. Then, cDNA was synthesized by using reverse transcriptase. The high capacity cDNA reverse transcription kit (#K1621, Fermentas, Waltham, MA, United States) was used for RNA conversion to cDNA. Real-time quantitative PCR (qPCR) amplification (SYBR green I) and Applied Biosystem with software version 3.1 (StepOne®, United States) was used for the detection and analysis of the results. A 10 μl qPCR reaction mixture was formed of the following: cDNA 1 μl, forward primer (10 μM) 0.5 μl, reverse primer (10 μM) 0.5 μl, SYBR qPCR mix 5 μl, ddH_2O 3 μl. Cycling conditions included initial denaturation for 10 min at 95°C, then 40 cycles of denaturation for 15 s at 95°C and annealing at which the temperature was lowered to 60°C for 1 min were done till the separation of all complex targets (dsDNA) (Song and Tan, 2018). The primer sequence for FKBP52 was obtained from Sangon Biotech Co., Ltd., Shanghai, China as shown in **Table 1**. The accession ID of HOXA-10 is XM_008762949.1, and for the house keeping gene Beta-actin is NM_031144.3.

All cDNAs of the prepared samples (for HOXA-10 and FKBP52), internal control (housekeeping gene of Beta-actin gene

expression), and non-template control (water to confirm the absence of DNA contamination in the reaction mixture) were obtained in duplicates (**Table 1**).

Assessment of Uterine Contractility

The caudal halves of the uterine right horns were excised and cleaned of adhering fat and mesentery. Whole tissue strips were cut from the area in between the implantation sites into 10-mm length streps and mounted in organ baths (ADInstruments, Sydney, NSW, Australia, ML1110). The bath contained about 20 ml of Krebs' solution buffer 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 1.16 mM $CaCl_2$, 14.9 mM $NaHCO_3$, and 5.5 mM dextrose (Sigma Aldrich, St. Louis, MO, United States) (Darios et al., 2012). The solution was maintained at 37°C and aerated with 95% O_2/5% CO_2 throughout the experimental period with washes every 15 min. The strips were initially tensioned to 1 gm and connected to an isometric force transducer (ADInstruments, Sydney, NSW, Australia, TRI210) by a silk thread. The latter was connected to a bridge amplifier (ADInstruments, Sydney, NSW, Australia, ML221), to amplify and convert the tension force generated by the contractions. The measurements were recorded by Powerlab (ADInstrument, Sydney, NSW, Australia, ML866) and analyzed by using LabChart 8 version. Preparations were allowed to equilibrate for about 60 min to obtain the spontaneous phasic contractions. After that, a continuous curve was recorded for 10 min (Aley et al., 2010).

Histological Analysis

The pregnant uteri demonstrated scattered implantation chambers through which transverse cuts were taken at their widest area. Then, the specimens were placed in Bouin's solution overnight, and paraffin blocks were prepared. Sections of 5-μm thickness were obtained and processed to the following histological studies:

1. H&E staining to demonstrate the structural changes in the decidual cells at the mesometrial region.
2. Immunohistochemical staining for PGRs and osteopontin (OSN), as a marker of the endometrial decidualization. Anti-PGRs monoclonal Ab (Thermo Scientific, Waltham, MA, United States, MA1-10202) and anti-OSN polyclonal antibody (Ab) (Thermo Scientific, Waltham, MA, United States, RB-9097-R7) were used. The application of the primary Abs was followed by incubation in a humidity chamber for an hour at room temperature. The sections were costained with Meyer hematoxylin to visualize the nuclei.

TABLE 1 | Primer sequence of Homeobox transcription factor-10 (HOXA-10), FK506-binding protein 52 (FKBP52), and the housekeeping gene Beta-actin.

	Primer sequence
HOXA-10	**Sense:** 5′ AGGACTCCCTGGGCAATTC 3′
	Antisense: 5′ GTAAGGGCAGCGTTTCTTCC 3′
FKBP52	Sense: 5′ CACTACACTGGCTGGCTGCT 3′
	Antisense: 5′ TGGTTGCCACAGCAATATCC 3′
Beta actin	**Sense:** 5′ GTAGCCATCCAGGCTGTGTTG 3′
	Antisense: 5′ TGCCAGTGGTACGACCAGAG 3′

Morphometric Assessment

Using the "Leica Qwin 500 C" image analyzer (Cambridge, United Kingdom), the area% of + ve PGR immunostaining in addition to the area% of + ve OSN at the mesometrial region, in 10 non-overlapping high-power fields (HPF) (×400)/rat were estimated.

Statistical Analysis

Quantitative data were summarized as means ± SDs. Data were normally distributed (attached **Supplementary File 1**) and compared using the ANOVA test followed by Tukey's *post hoc* test for multiple comparisons between the groups. Pearson's correlation test was done to detect the relationship between the parameters among groups. The probability value < 0.05 was considered statistically significant. Calculations were made on Statistical Package of Social Science software (SPSS), version 25 (Chicago, CA, United States).

RESULTS

Serum Vitamin D and Progesterone Level Changes Between Groups

The serum VD levels were significantly increased in the control group received the vitamin compared to that of control group. The induced VD deficiency model succeeded to reduce ($p < 0.01$) the serum vitamin levels compared to the control group. In the treated groups following deficiency, there was a significant ($p < 0.05$) progressive dose-dependent elevation in the serum VD level as demonstrated in **Figure 2B**.

Interestingly, the progesterone level followed the fluctuation in the VD level that was observed among the deficient and treated groups. Although the serum VD level was significantly higher ($p < 0.05$) in the group received 10,000 doses than the rats treated with 4,000 doses, the progesterone level showed no further elevation (data are shown in **Figure 2C**).

The Total Number of Implantation Sites Was Diminished by the Model Establishment and Improved by Vitamin D Supplementation

Vitamin D-deficient rats demonstrated a significant decreased ($p < 0.001$) number of implantation sites (4.25 ± 1.03) compared to control and control-D400 groups (10.13 ± 1.1 and 10.2 ± 0.99, respectively). A progressive increase ($p < 0.05$) in the number of implantation beads was detected by VD replacement in a dose-dependent effect in the groups DEF-D400, DEF-D4000, and DEF-D10000 (6.37 ± 1.06, 8.6 ± 1.3, and 9.4 ± 1.06, respectively) (**Figure 2D**).

Serum Calcium and Phosphate Levels

As demonstrated in **Figures 3A,B**, maintained serum calcium and phosphate levels were achieved through diet adjustment

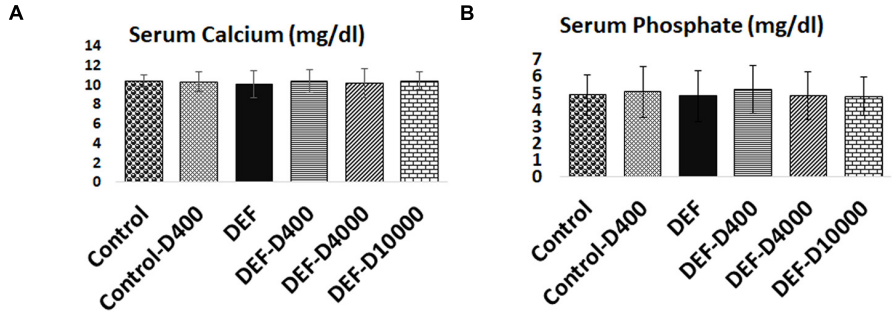


FIGURE 3 | Representation of maintained serum **(A)** calcium and **(B)** phosphate within the normal range in all the groups. D: refers to VD supplementation in different doses equivalent to 400, 4,000, and 10,000 IU. DEF: refers to VD deficiency. No statistical significance between the groups.

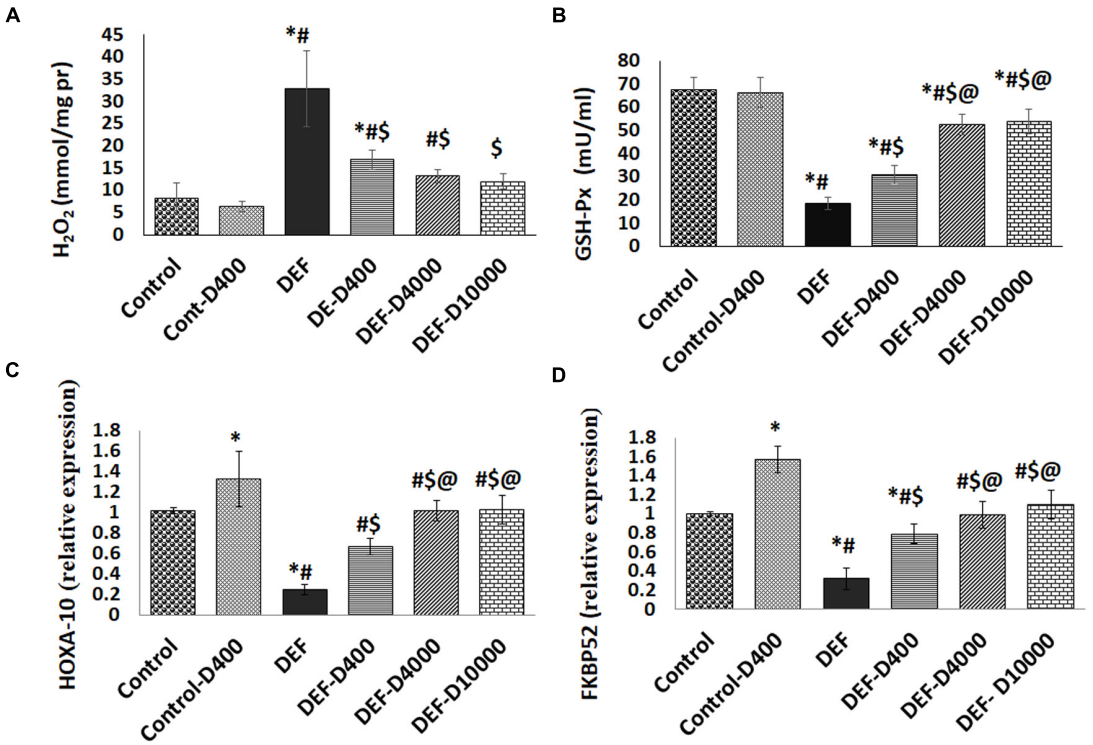


FIGURE 4 | Represents the levels of **(A)** H_2O_2, **(B)** Glutathione peroxidase activity (GSH-Px) in uterine tissues, and the expression levels of **(C)** Homeobox transcription factor (HOXA-10), as well as **(D)** Immunophilin FK506-binding protein 52 (FKBP52). D: refers to vitamin D supplementation in different doses equivalent to 400, 4,000, and 10,000 IU. DEF: refers to vitamin D deficiency. *: Statistically significant sign compared to the control group, #: compared to control-400, $: compared to deficiency group, and @: compared to DEF-400 ($P < 0.05$).

to exclude their effects on the uterine contractility. There was no significant difference among groups in neither serum calcium nor phosphate.

Oxidative Stress Markers in the Uterine Tissues in the Studied Groups

The uterine GSH-Px activity and H_2O_2 content were measured to trace the oxidative status in the uterine tissues. In the VD-deficient group (DEF), there was a marked ($p < 0.001$) increase in H_2O_2 and diminished GSH-Px activity compared to controls. In VD-deficient rats with supplementation, we detected a dose-dependent correction in both H_2O_2 and the GSH-Px activity data as demonstrated in **Figures 4A,B**. No significant change was detected between the group treated with VD 4,000 and 10,000 doses.

Assessment of the Uterine Receptivity Marker HOXA-10

The gene expression level of HOXA-10 was significantly ($p < 0.05$) increased in the normal 400 VD dose treatment

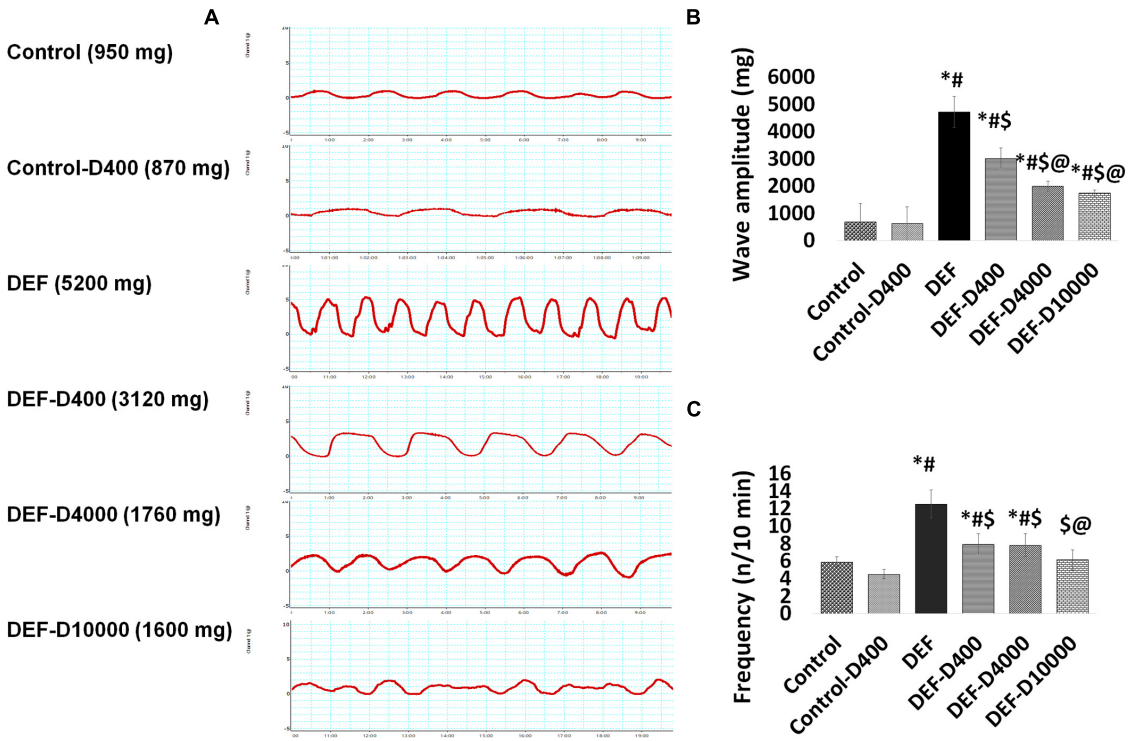


FIGURE 5 | The figure represents **(A)** the curves of the uterine contraction traced during 10 min after equilibration for 60 min, the scale of the *Y* axis is 15 g and the axis is 10 min. **(B)** The amplitude (mg) and **(C)** frequency (n/10 min) of the wave were calculated. D: refers to vitamin D supplementation in different doses equivalent to 400, 4,000, and 10,000 IU. DEF: means vitamin D deficiency. *: Statistically significant sign compared to the control group, #: compared to control-400, $: compared to the deficiency group, and @: compared to DEF-400 ($P < 0.05$).

group compared to the control group. In VD-deficient rats, we reported a marked reduction ($p < 0.001$) in the HOXA-10 levels. By treating the deficient rats with VD, data showed progressive elevated HOXA-10 levels with normalization in the group supplemented with 4,000 and 10,000 VD doses (**Figure 4C**).

The Modulation of Uterine Tissue Expression of FKBP52

Interestingly, the PGR co-chaperone protein FKBP52 expression levels were linked to the VD level changes. FKBP52 that was elevated ($p < 0.05$) in the normal group received 400 VD compared to the control levels (1 ± 0.02). VD deficiency resulted in a marked reduction ($p < 0.001$). FKBP52 was elevated with VD treatment in the group DEF-400 to reach the normal levels in the group treated doses equivalent to 4,000 and 10,000 (**Figure 4D**).

The *in vitro* Uterine Contractility Assessment

Uterine contraction parameters represented by the wave amplitude and frequency of contraction in 10 min gave an idea about the uterine smooth muscle excitability status. We recorded a significant ($p < 0.001$) increase in the wave amplitude and frequency from uterine specimens obtained from the VD DEF compared to normal VD rats. In the treated groups, we noticed a significant ($p < 0.05$) progressive dose-dependent decrease in both the wave amplitude and frequency in the all administered doses compared to waves recorded from the uteri of the VD-deficient rats. The dose 10,000 did not give significant wave parameter changes from that recorded from the 4,000 dose-treated rats (**Figures 5A–C**).

Morphological Assessment of the Decidualization

Histological assessment of the antimesometrial region of the rat uteri at the 8th day of pregnancy using H&E revealed proper decidualization in the control and control-D400 groups. The decidua exhibited variable-sized active decidual cells. Meanwhile, defective decidualization was detected in DEF that was evident by an impaired differentiation of stromal cells into decidual cells. The treatment with VD in DEF-D400 illustrated an impaired differentiation of some stromal cells into decidual cells. However, DEF-D4000 and DEF-D10000 displayed an obvious successful decidualization, which was comparable to the control group (**Figure 6**).

Immunohistochemical Assessment of Progesterone Receptor

Immunohistochemical staining of PGR in the mesometrial region of the rat uteri at the 8th day of pregnancy for control and control-D400 groups displayed a strong nuclear reaction in

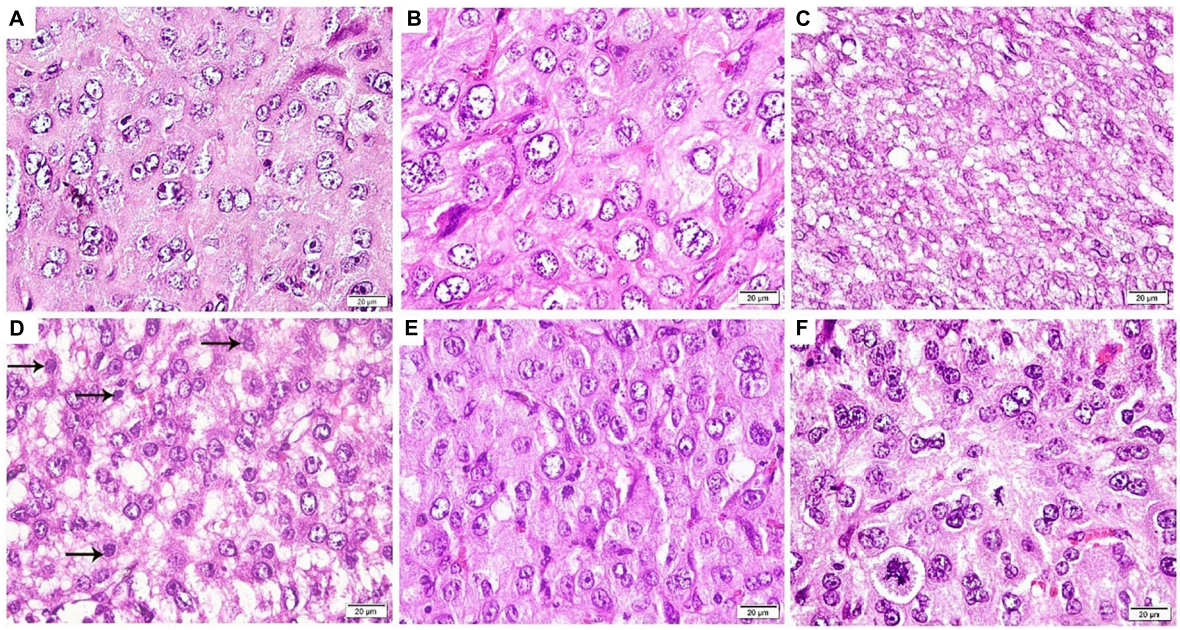


FIGURE 6 | Photomicrograph of an antimesometrial region of the rat uteri at the 8th day of pregnancy (H&E ×400): **(A)** Control and **(B)** Control-D400 revealing different sized rounded decidual cells illustrating eosinophilic cytoplasm, vesicular nuclei, and numerous prominent nucleoli. **(C)** DEF displaying an impaired differentiation of stromal cells into decidual cells. **(D)** DEF-D400 illustrating alternating decidual cells and impaired differentiated stromal cells (arrows) into decidual cells. **(E)** DEF-D4000 and **(F)** DEF-D10000 revealing an obvious differentiation of stromal cells into different sized rounded decidual cells. Scale bar: 20 μm.

the stromal cells and endothelial cells. This was significantly decreased in the DEF group. In DEF-D400, moderate nuclear immunostaining of PGR was detected in some stromal cells with a significant increase as compared to DEF but still significantly decreased as compared to control. DEF-D4000 and DEF-D10000 revealed substantial strong nuclear immunostaining that was comparable to the control group (**Figure 7**).

Immunohistochemical Assessment of Osteopontin

Osteopotin immunostaining of the antimesometrial region in control and control-D400 groups revealed strong cytoplasmic immunostaining of OSN in many decidual cells. In the DEF group, significant weak cytoplasmic immunostaining of OSN was detected in few cells compared to the control group. DEF-D400 illustrated a significant increase in the mean area% of OSN immunostaining compared to DEF. DEF-D4000 and DEF-D10000 groups demonstrated substantial strong cytoplasmic immunostaining of OSN in numerous decidual cells compared to the control group (**Figure 8**).

Correlations

To detect the association between the serum VD level and PGR expression and the uterine receptivity and decidualization, we revealed a strong positive correlation between the VD level and serum progesterone (r = 0.809), uterine PGR expression (r = 0.854). VD was directly correlated to the uterine PGR co-chaperone protein FKBP52 (r = 0.816), and the PGR expression was correlated significantly to FKBP52 (r = 0.778). The serum vitamin level was positively correlated to the HOXA-10 (r = 0.857) and to the GSH-Px levels (r = 0.809). However, a significant ($p < 0.001$) negative correlation was detected between the serum VD level and uterine contractility determined by the wave amplitude (r = − 0.877) and frequency of contraction (r = −0.751) (**Figures 9**, **10**).

DISCUSSION

The main objectives of this work were to investigate the potential efficacy of VD supplement at different doses on endometrial decidualization and uterine receptivity as well as myometrial contractility. We monitored the tissue oxidative stress, the uterine expression of HOXA-10/FKBP52 axis, as well as the progesterone level and PGR expression in VD deficiency that was induced in rats. Here, we report that the induction of VD deficiency in female rats prior to pregnancy was associated with the decreased expression of HOXA-10/FKBP52, and PGR, a substantial augmentation of oxidative stress, and the uterine contractility. These findings were dose dependently improved by VD intake.

The proper endometrial priming with hormones is required for the successful implantation and continuation of pregnancy (Varayoud et al., 2014). Early in pregnancy, progesterone mediates an antiproliferative and anti-inflammatory role in uterine epithelium, which is being necessary for proper implantation and subsequent decidualization (Wetendorf and DeMayo, 2012). PGRs are located in the cytoplasm bound by chaperone proteins. However, upon binding to its ligand,

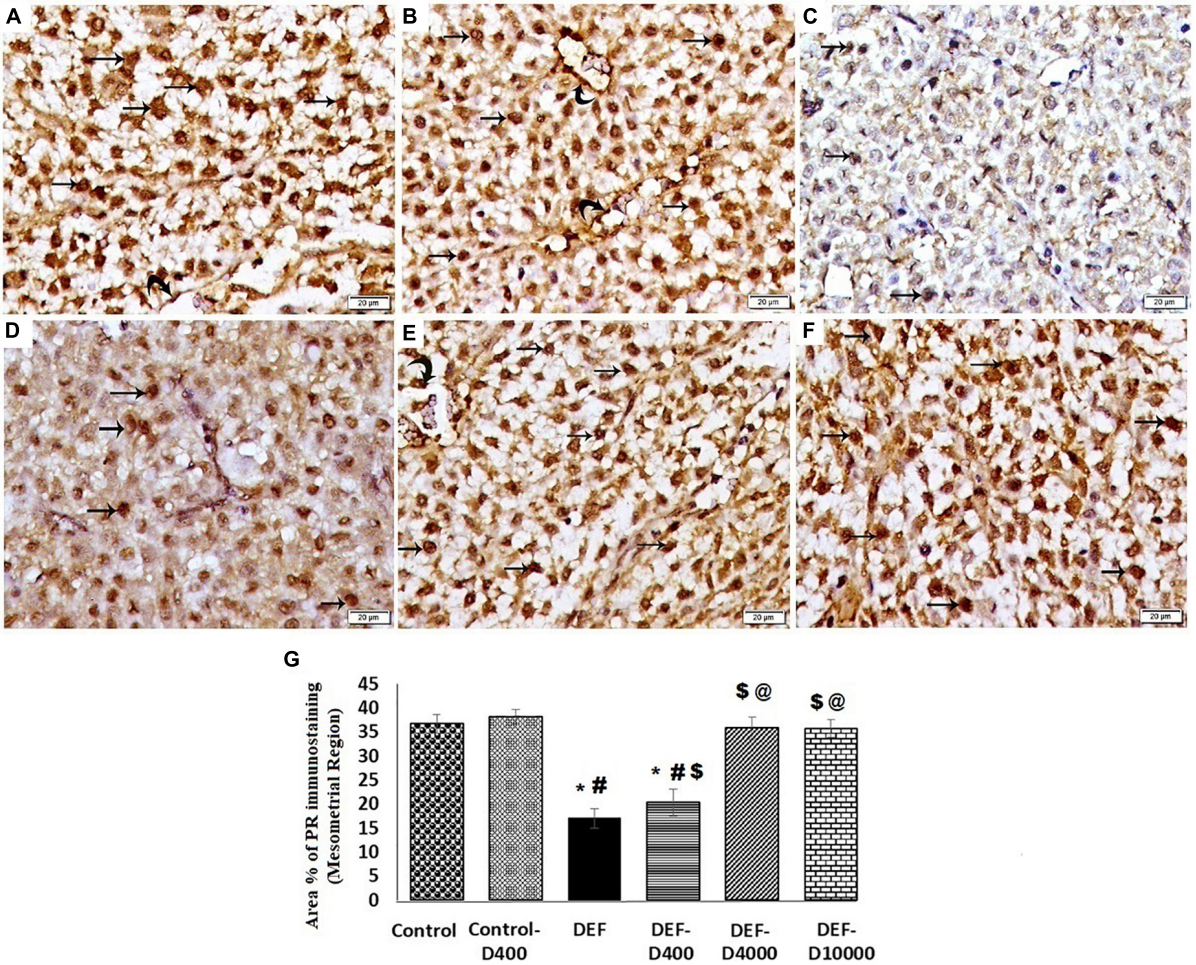


FIGURE 7 | Photomicrograph of the progesterone receptor (PGR) immunostained sections in the mesometrial region of the rat uteri at 8th day of pregnancy (×400): **(A)** Control and **(B)** Control-D400 revealing strong nuclear immunostaing of PGR in the stromal cells (arrows) and vascular endothelium (curved arrows). **(C)** DEF displaying weak nuclear immunostaining of PGR in few stromal cells. **(D)** DEF-D400 illustrating moderate nuclear immunostaining of PGR in some stromal cells. **(E)** DEF-D4000 and **(F)** DEF-D10000 demonstrating obvious strong nuclear immunostaing of PGR in the stromal cells (arrows) and vascular endothelium (curved arrows). Scale bar 20 μm. **(G)** Quantification of the mean area % of PGR positive immunostaining in the mesometrial region of the rat uteri at 8th day of pregnancy. D: refers to vitamin D supplementation in different doses equivalent to 400, 4,000 and 10,000 IU. DEF: refers to vitamin D deficiency *: Statistically significant sign compared to the control group, #: compared to control-400, $: compared to the deficiency group, and @: compared to DEF-400 ($P < 0.05$).

PGRs dimerize and translocate to the nucleus, then binds to stocktickerDNA, thus, it could regulate the expression of different target genes (Wetendorf and DeMayo, 2012).

It is worth mentioning that calcitriol stimulated progesterone secretion from cultured human syncytiotrophoblasts (Barrera et al., 2007) and cumulus granulosa cells by enhancing 3β-hydroxysteroid dehydrogenase mRNA expression levels. Thereby, it could increase progesterone release (Merhi et al., 2014). In Hong et al. (2016), the authors documented decreased progesterone production from porcine ovarian granulosa cells. However, these cells were extracted from immature pigs. The relation between circulating VD levels and the outcome of pregnancy was assessed in several studies; some of them demonstrated a positive correlation between VD levels and the continuation of pregnancy. Moreover, it was reported that VD could enhance granulosa cell luteinization and increase progesterone production providing better endometrium and helping decidualization (Irani and Merhi, 2014). Herein, the current results demonstrated enhanced progesterone production *in vivo* with VD supplementation dose dependently. Therefore, VD functions on luteinization potentiation and helps decidualization. Our results showed an increased area% of PR immunostaining dose dependently in VD supplemented groups. These results were in accordance with the Hosseinirad et al.'s (2020) study, where the authors reported that VD significantly increased the expression of PGRs mRNA, protein level, and their phosphorylation in cultured endometrial stromal cells (Hosseinirad et al., 2020). FKBP52 regulates decidualization *via* promoting PGR transcriptional activity. It has been revealed that FKBP52 modulates PGR activity upon a complex formation

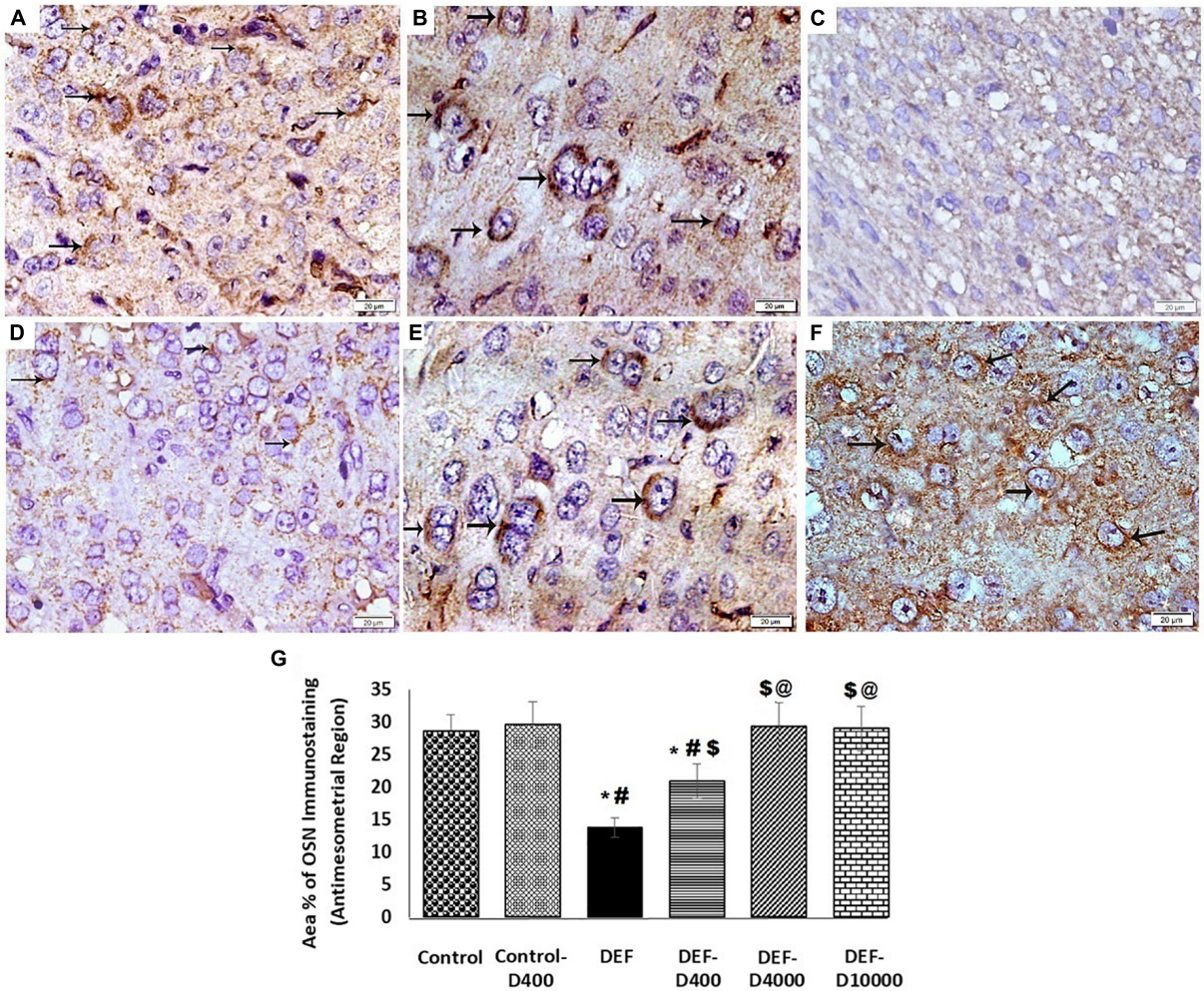


FIGURE 8 | Photomicrograph of osteopontin (OSN) immunostained sections of the antimesometrial region of the rat uteri at 8th day of pregnancy (×400): **(A)** Control and **(B)** Control-D400 revealing strong cytoplasmic immunostaing of OSN in many decidual cells (arrows). **(C)** DEF displaying weak cytoplasmic immunostaining of OSN in few cells. **(D)** DEF-D400 illustrating moderate cytoplasmic immunostaining of OSN in some cells. **(E)** DEF-D4000 and **(F)** DEF-D10000 demonstrating strong cytoplasmic immunostaing of OSN in many decidual cells (arrows). Scale bar 20 μm. **(G)** Quantification of the mean area % of OSN positive immunostaining in the antimesometrial region of the rat uteri at 8th day of pregnancy. D: refers to vitamin D supplementation in different doses equivalent to 400, 4,000 and 10,000 IU. DEF: refers to vitamin D deficiency *: Statistically significant sign compared to the control group, #: compared to control-400, $: compared to the deficiency group, and @: compared to DEF-400 ($P < 0.05$).

following its binding to heat shock protein 90 (Hsp90) together with PGR (Zgajnar et al., 2019).

In FKBP52 knockout female rats, the lowered degree of decidualization was associated with a diminished progesterone response (Yang et al., 2012). Furthermore, Tranguch et al. (2007) studied the uterine changes in FKBP52 null mice prior to implantation, and found that the failure of the embryo to implant was the cause of sterility, even with normal ovulation, and was related to progesterone hormone resistance (Hirota et al., 2010). Thus, FKBP52 governs the uterine functions of PGR (Davies et al., 2005).

Many researches support the theory that the VD function in fertility is mainly implicated in an initial process of implantation (Paffoni et al., 2019). VD can modulate the expression of genes important for embryo implantation and could even interact with the local cytokines in human endometrial cells (Cermisoni et al., 2018). Abedi et al. (2019) also reported improved endometrial thickness in women subjected to a randomized trial of VD intake.

In a study conducted on human myometrial cells infected with lentivirus to stably express HOXA-10, enhanced HOXA-10 strongly suppressed the expression of contraction-associated proteins, such as connexin 43 (Cx43) and cyclooxygenase 2 (Cox2). Cx43 is responsible for cell-to-cell connections in myometrial tissues and is required for the development of synchronized myometrial contractions, whereas Cox2 catalyzes the formation of prostaglandins that could stimulate myometrial contractility following the binding to its receptors (Ramathal et al., 2010). At present, the decreased VD levels in the DEF group was associated with defective decidualization indicated by the decreased area% of OSN and a significant decrease

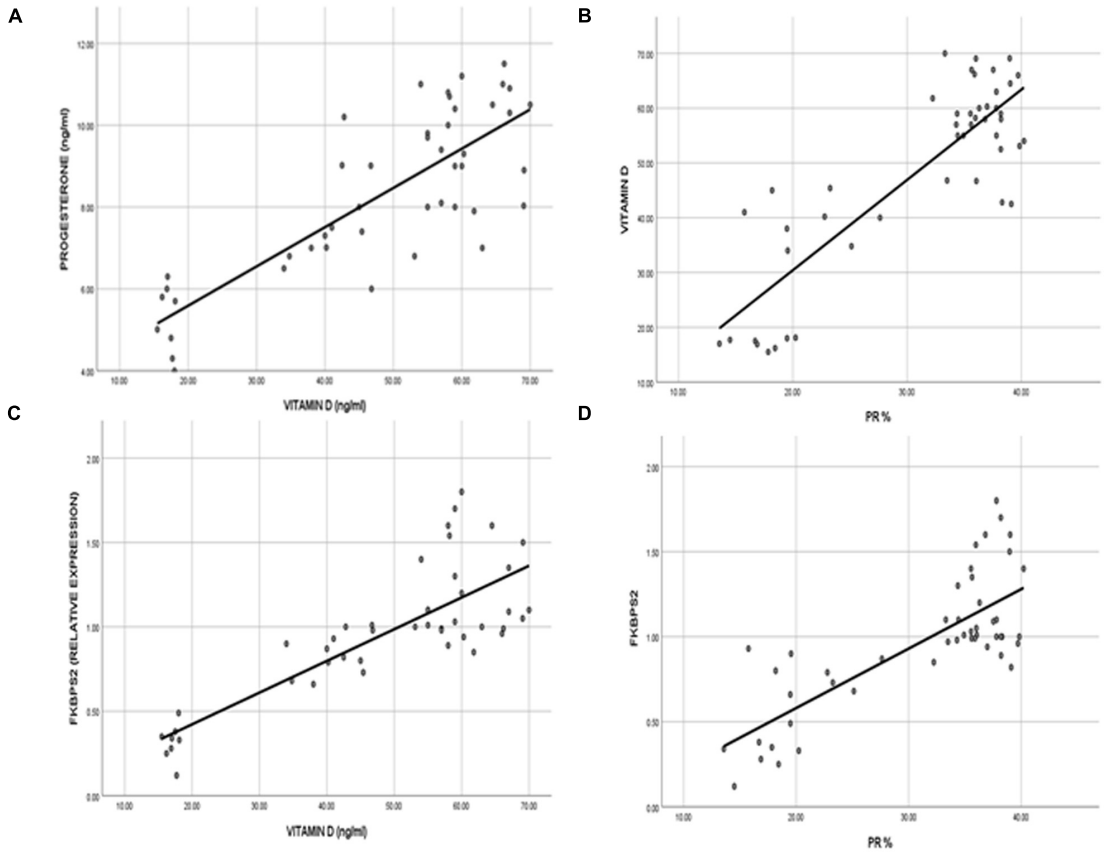


FIGURE 9 | The correlation represented the direct relationship between VD levels and **(A)** the serum progesterone level and **(B)** the uterine progesterone receptor expression. VD revealed a direct correlation to the **(C)** uterine immunophilin FK506-binding protein 52 (FKBP52), which was correlated significantly to **(D)** the progesterone receptor expression in the uterine tissues.

in the mean area% of PGR; indicating the role of VD in uterine priming. All the results were reflected on a decreased number of implanted beads and FKBP52 expression levels in the DEF group as compared to the normal control rats. OSN is a member of extracellular matrix protein family implicated in cell adhesion and invasion during the implantation process (Liu et al., 2013). OSN has been shown to be expressed and upregulated 8- to 12-fold between the early and mid-secretory phase of the menstrual cycle, which is consistent with the decidualization time suggesting to be served as a marker of receptivity (Wang et al., 2018).

It was revealed that the expression and colocalization of both FKBP52 and PGR on days 4 and 5 of implantation suggesting the role of FKBP52 in decidualization as well as in modulating PGR activity during implantation (Tranguch et al., 2007).

HOXA-10 regulates the uterine levels of FKBP52, and it appears in stromal cells on gestational day 3.5 under the controlling effects of both estrogen and progesterone (Ramathal et al., 2010). HOXA-10 persists through day 4.5 when decidua starts to develop on gestational day 4.5 at the site of embryo attachment, then proliferate and differentiate extensively under controlling mechanisms of progesterone (Lim et al., 1999; Ramathal et al., 2010). The expression of HOXA-10 was found to be regulated by 1, 25(OH) D_3 in human stromal cells. VDR and HOXA-10 protein expressions were substantially elevated in pregnant women cells compared to non-pregnant women; and VDR protein levels were positively correlated with HOXA-10 levels (Guo et al., 2020). In addition, it was documented that the intake of active VD in a dose-dependent way induced the upregulation of HOXA-10 gene expression in *in vitro* study conducted on a human endometrial stromal cell line (Du et al., 2005). HOXA10 has emerged to be a key player in both uterine development and its optimal functioning in adulthood (Ashary et al., 2020). Our results support the hypothesis that the VD-VDR system performs a role in the expression of HOXA-10. In this study, the upregulated expressional response of HOXA-10 and an improvement of the endometrial decidualization were observed to follow the intake of VD in a dose-dependent manner. An obvious successful decidualization was noted in DEF-D4000 and DEF-D10000 groups.

FKBP52 is the downstream of HOXA-10, thus the overexpressed HOXA-10 enhances FKBP52 mRNA and its protein levels (Yang et al., 2012), and eventually leading to the maintenance of PGR competency and improving its transcriptional activity (Large and DeMayo, 2012).

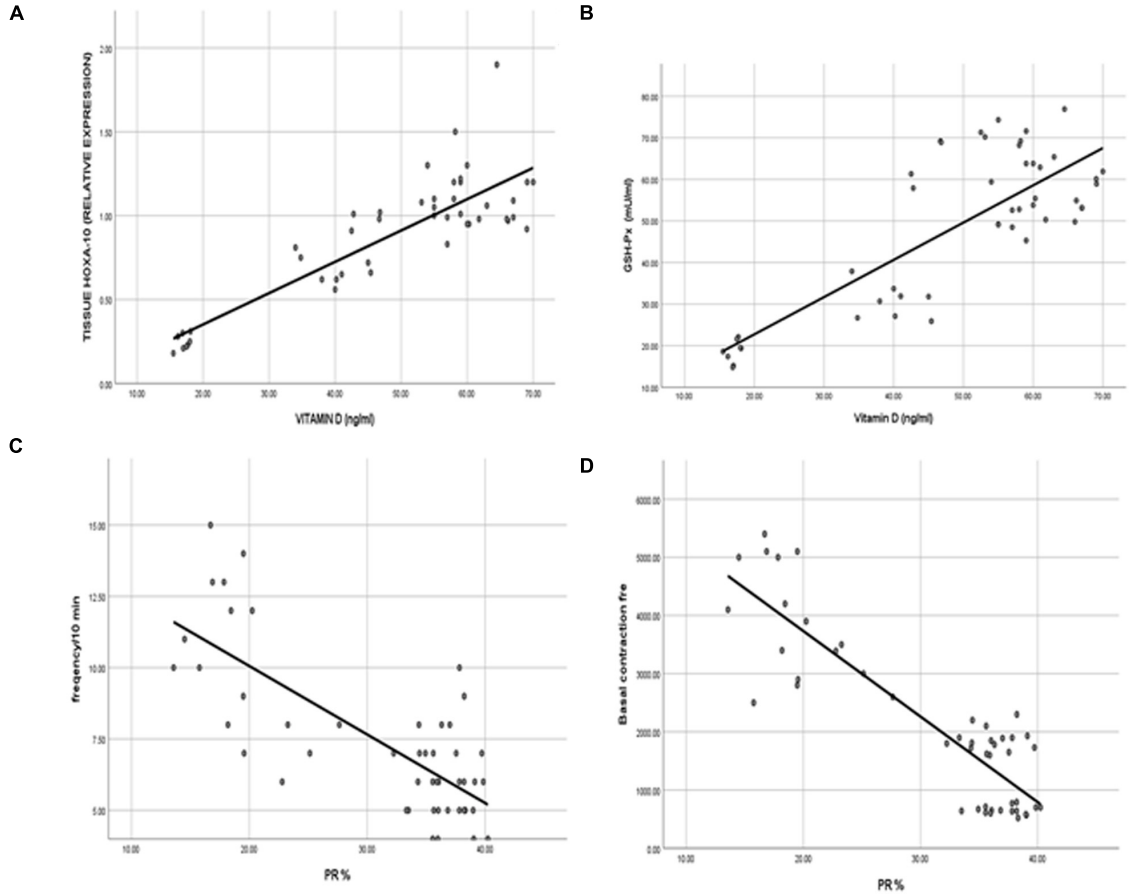


FIGURE 10 | Representation of strong positive correlations between the VD levels and **(A)** HOXA-10 and **(B)** GSH-Px. Interestingly, the uterine contractility represented by **(C)** the contraction wave amplitude and **(D)** the frequency of contractions was negatively correlated to VD status.

The present work showed that a progressive increase in HOXA-10/FKBP52 expression levels was reflected on the PGR area% and supported uterine receptivity in all the DEF groups treated with 400, 4,000, and 10,000 IU/day. However, this increase was not significant in DEF-10000 compared to the DEF-4000 group.

The potential scavenger capacity of VD was observed in the current results and evident by an increased tissue level of GSH-px in all the supplemented groups. Increased serum level of GSH-px emphasizes the major beneficial antioxidant mechanisms of VD that could support invading trophoblasts (Xu et al., 2012; Alwarfaly et al., 2014).

The oxidative stress has a negative relationship to the uterine receptivity. Hence, proper antioxidant system is essential for a successful decidualization. Decidualization is a response of maternal cells to progesterone. Human studies confirmed the role of the oxidative state and the pregnancy outcome. Under physiologic condition, progesterone enhances the activity of superoxide dismutase (SOD) in the human endometrium in early pregnancy, which in turn suppresses the production of reactive oxygen species and prostaglandin (Sugino et al., 1996). Successful implantation was correlated to a higher endometrial antioxidant; SOD, catalase (CAT) activities and the total antioxidant power (TAP), and lower lipid peroxidation (LPO) and the assessed total thiol groups (TTG) (Rahiminejad et al., 2016). Glutathione is one of the cornerstones in the intracellular ant*ioxidant system. It plays a key role in H_2O_2 metabolism into water and oxygen* (Rizk et al., 2013).

The regulation of oxidative stress is characterized by FKBP52. Uterine levels of the antioxidant peroxiredoxin-6 (PRDX6) were significantly diminished in the FKBP52-deficient mice leading to the implantation failure even with proper progesterone supplementation. FKBP52-deficient mice promotes H_2O_2-induced cell death (Hirota et al., 2010). Furthermore, induced oxidative stress downregulates HOXA-10 expression in the FKBP52-deficient mice. HOXA-10 is a crucial regulator of implantation. It mediates stromal cell proliferation and local immunosuppression (Yao et al., 2003). Thus, the low level of VD during pregnancy may enhance oxidative stress and decreases progesterone level and HOXA-10 expression (Tarcin et al., 2009). Progesterone signaling maintains uterine quiescence by suppressing the myometrial response to prostaglandin- and oxytocin-mediated inflammatory and contractile activities. Combing together, either decreasing progesterone levels or the

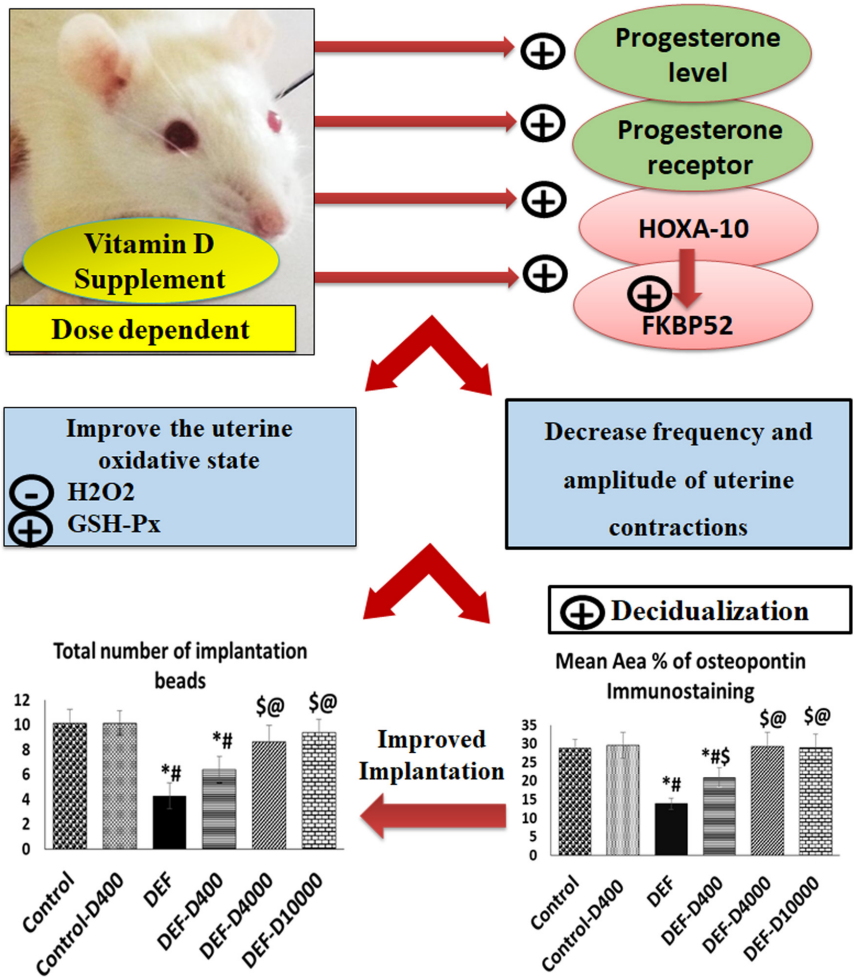


FIGURE 11 | A schematic representation demonstrating the role of VD level in early pregnancy and implantation. VD-deficient rat uteri revealed a small number of uterine implantation sites (beads). By VD supplementation and in a dose-dependent effect, enhanced progesterone secretion, uterine progesterone receptor, and its co-chaperone FKBP52 expression associated with HOXA-10 upregulation were obtained. This causes improving the oxidative stress markers; GSH-px and the H_2O_2 levels and favors decasualization and controlled uterine contractions, thus improved uterine receptivity and promoted the implantation process. The role of VD on increased receptivity was mediated through the modulation of the immunophilin FKBP52, HOXA-10.

alteration of PGR signaling may evoke uterine contractions, thus allowing the myometrium to adopt contractility (Wu and DeMayo, 2017). The results of this work revealed an increased frequency of the uterine contractility in the DEF group compared to normal one. In addition, VD supplementation could decrease the uterine contractility in a dose-dependent way. Reduced uterine peristalsis could enhance embryo implantation (Bellver and Simón, 2018). The highest prevalence of spontaneous preterm birth was detected among winter conceptions than in spring. In this context, Peng (2016) reported that VD supplementation could decrease the risk of spontaneous preterm birth by maintaining myometrial quiescence. In an *in vitro* study of human myometrial cells cocultured with monocytes, the incubation with VD decreased the expression of contractile-associated factors (Thota et al., 2014).

In conclusion, VD deficiency during the critical period of decidualization could decrease uterine receptivity and contribute to a decreased PGR level that was reflected on endometrial priming. Therefore, as demonstrated in **Figure 11**, an improved VD status through supplementation in the early prenatal period enhanced the expression level of HOXA-10/FKBP52, elevated immunostaining of PGR, increased glutathione activity, enhanced decidualization, and reduced the uterine contractility, and consequently improving the implantation process. As the biology of rats is consistent with that of human, VD supplementation in case of deficiency is a potential strategy for successful implantation.

Limitations of This Study and Recommendations

Future experiments are recommended to determine the underlying mechanisms of VD-induced myometrial relaxation

and ovarian progesterone secretion. An investigation of the endometrial molecular signals, which mediate the action of VD on the PGR expression and its co-chaperone, is needed. We recommend future experiments using the knockout models of HOXA-10/FKBP52 to determine decisively whether the role of VD in the regulation of PR receptors is mediated *via* the HOXA-10/FKBP52 axis or possibly the direct genomic action has an effect. Future researches are needed to employ the selected dose of VD for implementation as an efficient therapeutic strategy in human studies.

DATA AVAILABILITY STATEMENT

Data used and/or analyzed during the current study are available from the corresponding author and also found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, XM_008762949.1; https://www.ncbi.nlm.nih.gov/, 1; and https://www.ncbi.nlm.nih.gov/genbank/, NM_031144.3.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC) (approval NO. CU/III/F/70/17).

AUTHOR CONTRIBUTIONS

AS and HAs: conceptualization. LR, RH and SK: methodology. LR: validation. LR, RH, HAt, MM, NS, and SK: investigation. HAs, NS, SG, and AS: data processing. AS, HAs, and SK: writing original draft preparation. SG, MM, and AS: writing, review, and editing. HAs: supervision. AS: project administration. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by Kasralainy Faculty of Medicine, Cairo University Laboratories. The authors express their gratitude to King Khalid University for providing funding through the General Research Project Grant (# RGP.1/312/42).

ACKNOWLEDGMENTS

All authors offer sincere appreciation for the Experimental Animal Unit, RIO for providing rats.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://doi.org/10.6084/m9.figshare.16818805.v1

REFERENCES

Abedi, S., Taebi, M., and Esfahani, M. H. N. (2019). Effect of vitamin D supplementation on intracytoplasmic sperm injection outcomes: a randomized double-blind placebo-controlled trial. *Int. J. Fertil. Steril.* 13, 18–23. doi: 10.22074/ijfs.2019.5470

Alaee, S., Novin, M. G., and Yeganeh, F. (2014). FKBP51 and FKBP52 as potential biomarkers for predicting endometrial receptivity and embryo implantation in assisted reproductive technologies. 2, 77–81.

Aley, P. K., Noh, H. J., Gao, X., Tica, A. A., Brailoiu, E., and Churchill, G. C. (2010). A functional role for nicotinic acid adenine dinucleotide phosphate in oxytocin-mediated contraction of uterine smooth muscle from rat. *J. Pharmacol. Exp. Ther.* 333, 726–735. doi: 10.1124/jpet.110.165837

Alwarfaly, S., Abdulsid, A., Hanretty, K., and Lyall, F. (2014). Paraoxonase 2 Protein Is Spatially Expressed in the Human Placenta and Selectively Reduced in Labour. *PLoS One* 9:e96754. doi: 10.1371/journal.pone.0096754

Ashary, N., Laheri, S., and Modi, D. (2020). Homeobox genes in endometrium: from development to decidualization. *Int. J. Dev. Biol.* 64, 227–237.

Barrera, D., Avila, E., Hernández, G., Halhali, A., Biruete, B., Larrea, F., et al. (2007). Estradiol and progesterone synthesis in human placenta is stimulated by calcitriol. *J. Steroid Biochem. Mol. Biol.* 103, 529–532. doi: 10.1016/j.jsbmb.2006.12.097

Bellver, J., and Simón, C. (2018). Implantation failure of endometrial origin: what is new? *Curr. Opin. Obstet. Gynecol.* 30, 229–236. doi: 10.1097/GCO.0000000000000468

Cermisoni, G., Alteri, A., Corti, L., Rabellotti, E., Papaleo, E., Viganò, P., et al. (2018). Vitamin D and endometrium: a systematic review of a neglected area of research. *Int. J. Mol. Sci.* 19:2320. doi: 10.3390/ijms19082320

Chen, H.-Y., Li, O.-Y., Pang, L.-H., Xu, H., Fan, X.-J., Liang, H.-F., et al. (2015). Expression of FK506-binding protein 52 (FKBP52) in chorionic villi with early recurrent spontaneous abortion. *J. Matern. Fetal Neonatal. Med.* 28, 1165–1169. doi: 10.3109/14767058.2014.947572

Cheng, F., Li, T., Wang, Q. L., Zhou, H. L., Duan, L., and Cai, X. (2015). Effects of hydrosalpinx on ultrasonographic parameters for endometrial receptivity during the window of implantation measured by power color doppler ultrasound. *Int. J. Clin. Exp. Med.* 8, 6103–6108.

Daikoku, T., Tranguch, S., Friedman, D. B., Das, S. K., Smith, D. F., and Dey, S. K. (2005). Proteomic Analysis Identifies Immunophilin FK506 Binding Protein 4 (FKBP52) as a Downstream Target of Hoxa10 in the Periimplantation Mouse Uterus. *Mol. Endocrinol.* 19, 683–697.

Darios, E. S., Seitz, B., and Watts, S. W. (2012). Smooth Muscle Pharmacology in the Isolated Virgin and Pregnant Rat Uterus and Cervix. *J. Pharmacol. Exp. Ther.* 341, 587–596. doi: 10.1124/jpet.111.191031

Davies, S. C., Jewell, T., McBride, M., and Burns, H. (2012). *Vitamin D-Advice on Supplements for at Risk Groups*. Available online at: tinyurl.com/pwaqxt2

Davies, T. H., Ning, Y.-M., and Sánchez, E. R. (2005). Differential control of glucocorticoid receptor hormone-binding function by Tetratricopeptide Repeat (TPR) Proteins and the Immunosuppressive Ligand FK506 †. *Biochemistry* 44, 2030–2038. doi: 10.1021/bi048503v

Du, H., Daftary, G. S., Lalwani, S. I., and Taylor, H. S. (2005). Direct Regulation of HOXA10 by 1,25-(OH) 2 D 3 in Human Myelomonocytic Cells and Human Endometrial Stromal Cells. *Mol. Endocrinol.* 19, 2222–2233.

Farzadi, L., Khayatzadeh Bidgoli, H., Ghojazadeh, M., Bahrami, Z., Fattahi, A., Latifi, Z., et al. (2015). Correlation between follicular fluid 25-OH vitamin D and assisted reproductive outcomes. *Iran. J. Reprod. Med.* 13, 361–366.

Fernando, M., Coster, T. G., Ellery, S. J., de Guingand, D., Lim, S., Harrison, C. L., et al. (2020). Relationships between total, free and bioavailable vitamin D and Vitamin D binding protein in early pregnancy with neonatal outcomes: a retrospective cohort study. *Nutrients* 12:2495. doi: 10.3390/nu12092495

Ganguly, A., Tamblyn, J. A., Finn-Sell, S., Chan, S.-Y., Westwood, M., Gupta, J., et al. (2018). Vitamin D, the placenta and early pregnancy: effects on trophoblast function. *J. Endocrinol.* 236, R93–R103.

Guo, J., Liu, S., Wang, P., Ren, H., and Li, Y. (2020). Characterization of VDR and CYP27B1 expression in the endometrium during the menstrual cycle

before embryo transfer: implications for endometrial receptivity. *Reprod. Biol. Endocrinol.* 18:24. doi: 10.1186/s12958-020-00579-y

Harms, L., Eyles, D., Mcgrath, J., Mackaysim, A., and Burne, T. (2008). Developmental vitamin D deficiency alters adult behaviour in 129/SvJ and C57BL/6J mice. *Behav. Brain Res.* 187, 343–350. doi: 10.1016/j.bbr.2007.09.032

Heaney, R. P., Davies, K. M., Chen, T. C., Holick, M. F., and Barger-Lux, M. J. (2003). Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. *Am. J. Clin. Nutr.* 77, 204–210. doi: 10.1093/ajcn/77.1.204

Hewison, M. (2018). The earlier the better: preconception vitamin D and protection against pregnancy loss. *Lancet Diabetes Endocrinol.* 6, 680–681.

Hirota, Y., Acar, N., Tranguch, S., Burnum, K. E., Xie, H., Kodama, A., et al. (2010). Uterine FK506-binding protein 52 (FKBP52)-peroxiredoxin-6 (PRDX6) signaling protects pregnancy from overt oxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15577–15582. doi: 10.1073/pnas.10093 24107

Hollis, B. W., Johnson, D., Hulsey, T. C., Ebeling, M., and Wagner, C. L. (2011). Vitamin D supplementation during pregnancy: and effectiveness. *J. Bone Miner Res.* 26, 2341–2357. doi: 10.1002/jbmr.498

Hong, S. H., Lee, J. E., Kim, H. S., Jung, Y. J., Hwang, D. Y., Lee, J. H., et al. (2016). Effect of vitamin D3 on production of progesterone in porcine granulosa cells by regulation of steroidogenic enzymes. *J. Biomed. Res.* 30, 203–208. doi: 10.7555/JBR.30.2016K0012

Hosseinirad, H., Novin, M. G., Hosseini, S., Nazarian, H., Amidi, F., Paktinat, S., et al. (2020). Effect of 1,25(OH)2-vitamin D3 on expression and phosphorylation of progesterone receptor in cultured endometrial stromal cells of patients with repeated implantation failure. *Acta Histochem.* 122:151489. doi: 10.1016/j.acthis.2019.151489

Hou, H., Zhang, J. Y., Chen, D., Deng, F., Morse, A. N., Qiu, X., et al. (2020). Altered decidual and placental catabolism of vitamin D may contribute to the aetiology of spontaneous miscarriage. *Placenta* 92, 1–8.

Irani, M., and Merhi, Z. (2014). Role of vitamin D in ovarian physiology and its implication in reproduction: A systematic review. *Fertil. Steril.* 102, 460–468.e3. doi: 10.1016/j.fertnstert.2014.04.046

Kollenkirchen, U., Fox, J., and Walters, M. R. (2009). Normocalcemia without hyperparathyroidism in vitamin D-deficient rats. *J. Bone Miner. Res.* 6, 273–278. doi: 10.1002/jbmr.5650060309

Large, M. J., and DeMayo, F. J. (2012). The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. *Mol. Cell. Endocrinol.* 358, 155–165. doi: 10.1016/j.mce.2011.07.027

Lim, H., Ma, L., Ma, W., Maas, R. L., and Dey, S. K. (1999). Hoxa-10 regulates uterine stromal cell responsiveness to progesterone during implantation and decidualization in the mouse. *Mol. Endocrinol.* 13, 1005–1017. doi: 10.1210/mend.13.6.0284

Liu, N., Zhou, C., Chen, Y., and Zhao, J. (2013). The involvement of osteopontin and β3 integrin in implantation and endometrial receptivity in an early mouse pregnancy model. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 170, 171–176.

Liu, X., Zhang, W., Xu, Y., Chu, Y., Wang, X., Li, Q., et al. (2019). Effect of vitamin D status on normal fertilization rate following in vitro fertilization. *Reprod. Biol. Endocrinol.* 17:59. doi: 10.1186/s12958-019-0500-0

Merhi, Z., Doswell, A., Krebs, K., and Cipolla, M. (2014). Vitamin D Alters Genes Involved in Follicular Development and Steroidogenesis in Human Cumulus Granulosa Cells. *J. Clin. Endocrinol. Metab.* 99, E1137–E1145.

Mumford, S. L., Garbose, R. A., Kim, K., Kissell, K., Kuhr, D. L., Omosigho, U. R., et al. (2018). Association of preconception serum 25-hydroxyvitamin D concentrations with livebirth and pregnancy loss: a prospective cohort study. *Lancet Diabetes Endocrinol.* 6, 725–732.

Ochoa-Bernal, M. A., and Fazleabas, A. T. (2020). Physiologic Events of Embryo Implantation and Decidualization in Human and Non-Human Primates. *Int. J. Mol. Sci.* 21:1973. doi: 10.3390/ijms21061973

Paffoni, A., Somigliana, E., Sarais, V., Ferrari, S., Reschini, M., Makieva, S., et al. (2019). Effect of vitamin D supplementation on assisted reproduction technology (ART) outcomes and underlying biological mechanisms: protocol of a randomized clinical controlled trial. The "supplementation of vitamin D and reproductive outcome" (SUNDRO) study. *BMC Pregnancy Childbirth* 19:395. doi: 10.1186/s12884-019-2538-6

Peng, D. (2016). *World's largest Science, Technology & Medicine Open Access book publisher c. Agricultural and Biological Sciences Grain Legumes*. doi: 10.5772/711

Rahiminejad, M. E., Moaddab, A., Ganji, M., Eskandari, N., Yepez, M., Rabiee, S., et al. (2016). Oxidative stress biomarkers in endometrial secretions: a comparison between successful and unsuccessful in vitro fertilization cycles. *J. Reprod. Immunol.* 116, 70–75. doi: 10.1016/j.jri.2016.05.003

Ramathal, C., Bagchi, I., Taylor, R., and Bagchi, M. (2010). Endometrial decidualization: of mice and Men. *Semin. Reprod. Med.* 28, 017–026. doi: 10.1055/s-0029-1242989

Rizk, B., Badr, M., and Talerico, C. (2013). "Oxidative stress and the endometrium," in *Studies on Women's Health*, eds A. Agarwal (New York, NY: Springer), 61–74. doi: 10.1007/978-1-62703-041-0_3

Sharef, A. A.-R., Hussien, S. S., and Noori, F. M. (2020). Vitamin D3 deficiency and early pregnancy loss. *World Fam. Med.* 18, 76–80. doi: 10.5742/mewfm.2020.93732

Shin, J. S., Choi, M. Y., Longtine, M. S., and Nelson, M. (2011). Vitamin D Effects on Pregnancy and the Placenta. 193, 118–125. doi: 10.1016/j.jneumeth.2010.08.011.Autogenic

Song, S., and Tan, Y. (2018). Expression of FKBP52 in the ovaries of PCOS rats. *Int. J. Mol. Med.* 43, 868–878. doi: 10.3892/ijmm.2018.3998

Stavenuiter, A. W. D., Arcidiacono, M. V., Ferrantelli, E., Keuning, E. D., Vila Cuenca, M., ter Wee, P. M., et al. (2015). A novel rat model of vitamin d deficiency: safe and rapid induction of Vitamin D and Calcitriol Deficiency without Hyperparathyroidism. *Biomed Res. Int.* 2015:604275. doi: 10.1155/2015/604275

Sugino, N., Shimamura, K., Takiguchi, S., Tamura, H., Ono, M., Nakata, M., et al. (1996). Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. *Hum. Reprod.* 11, 1073–1078. doi: 10.1093/oxfordjournals.humrep.a019299

Tarcin, O., Yavuz, D. G., Ozben, B., Telli, A., Ogunc, A. V., Yuksel, M., et al. (2009). Effect of Vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. *J. Clin. Endocrinol. Metab.* 94, 4023–4030.

Thota, C., Laknaur, A., Farmer, T., Ladson, G., Al-Hendy, A., and Ismail, N. (2014). Vitamin D regulates contractile profile in human uterine myometrial cells via NF-κB pathway. *Am. J. Obstet. Gynecol.* 210, 347.e1–347.e10. doi: 10.1016/j.ajog.2013.11.027

Tranguch, S., Daikoku, T., Guo, Y., Wang, H., and Dey, S. K. (2005). Molecular complexity in establishing uterine receptivity and implantation. *Cell. Mol. Life Sci.* 62, 1964–1973.

Tranguch, S., Wang, H., Daikoku, T., Xie, H., Smith, D. F., and Dey, S. K. (2007). FKBP52 deficiency–conferred uterine progesterone resistance is genetic background and pregnancy stage specific. *J. Clin. Invest.* 117, 1824–1834. doi: 10.1172/JCI31622

Varayoud, J., Ramos, J. G., Muñoz-de-Toro, M., and Luque, E. H. (2014). Long-lasting effects of neonatal bisphenol A exposure on the implantation process. *Vitam. Horm.* 94, 253–275.

Vitiello, D., Pinard, R., and Taylor, H. S. (2008). Gene expression profiling reveals putative HOXA10 Downstream Targets in the Periimplantation Mouse Uterus. *Reprod. Sci.* 15, 529–535. doi: 10.1177/1933719108316911

Von Websky, K., Abdallah, A., Reichetzeder, C., and Tsuprykov, O. (2018). Impact of vitamin D on pregnancy-related disorders and on o ff spring outcome. *J. Steroid Biochem. Mol. Biol.* 180, 51–64. doi: 10.1016/j.jsbmb.2017.11.008

Wang, X., Qi, Q., Wu, K., and Xie, Q. (2018). Role of Osteopontin in decidualization and pregnancy success. *Reproduction* 155, 423–432.

Wetendorf, M., and DeMayo, F. J. (2012). The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. *Mol. Cell. Endocrinol.* 357, 108–118. doi: 10.1016/j.mce.2011.10.028

Wu, S.-P., and DeMayo, F. J. (2017). Progesterone receptor signaling in uterine myometrial physiology and preterm birth. *Curr. Top. Dev. Biol.* 125, 171–190. doi: 10.1016/bs.ctdb.2017.03.001

Xu, X., Liu, T., Zhang, A., Huo, X., Luo, Q., Chen, Z., et al. (2012). Reactive oxygen species-triggered trophoblast apoptosis is initiated by endoplasmic reticulum stress via activation of caspase-12, CHOP, and the JNK pathway in *Toxoplasma gondii* infection in mice. *Infect. Immun.* 80, 2121–2132. doi: 10.1128/IAI.06295-11

Yang, H., Zhou, Y., Edelshain, B., Schatz, F., and Lockwood, C. J. (2012). FKBP52 is regulated by HOXA10 during decidualizaton and in endometriosis. *Reproduction* 143, 531–538. doi: 10.2135/cropsci2013.07.0499

Yao, M. W. M., Lim, H., Schust, D. J., Choe, S. E., Farago, A., Ding, Y., et al. (2003). Gene expression profiling reveals progesterone-mediated cell cycle and immunoregulatory roles of Hoxa-10 in the Preimplantation Uterus. *Mol. Endocrinol.* 17, 610–627.

Zgajnar, N., De Leo, S., Lotufo, C., Erlejman, A., Piwien-Pilipuk, G., and Galigniana, M. (2019). Biological actions of the Hsp90-binding Immunophilins FKBP51 and FKBP52. *Biomolecules* 9:52. doi: 10.3390/biom9020052

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Citation: Ashour H, Gamal SM, Sadek NB, Rashed LA, Hussein RE, Kamar SS, Ateyya H, Mehesen MN and ShamsEldeen AM (2021) Vitamin D Supplementation Improves Uterine Receptivity in a Rat Model of Vitamin D Deficiency: A Possible Role of HOXA-10/FKBP52 Axis. Front. Physiol. 12:744548. doi: 10.3389/fphys.2021.744548

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 26 November 2021
doi: 10.3389/fendo.2021.746329

Postpartum Thyroid Dysfunction in Women With Known and Newly Diagnosed Hypothyroidism in Early Pregnancy

*Xiaotong Gao, Xichang Wang, Yutong Han, Haoyu Wang, Jiashu Li, Yuanyuan Hou, Yang Yang, Huiru Wang, Weiping Teng and Zhongyan Shan**

Department of Endocrinology and Metabolism, Institute of Endocrinology, National Health Commission (NHC) Key laboratory of Diagnosis and Treatment of Thyroid Diseases, The First Affiliated Hospital of China Medical University, Shenyang, China

OPEN ACCESS

Edited by:
Qiongjie Zhou,
Fudan University, China

Reviewed by:
Salvatore Benvenga,
University of Messina, Italy
Giovanna Motta,
University Hospital of the City of Health and Science of Turin, Italy
Dongfang Liu,
Chongqing Medical University, China

***Correspondence:**
Zhongyan Shan
cmushanzhongyan@163.com

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *23 July 2021*
Accepted: *08 November 2021*
Published: *26 November 2021*

Citation:
Gao X, Wang X, Han Y, Wang H, Li J, Hou Y, Yang Y, Wang H, Teng W and Shan Z (2021) Postpartum Thyroid Dysfunction in Women With Known and Newly Diagnosed Hypothyroidism in Early Pregnancy. Front. Endocrinol. 12:746329. doi: 10.3389/fendo.2021.746329

Background: Hypothyroidism in the first trimester of pregnancy (T1) has great adverse effects on mothers and foetuses. However, few studies have investigated the influence on postpartum thyroid dysfunction. This study aimed to evaluate their long-term effect on postpartum thyroid function within one year after delivery.

Methods: In total, 151 women were recruited from 1496 participants and were classified as newly diagnosed subclinical hypothyroidism (SCH) in T1 (ND-SCH, n=50), previously known SCH before pregnancy (PK-SCH, n=51) and previously known overt hypothyroidism (PK-OH, n=50). Their thyroid functions were dynamically monitored from pre-conception to one-year postpartum.

Results: During pregnancy, the first thyroid functions' test time in T1 were 5-8 gestational weeks. After delivery, the prevalence of postpartum thyroiditis (PPT) was comparable in women with previously known and newly diagnosed hypothyroidism [ND-SCH 62.0% *vs* PK-SCH 64.7% *vs* PK-OH 64.0%, P=0.96]. For the ND-SCH group, PPT was significantly related with thyroid-stimulating hormone (TSH) >4.0 mU/L occurring at <8 gestational weeks [OR=8.06, 95% CI, 2.08-31.29] and TSH levels outside 1.0-2.5 mU/L near childbirth [OR=3.73, 95% CI, 1.04-13.41]. For patients with known hypothyroidism before pregnancy (PK-SCH and PK-OH), TSH>2.5 mU/L in T1 [OR=3.55, 95% CI, 1.43-8.81] and TPOAb≥300 μIU/mL [OR=6.58, 95% CI, 2.05-21.12] were associated with PPT. Regardless of whether SCH was diagnosed before pregnancy or in T1, the levothyroxine (LT4) treatment was discontinued at delivery. More than 50% of the patients had to face the hypothyroidism phase of postpartum and restarted LT4 treatment in the first-year follow-up. The logistic regression analysis revealed that TSH elevation occurring at <8 gestational weeks [OR=2.48, 95% CI, 1.09-5.6], TSH levels outside 1.0-2.5 mU/L near childbirth [OR=3.42, 95% CI, 1.45-8.05], and TPOAb≥300 μIU/mL [OR=6.59, 95% CI, 1.79-24.30] were the risk factors.

Conclusion: TSH elevation at <8 gestational weeks was associated with PPT after delivery in women with known and newly diagnosed hypothyroidism. Especially for SCH patients who stopped LT4 treatment at delivery, unsatisfactory TSH level at <8 gestational weeks and near childbirth, TPOAb≥300 μIU/mL were the risk factors for LT4 retreatment in one-year postpartum.

Keywords: hypothyroidism, early pregnancy, postpartum thyroiditis, thyroid-stimulating hormone, levothyroxine

INTRODUCTION

Hypothyroidism in pregnancy, even subclinical hypothyroidism (SCH), is associated with an increased risk of adverse pregnancy outcomes (1–4). The maternal requirement for thyroid hormones is enhanced during pregnancy (5–7), and begins at 4-6 gestational weeks (8). Endogenous thyroid hormone variation is affected by residual thyroid tissue and hormone synthesis capacity, human chorionic gonadotropin (hCG), thyroxine-binding globulin, thyroid peroxidase antibody (TPOAb)/thyroglobulin antibody (TgAb), iodine nutrition and other factors (9–12). However, in the condition of hypothyroidism, the sensitivity of thyroid function to hCG stimulation is reduced. Therefore, supplementation with exogenous thyroid hormones to meet maternal and foetal needs is critical for pregnancy, especially during early pregnancy.

According to the 2017 American Thyroid Association (ATA) guidelines, 4.0 mU/L of thyroid-stimulating hormone (TSH) could be regarded as the upper limit cut-off point for the diagnosis of SCH during pregnancy (13). Among all hypothyroidism patients, TSH<2.5 mU/L should be regarded as the control target. Since pregnancy can lead to an increased demand for levothyroxine (LT4), after delivery, pre-pregnancy LT4 isodose should be restored in patients with overt hypothyroidism (OH) diagnosed before pregnancy. Patients with SCH newly diagnosed during pregnancy are candidates for discontinuing LT4. The TSH levels should be evaluated at 6 weeks postpartum (13).

Postpartum thyroiditis (PPT) is an irregular thyroid condition that occurs within one year after delivery (14). The prevalence of PPT in the general population is 1-22% (15). Among women with hypothyroidism, especially those with autoimmune thyroiditis (AIT), the prevalence reached to 62.1-68.0% (16–18). However, few studies have reported the prevalence of PPT among women with SCH. In one study, 24.6% of patients with SCH diagnosed at 28 gestational weeks showed a sustained TSH elevation at 4.9 years postpartum (19). In another study, SCH was diagnosed at 22 gestational weeks, and 38.9% of women still maintained hypothyroidism at 7-19 months (20).

However, few studies focused on the postpartum prognosis of thyroid function when hypothyroidism occurs during early pregnancy. The aim of our study was to investigate the outcome of thyroid dysfunction within one year after delivery in women with thyroid dysfunction detected in the first trimester of gestation (both as newly diagnosed and previously known hypothyroidism).

MATERIALS AND METHODS

Participants and Study Design

The investigators performed a chart review of 1496 pregnant women in the First Affiliated Hospital of China Medical University during a ten-year period (2010-2020), and the study was approved by the hospital ethics committee. All candidates signed informed consent forms and completed the questionnaire survey. They had been tested thyroid function routinely before pregnancy and had been treated once they were diagnosed with hypothyroidism. On the basis of their pre-pregnancy thyroid function, 409 women were diagnosed with hypothyroidism prior to conception from 2010 to 2020, and 108 women were consistently euthyroid pre-pregnancy but developed SCH in early pregnancy from 2017 to 2020, which was defined as TSH>4.0 mU/L with normal FT4 levels according to the 2017 ATA guidelines (13).

For the purposes of our study, we only included women who strictly met the following inclusion criteria: ① serum TSH, free triiodothyronine (FT3) and free thyroxine (FT4) monitored within 6 months before pregnancy (BP); in early (T1: 0 to 13^{+6} weeks), middle (T2: 14 to 27^{+6} weeks) and late (T3: 28 to 40 weeks) pregnancy; and in the first (A1: 6 weeks to 3 months), second (A2: 3 to 6 months) and third (A3: 6 months to 1 year) stages after delivery; ② serum TPOAb and TgAb monitored in T1; ③ complete and detailed clinical data covered by the questionnaire, i.e., age at the current pregnancy; pre-pregnancy body mass index (BMI); gestational weight gain (GWG, weight at delivery minus pre-pregnancy weight) (21); family history of thyroid disease; delivery history; abortion history; and LT4 replacement before, during and after pregnancy; ④ no history of diseases or medications affecting thyroid function (except for LT4 and progesterone); and ⑤ living in an iodine-sufficient area.

Based on the above criteria, in total, 151 women were recruited (**Figure 1**). According to the diagnosis time, disease degree and aetiology of hypothyroidism, the patients were further classified as follows: SCH newly diagnosed in the first trimester of pregnancy (ND-SCH, n=50); Previously known autoimmune SCH (PK-SCH, n=51); and previously known autoimmune overt hypothyroidism(PK-OH, n=50). All women with previously known hypothyroidism received adequate LT4 replacement within 6 months before pregnancy. The pregnant women in the ND-SCH group started LT4 intervention once they were diagnosed (22). The woman's thyroid function was evaluated within 8 gestational weeks, followed by monitoring every 4-6 weeks to adjust the LT4 dose. The serum TSH target

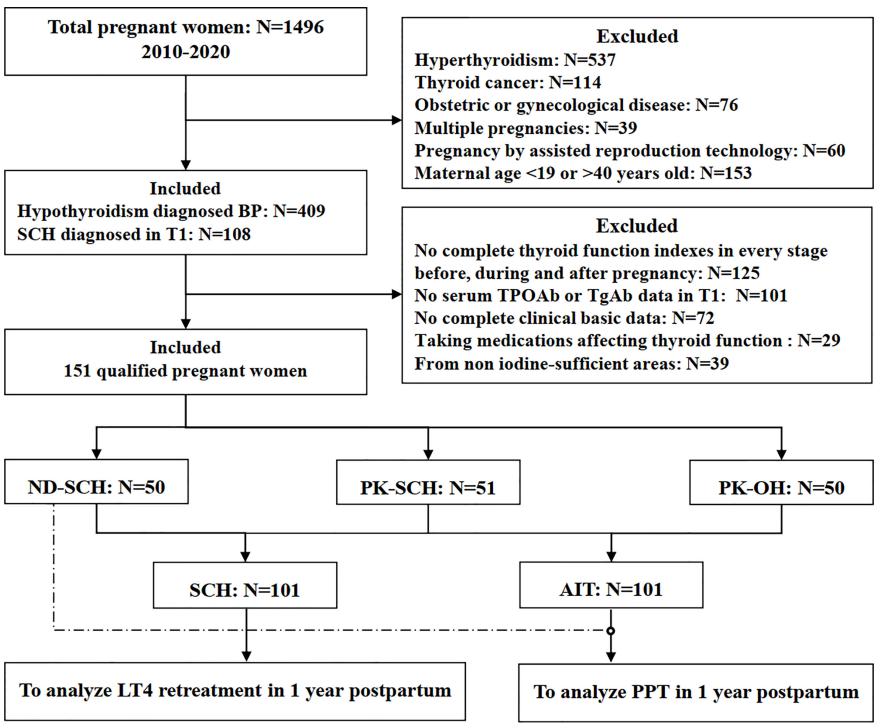


FIGURE 1 | Flow chart of the study population. BP, before pregnancy; T1, early pregnancy; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; SCH, subclinical hypothyroidism; ND-SCH, subclinical hypothyroidism newly diagnosed in early pregnancy; PK-SCH, previously known subclinical hypothyroidism before pregnancy; PK-OH, previously known autoimmune overt hypothyroidism; AIT, autoimmune hypothyroidism diagnosed before pregnancy; PPT, postpartum thyroiditis.

was maintained between the lower limit of the pregnancy-specific reference range and 2.5 mU/L throughout pregnancy.

The pregnant women with previously known and newly diagnosed SCH discontinued LT4 treatment after delivery. The pregnant women with PK-OH continued LT4 treatment after delivery, and the LT4 dose was adjusted according to the dose before delivery.

Definition of Postpartum Thyroiditis

Among the SCH patients, PPT was diagnosed based on the occurrence of any of the following three possibilities within the first year after delivery ① transient thyrotoxicosis (TSH<0.27 mU/L, which is the lower limit of reference range in nonpregnant women) accompanied by transient hypothyroidism (TSH>4.20 mU/L, which is the upper limit of reference range in nonpregnant women) (biphasic PPT); ② only transient thyrotoxicosis; or ③ only transient hypothyroidism. Among the PK-OH patients, 'only transient thyrotoxicosis' and 'only transient hypothyroidism' were probably due to a surplus or deficit in the LT4 intervention, respectively. Therefore, PPT was diagnosed only in the following two cases: ① when the optimal postpartum LT4 dose was chosen, there was an occurrence of hypothyroidism conversed by transient thyrotoxicosis in the first postpartum year; or ② under the appropriated LT4 treatment after delivery, although the thyroid function was normal at 6 postpartum weeks, there were a remarkable thyroid dysfunction in the next 3-12 postpartum months.

Measurement of Thyroid Function and Thyroid Autoimmunity

Thyroid function indicators were measured by electrochemiluminescence immunoassay system assay (Cobas; Roche Diagnostics, Basel, Switzerland). The reference ranges for nonpregnant women are as follows: FT3 3.1-6.8 pmol/L; FT4 9.0-22.0 pmol/L; TSH 0.27-4.20 mU/L; TPOAb<34 μIU/mL; and TgAb<115 μIU/mL. There were no changes in testing methodology during our study period. The gestational trimester-specific reference intervals tested in our laboratory were applied in the whole period of gestation (23). The intra- and interassay variation coefficients of the above variations were less than 10%. In our study, TPOAb≥300 μIU/mL indicates TPOAb positivity, and TgAb≥300 μIU/mL indicates TgAb positivity (24).

Statistical Analysis

SPSS statistics for Windows v20.0 (SPSS, Inc., Chicago, IL) was used for the statistical analysis. Continuous variables are described as the mean ± standard deviation (M ± SD) or median and quartile difference (IQR), and categorical variables are described as numbers and corresponding percentages.

For continuous variables, one-way ANOVA or the Kruskal-Wallis test was utilized to compare the baseline characteristics of multiple groups, and *post hoc* Bonferroni correction was performed. For categorical variables, the chi-square test, Pearson's chi-square test or Fisher's exact test was used to compare frequencies.

A logistic regression model was used to estimate the prevalence of PPT and the prevalence of postpartum LT4 retreatment, to analyse the correlation with multiple variables and confounding factors. Maternal age and family history of thyroid disease were controlled as covariates (25). The results were presented as odds ratios (ORs), 95% confidence intervals (95%CIs) and significance. P<0.05 was considered statistically significant.

RESULTS

Clinical Characteristics of Pregnant Women With Different Types of Hypothyroidism

The clinical characteristics of the ND-SCH, PK-SCH, and PK-OH groups are shown in **Table 1**. There were no significant differences in most baseline clinical data, namely, maternal age, pre-pregnancy BMI, the proportion of GWG>15 kg (21), family history of thyroid disease, abortion history among the three groups.

Among the women with ND-SCH, the gestational age at SCH diagnosis was 6.6 (5.0-12.0) weeks, and the initial TSH level was 5.56 (4.39-7.12) mU/L. Women with previously known

TABLE 1 | Descriptive statistics of women grouped according to thyroid function (n = 151).

	ND-SCH	*PK-SCH*	*PK-OH*	*P*
• *Clinical basic data*				
N	50	51	50	*0.990*
Maternal age (years)	30.98 ± 4.00	30.00 ± 3.09	31.02 ± 4.26	*0.315*
Pre-pregnancy BMI (kg/m2)	21.19 ± 3.04	22.55 ± 3.70	22.02 ± 2.99	*0.111*
GWG≥15 kg, n (%)	27 (54.0)	25 (49.0)	27 (54.0)	*0.845*
Family history of thyroid disease, n (%)	10 (20.0)	5 (9.8)	8 (16.0)	*0.356*
Abortion history, n (%)	15 (30.0)	16 (31.4)	16 (32.0)	*0.976*
• *Thyroid function and thyroid autoantibodies*				
I. The period before pregnancy (BP)				
*FT4 (pmol/L)**	11.27 (10.34-12.99)[bc]	16.38 (14.96-18.74)[ac]	13.48 (11.68-15.05)[ab]	*0.000*
*FT3 (pmol/L)**	3.87 (3.67-4.36)[c]	4.13 (3.81-4.50)	4.20 (3.79-4.53)[a]	*0.035*
TSH (mU/L)	1.65 (1.24-2.56)	2.18 (1.03-3.19)	2.00 (0.74-3.17)	*0.089*
II. The first trimester of pregnancy (T1)				
*Gestational age (weeks)**	6.6 (5.0-12.0)[bc]	6.0 (5.0-7.0)[a]	6.0 (5.0-7.1)[a]	*0.000*
*FT4 (pmol/L)**	13.26 (11.84-15.98)[bc]	16.24 (14.67-18.42)[a]	14.67 (13.33-17.13)[a]	*0.000*
FT3 (pmol/L)	4.29 (3.75-4.55)	4.17 (3.71-4.61)	4.26 (3.99-4.58)	*0.692*
*TSH (mU/L)**	5.56 (4.39-7.12)[bc]	2.78 (1.73-3.63)[a]	2.66 (1.95-4.14)[a]	*0.000*
*TPOAb≥300 μIU/mL, n (%)**	10 (20.0)	14 (27.5)	24 (48.0)	*0.008*
TgAb≥300 μIU/mL, n (%)	13 (26.0)	16 (31.4)	16 (32.0)	*0.771*
TPOAb/TgAb +, n (%)	19 (38.0)	22 (43.1)	27 (54.0)	*0.260*
III. The second trimester of pregnancy (T2)				
Gestational age (weeks)	20.1 (19.0-21.3)	20.0 (19.0-21.0)	20.0 (19.0-21.5)	*0.822*
*FT4 (pmol/L)**	12.03 (11.10-13.71)[b]	13.50 (11.50-15.10)[a]	12.58 (10.85-14.23)	*0.039*
*FT3 (pmol/L)**	4.03 (3.63-4.45)	4.08 (3.37-4.54)[c]	4.22 (3.85-4.72)[b]	*0.031*
*TSH (mU/L)**	1.86 (1.28-3.13)[c]	1.78 (1.17-2.51)	1.30 (0.75-2.07)[a]	*0.009*
IV. The third trimester of pregnancy (T3)				
Gestational age (weeks)	35.0 (33.0-36.3)	35.0 (32.0-37.0)	35.0 (33.0-37.0)	*0.831*
*FT4 (pmol/L)**	11.27 (10.18-13.34)	12.71 (10.53-13.74)[c]	11.27 (10.23-12.74)[b]	*0.020*
FT3 (pmol/L)	3.89 (3.47-4.18)	4.09 (3.68-4.37)	4.08 (3.58-4.44)	*0.137*
TSH (mU/L)	1.56 (0.94-2.53)	1.65 (1.05-2.55)	1.36 (0.81-2.00)	*0.273*
• *Postpartum thyroid function status*				
Euthyroid, n (%)	18 (36.0)	17 (33.3)	15 (30.0)	*0.815*
Abnormal thyroid function, n (%)	32 (64.0)	34 (66.7)	35 (70.0)	*0.815*
PPT, n (%)	31 (62.0)	33 (64.7)	32 (64.0)	*0.958*
*Only transient hypothyroidism, n (%)**	23 (46.0)[c]	25 (49.0)[c]	2 (4.0)[ab]	*0.000*
Only transient thyrotoxicosis, n (%)	3 (6.0)	3 (5.9)	1 (2.0)	*0.254*
Total hypothyroidism phase, n (%)	28 (56.0)	30 (58.8)	34 (68.0)	*0.437*
Hyperthyroidism, n (%)	1 (2.0)	1 (2.0)	——	*0.989*

Data were presented as the median (interquartile range: 25-75%), M ± SD or n (%) as appropriate.

**presented as P<0.05; [a]presented that there was significant difference in the group with the ND-SCH group, P<0.05; [b]presented that there was significant difference in the group with the PK-SCH group, P<0.05; [c]presented that there was significant difference in the group with the PK-OH group, P<0.05.*

ND-SCH, subclinical hypothyroidism newly diagnosed in early pregnancy; PK-SCH, previously known subclinical hypothyroidism; PK-OH, previously known autoimmune overt hypothyroidism; TSH, thyroid stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; BMI, body mass index; GWG, gestational weight gain; PPT, postpartum thyroiditis.

The follow-up time points were as followed: BP, 5.39 ± 1.84 months; T1, 6.72 ± 2.11 gestational weeks; T2, 20.38 ± 1.73 gestational weeks; T3, 34.93 ± 2.29 gestational weeks; the first stage after delivery (A1), 8.13± 2.69 weeks; the second stage after delivery (A2), 5.62± 2.04 months; the third stage after delivery (A2), 10.47± 4.36 months. Thyroid function indicators were measured by Roche, and during pregnancy, the trimester-specific reference intervals tested in our laboratory were applied (23).

hypothyroidism already received adequate LT4 replacement before conception, and their TSH levels were maintained below 2.5 mU/L as shown in **Table 1**. During pregnancy, the first monitoring time in T1 was within 8 gestational weeks, and no differences were observed among these three groups. In the whole cohort, LT4 adjustments were applied individually to maintain TSH<2.5 mU/L until birth [ND-SCH 1.56 (0.94-2.53) mU/L, PK-SCH 1.65 (1.05-2.55) mU/L, PK-OH 1.36 (0.81-2.00) mU/L]. After delivery, thyroid function was tested at least three times [A1: 8.13 ± 2.69 weeks, A2: 5.62 ± 2.04 months and A3: 10.47 ± 4.36 months].

As shown in **Figure 2**, the LT4 dose was remarkably correlated with the impairment degree of hypothyroidism. The LT4 doses in the PK-OH group were obviously higher than those in the ND-SCH and PK-SCH groups. However, regardless of the cause of hypothyroidism, the LT4 dose first presented an increasing trend, followed by a decreasing change in the following progression of gestation. In late pregnancy, the LT4 dose returned to the initial dose in early pregnancy in the ND-SCH group and the original pre-pregnancy dose in the other two groups [initial dose *vs* final dose: ND-SCH 0.83 ± 0.43 mcg/kg/d *vs* 0.85 ± 0.34 mcg/kg/d, PK-SCH 0.83 ± 0.27 mcg/kg/d *vs* 0.80 ± 0.37 mcg/kg/d, PK-OH 0.97 ± 0.49 mcg/kg/d *vs* 1.11 ± 0.39 mcg/kg/d, P>0.05].

Outcomes and Influencing Factors of PPT in ND-SCH Women

After delivery, LT4 treatment was suspended in the ND-SCH group. During the following one year, 32 (64.0%) patients developed thyroid dysfunction, including 31 (62.0%) cases of PPT and 1 (2.0%) case of hyperthyroidism (**Table 1**). A comparison of the clinical characteristics of the postpartum-euthyroid group (n=18) and PPT group (n=31) showed that the gestational age at diagnosis in the postpartum-euthyroid group was apparently later than that in the PPT group [12.0 (6.4-14.9) weeks *vs* 6.0 (5.0-9.0) weeks, P=0.001]. The rate of postpartum-euthyroid women whose TSH level was controlled at 1.0-2.5 mU/L in late pregnancy was 1.72-fold greater than that in the PPT group [13/18 (72.2%) *vs* 13/31 (41.9%), P=0.041] (**Supplementary Table 1**).

The logistic regression analysis further confirmed that gestational SCH diagnosed at <8 weeks was a risk factor for developing PPT [OR=8.063, 95% CI, 2.078-31.285; P=0.003]. TSH levels outside the 1.0-2.5 mU/L range before childbirth were also a risk factor [OR=3.725, 95% CI, 1.035-13.411; P=0.044]. There were no apparent correlations between PPT and other adjusted factors (**Table 2**).

Outcomes and Influencing Factors of PPT in Women With Previously Known Hypothyroidism

After delivery, the PK-SCH women also discontinued LT4 treatment. Thirty-four (66.7%) patients developed thyroid dysfunction in the first year postpartum, including 33 (64.7%) cases of PPT and 1 (2.0%) case of hyperthyroidism. In the PK-OH group, 35 (70.0%) patients developed thyroid dysfunction, including 32 (64.0%) cases of PPT, 2 (4.0%) cases of transient hypothyroidism and 1 (2.0%) case of transient thyrotoxicosis (**Table 1**).

The main aetiology for both PK-SCH and PK-OH groups was AIT, and the prevalence of PPT was also similar [PK-SCH 33/51 (64.7%) *vs* PK-OH 32/50 (64.0%), P=0.941] (**Table 1**). Hence, these two groups were combined as the AIT group (n=101) for further analysis.

A comparison of the clinical characteristics of the postpartum-euthyroid group (n=32) and PPT group (n=65) showed that the rate of postpartum-euthyroid women with a TSH level>2.5 mU/L in T1 was obviously lower than that in the PPT group [11/32 (34.4%) *vs* 43/65 (66.2%), P=0.003]. The positive rate of TPOAb≥300 μIU/mL among the postpartum-euthyroid women was a quarter of that in the PPT group [4/32 (12.5%) *vs* 32/65 (49.2%), P=0.001] (**Supplementary Table 2**).

The logistic regression analysis also confirmed that TSH>2.5 mU/L in T1 was a risk factor for developing PPT [OR=3.551, 95% CI, 1.430-8.814; P=0.006], and TPOAb≥300 μIU/mL was another risk factor [OR=6.578, 95% CI, 2.049-21.122; P=0.002]. There were no apparent correlations between PPT and the other adjusted factors (**Table 3**).

Outcomes and Influencing Factors of LT4 Retreatment After Delivery in SCH Women

Regardless of whether transient thyrotoxicosis occurred during the first-year follow-up after delivery, once the hypothyroidism phase occurred, LT4 replacement was restarted to prevent a further decline in postpartum thyroid function in all SCH women. The prevalence of LT4 retreatment was comparable between the ND-SCH and PK-SCH groups [28/50 (56.0%) *vs*

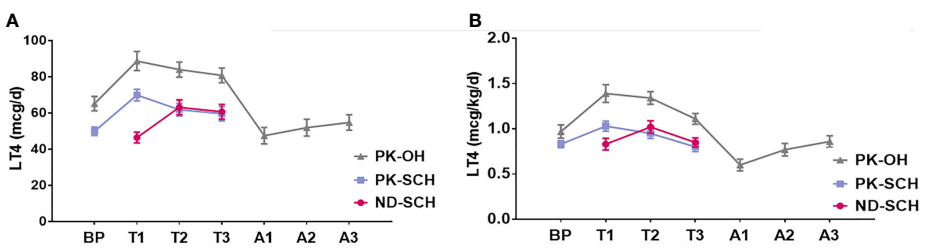


FIGURE 2 | LT4 dose adjustment before, during and after pregnancy. The whole pregnant women were followed up in every stage before, during and after pregnancy. Pregnant women with SCH stopped taking LT4 once they gave birth; As for pregnant women with PK-OH, LT4 dose were adjusted according to the dose before childbirth. Then adjusted LT4 dose accordingly in the light of the thyroid function postpartum. **(A)** shows the variations of daily LT4 dose (mcg/d) in each group and **(B)** shows weight adjusted LT4 dose (mcg/kg/d) in each group.

TABLE 2 | Logistic regression model for the risk factors of developing PPT in women with ND-SCH (n = 50).

ND-SCH group	OR	95%CI		P
Maternal age (years)	0.966	0.835	1.117	0.638
Family history of thyroid disease (yes vs no)	2.783	0.521	14.866	0.231
Initial TSH level in T1 (mU/L)	1.128	0.911	1.398	0.269
SCH diagnosed < 8 weeks (yes vs no)*	8.063	2.078	31.285	0.003
TSH <1.0 mU/L or >2.5 mU/L in T3 (yes or no)*	3.725	1.035	13.411	0.044
TPOAb≥300μIU/mL (yes or no)	1.295	0.279	6.024	0.741
TgAb≥300μIU/mL (yes or no)	1.434	0.360	5.715	0.609

**P < 0.05. The crude OR for maternal age and family history of thyroid disease were calculated first and we found no apparent correlations between PPT and them. Subsequently, the OR value for the potential influencing factors were adjusted by these two basic factors.*

TSH, thyroid-stimulating hormone; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; PPT, postpartum thyroiditis; T1, the first trimester of pregnancy; T3, the third trimester of pregnancy.

30/51 (58.8%), P=0.774]. Hence, these groups were combined as the SCH group (n=101) for further analysis.

In the SCH group, there were 41 women completely stop LT4 replacement in the first year after delivery (LT4-discontinuation group), and 58 women restarted LT4 treatment due to the occurrence of hypothyroidism (LT4-retreatment group). A comparison of the clinical characteristics showed that the rate of women with a TSH level>2.5 mU/L within 8 gestational weeks in the LT4-retreatment group was 1.54-fold greater than that in the LT4-discontinuation group [37/58 (63.8%) *vs* 17/41 (41.5%), P=0.028]. The rate of women whose TSH level was controlled at 1.0-2.5 mU/L in late pregnancy in the LT4-retreatment group was three-fifth of that in the LT4-discontinuation group [24/58 (41.4%) *vs* 29/41 (70.7%), P=0.004], and the positive rate of TPOAb≥300 μIU/mL in the LT4-retreatment group was 4.96-fold greater than that in the LT4-discontinuation group [20/58 (34.5%) *vs* 3/41 (7.3%), P=0.002] (**Supplementary Table 3**).

The logistic regression analysis also confirmed that hypothyroidism occurring at <8 gestational weeks [OR=2.478, 95% CI, 1.087-5.648; P=0.031], TSH levels outside 1.0-2.5 mU/L near childbirth [OR=3.418, 95% CI, 1.451-8.049; P=0.005], and TPOAb≥300 μIU/mL [OR=6.589, 95% CI, 1.787-24.302; P=0.005] were the risk factors for LT4 retreatment at one year postpartum (**Table 4**).

DISCUSSION

In this retrospective study, 151 hypothyroid women were recruited to analyse the postpartum prognosis of thyroid function in the first year after delivery and the influencing factors during pregnancy. We found that among the ND-SCH women, when LT4 intake was stopped after delivery, the prevalence of PPT was 62.0% during the 12-months monitoring of thyroid function after delivery. The patients with SCH occurring at <8 gestational weeks or whose TSH levels were outside the range of 1.0-2.5 mU/L in late pregnancy were more likely to suffer from PPT; Women with PK-SCH also discontinued LT4 intake after delivery. However, both TSH>2.5 mU/L in T1 and TPOAb≥300 μIU/mL were the risk factors for developing PPT in one year postpartum in PK-SCH and PK-OH patients, and the prevalences were separately 64.7% and 64.0%; Regardless of whether SCH occurred before pregnancy or during early pregnancy, the prevalence of the postpartum hypothyroidism phase was similar (PK-SCH 58.8% *vs* ND-SCH 56.0%), leading to LT4 retreatment after delivery. By combining all these SCH women into a unified group, we found that unideal TSH elevation at <8 gestational weeks, TSH levels outside the range of 1.0-2.5 mU/L in late pregnancy and TPOAb≥300 μIU/mL were the risk factors for LT4 retreatment in the first postpartum year.

Early gestation is a time of rapid development of the central nervous system in the foetus. During this time, the mother is the only source of foetal thyroid hormones (7). Therefore, it is the most critical period for monitoring thyroid diseases during pregnancy. To date, no studies reported the influence of newly occurred SCH in early pregnancy on thyroid function postpartum, especially within 8 gestational weeks. And no studies assessed the influence of SCH occurred before or in early pregnancy on the LT4 strategy after delivery.

TABLE 3 | Logistic regression model for the risk factors of developing PPT in women with previously known hypothyroidism caused by AIT (n = 101).

AIT group	OR	95%CI		P
Maternal age (years)	0.967	0.864	1.082	0.559
Family history of thyroid disease (yes vs no)	3.056	0.635	14.707	0.164
TSH in T1 >2.5 mU/L (yes vs no)*	3.551	1.430	8.814	0.006
TSH <1.0 mU/L or >2.5 mU/L in T3 (yes or no)	0.619	0.259	1.476	0.279
TPOAb≥300μIU/mL (yes or no)*	6.578	2.049	21.122	0.002
TgAb≥300μIU/mL (yes or no)	2.005	0.744	5.407	0.169

**P < 0.05. The crude OR for maternal age and family history of thyroid disease were calculated first and we found no apparent correlations between PPT and them. Subsequently, the OR value for the potential influencing factors were adjusted by these two basic factors.*

The main aetiology of previously known SCH and OH was autoimmune hypothyroidism, so combined these two groups to further analysis (PK-SCH & PK-OH).

TSH, thyroid stimulating hormone; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; PPT, postpartum thyroiditis; T1, the first trimester of pregnancy; T3, the third trimester of pregnancy.

TABLE 4 | Logistic regression model for the risk factors of LT4 retreatment in all SCH women (n = 101).

SCH group	OR	95%CI		P
Maternal age (years)	0.999	0.894	1.118	0.991
Family history of thyroid disease (yes vs no)	1.500	0.472	4.772	0.492
Hypothyroidism within 8 weeks (yes vs no)*	2.478	1.087	5.648	0.031
TSH <1.0 mU/L or >2.5 mU/L in T3 (yes or no)*	3.418	1.451	8.049	0.005
TPOAb≥300μIU/mL (yes or no)*	6.589	1.787	24.302	0.005
TgAb≥300μIU/mL (yes or no)	1.526	0.619	3.763	0.358

**P < 0.05. The crude OR for maternal age and family history of thyroid disease were calculated first and we found no apparent correlations between postpartum LT4 retreatment and them. Subsequently, the OR value for the potential influencing factors were adjusted by these two basic factors.*

Regardless of whether SCH was newly diagnosed in early pregnancy or previously known, they were combined as SCH group to further analysis (ND-SCH & PK-SCH).

TSH, thyroid stimulating hormone; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody; T1, the first trimester of pregnancy; T3, the third trimester of pregnancy.

According to the 2017 ATA guidelines, if TSH >4.0 mU/L with normal serum T4, SCH could be diagnosed in early pregnancy and should start LT4 treatment, and the treatment might be considered to stop after delivery (13). In our study, the ND-SCH women who developed PPT in the first postpartum year had an earlier gestational week at diagnosis than those who did not develop PPT [6.0 (5.0-9.0) weeks *vs* 12.0 (6.4-14.9) weeks, P=0.001]. SCH appeared earlier, indicating that thyroid function was even worse during pregnancy, therefore, impaired thyroid function was difficult to recover after delivery. This phenomenon is consistent with another study conducted by Li et al. (20). Although the average gestational age of SCH in that study was 22 weeks, the patients who suffered from postpartum hypothyroidism had an earlier diagnosis time than those who did not [19 (14–26) weeks *vs* 22 (16–28) weeks, P<0.05]. In the long-term follow-up in our study, regardless of whether transient thyrotoxicosis occurred, the prevalence of the postpartum hypothyroidism phase was 56.0% in the ND-SCH group, which is higher than that in the previously mentioned study (38.9%). This finding also indicated that the recovery of thyroid function after delivery among SCH newly diagnosed patients in early pregnancy was worse than that of SCH patients diagnosed in middle pregnancy. Adequate LT4 replacement was a protective factor against PPT and controlling TSH levels at 1.0-2.5 mU/L in late pregnancy could reduce its occurrence. However, the TPOAb-positive rate in the ND-SCH group was only 20.0% in our study; therefore, AIT was not the main cause of the abnormal TSH levels. To date, mandatory universal salt iodization (USI) has been carried out in China for twenty years, and residents' TSH levels are generally enhanced by an increase in iodine intake (26). All recruited women in our study were from Shenyang, which is an iodine-sufficient city. Therefore, iodine-induced nonautoimmune SCH was probably the main cause of ND-SCH.

Among known SCH and OH resulting from AIT, the overall prevalence of PPT was 65/101 (64.5%), which is similar to the prevalence reported in previous studies [Caixas et al. (16): 12/18 (66.7%); Galofre et al. (17): 18/29 (62.1%); Sergi et al. (18): 66/97 (68.0%)]. Stagnaro-Green indicated that more than 50% of TPOAb-positive women suffered from PPT after delivery, and the higher the TPOAb titre, the greater the risk of PPT (27). In our study, AIT hypothyroid women with TPOAb≥300 μIU/mL were more likely to develop PPT than those with TPOAb<300 μIU/mL [32/36 (88.9%) *vs* 33/61 (54.1%), P=0.000]. Similar to the results reported by Sergi et al., the prevalences of PPT among the TPOAb-positive and TPOAb-negative patients were 83% and 44%, respectively (18). Recently, Moleti et al. (28), found that three-quarters of PPT was biphasic in AIT-euthyroid women. Regarding AIT-hypothyroidism, the diagnostic criteria for PPT included only biphasic PPT, and the prevalence rate was only 18/98 (18.4%). The authors insisted that the euthyroid status in early pregnancy implied more viable thyroid tissue exposed to postpartum autoimmune attack. In contrast to the study focusing on biphasic PPT, our study found that TSH>2.5 mU/L in early pregnancy was a risk factor for the development of PPT in women with previously known AIT hypothyroidism. The prevalences of the only one-way hypothyroidism of PPT were 75% and 35% in the PK-SCH and PK-OH groups, respectively. TSH>2.5 mU/L implies that thyroid function deterioration might be more severe during early pregnancy and the LT4 replacement was not adequate; thus, thyroid function could be more prone to further decline postpartum and could not be restored to the pre-pregnancy levels. Therefore, more patients experienced the hypothyroidism phase of PPT.

In our study, we suspended the LT4 treatment after delivery, regardless of whether SCH was diagnosed before or during early pregnancy. The occurrence of the postpartum hypothyroidism phase is a signal for LT4 retreatment. We first compared the incidence of LT4 retreatment between the ND-SCH and PK-SCH groups and found no apparent difference. In total, approximately more than 50% of the SCH women eventually restarted LT4 replacement in the first year after delivery. During the first 8 gestational weeks, we considered both TSH>4.0 mU/L in the ND-SCH group and TSH>2.5 mU/L in the PK-SCH group as unideal TSH elevations in early pregnancy. And we found that these uncontrolled TSH elevations at < 8 gestational weeks was actually a risk factor to suffer from postpartum hypothyroidism and restarting LT4 replacement in the first postpartum year. Moreover, among SCH women, especially those with strong TPOAb positivity, should undergo frequent thyroid function evaluations after delivery to prevent the occurrence of postpartum hypothyroidism. In late pregnancy, the stimulating effect of hCG on thyroid function was decreased (29). During the time, TSH <1.0 mU/L may indicate the destruction of thyroid follicles with release of hormone (30), which would be one reason for SCH women being more likely to suffer hypothyroidism phase in postpartum. So that controlling TSH levels at 1.0-2.5 mU/L before delivery was advised.

Our study first focused on the influence of newly diagnosed SCH in early pregnancy on postpartum thyroid function. Regarding previously known SCH, they also discontinued LT4 treatment after delivery. Although the risk factors for the occurrence of PPT were differ, after combined analysis of these two types of SCH, we evaluated the shared risk factors for LT4 retreatment in the first year after delivery.

However, there are some limitations in our study. The sample size was small because the time points of detection were throughout the whole process, namely, before, during and after pregnancy, and the inclusion criteria were strict such that patient compliance would be limited. During our analysis, we selected women with a relatively high level of thyroid autoimmunity as TPOAb/TGAb positive and determined this as one of the risk factors in our study. All patients were regarded as having adequate iodine nutrition according to the local iodine-sufficient nutrition situation, and we did not detect urinary iodine. We look forward to large-scale studies in the future.

Overall, regardless of whether SCH is diagnosed before pregnancy or newly diagnosed during early pregnancy, uncontrolled TSH elevation within 8 gestational weeks is a shared risk factor for PPT and LT4 retreatment in the first year after delivery. Therefore, it is essential to monitor the thyroid function of pregnant women during the first 8 gestational weeks in early pregnancy and for them to undergo frequently thyroid function evaluations within one year postpartum to identify thyroid dysfunction in a timely manner and adjust the LT4 strategy accordingly.

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 China Medical University (Ethics Committee of China Medical University [2012] 2011-32-4). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

We sincerely appreciate the strong support and cooperation of members responsible for the patients' recruitment and data collection, namely, XG, XW, YTH, YYH, YY, and HuW. ZS, HaW, JL, and XG responsible for the patient's treatment strategy. XG followed up the whole pregnant women and wrote the article and ZS guided and supervised the whole process. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the National Natural Science Foundation of China (81970682, 81570709); the National Key Research and Development Program of China (2017YFC0907403); Health and Medicine Research Foundation in Shenyang City (17-230-9-02); The central government guided local special funds for scientific and technological development (2019416021).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.746329/full#supplementary-material

REFERENCES

1. Casey BM, Dashe JS, Wells CE, McIntire DD, Byrd W, Leveno KJ, et al. Subclinical Hypothyroidism and Pregnancy Outcomes. *Obstet Gynecol* (2005) 105:239–45. doi: 10.1097/01.AOG.0000152345.99421.22
2. Cleary-Goldman J, Malone FD, Lambert-Messerlian G, Sullivan L, Canick J, Porter TF, et al. Maternal Thyroid Hypofunction and Pregnancy Outcome. *Obstet Gynecol* (2008) 112:85–92. doi: 10.1097/AOG.0b013e3181788dd7
3. Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J, et al. Maternal Thyroid Deficiency During Pregnancy and Subsequent Neuropsychological Development of the Child. *N Engl J Med* (1999) 341:549–55. doi: 10.1056/NEJM199908193410801
4. Li Y, Shan Z, Teng W, Yu X, Li Y, Fan C, et al. Abnormalities of Maternal Thyroid Function During Pregnancy Affect Neuropsychological Development of Their Children at 25-30 Months. *Clin Endocrinol (Oxf)* (2010) 72:825–9. doi: 10.1111/j.1365-2265.2009.03743.x
5. Mandel SJ, Spencer CA, Hollowell JG. Are Detection and Treatment of Thyroid Insufficiency in Pregnancy Feasible? *Thyroid* (2005) 15:44–53. doi: 10.1089/thy.2005.15.44
6. Kester MH, Martinez de Mena R, Obregon MJ, Marinkovic D, Howatson A, Visser TJ, et al. Iodothyronine Levels in the Human Developing Brain: Major Regulatory Roles of Iodothyronine Deiodinases in Different Areas. *J Clin Endocrinol Metab* (2004) 89:3117–28. doi: 10.1210/jc.2003-031832
7. Fisher DA, Klein AH. Thyroid Development and Disorders of Thyroid Function in the Newborn. *N Engl J Med* (1981) 304:702–12. doi: 10.1056/NEJM198103193041205
8. Alexander EK, Marqusee E, Lawrence J, Jarolim P, Fischer GA, Larsen PR. Timing and Magnitude of Increases in Levothyroxine Requirements During Pregnancy in Women With Hypothyroidism. *N Engl J Med* (2004) 351:241–9. doi: 10.1056/NEJMoa040079
9. Glinoer D. The Regulation of Thyroid Function in Pregnancy: Pathways of Endocrine Adaptation From Physiology to Pathology. *Endocr Rev* (1997) 18:404–33. doi: 10.1210/edrv.18.3.0300
10. Korevaar TIM, Pop VJ, Chaker L, Goddijn M, de Rijke YB, Bisschop PH, et al. Dose Dependency and a Functional Cutoff for TPO-Antibody Positivity During Pregnancy. *J Clin Endocrinol Metab* (2018) 103:778–89. doi: 10.1210/jc.2017-01560
11. Hou Y, Liu A, Li J, Wang H, Yang Y, Li Y, et al. Different Thyroidal Responses to Human Chorionic Gonadotropin Under Different Thyroid Peroxidase Antibody and/or Thyroglobulin Antibody Positivity Conditions During the First Half of Pregnancy. *Thyroid* (2019) 29:577–85. doi: 10.1089/thy.2018.0097
12. Shi X, Han C, Li C, Mao J, Wang W, Xie X, et al. Optimal and Safe Upper Limits of Iodine Intake for Early Pregnancy in Iodine-Sufficient Regions: A Cross-Sectional Study of 7190 Pregnant Women in China. *J Clin Endocrinol Metab* (2015) 100:1630–8. doi: 10.1210/jc.2014-3704

13. Alexander EK, Pearce EN, Brent GA, Brown RS, Chen H, Dosiou C, et al. 2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum. *Thyroid* (2017) 27:315–89. doi: 10.1089/thy.2016.0457
14. Epp R, Malcolm J, Jolin-Dahel K, Clermont M, Keely E. Postpartum Thyroiditis. *BMJ* (2021) 372:n495. doi: 10.1136/bmj.n495
15. Benvenga S. Targeted Antenatal Screening for Predicting Postpartum Thyroiditis and Its Evolution Into Permanent Hypothyroidism. *Front Endocrinol (Lausanne)* (2020) 11:220. doi: 10.3389/fendo.2020.00220
16. Caixas A, Albareda M, Garcia-Patterson A, Rodriguez-Espinosa J, de Leiva A, Corcoy R. Postpartum Thyroiditis in Women With Hypothyroidism Antedating Pregnancy? *J Clin Endocrinol Metab* (1999) 84:4000–5. doi: 10.1210/jcem.84.11.6144
17. Galofre JC, Haber RS, Mitchell AA, Pessah R, Davies TF. Increased Postpartum Thyroxine Replacement in Hashimoto's Thyroiditis. *Thyroid* (2010) 20:901–8. doi: 10.1089/thy.2009.0391
18. Sergi M, Tomlinson G, Feig DS. Changes Suggestive of Post-Partum Thyroiditis in Women With Established Hypothyroidism: Incidence and Predictors. *Clin Endocrinol (Oxf)* (2015) 83:389–93. doi: 10.1111/cen.12604
19. Shields BM, Knight BA, Hill AV, Hattersley AT, Vaidya B. Five-Year Follow-Up for Women With Subclinical Hypothyroidism in Pregnancy. *J Clin Endocrinol Metab* (2013) 98:E1941–5. doi: 10.1210/jc.2013-2768
20. Li N, Yang J, Chen X, Huang J, Lai M, Fang F, et al. Postpartum Follow-Up of Patients With Subclinical Hypothyroidism During Pregnancy. *Thyroid* (2020) 30:1566–73. doi: 10.1089/thy.2019.0714
21. Daemers DOA, Wijnen HAA, van Limbeek EBM, Bude LM, Nieuwenhuijze MJ, Spaanderman MEA, et al. The Effect of Gestational Weight Gain on Likelihood of Referral to Obstetric Care for Women Eligible for Primary, Midwife-Led Care After Antenatal Booking. *Midwifery* (2016) 34:123–32. doi: 10.1016/j.midw.2015.12.006
22. Yu X, Chen Y, Shan Z, Teng W, Li C, Zhou W, et al. The Pattern of Thyroid Function of Subclinical Hypothyroid Women With Levothyroxine Treatment During Pregnancy. *Endocrine* (2013) 44:710–5. doi: 10.1007/s12020-013-9913-2
23. Zhang X, Yao B, Li C, Mao J, Wang W, Xie X, et al. Reference Intervals of Thyroid Function During Pregnancy: Self-Sequential Longitudinal Study Versus Cross-Sectional Study. *Thyroid* (2016) 26:1786–93. doi: 10.1089/thy.2016.0002
24. Teng X, Shan Z, Chen Y, Lai Y, Yu J, Shan L, et al. More Than Adequate Iodine Intake may Increase Subclinical Hypothyroidism and Autoimmune Thyroiditis: A Cross-Sectional Study Based on Two Chinese Communities With Different Iodine Intake Levels. *Eur J Endocrinol* (2011) 164:943–50. doi: 10.1530/EJE-10-1041
25. Babyak MA. What You See May Not be What You Get: A Brief, Nontechnical Introduction to Overfitting in Regression-Type Models. *Psychosom Med* (2004) 66:411–21. doi: 10.1097/01.psy.0000127692.23278.a9
26. Li Y, Teng D, Ba J, Chen B, Du J, He L, et al. Efficacy and Safety of Long-Term Universal Salt Iodization on Thyroid Disorders: Epidemiological Evidence From 31 Provinces of Mainland China. *Thyroid* (2020) 30:568–79. doi: 10.1089/thy.2019.0067
27. Stagnaro-Green A. Approach to the Patient With Postpartum Thyroiditis. *J Clin Endocrinol Metab* (2012) 97:334–42. doi: 10.1210/jc.2011-2576
28. Moleti M, Mauro MD, Alibrandi A, Vita R, Benvenga S, Vermiglio F. Postpartum Thyroiditis in Women With Euthyroid and Hypothyroid Hashimoto's Thyroiditis Antedating Pregnancy. *J Clin Endocrinol Metab* (2020) 105(7):e2421–28. doi: 10.1210/clinem/dgaa197
29. Hershman JM. Physiological and Pathological Aspects of the Effect of Human Chorionic Gonadotropin on the Thyroid. *Best Pract Res Clin Endocrinol Metab* (2004) 18:249–65. doi: 10.1016/j.beem.2004.03.010
30. Cooper DS, Laurberg P. Hyperthyroidism in Pregnancy. *Lancet Diabetes Endocrinol* (2013) 1:238–49. doi: 10.1016/S2213-8587(13)70086-X

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

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Endocrinology

SYSTEMATIC REVIEW
published: 17 February 2022
doi: 10.3389/fendo.2022.759064

Levothyroxine Supplementation in Euthyroid Pregnant Women With Positive Autoantibodies: A Systematic Review and Meta-Analysis

Raffaella Di Girolamo, Marco Liberati, Claudia Silvi and Francesco D'Antonio *

Center for Fetal Care and High-Risk Pregnancy, Department of Obstetrics and Gynecology, University of Chieti, Chieti, Italy

OPEN ACCESS

Edited by:
Carlo Alviggi,
University of Naples Federico II, Italy

Reviewed by:
Ezequiel Lacunza,
Universidad Nacional de La Plata, Argentina
Renata Simona Auriemma,
University of Naples Federico II, Italy

***Correspondence:**
Francesco D'Antonio
francesco.dantonio@unich.it
orcid.org/0000-0002-5178-3354

Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 August 2021*
Accepted: *13 January 2022*
Published: *17 February 2022*

Citation:
Di Girolamo R, Liberati M, Silvi C and D'Antonio F (2022) Levothyroxine Supplementation in Euthyroid Pregnant Women With Positive Autoantibodies: A Systematic Review and Meta-Analysis. Front. Endocrinol. 13:759064. doi: 10.3389/fendo.2022.759064

Objectives: To explore the role of levothyroxine (LT4) supplementation in affecting the outcome of pregnant euthyroid women with thyroperoxidase (TPO) antibodies.

Methods: MEDLINE, EMBASE, Google Scholar, and the Web of Science databases were searched. The primary outcome was pre-term birth (PTB), defined as live birth before 37 weeks of gestation; secondary outcomes were gestational hypertension, pre-eclampsia (PE), placental abruption, miscarriage, intra-uterine death (IUD), and admission to neonatal intensive care unit (NICU). All these outcomes were explored in euthyroid women with TPO antibodies receiving compared to those not receiving LT4 supplementation in pregnancy. Random-effect meta-analyses were used to analyze the data and results reported as pooled odds ratios (OR) with their 95% confidence intervals (CI).

Results: The risk of PTB was lower in women with TPO antibodies receiving compared to those not receiving LT4 supplementation (OR of 0.60 (95% CI 0.4-0.9). However, this association came mainly from observational studies (OR: 0.29, 95% CI 0.1-0), while RCTs did not show any beneficial effect of LT4 supplementation in affecting such outcomes. Conversely, there was no difference in the risk of gestational hypertension, preeclampsia, placental abruption, miscarriage, and admission to NICU between the two groups.

Conclusions: LT4 supplementation in TPO euthyroid women is not associated with a reduced risk of PTB in TPO-positive women with normal thyroid function.

Keywords: thyroid disorders in pregnancy, preterm birth (PTB), thyroid disorders in IVF, thyroid autoimmune status, euthyroid pregnant women

Abbreviations: COS, controlled ovarian stimulation; GDM, gestational diabetes mellitus; IVF, *in vitro* fertilization; LT4, levothyroxine; LT4-pregnant, women treated with levothyroxine; (n)LT4-pregnant, women not treated with levothyroxine; RCT, randomized clinical trial; TgAb, thyroglobulin antibody; TPOAb, thyroid peroxidase antibody; SCH, sub-clinical hypothyroidism; TAI, thyroid auto-immune.

INTRODUCTION

Thyroid diseases affect up to 4% of all pregnancies with primary hypothyroidism being the most prevalent disease (1). Inadequately treated or subclinical hypothyroidism increases are associated with an increased risk of adverse pregnancy outcomes, including placental abruption, preterm birth, miscarriage, preeclampsia, gestational diabetes, and sub-optimal neurocognitive development (2, 3). However, not all studies have found an increased risk of adverse outcomes with hypothyroidism and the pathophysiology of the association between altered thyroid function and adverse pregnancy outcome has not been completely elucidated yet. Despite that, American Thyroid Association recommends universal treatment of maternal hypothyroidism, irrespective of antibody status (4). More recently, several observational studies reported that thyroperoxidase antibody (TPOAb)-positive women with a normal thyroid function are at also increased risk of experiencing adverse pregnancy outcomes (5). However, there is still insufficient whether levothyroxine (LT4) supplementation may improve pregnancy outcomes in these women (6, 7) The aim of this systematic review was to explore the role of LT4 supplementation in affecting maternal and perinatal outcomes in euthyroid women with TPO antibodies.

METHODS

Protocol, Information Sources, and Literature Search

This systematic review was performed according to an *a priori* protocol recommended for systematic reviews and meta-analysis. PRISMA guidelines were followed (8).

MEDLINE, EMBASE, Google Scholar, and the Web of Science databases were searched electronically up to August 14, 2021, using the following search terms (as words in the title/abstract), and combinations of the relevant medical subject heading (MeSH) terms, keywords, and word variants for "Euthyroid women", "LT4 supplementation", "Perinatal outcome" and "Maternal outcome". Studies including euthyroid pregnant women with thyroid autoantibodies treated compared to those not treated with levothyroxine were included. The search and selection criteria were restricted to the English language. Reference lists of relevant articles and reviews were hand-searched for additional reports.

Outcomes Measures, Study Selection, and Data Collection

The primary outcome was pre-term birth (PTB), defines as birth before 37 weeks of gestation.

The secondary outcomes were:

- Gestational hypertension, defined as blood pressure ≥140/90 mmHg
- Pre-eclampsia (PE), defined as gestational hypertension accompanied by one or more of the following new-onset conditions at or after 20 weeks gestation: proteinuria, other maternal organ dysfunction, including acute kidney injury, liver involvement, neurological or hematological complications, or uteroplacental dysfunction (such as fetal growth restriction, abnormal umbilical artery Doppler waveform analysis, or stillbirth)
- Placental abruption, defined as the separation of the placenta from the wall of the uterus during pregnancy
- Miscarriage, defined as the loss of an embryo or fetus before the 20th week of pregnancy
- Intra-uterine death, defined as a fetal death detected at ≥24 weeks
- Admission to neonatal intensive care unit (NICU)

All these outcomes were explored in the euthyroid women with TPO antibodies receiving compared to those not receiving LT4 supplementation in pregnancy.

Two authors (RDG, FDA) reviewed all abstracts independently. Agreement regarding potential relevance was reached by consensus. Full-text copies of those papers were obtained, and the same two reviewers independently extracted relevant data regarding study characteristics and maternal and perinatal outcomes with LT4 supplementation. Inconsistencies were discussed by the reviewers and consensus was reached by discussion with the senior authors (FDA).

Risk of Bias and Statistical Analysis

Quality assessment of the included studies was assessed using the Revised Cochrane risk-of-bias tool for randomized trials (RoB 2) (9). According to this tool, the risk of bias of each included study is judged according to five domains: bias arising from the randomization process, bias due to deviations from intended interventions, bias due to missing outcome data, bias in the measurement of the outcome, and bias in the selection of the reported result. Although the RoB 2 tool does not provide an overall risk of bias assessment, the overall risk of bias was considered low if four or more domains were rated as low risk (not counting 'other biases'), with at least one of them being sequence generation or allocation concealment, according to what reported in previous systematic reviews of intervention. Finally, the quality of evidence and strength of recommendations were assessed using the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) methodology (GRADE pro, Version 20. McMaster University, 2014) (10).

Conversely, quality assessment for non-randomized trials was assessed using the Newcastle-Ottawa Scale (NOS) for case-control studies. According to the NOS, each study is judged on three broad perspectives: the selection of the study groups, the comparability of the groups, and the ascertainment outcome of interest. The assessment of the selection of a study includes the evaluation of the representativeness of the exposed cohort, selection of the non-exposed cohort, ascertainment of exposure, and the demonstration that the outcome of interest was not present at the start of the study. The assessment of the comparability of the study includes the evaluation of the

comparability of cohorts based on the design or analysis. Finally, the ascertainment of the outcome of interest includes the evaluation of the type of the assessment of the outcome of interest and the length and the adequacy of follow-up. According to the NOS, a study can be awarded a maximum of one star for each numbered item within the Selection and Outcome categories. A maximum of two stars can be given for Comparability (11).

Small study effects (potentially caused by publication bias) were assessed using funnel plots and formally tested through the Egger regression asymmetry test for those meta-analyses including ≥10 studies. When less than 10 studies are included, the available tests are at a very high risk of bias because of the lack of statistical power (12).

Head-to-head meta-analyses using the random-effect model were used to analyze the data and results reported as pooled odds ratios (OR) with their 95% confidence intervals (CI). RevMan 5.3 (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014) Stata, version 13.1 (Stata Corp., College Station, TX, 2013) and Comprehensive Meta-analysis (Biostat, Englewood NJ, 07361) were used to analyze the data.

RESULTS

Study Selection and Characteristics

There were 39 articles initially selected, of which 26 were assessed with respect to their eligibility for inclusion and 9 (2, 3, 5, 13–18) were included in the systematic review (**Supplementary Table 1**). There were 4 randomized clinical trials (RCTs), 3 were prospective, and 2 were retrospective series (**Table 1**, **Figure 1**). When considering the RCTs, the studies by Dhillon-Smith et al. (14) and Wang et al. (15) explored the role of LT4 supplementation before conception in women undergoing *in vitro* fertilization and in those with a history of miscarriage and infertility, respectively, while those by Nigro et al. (16) and Narzapour et al. (5) evaluated obstetric complications in pregnancy as their primary outcome.

Overall, these nine studies included 1884 pregnant TPO Ab positive euthyroid women, aged between 16 and 41 [mean ± standard deviation (SD)=30.75 ± 4.56]; out of these, 935 were treated with LT4 supplementation. TSH levels were 2.5 ± 0.5 and 2.2 ± 0.5 in women treated compared to those not treated with LT4 in RCTs, while the corresponding figures for observational studies were 3.2 ± 1.6 and 1.19 ± 1.2, respectively.

Assessment of the RCTs included in the present systematic review using GRADE showed a low quality of evidence for the primary outcome.

The results of the quality assessment of the included studies using the NOS for observational studies are presented in **Supplementary Table 2**. The included studies showed an overall good score regarding the selection and comparability of the study groups, and for ascertainment of the outcome of interest. The main weaknesses of these studies were their non-randomized design, small sample size, and heterogeneity in outcome definition and assessment.

Synthesis of the Results

When considering RCTs and observational studies together, the risk of PTB was lower in TPO-positive women with normal thyroid function receiving compared to those not receiving LT4 supplementation with an OR of 0.60 (95% CI 0.4-0.9; I^2: 53.9%) (**Table 2**). Likewise, the risk of admission to NICU was lower in these women (OR: 0.14, 95% CI 0.03-0.7; I^2: 0%), while there was no difference in the risk of gestational hypertension (p=0.23), preeclampsia (p=0.48), placental abruption (p=0.11), miscarriage (p=0.37), between the two groups.

When considering only RCTs, there was no difference in the risk of PTB (p= 0.311), placental abruption (p= 0.994), miscarriage (p= 0.999), or IUD (p= 0.489) between TPO-positive women treated compared to those not treated with LT4, while the risk of gestational hypertension and PE was not assessed in any of the included RCT. The risk of admission to NICU was lower in TPO-positive women treated with LT4 supplementation, but this outcome was assessed only in one trial.

The risk of PTB was lower in TPO-positive women undergoing LT4 supplementation when considering only observational studies (OR: 0.29, 95% CI 0.1-0.6; I^2: 37.3%), while there was no difference in any of the other outcomes explored in the present systematic review.

DISCUSSION

Summary of the Main Findings

The findings from this systematic review show that, in TPO-positive women with normal thyroid function, LT4 supplementation does not significantly affect pregnancy and perinatal outcomes. The reduced risk of PTB observed in this systematic review came mainly from observational studies, while RCTs did not demonstrate any beneficial effect of LT4 supplementation in these women. However, not all the RCTs included in the present systematic review were specifically designed to report the effect of LT4 supplementation in affecting the primary outcome, thus limiting the clinical applicability of these findings, and highlighting the need for appropriately designed and powered studies.

Strengths and Limitations

Thorough literature search, multitude of outcomes explored and stratification of the analysis according to the type of study (RCTs and observational studies) are the main strengths of the present systematic review.

The small number of included studies for some of the observed outcomes, dissimilarity in inclusion criteria, outcome definition and gestational age at treatment are the main weakness of the present review. Furthermore, we could not perform comprehensive sub-group analyses considering only women with specific risk factors for PTB, type of PTB and IVF status, this is likely to represent a major bias. Finally, not all the included RCTs were specifically designed to assess the role of LT4 supplementation during pregnancy, with two of them rather exploring the reproductive outcomes of TPO-positive women

TABLE 1 | General characteristics of the included studies.

First Author	Year	Study Design	Period Considered	Outcomes Observed (Maternal)	Outcomes Observed (Fetal)	Reference Values For Thyroid Status*	Starting Dose LT4 Supplementation (μg/d)	Women (n)
Dhillon-Smith et al. (14)	2019	RCT	NS	Miscarriage, preterm birth, clinical pregnancy rate and live birth	None	TSH: (0.44-3.63) FT4: (0.0-21.0) **	50	266
Narzapour et al. (5)	2017	RCT	NS	Placental abruption, Miscarriage, Preterm birth	Admission to NICU, Stillbirth, Birthweight	TSH (0.1–2.5): FT4: (1–4.5) TPO: (<50)	0.5 μg/kg/d (TSH <1.0 μIU/mL) 0.75 μg/kg/d (TSH 1.0–2.0 μIU/mL) 1 μg/kg/d (TSH >2.0 μIU/mL)	131
Wang et al. (15)	2017	RCT	2012-2016	Miscarriage, preterm birth, clinical pregnancy rate and live birth	None	TSH: (0.5-4.78) TPOAb: (<60)	50 (TSH ≥2.5mIU/L) 25 (If TSH <2.5 mIU/L)	282
Stoian et al. (2)	2016	Prospective cohort	NS	Spontaneous miscarriage	APGAR score, Week of Gestation; Birth Length (cm); Birthweight (kg)	TSH: (>2.5)	25 (TSH ≥4.5mIU/L) 12.5 (If TSH <4.5 mIU/L)	107
Negro et al. (16)	2016	RCT	2011-2014	Miscarriage, preterm birth	None	TSH: (0.5-2.5) TPOAb: (≤16)	0.5 μg/kg/d (TSH 0.5-1.5 μIU/mL) 1 μg/kg/d (TSH 1.5–2.5 μIU/mL)	198
Lata et al. (13)	2013	Prospective cohort	2010-2011	Miscarriage, Preterm labor (PTL), Gestational hypertension, Preterm premature rupture of membranes, Intrauterine growth retardation	None	TSH: (0.27–4.2) FT3: (1.7–4.2) FT4: (0.7–1.8) anti-TPO: (<34)	25	31
Lepoutre et al. (18)	2012	Retrospective	2008-2009	Miscarriage, preterm birth and placental abruption	None	TSH: (0.2–3.5) FT4: (0.6–1.4) TPO-Ab: (<9)	NS: the initial LT4 dose started as soon as TPOAb was detected and TSH >1 mU/l	49
Revelli et al. (17)	2009	Retrospective	2004-2008	Miscarriage, pregnancy rate, ongoing pregnancy rate	None	TPO-Ab: (0–40) Tg-Ab: (0–35)	50	55
Negro et al. (3)	2006	Prospective cohort	2002-2004	Hypertension, Preeclampsia, Placental abruption, Miscarriage, Preterm birth	None	TSH: (0.27–4.2) FT4: (9.3–18.0) TPO-Ab: (<100)	0.5 μg/kg/d (TSH <1.0 μIU/mL) 0.75 μg/kg/d (TSH 1.0–2.0 μIU/mL) 1 μg/kg/d (TSH >2.0 μIU/mL)	115

NS, not specified; RCT, Randomized Controlled Trial.
**Values are expressed in:*
1. IU/L for TPO and Tg antibodies.
2. mIU/L for TSH.
3. ng/ml for FT3 and FT4.
***Value expressed in mol/L.*

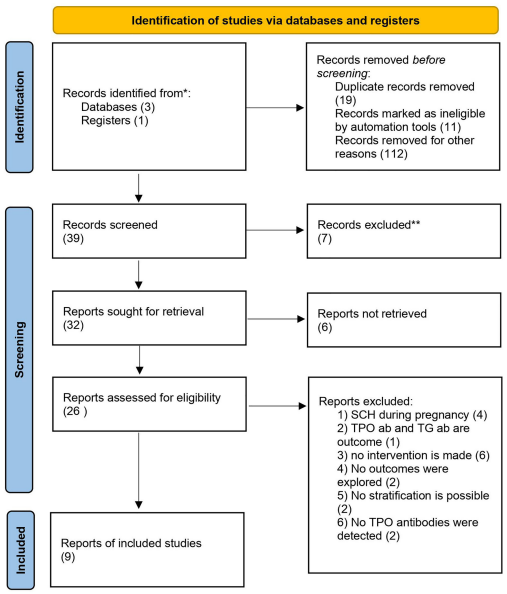
with normal thyroid function undergoing IVF. In this scenario, it is likely that this systematic review might be underpowered for some of the outcomes explored.

Interpretation of the Study Findings, Clinical and Research Implications

Thyroid disorders are among the most common medical complications occurring in pregnancy. Previous systematic reviews reported a higher risk of obstetric complications in women with thyroid autoimmunity and sub-clinical hypo- or hyper-thyroidism. However, there is still conflicting evidence on whether these women should receive LT4 supplementation.

In the systematic review by Rao et al. (6), the authors reported a beneficial effect of LT4 supplementation in affecting pregnancy loss and pre-term birth rate in women with sub-clinical hypothyroidism (SCH) and thyroid auto-immune (TAI) status. Conversely, Sun et al. (19) did not report any efficacy of LT4 supplementation in affecting pregnancy and perinatal outcome in euthyroid women with thyroid autoimmunity.

In the present systematic review, we could not find any beneficial role of LT4 supplementation in affecting pregnancy and perinatal outcomes improvement TPO-positive women with normal thyroid function. This lack of association between LT4 supplementation and reduced risk of adverse pregnancy outcome, mainly PTB, can be explained on different basis. First, the included trials were not specifically designed to elucidate the role of LT4 supplementation in affecting PTB. Furthermore, the large majority of included cases were IVF pregnancies, which have been reported to be at higher risk of PTB irrespective of thyroid status. Finally, it was not possible to extrapolate whether the included cases had other risk factors for PTB.



*Consider, if feasible to do so, reporting the number of records identified from each database or register searched (rather than the total number across all databases/registers).

**If automation tools were used, indicate how many records were excluded by a human and how many were excluded by automation tools.

SCH: subclinical hypotiroidism

FIGURE 1 | Flow diagram of the systematic review.

TABLE 2 | Pooled Odds ratio (OR) for the different categorical outcome explored in the present systematic review in euthyroid women having compared to those not having LT4 supplementation.

Outcome	Studies	Pregnancies	Pooled OR (95% CI)	I^2 (%)	p-value (*P*<0.05)
			All studies		
Pre-term birth	6	59/871vs 93/903	0.60 (0.4-0.9)	53.9	**0.008**
Gestational Hypertension	3	8/137 vs 13/154	0.56 (0.2-1.4)	0	0.23
Pre-eclampsia	2	4/106 vs 5/85	0.61 (0.1-2.3)	0	0.48
Placenta abruption	3	1/171 vs 1/151	0.85 (0.08-8.5)	7,7	0.11
Miscarriage	9	121/935 vs 142/949	0.82 (0.5-1.2)	28.1	0.37
Intra-uterine death	3	1/438 vs 0/449	3.11 (0.1-76.5)	0	0.488
Admission to NICU	2	2/163 vs 12/167	0.14 (0.03-0.7)	0	**<0.001**
			Randomized controlled trials		
Pre-term birth	3	50/627 vs 65/640	0.74 (0.4-1.3)	50.2	0.311
Gestational Hypertension	0	–	–	–	–
Pre-eclampsia	0	–	–	–	–
Placenta abruption	1	0/65 vs 0/66	1.02 (0.01-51.9)	–	0.994
Miscarriage	4	112/636 vs 126/648	0.88 (0.7-1.2)	0	0.999
Intra-uterine death	1	1/266 vs 0/274	3.10 (0.1-76.5)	–	0.489
Admission to NICU	1	2/56 vs 12/58	0.14 (0.03-0.7)	–	**0.013**
			Observational studies		
Pre-term birth	3	9/244 vs 28/263	0.29 (0.1-0.6)	37.3	***0.002***
Gestational Hypertension	3	8/137 vs 13/154	0.57 (0.2-1.4)	0	0.233
Pre-eclampsia	2	4/106 vs 5/85	0.62 (0.2-2.4)	0	0.483
Placenta abruption	2	1/106 vs 1/85	0.85 (0.01-8.6)	7.7	0.892
Miscarriage	5	9/299 vs 16/301	0.72 (0.1-3.6)	60	0.692
Intra-uterine death	1	0/107 vs 0/109	1.02 (0.02-51.8)	–	0.993
Admission to NICU	1	0/107 vs 0/109	1.02 (0.02-51.8)	–	0.993

NICU, neonatal intensive care unit. Bold values are statistically significant.

CONCLUSION

LT4 supplementation in TPO euthyroid women does not affect pregnancy and perinatal outcomes in TPO-positive women with normal thyroid function. However, the small number of included cases and dissimilarity in inclusion criteria, therapeutic strategies, and populations analyzed highlights the need for properly designed RCTs aimed at elucidating whether treatment with LT4 may reduce the risk of adverse obstetric outcomes in these women.

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

RDG and FD'A contributed to the conception and design of the study. RDG and CS reviewed the literature and extracted the data from the article. ML and FD'A performed data analysis. All authors participated to the drafting of the article and critically revised the work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.759064/full#supplementary-material

REFERENCES

1. Dallas JS. Autoimmune Thyroid Disease and Pregnancy: Relevance for the Child. *Autoimmunity* (2003) 36(6-7):339–50. doi: 10.1080/0891693031000160299 3
2. Stoian D, Pantea S, Margan M, Timar B, Borcan F, Craina M, et al. Individualized Follow-Up of Pregnant Women With Asymptomatic Autoimmune Thyroid Disease. *Int J Mol Sci* (2016) 17(1):88. doi: 10.3390/ijms17010088
3. Negro R, Formoso G, Mangieri T, Pezzarossa A, Dazzi D, Hassan H. Levothyroxine Treatment in Euthyroid Pregnant Women With Autoimmune Thyroid Disease: Effects on Obstetrical Complications. *J Clin Endocrinol Metab* (2006) 91(7):2587–91. doi: 10.1210/jc.2005-1603
4. Alexander EK, Pearce EN, Brent GA, Brown RS, Chen H, Dosiou C, et al. 2017 Guidelines of the American Thyroid Association for the Diagnosis and Management of Thyroid Disease During Pregnancy and the Postpartum. *Thyroid* (2017) 27(3):315–89. doi: 10.1089/thy.2016.0457. Erratum in: Thyroid. 2017 Sep;27(9):1212.
5. Nazarpour S, Ramezani Tehrani F, Simbar M, Tohidi M, Alavi Majd H, Azizi F. Effects of Levothyroxine Treatment on Pregnancy Outcomes in Pregnant Women With Autoimmune Thyroid Disease. *Eur J Endocrinol* (2017) 176 (2):253–65. doi: 10.1530/EJE-16-0548
6. Rao M, Zeng Z, Zhou F, Wang H, Liu J, Wang R, et al. Effect of Levothyroxine Supplementation on Pregnancy Loss and Preterm Birth in Women With Subclinical Hypothyroidism and Thyroid Autoimmunity: A Systematic Review and Meta-Analysis. *Hum Reprod Update* (2019) 25(3):344–61. doi: 10.1093/humupd/dmz003
7. De Leo S, Pearce EN. Autoimmune Thyroid Disease During Pregnancy. *Lancet Diabetes Endocrinol* (2018) 6(7):575–86. doi: 10.1016/S2213-8587(17)30402-3
8. Zorzela L, Loke YK, Ioannidis JP, Golder S, Santaguida P, Altman DG, et al. PRISMA Harms Group. PRISMA Harms Checklist: Improving Harms Reporting in Systematic Reviews. *BMJ* (2016) 352:i157. doi: 10.1136/bmj.i157
9. Minozzi S, Cinquini M, Gianola S, Gonzalez-Lorenzo M, Banzi R. The Revised Cochrane Risk of Bias Tool for Randomized Trials (RoB 2) Showed Low Interrater Reliability and Challenges in its Application. *J Clin Epidemiol* (2020) 126:37–44. doi: 10.1016/j.jclinepi.2020.06.015
10. Guyatt GH, Oxman AD, Kunz R, Woodcock J, Brozek J, Helfand M, et al. GRADE Guidelines: 7. Rating the Quality of Evidence–Inconsistency. *J Clin Epidemiol* (2011) 64(12):1294–302. doi: 10.1016/j.jclinepi.2011.03.017
11. Lo CK, Mertz D, Loeb M. Newcastle-Ottawa Scale: Comparing Reviewers' to Authors' Assessments. *BMC Med Res Methodol* (2014) 14:45. doi: 10.1186/1471-2288-14-45
12. NHS Centre for Reviews and Dissemination. *Systematic Reviews: CRD's Guidance for Undertaking Reviews in Health Care*. York (UK: University of York (2009). Available at: https://www.york.ac.uk/media/crd/Systematic_Reviews.pdf.
13. Lata K, Dutta P, Sridhar S, Rohilla M, Srinivasan A, Prashad GR, et al. Thyroid Autoimmunity and Obstetric Outcomes in Women With Recurrent Miscarriage: A Case-Control Study. *Endocr Connect* (2013) 2(2):118–24. doi: 10.1530/EC-13-0012
14. Dhillon-Smith RK, Middleton LJ, Sunner KK, Cheed V, Baker K, Farrell-Carver S, et al. Levothyroxine in Women With Thyroid Peroxidase Antibodies Before Conception. *N Engl J Med* (2019) 380(14):1316–25. doi: 10.1056/NEJMoa1812537
15. Wang H, Gao H, Chi H, Zeng L, Xiao W, Wang Y, et al. Effect of Levothyroxine on Miscarriage Among Women With Normal Thyroid Function and Thyroid Autoimmunity Undergoing *In Vitro* Fertilization and Embryo Transfer: A Randomized Clinical Trial. *JAMA* (2017) 318 (22):2190–8. doi: 10.1001/jama.2017.18249
16. Negro R, Schwartz A, Stagnaro-Green A. Impact of Levothyroxine in Miscarriage and Preterm Delivery Rates in First Trimester Thyroid Antibody-Positive Women With TSH Less Than 2.5 mIU/L. *J Clin Endocrinol Metab* (2016) 101(10):3685–90. doi: 10.1210/jc.2016-1803
17. Revelli A, Casano S, Piane LD, Grassi G, Gennarelli G, Guidetti D, et al. A Retrospective Study on IVF Outcome in Euthyroid Patients With Anti-Thyroid Antibodies: Effects of Levothyroxine, Acetyl-Salicylic Acid and Prednisolone Adjuvant Treatments. *Reprod Biol Endocrinol* (2009) 7:137. doi: 10.1186/1477-7827-7-137
18. Lepoutre T, Debiève F, Gruson D, Daumerie C. Reduction of Miscarriages Through Universal Screening and Treatment of Thyroid Autoimmune Diseases. *Gynecol Obstet Invest* (2012) 74(4):265–73. doi: 10.1159/000343759
19. Sun X, Hou N, Wang H, Ma L, Sun J, Liu Y. A Meta-Analysis of Pregnancy Outcomes With Levothyroxine Treatment in Euthyroid Women With Thyroid Autoimmunity. *J Clin Endocrinol Metab* (2020) 105(4):dgz217. doi: 10.1210/clinem/dgz217

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership